Flavonoid quercetin modulates glucose metabolism by up-regulating the methylation status of genes as Kcnj11, Gys1 and Erp29 in liver of Wistar rats

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Abstract

Background: Quercetin is a flavonoid that exists extensively in vegetables and fruits, and has many biological activities. It is reported that quercetin participates in the regulation of glucose metabolism through various mechanisms. However, whether DNA methylation is involved in those regulatory effects remains unclear. As liver is one of the main organ involved in methyl and glucose metabolism, DNA methylation targets related to glucose metabolism were identified in liver of Wistar rats upon quercetin exposure.

Methods: The rats were fed a control diet or a 0.5% quercetin-supplemented diet for 6 weeks. Arraystar Rat 4 × 180K RN4 RefSeq Promoter Arrays were used for a genome-wide survey of DNA methylation in rat livers. NimbleScan v2.5 software was used to process microarray data. DAVID software was used to perform GO and Pathway analysis of regulatory networks. Gene promoter methylation status was examined by the ChIP-quantitative PCR assay, and hepatic levels of methylated Kcnj11, Erp29, Gys1, Flot1 and GAPDH were evaluated. Gene expression was assayed by quantitative PCR, and hepatic mRNA expression levels of Kcnj11, Gys1 and Erp29 were estimated.

Results: Quercetin induced specific changes in DNA methylation. A total of 1,263 differentially expressed genes were found in 22 chromosomes, particularly on chromosomes 1, 3, 5, 7, 8, and 10. According to GO functional analysis, differential genes have focused on organic substance, cellular and primary metabolic process. According to pathway analysis, the most enrichment pathways included Type 2 diabetes mellitus, insulin signaling pathway and protein processing in endoplasmic reticulum. Nineteen up-methylated genes were found among several biological pathways after quercetin treatment. Critical genes and pathways associated with glucose metabolism (Kcnj11 and Gys1) and protein processing in the endoplasmic reticulum (Erp29) were changed significantly. Promoter methylation levels of Kcnj11, Gys1, and Erp29 were significantly increased, and the mRNA expression of those genes significantly decreased simultaneously upon quercetin exposure.

Conclusions: Quercetin changed the promoter methylation status and expression of Kcnj11, Gys1, and Erp29, which are mainly related to glucose metabolism. The gene Kcnj11, Gys1, and Erp29 could be novel epigenetic targets of quercetin in regulating glucose metabolism.

Introduction

Epigenetic changes are heritable changes to gene expression independent of changes to the DNA sequence [1]. DNA methylation is one of important form of epigenetic modification, and generally occurs at the cytosine residue in cytosine-guanine dinucleotide pairs to form 5-methylcytosine [2]. Unlike genetic modifications, epigenetic modulation is reversible and can be altered by certain factors. This characteristic of epigenetic modulation may allow regulation of physiological responses to diet and environmental stimuli [3, 4]. Evidence has shown that dietary polyphenols present in fruits, vegetables and beverages are potential epigenetic regulators [5, 6]. It has been reported that the compound quercetin,

which belongs to the flavonoid subclass of polyphenols, may affect DNA methylation in therapy for cancer and chronic inflammatory disorders [7, 8].

Quercetin is a representative compound of flavonoid, and is ubiquitously present in Chinese diet such as fruits and vegetables. Quercetin has been reported to exert a wide range of biological effects, including antioxidant, anticarcinogenic, anti-inflammatory, and combatting gut dysbiosis activities [9–11]. Besides the above properties, quercetin also participates in regulating glucose metabolism. This compound modulates hyperglycemia by improving the pancreatic enzymes activities linked with glucose metabolism in rats with diabetes [12], and alters glucose homeostasis through insulin-dependent and -independent mechanism in the brain of diabetic rats [13]. In vitro experiments demonstrated that a mixture containing quercetin had the potential to modulate cellular glucose metabolism in human HepG2 cells [14], and could stimulate insulin release in rat INS-1 beta-cells [15, 16]. However, whether DNA methylation plays a role in those effects of quercetin is not clear.

The liver plays a pivotal role in controlling glucose metabolism [17]. It can store glucose in the form of glycogen with feeding, assemble glucose via the gluconeogenic pathway in response to fasting, and also participates in metabolizing and releasing glucose [18]. The liver is also an important organ in which quercetin is metabolized to its methylated compounds [19, 20]. It has been demonstrated that DNA methylation patterns exhibit a tissue specificity across human and mammalian animals [21, 22]. The liver has the highest DNA methylation rate in sika deer [22], while the brain contains the highest levels of DNA methylation in human [23]. Several studies have revealed that DNA methylation plays a much larger role in regulation of tissue-specific expression for genes [24–26]. Otherwise, quercetin improves the ultrastructure of hepatocytes and serum markers of liver injury in diabetic rats [27], and reduces the liver damage induced by drugs [28, 29], chemicals [30–34], and physical factors [35]. As liver is one of the main organ involved in glucose and methyl metabolism, we want to identify DNA methylation targets related to glucose metabolism in liver of rats upon quercetin exposure.

Although nutrition affected global DNA methylation status throughout lifespan in mammals [36], limited studies have reported how dietary quercetin modulates the tissue specific epigenetic profile. In this study, using rat DNA methylation promoter microarrays, we performed a genome-wide survey of DNA methylation in rat livers exposed to quercetin, and evaluated epigenetic alterations in specific gene promoters by ChIP-quantitative PCR methods. We hypothesized that quercetin would induce the methylations of genes related to glucose metabolism, which could affect gene expression in liver of Wistar rats.

Materials And Methods

Animals handling

The animal experiments were approved by the Ethical Committee of the Department of Scientific Management of the Institute. After adaptive feeding with AIN-93M formula [37] for 5 days, 24 Wistar rats were divided randomly into control or 0.5% quercetin groups according to body weight and maintained on

an AIN-93 diet (control) or 0.5% quercetin-supplemented (Sigma-Aldrich, St. Louis, MO, SA) AIN-93 diet (0.5%Q) for 6 weeks. Each group consisted of 6 female and 6 male rats, whose initial weights ranged from 180 to 200 g. Dietary intake was recorded daily and body weight weekly. The animal source, handling methods, and environmental conditions matched a previously reported protocol [20]. At the end of the experiment, all rats were fasted overnight. The animals were sacrificed by cervical dislocation. Liver tissues were sampled immediately, washed in ice-cold saline, and frozen in liquid nitrogen until use.

DNA preparation and methylated DNA immunoprecipitation-ChIP analysis

Liver samples from 2 female and 2 male rats in each group were randomly selected for genome-wide methylation analysis. Genomic DNA (gDNA) extraction, purification, quantification, and quality assessments were conducted according to the procedure of Aksomics, Ltd. (Shanghai, China). Sonicated gDNA was used for immunoprecipitation with a mouse monoclonal anti-5-methylcytosine antibody (Diagenode, Liège, Belgium). Methylated DNA immunoprecipitation DNA (MeDIP DNA) was purified and quantified using Qiagen MinElute columns (Qiagen, Hilden, Germany) and a nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A NimbleGen Dual-Color DNA Labeling Kit was used for DNA labeling according to the NimbleGen MeDIP-chip protocol (Nimblegen Systems, Inc., Madison, WI, USA). The microarrays were then hybridized with Cy3/5- labelled DNA and washed using the Nimblegen Wash Buffer Kit (Nimblegen Systems, Inc.).

The Arraystar Rat 4 × 180K RN4 RefSeq Promoter Array (Aksomics, Shanghai, China) is designed to investigate DNA methylation and transcription factor binding sites within RefSeq Gene promoter regions and includes 15,987 gene promoter regions (from about - 1300 bp to + 500 bp of the transcript start sequences) covered by approximately 180,000 probes with approximately 158 bp spacing.

Data Normalization and Analysis

The MeDIP-chip data were analyzed by sliding-window (1500 bp) peak-finding algorithm provided by NimbleScan v2.5 (Roche-NimbleGen Inc.) from the normalized log₂ ratio data. NimbleScan detects peaks by searching for at least two probes above a p-value minimum cutoff (-log₁₀) of 2 and maximum spacing of 500 bp between nearby probes within the peaks. To compare differentially enriched regions between the 0.5%Q group and the control group, the log₂ ratios were averaged and then used to calculate M₀ for each probe: $M_0 = \text{Average}(\log_2^{\text{MeDIP}(1\% \text{Met})/\text{Input}(1\% \text{Met})}) - \text{Average}(\log_2^{\text{MeDIP}(\text{control})/\text{Input}(\text{control})})$. The NimbleScan sliding-window peak-finding algorithm was run on these data to find the differential enrichment peaks (DEPs). The DEPs, identified by the NimbleScan algorithm, were filtered according to the following criteria: (1) At least one of the two groups has a median log₂ MeDIP/Input ≥ 0.3 and a median M₀ > 0. (2) At least half of the probes in a peak may have a CV ≤ 0.8 in both groups. To separate strong CpG islands from weak CpG islands, promoters were categorized into three levels: high CpG promoters/regions (HCP, high CpG density promoter), intermediate CpG promoters/regions (ICP, intermediate CpG density promoter), and low CpG promoters/regions (LCP, low CpG density promoter).

Microarray data processing and Gene Ontology (GO) and Pathway analysis

Raw microarray data were normalized by the Bioconductor packages Ringo, limma, and MEDME. Normalized MeDIP-chip data were analyzed by NimbleScan v2.5 (NimbleGen). DAVID software was used to perform GO and Pathway analysis of regulatory networks. The GO project provides a controlled vocabulary to describe gene and gene product attributes in any organism and covers three domains: Biological Process, Cellular Component and Molecular Function. Pathway analysis is functional analysis that maps genes to KEGG pathways. The p-value (EASE-score, Fisher-Pvalue or Hypergeometric-Pvalue) denotes the significance of the Pathway correlated to the conditions. Lower the p-value, more significant is the Pathway (The recommend p-value cut-off is 0.05), using an unbiased, automated survey of published scientific literature (Global Literature Analysis). This analysis identifies functional relations among genes, such as direct binding, up-regulation or down-regulation and also builds subnetworks of genes and cellular processes based on their interconnections.

ChIP-quantitative PCR assay

A MeDIP assay combined with real-time quantitative PCR (qPCR) was used to evaluate the methylation status of candidate genes in the rat liver. MeDIP was performed as described above. Quantitative PCR was used to analyze the expression of purified DNA with an Applied Biosystems 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression levels of methylated Flot1, Kcnj11, Gys1, Erp29, and GAPDH were evaluated. The primers used for ChIP validation are shown in Table 1.

Table 1
Sequences of the primers used in qChIP analyses

Genes	Primer sequence	Product length (bp)
Flot1	F:5' GGCGACTCGACCTCTTGCT 3'	104
	R:5' CGTGCGTCCGAACACTTCT 3'	
Kcnj11	F:5' TGTGCCCCGGATTTCCCAT 3'	121
	R:5' GGTTGCAGGGTGACTCGAAGG 3'	
Gys1	F:5' TCCCGGTCATCCAGTCATCT 3'	106
	R:5' CACCCGCTTTCCAATTTAGC 3'	
Erp29	F:5' GCTATGATCCCTGTGTCTTCTCCAG 3'	56
	R:5' GTGATAAAGGCTCGAAGGAATGAA 3'	
GAPDH	F:5'GGACCCTGTGGTGCTTCATCT3'	181
	R:5'GGGCAGTAAGTGCTCCTAATCG3'	

Quantitative PCR analysis

Hepatic mRNA expressions of genes were determined by quantitative PCR (qPCR) [38]. Total hepatic RNA was isolated using the TRIzol reagent. The first cDNA strand was synthesized using a cDNA synthesis kit. Quantitative PCR was performed using a FastStart Universal SYBR Green Master Mix kit. TRIzol reagent, cDNA synthesis kit, and FastStart Universal SYBR Green Master Mix kit were purchased from Roche, Ltd. (Basel, Switzerland). Finally, melting curve analysis was performed by slowly cooling the PCR mixture with simultaneous measurement of the SYBR Green I signal intensity using an ABI Real-time PCR System (Applied Biosystems). The Δ threshold cycle (Ct) method was used to evaluate relative quantification, and GAPDH was used as a reference. The primers used for qPCR validation are shown in Table 2.

Table 2
Sequences of the primers used in qPCR analyses

Genes	Primers sequence	Product length (bp)
Erp29	F:5' GACAAGAAGTGGGCCAGTCA 3' R :5' GAAGGCGGTGAGGATGTTGA 3'	168
Kcnj11	F: 5' GTCAGGGGCTCAGTAAGCAA 3' R :5' CTTGCACCAACCTCTGGACT 3'	105
Gys1	F:5' GAGGGCAGAATGTCGGTCAA 3' R :5' GTACACGTGGGGCTTCAAGA 3'	172
GAPDH	F: 5'CCCCCAATGTATCCGTTGTG 3' R :5'TAGCCCAGGATGCCCTTTAGT 3'	192

Statistical analysis

The one-sided Kolmogorov-Smirnov test was applied to analyze the microarray data. Fisher’s exact test was used to perform GO and Pathway analysis. ChIP-quantitative PCR and qPCR data are presented as means ± standard deviation. Statistical analysis was performed using the SPSS 10.01 software (SPSS Inc., Chicago, IL, USA). Student’s t-test was used to compare differences between two groups. Differences between two groups were considered statistically significant at $p < 0.05$.

Results

Quercetin does not change body weight or dietary intake

No significant difference was found in body weight or food intake between the control and quercetin groups (Fig. 1).

Quercetin alters DNA methylation markers of genes involved in biological process

Genome-wide DNA methylation analysis indicated that quercetin induced specific changes in DNA methylation. A total of 1,263 differentially methylated promoters (DMRs) were found in 22 chromosomes (Fig. 2), particularly on chromosomes 1, 3, 5, 7, 8, and 10. Among these DMRs, 670 (53.05%) were located in high CpG density promoters (HCP), 309 (24.47%) in intermediate CpG density promoters (ICP), and 284 (22.49) in low CpG density promoters (LCP) (Fig. 2). Nine hundred twenty one hypermethylated DMRs (72.92%) and 342 hypomethylated DMRs (37.08%) were found in the 0.5% quercetin group compared with the DMRs of the control group.

In the up regulation of GO biological process, differential genes have focused on cellular metabolic process and primary metabolic process (Fig. 3). In the down regulation of GO biological process, differential genes have focused on organic substance, cellular and primary metabolic process (Fig. 4). By pathway analysis of up regulation, the most enrichment pathways included Type 2 diabetes mellitus, insulin signaling pathway and protein processing in endoplasmic reticulum (Fig. 5).

Quercetin alters promoter methylation of specific genes in biological pathways

When Peak Score was set at ≥ 3 , Peak Length ≥ 500 , and Peak M value higher than the median, we found that the promoters methylation status of 169 genes was changed by quercetin treatment, including 35 down-methylated genes and 134 up-methylated genes. When those 169 genes were screened against genes in differential biological pathways, 19 up-methylated genes were identified after quercetin treatment (Table 3). Five of the genes are associated with insulin and its signaling pathway, including Kcnj11 in type 2 diabetes mellitus, and Irs2, Flot1, Gys1, and Foxo1 in the insulin signaling pathway. Erp29 is involved in protein processing in the endoplasmic reticulum.

Table 3
Differential genes in biological pathways

Gene	Promoter Classification	Peak score (0.5%Q group vs control)	Pathways
Tnni3	LCP	3.85	Adrenergic signaling in cardiomyocytes
Sstr2	ICP	3.51	Neuroactive ligand-receptor interaction
Slc6a3	ICP	3.16	Dopaminergic synapse
Rrm2b	HCP	3.42	Purine metabolism
Ptpn6	LCP	4.01	Proteoglycans in cancer
			B cell receptor signaling pathway
			T cell receptor signaling pathway
Polr2c	HCP	3.14	Purine metabolism
Orai3	LCP	3.03	Calcium signaling pathway
Mrvi1	LCP	4.08	Vascular smooth muscle contraction
Kcnj11	HCP	3.37	Type 2 diabetes mellitus
Irs2	HCP	3.39	Insulin signaling pathway
			FoxO signaling pathway
Gys1	HCP	3.96	PI3K-Akt signaling pathway
			Insulin signaling pathway
Gpr35	LCP	3.55	Neuroactive ligand-receptor interaction
Gpc3	HCP	3.77	Proteoglycans in cancer
Foxo1	HCP	3.44	Thyroid hormone signaling pathway
			Insulin signaling pathway
			Prostate cancer
			FoxO signaling pathway
			Pathways in cancer
Flot1	ICP	3.05	Insulin signaling pathway

Abbreviation: HCP: high CpG density promoter; ICP: intermediate CpG density promoter; LCP: low CpG density promoter

Gene	Promoter Classification	Peak score (0.5%Q group vs control)	Pathways
Erp29	HCP	3.96	Protein processing in endoplasmic reticulum
Dolk	HCP	3.88	N-Glycan biosynthesis
Bid	ICP	3.44	Apoptosis
			Pathways in cancer
Aqp3	HCP	3.54	Vasopressin-regulated water reabsorption
Abbreviation: HCP: high CpG density promoter; ICP: intermediate CpG density promoter; LCP: low CpG density promoter			

Quercetin increases methylation levels and decreases mRNA expression of Erp29, Gys1, and Kcnj11

The promoter methylation levels of Kcnj11, Gys1, and Erp29 were significantly increased in the 0.5% quercetin group (Fig. 6), whereas the mRNA expression of those genes was notably decreased (Fig. 7). No difference was found in methylation status of the genes coding for Flot1 (Fig. 6).

Discussion

Our genome-wide analysis of the DNA methylation landscape in rats revealed that some genes were changed significantly by quercetin. According to GO functional analysis, differential genes have focused on organic substance, cellular and primary metabolic process. According to pathway analysis, the most enrichment pathways of Type 2 diabetes mellitus, insulin signaling pathway and protein processing in endoplasmic reticulum were changed by quercetin. Differentially methylated CpG sites were up-methylated in response to quercetin. Those sites were mainly located in genes involved in insulin metabolism, including Kcnj11, Irs2, Gys1, Flot1, and Foxo1. These results suggest that quercetin may regulate peptide hormone metabolism, especially insulin metabolism, by epigenetic mechanisms.

In the present study, we found for the first time that Kcnj11, Gys1, and Erp29 were significantly up-methylated, while their mRNA expression levels were down-regulated in rat livers. Kcnj11, the potassium inwardly-rectifying channel, subfamily J, member 11 gene, encodes Kir6.2, a subunit of ATP-sensitive potassium (K_{ATP}) channels in mammalian cells. In pancreatic beta cells, K_{ATP} channels play a pivotal role in glucose-stimulated insulin secretion [39–41]. It has been reported that Kcnj11 gene methylation might be associated with type 2 diabetes mellitus (T2DM). In patients with T2DM, one or more locus of the Kcnj11 gene was significantly up-methylated, and this alteration may mimic genetic defects [42, 43]. However, experiments in vitro got different results. Insulin secretion was continuously increased in

Kir6.2^{-/-} beta cells under unstimulated conditions [44]. Increased insulin secretion was also observed in cultured islets isolated from Kir6.2^{-/-} mice [45]. In vivo experiments confirmed that the hyperinsulinemic phenotype was based on incomplete loss of K_{ATP} in beta cells [46]. For example, methylation at a specific locus of the Kcnj11 gene reduced expression of the Kir6.2 subunit. In our experiment, we found that hepatic Kcnj11 was up-methylated and its mRNA expression down-regulated after quercetin treatment, indicating that Kcnj11 expression was incompletely lost, which might account for the hyperinsulinemic phenotype. Actually, quercetin stimulated insulin release in rat INS-1 beta-cells [15, 16], and transient K_{ATP} channel inhibition induced by DNA methylation may be one of the mechanisms.

The Erp29 gene encodes endoplasmic reticulum protein 29 (ERp29). ERp29 is a molecular chaperone that plays a pivotal role in protein secretion, folding, and trafficking. ERp29 is highly expressed in secretory tissues, including in the pancreas and liver [47]. High ERp29 protein expression was found in islets of transgenic MKR mice, and was associated with T2DM development [48]. Our results show that quercetin epigenetically down-regulates Erp29 expression in the rat liver. Therefore, we inferred that the Erp29 gene may be an important target of quercetin in preventing T2DM development. The Gys1 gene encodes glycogen synthase, which catalyzes the key step of glycogen synthesis in liver and muscle cells [49, 50]. Under fasting conditions, lower glycemia and higher insulinemia were found in Gys1-knockout mice. However, under normal conditions, the mice showed no abnormalities in glucose tolerance, insulin secretion, or basal glycemia [51]. In our research, we found that the Gys1 gene was epigenetically down-regulated in healthy rats upon quercetin treatment. Considering the results from Gys1-knockout mice, we speculate that the effects of quercetin on the Gys1 gene may not cause adverse effects to healthy rats.

Conclusions

In the present study, we showed that quercetin is involved in the regulation of glucose metabolism by regulating the methylation level of Kcnj11, Gys1, and Erp29 genes, and also modulating related glucose metabolism pathways. Quercetin may be helpful to maintain the balance of blood glucose, and consumption of functional food and nutraceutical rich in quercetin could be a cheap and affordable method for the improvement of hyperglycemia.

We reported for the first time that the effects of quercetin on methylation status of genes participating in the glucose metabolism in Wistar rats. To our knowledge, intervention studies of dietary factors on DNA methylation patterns in humans are very limited besides the study on folic acid [52]. Our results will provide a reference for future studies that examining the interplay of epigenetics and environmental factors in humans.

Abbreviations

gDNA: Genomic DNA; MeDIP DNA: methylated DNA immunoprecipitation DNA; GO: Gene Ontology; qPCR: real-time quantitative PCR; DMRs: differentially methylated promoters; HCP: high CpG density promoters;

ICP: intermediate CpG density promoters; LCP: low CpG density promoters; KATP channels: ATP-sensitive potassium channels; T2DM: type 2 diabetes mellitus; ERp29: endoplasmic reticulum protein 29.

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in the article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable. .

Authors' contributions

WG, LP, YM, YY, TS and ZY designed and performed the experiments, XB analyzed the data. WG, LP, and CG drafted and reviewed the article. All authors revised and approved the final version of the article. The authors have declared no conflict of interest.

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Figures

Figure 1

Changes of body weight and dietary intake during experimental period ($\pm$ SE, $n=12$). (A) Body weight change of rats; (B) food intake change of rats. Wistar rats were fed with control and diet supplemented with 0.5% quercetin for 6 weeks.

Figure 2

Differentially methylated promoters between 0.5% quercetin group and control group. (A) CpG density of differentially methylated promoters. (B) Chromosomal distribution of differentially methylated promoters. Blue, differentially hypomethylated promoters; red, differentially hypermethylated promoters. Classification of all promoters with high (HCP, high CpG density promoter), intermediated (ICP, intermediate CpG density promoter), and low (LCP, low CpG density promoter) CpG content.

Figure 3

Quercetin group compared to control group of biological process up regulation of GO Biological Process Classification.

Figure 4

Quercetin group compared to control group of biological process down regulation of GO Biological Process Classification

Figure 5

Quercetin group compared to control group of biological process up regulation of pathway analysis

Figure 6

Quantitative ChIP results of differential genes. (A) Flot1; (B) Kcnj11; (C) Gys1; (D) Erp29. Data are expressed as the mean $\pm$ SEM. Data with superscript letters (*) are significantly different at $p < 0.05$ according to Student's t-test. Wistar rats were fed a AIN93M diet supplemented with or without quercetin for 6 weeks.

Figure 7

Hepatic mRNA expression of Kcnj11, Erp29 and Gys1 in rats. (A) Kcnj11; (B) Erp29; (C) Gys1. Data are expressed as the mean $\pm$ SEM. Data with superscript letters (*) are significantly different at $p < 0.05$ according to Student's t-test. Wistar rats were fed a AIN93M diet supplemented with or without quercetin for 6 weeks.