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Quercetin downregulates the expression of *IL15* in cancer cells through DNA methylation

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Abstract. – OBJECTIVE: This study aimed to investigate the effect of quercetin on cellular immunity (*via IL15* expression) against cancer and to elucidate its regulatory mechanism.

MATERIALS AND METHODS: HeLa cells and A549 cells were cultured *in vitro* and were divided into control (DMSO treated) and experimental groups (treated with different concentrations of quercetin). Transcript levels of *IL15* and DNA methyltransferase (DNMTS) were measured using Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Genomic DNA was extracted, treated with bisulfite, and the promoter region of *IL15* was cloned. Finally, Sanger sequencing was used to detect the degree of promoter methylation.

RESULTS: Following quercetin treatment, the expression of *IL15* was significantly downregulated in HeLa and A549 cells. The methylation level of *IL15* promoter in HeLa cells was about twice that of the control group, and the methylation level of *IL15* promoter in A549 cells was about three times that of the control group.

CONCLUSIONS: Quercetin inhibits cancer cell proliferation while downregulating *IL15* expression, and this regulation is achieved by increasing the methylation of the *IL15* promoter.

Key Words:
Quercetin, IL15, A549 cells, HeLa cells, DNA methylation.

Introduction

Cancer is the preeminent difficult medical problem for the scientific community to overcome. Lung cancer has a particularly high death rate among various malignant tumors, and cervical cancer is one of the most common malignant tumors in women[1]. Chemotherapy remains the most common method of cancer treatment[2]. However, due to the emergence of cellular resistance and the bodily damage caused by chemotherapy, the effectiveness of chemotherapy is becoming less effective[3]. In recent years, herbal medicine has become considerably more effective in cancer treatment, and much effort has been made to better understand the anti-cancer components of herbal medicine[4-6]. Quercetin has been widely studied as a common active ingredient in Traditional Chinese Medicine[7,8]. Quercetin is a flavonoid with many benefits for people and animals and is found in large quantities in various fruits and vegetables, tea leaves, etc. It has a variety of biological activities such as anti-inflammation and antioxidation[9,10]. Both *in vivo* and *in vitro* experiments have shown that quercetin can exert anti-tumor effects by altering cell cycle progression, inhibiting cell proliferation, and promoting apoptosis[11]. Quercetin can induce G0/G1 phase changes in human osteosarcoma cells[12]. Similar results were found in the ovarian cancer cell line SKOV3, where cells were stalled in the S and G2/M phases with the reduction of cyclin D1[13]. It was found that quercetin induced ER stress and then promoted the release of p53, which inhibited the activity of CDK2, cell cycle protein A, and cell cycle protein B, thus arresting MCF-7 breast cancer cells in the S phase[14]. Quercetin activates caspases 3, 8, and 9, stimulates the expression of *Bax* and *Bad*, and downregulates anti-apoptotic proteins including Bcl-XL, Bcl-2, and Mcl-1 to induce apoptosis in cancer cells[15]. Previous studies[16] showed that quercetin inhibited the proliferation of and induced cell death in HeLa and A549 cells in a dose-dependent manner. Quercetin systematically alters the PI3K, MAPK, and WNT pathways by regulating the expression of TRAIL, TNF, caspase 9, cytochrome C proteins, AKT1, AKT2, and MTOR, thereby inhibiting the proliferation of cervical cancer (HeLa) cel-

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ls, causing cell cycle arrest, DNA damage, and apoptosis. In addition, another study[17] showed that quercetin can induce the production of NO, alter the redox environment, and thus promote the apoptosis of HeLa cells. This study also studied the expression of redox and NO pathway-related enzymes, forming a network diagram of quercetin-induced apoptosis of cancer cells. In addition, quercetin-induced apoptosis through BAX, BCL-2, and caspase3, showed an inhibitory effect on lung cancer cell proliferation and caused G2-M phase arrest in lung cancer cells[18]. Quercetin can upregulate the accumulation of p53 inducible gene 3 and ROS in human hepatoma HepG2 cells and reduce the mitochondrial membrane potential to activate the intrinsic mitochondrial apoptosis pathway, which ultimately leads to cellular apoptosis[19].

Studies[20-22] have shown that cancer cells are mostly infiltrated by multiple immune cells, as inflammation played a key role in tumorigenesis. The presence of an inflammatory microenvironment inevitably leads to a series of immune responses in which Interleukin 15 (*IL15*) is produced by a variety of cells other than T cells, including cancer cells. IL15 is a potential novel tool for cancer immunotherapy, as IL15 regulates the development, survival, and function of a variety of intrinsic and adaptive immune cells and has a dual role in anti-tumor immunity and the promotion of tumor cell growth[23-26]. The benefits of IL15 in tumor immunotherapy stem from its unique ability to activate important mechanisms of antitumor immunity, including the development and activity of NK cells and CD8+ T cells, and to promote durable immune responses through its activity on memory T cells[27,28]. In addition, IL15 also upregulates tumor necrosis factor-a (TNF-α) and interferon-γ (IFN-γ) produced by NK cells and T cells[29]. It was also found that IL15 increased the expression of intercellular adhesion molecules on the surface of A549 cells and the adhesion of neutrophils to these cells, which also indicates that IL15 has an important role in the immune response in lung cancer[30]. The potential use of IL15 as a cancer immunotherapeutic agent has been investigated in several mouse cancer models[31]. IL15 is also at the top of the list of agents listed by the National Cancer Institute as having the greatest potential in tumor immunotherapy[32]. However, it has also been shown that IL15 can protect tumor cells from apoptosis in certain contexts[33,34]. IL15 may increase the expression of the apoptosis inhibitor BCL2L1/BCL-x(L) through the transcriptional activation activity of STAT6, thereby preventing apoptosis[35]. Studies[36,37] using immunodeficient mice as a model showed that IL15 could be pro-tumorigenic by promoting tumor growth, invasion, and metastasis. *IL15* overexpression promotes the development of large granular lymphocytic leukemia. The specific role of IL15 in cancer immunotherapy still needs to be analyzed in greater detail, and different types of cancers have different cellular environments in the body, which will most likely lead to different effects of IL15. In conclusion, more research is needed to determine exactly how IL15 functions in the immunotherapy of cancer.

Many studies[38-40] conducted in recent years suggest that the combination of different approaches may lead to a new means of cancer treatment. Some outstanding questions include: what would be the relationship between the anti-cancer effects of quercetin and the action of human autoimmunity (by way of IL15) on cancer cells, and how do these two treatments relate to each other? Therefore, understanding the regulation of IL15 by quercetin will provide more information on the clinical effects of quercetin. Gene expression is influenced by various factors, and recent studies[41] have shown that quercetin can reactivate the expression of some cancer suppressor genes by regulating DNA methyltransferase (DNMT) expression and demethylating the promoters of these cancer suppressor genes in cells, thus exerting anti-cancer effects. However, whether quercetin can selectively demethylate the promoter remains to be studied. Therefore, how quercetin affects the expression of other genes while activating cancer suppressor genes is also of strong clinical value.

This study aimed to investigate the effect of quercetin on cellular immunity (IL15) during the inhibition of cancer cell proliferation. Finally, we found that quercetin inhibited the proliferation of A549 and HeLa cells while downregulating *IL15* expression by upregulating the degree of promoter methylation of *IL15*.

Materials and Methods

Cell Culture and Treatment with Quercetin

A549 cells (Catalog No. SCSP-503; Cell bank, Shanghai Institutes for Biology Science, Shanghai, China) was cultured in DME/F-12 1:1 me-

dium (SH30023.01, Cytiva) with 10% (v/v) fetal bovine serum (FBS) (10099141C, Gibco) and 1% (v/v) penicillin/streptomycin (15140-122, Gibco). HeLa cells (Catalog No. TCHu187) were cultured in MEM Alpha Modification Medium with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. All cells were incubated in a humidified 5% CO_2 air incubator at 37°C.

Cells were seeded in 6-well cell culture plates (140675, Thermo Fisher Scientific, Shanghai, China) approximately 1 d before treatment. Different concentrations of quercetin (B20527-20 mg, HPLC ≥ 98%, Yuanyeshengwu, Shanghai, China) were used to treat A549 or HeLa cells. Cells were harvested after 24 h of treatment for the next step of the analysis.

CCK8 Assays

Cells were seeded in 96-well culture plates at a density of about $3x10^3$ cells per well and incubated at 37˚C for 24 h. Then, they were treated with different concentrations of quercetin (20-60 μM). After incubation, Cell Counting Kit-8 reagents were added to each well and the plates were incubated at 37˚C for approximately 2 h. At the end of the incubation, the optical density was measured at 450 nm.

qRT-PCR

Total RNAs were isolated from cells of the indicated groups using Trizol Reagent (9109, TaKaRa Dalian China). Complementary DNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (RR047A, TaKaRa Dalian China) according to the manufacturer's protocol. qRT-PCR assays were performed to assess the relative abundances of the mRNA, using sequence-specific primers listed in Table I, stained with SYBR Green use Hieff® qPCR SYBR Green Master Mix (High Rox Plus) (11203ES03, YEASEN Shanghai China) on an ABI Step One Plus Real-Time PCR system to examine gene transcription. The relative abundances of the transcripts of the indicated genes were normalized to that of the housekeeping gene *GAPDH*, using the ΔΔCT method. All data were obtained from at least three independent experiments.

Bisulfite DNA Sequencing

Genomic DNA was extracted from cells of indicated groups using the Blood/Cell/Tissue Genome Extraction Kit (DP304, TIANGEN, Beijing, China) according to the manufacturer's protocol. Genomic DNA was treated with bisulfite using CpGenome Turbo Bisulfite Modification Kit according to the manufacturer's manual. The modified DNA was amplified using Platinum Taq DNA polymerase with the respective primer sets that recognize bisulfate-modified DNA only (primer sequences listed in Table II). Then, the PCR products were cloned into the pMD-19T vector (3271, TaKaRa, Dalian, China), followed by Sanger sequencing.

Statistical Analysis

Data are presented as the mean ± standard deviation of at least three experiments. Statistical analyses were performed as unpaired *t*-tests using GraphPad Prism (version 5 for Windows, GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered to indicate a statistically significant difference.

Table I. The primers for quantitative expression of multiple genes after quercetin treatment.

ID	Primer name	sequence (3′-5′)
1	Hs-IL15-RT-F1	TGTTCACCCCAGTTGCAAAG
2	Hs-IL15-RT-R1	TTGCATCTCCGGACTCAAGT
3	Hs-GAPDH-RT-F	TGTGGGCATCAATGGATTTGG
4	Hs-GAPDH-RT-R	ACACCATGTATTCCGGGTCAAT
5	HsDNMT1-RT-F	AGGCGGCTCAAAGATTTGGAA
6	HsDNMT1-RT-R	GCAGAAATTCGTGCAAGAGATTC
7	HsDNMT2-RT-F	GCCATTACCCTTTCAAGCCC
8	HsDNMT2-RT-R	CCAGAACACTGTATGCTGCC
9	HsDNMT3A-RT-F	ACGACCAGGAATTTGACCCT
10	HsDNMT3A-RT-R	AATGTAGCGGTCCACCTGAA
11	HsDNMT-3B-RT-F	AGGGAAGACTCGATCCTCGTC
12	HsDNMT-3B-RT-R	GTGTGTAGCTTAGCAGACTGG

Hs, quercetin; RT, reverse transcription; F, 5 ‘ primer; R, 3’ primer.

Table II. The primers for cloning of IL15 promoter after bisulfite treatment.

ID	Primer name	sequence (3′-5′)
1	HsIL15-BSP-F	TTAAAGGGTATTTAGTGGGAATTGA
2	HsIL15-BSP-R	TTCAACCCCCTTACAATTTTAATTA
3	HsIL15-BSP-F1	ATTTTTGTTTTTTATTTTAAAATTT
4	HsIL15-BSP-R1	AACAACCCCAAACTAATCCC

Hs, quercetin; BSP, bisulfite sequencing PCR; F, 5' primer; R, 3'primer.

Results

Quercetin Downregulates IL15 Transcription in HeLa Cells

To investigate the regulation of quercetin on *IL15* expression in cancer cells, HeLa cells were treated with different concentrations of quercetin and their *in vitro* antitumor activity (cytotoxic effects) on HeLa cells was examined. As shown in Figure 1A, the inhibitory effect of quercetin on HeLa cells was dose-dependent in the range of 0-40 μM according to the CCK8 assay, with an IC50 of 22.63 μM. The cell viability rate was already lower than 20% beyond the quercetin concentration of 20 μM, so we selected quercetin at concentrations of 5-30 μM to treat HeLa cells separately and examined the expression of *IL15* in HeLa cells. The transcript levels of *IL15* decreased with quercetin treatment but did not show a dose-dependence pattern (Figure 1B). This proposed that quercetin might indirectly regulate the expression of *IL15*.

Quercetin up-regulates DNMT3A and DNMT3B transcription in HeLa cells

We learned that quercetin regulates gene expression through DNA methylation. To investigate whether the downregulation of *IL15* by quercetin was regulated by DNA methylation, we performed qRT-PCR to detect the transcript levels of two important genes involved in DNA methylation, *DNMT3A* and *DNMT3B*[42-44]. As shown in Figures 2A and 2B, the transcript levels of *DNMT3A* and *DNMT3B* were significantly increased after treatment with different concentrations of quercetin compared to the DMSO control. In conclusion, these results suggest that the downregulation of the *IL15* gene by quercetin is most likely related to the change in DNA methylation levels.

Quercetin Increases IL15 Promoter DNA Methylation Levels in HeLa Cells

To confirm the molecular mechanism of quercetin-induced *IL15* downregulation, bisulfite sequencing PCR (BSP) was used to analyze the DNA

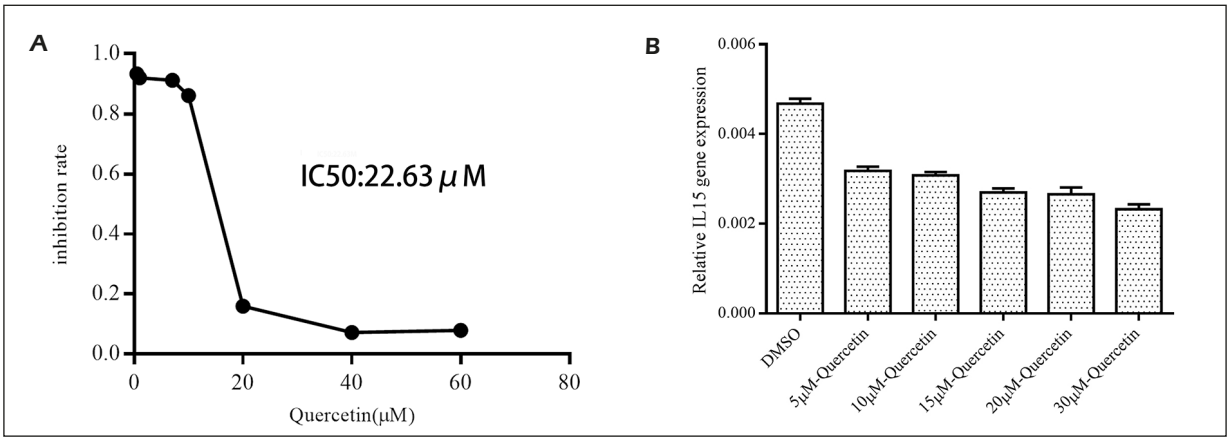

Figure 1. A, HeLa cells were treated with different concentration of quercetin for 1 day, then use CCK8 method to analyze the cytotoxic effect of quercetin on HeLa cell. **B,** HeLa cells were treated with different concentration of quercetin, qRT-PCR was used to analyze the relative transcription level of *IL15* after quercetin treatment for 1 day.

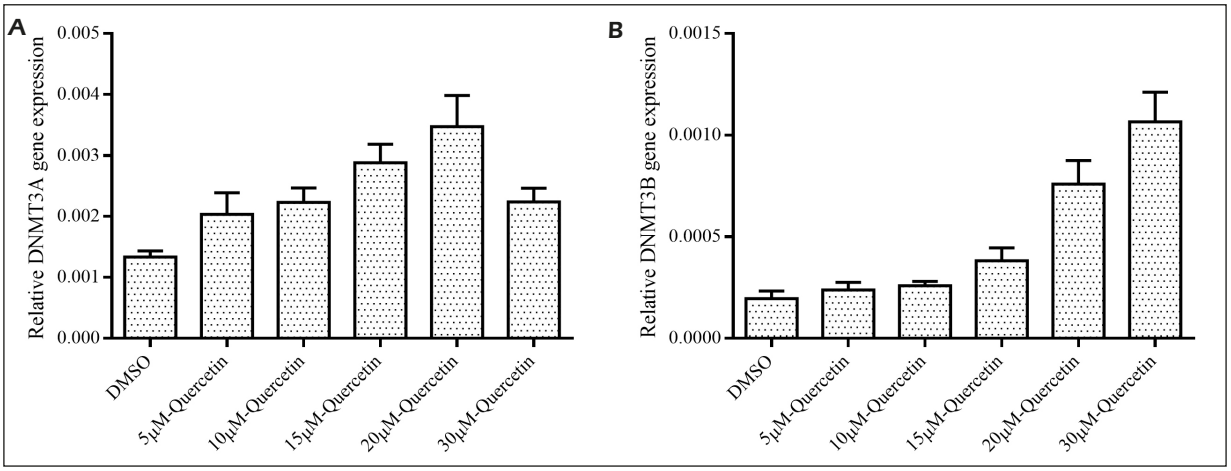

Figure 2. After HeLa cells treated with different concentration of quercetin for 1 day, qRT-PCR was used to analyze the relative transcription level of DNMT3A **(A)** and DNMT3B **(B)**.

methylation level of the *IL15* promoter region[45]. We examined the *IL15* promoter DNA methylation level to confirm that quercetin-induced *IL15* downregulation was caused by DNA methylation. As shown in Figure 3, the DNA methylation level of quercetin-treated cells was higher than DMSO controls (Figure 3A). The DNA methylation level increased from 0.9% to 1.7% post-treatment with quercetin. To confirm these results, we used azacytidine (a DNMT inhibitor) to treat HeLa cells. As shown in Figure 3B, *IL15* was downregulated upon treatment with quercetin, but was upregulated when treated with both quercetin and azacytidine. The transcription levels of *DNMT3A*

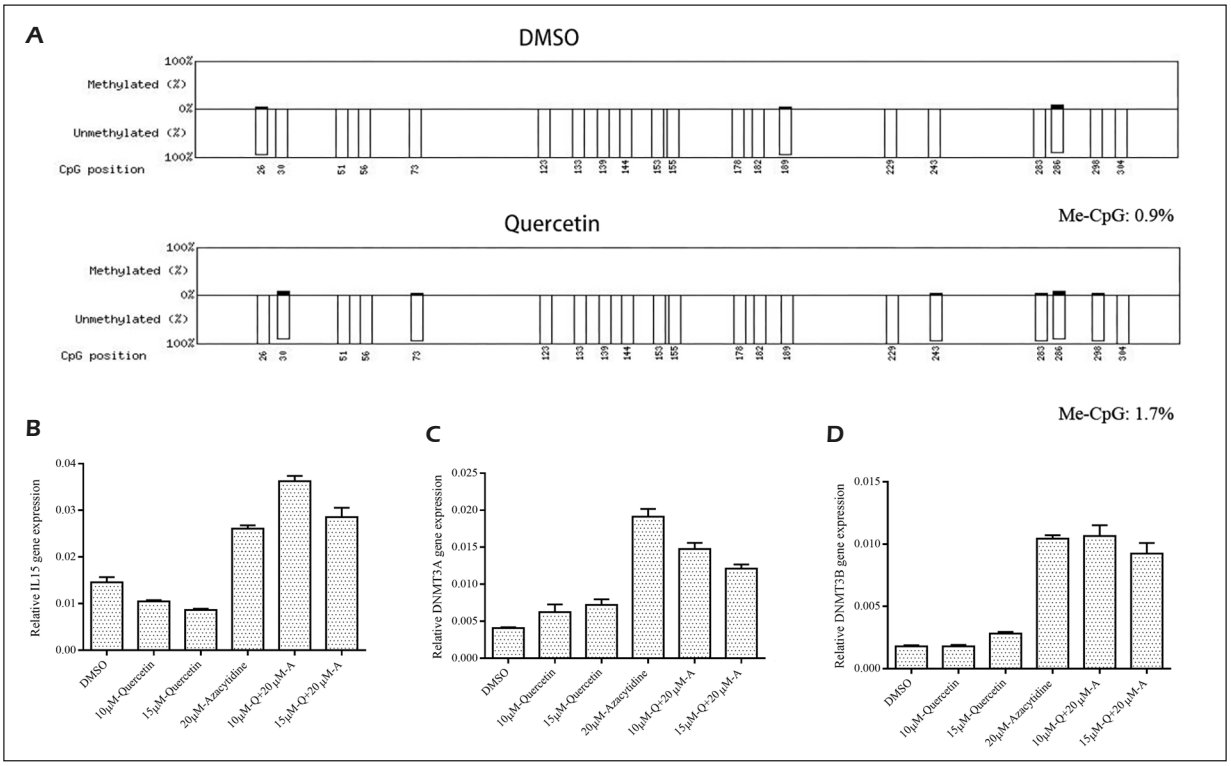

Figure 3. A, Bisulfite DNA sequencing method was used to analysis the *IL15* promoter DNA methylation level, the DMSO treated HeLa cell was as negative control; qRT-PCR was used to assay the transcription level of different genes IL15 **(B)**, DNMT3A **(C)** and DNMT3B **(D)**. 10 μM-Q+20 μM-A means HeLa cells treated with 10 μM quercetin and 20 μM azacitidine; 15 μM-Q+20 μM-A means HeLa cells treated with 150 μM quercetin and 20 μM Azacitidin.

and *DNMT3B* were significantly increased in both the quercetin-treated group and the quercetin and azacytidine combination-treated group. These results confirmed the hypothesis that quercetin downregulated *IL15* through DNA methylation.

Quercetin Downregulates IL15 and Up-Regulates DNMT3A/3B in A549 Cells

To confirm that the quercetin-induced *IL15* downregulation is not a cell line-specific effect, we verified the *IL15* transcription levels after quercetin treatment in the A549 cell line. As shown in Figure 4A, *IL15* was downregulated in A549 cells after treatment with quercetin, which was consistent with the results found in HeLa cells. We also checked the transcription levels of *DNMT1, 2, 3A,* and *3B* after quercetin treatment in A549. Results showed that the *DNMT* transcription level increased significantly after quercetin treatment compared to DMSO control in A549 cells (Figure 4B-E). These results indicate that the quercetin-induced *IL15* downregulation is not a cell line-specific effect.

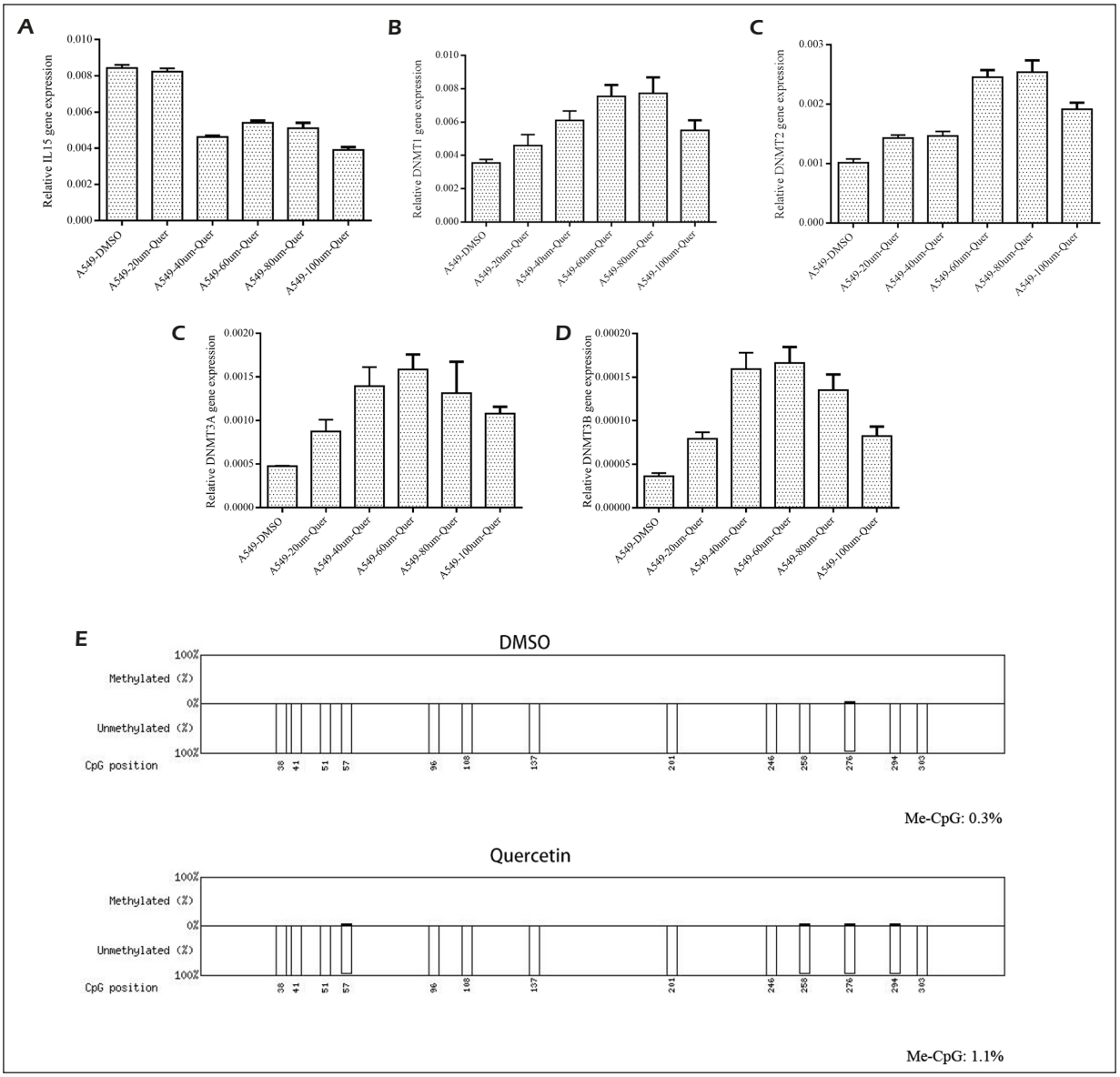

Figure 4. qRT-PCR was used to assay the transcription level of different genes *IL15* **(A)**, DNMT 1 **(B)**, DNMT2 **(C)**, DNMT3A **(D)**, and DNMT3B **(E)** post A549 cells were treated with different concentration of quercetin. **F,** Bisulfite DNA sequencing method was used to analysis the *IL15* promoter DNA methylation level, the DMSO treated A549 cell was as control.

In addition, we examined the DNA methylation level of the *IL15* promoter region in the A549 cell line. As shown in Figure 4D, the DNA methylation level increased after quercetin treatment in A549 cells. This is consistent with the results obtained from HeLa cells. Thus, *IL15* was downregulated in both A549 and HeLa cells through an increase in the DNA methylation level of the *IL15* promoter region.

Discussion

Many studies[46,47] have demonstrated that quercetin has anticancer and antioxidant functions. However, the functions of drugs in the human body are complex and their targets of action and activities can be diverse. Therefore, we need to carefully understand the pharmacological properties of quercetin to better utilize quercetin to treat various diseases. The main conclusion is that the natural product quercetin inhibits the growth of the cancer cell models HeLa and A549 cells, while effectively regulating *IL15* transcription in HeLa and A549 cells, which is achieved by increasing the DNA methylation of the *IL15* promoter region as the quercetin-induced downregulation of *IL15*. Given the wide distribution of quercetin in food and its potential function in the human diet, the diversified effects of quercetin intake on humans will be an ongoing area of research[48,49]. Our results showed that quercetin had an inhibitory effect on the cellular immune response (*via* IL15) while inhibiting the proliferation of cancer cells, which suggested that combination therapy employing quercetin and IL15 targeting drugs for cancer was not feasible.

In addition, based on the results of our *in vitro* experiments, we suggest that quercetin most likely negatively regulates *IL15* expression during the *in vivo* immune response, which provides some theoretical support for the anti-inflammatory activity of quercetin. Abnormal expression of *IL15* promotes a variety of autoimmune diseases in humans[50]. The mechanisms by which *IL15* promotes chronic inflammation and tissue-specific autoimmune diseases have been studied in detail in rheumatoid arthritis, inflammatory bowel disease, vitiligo, and celiac disease[29,51-56]. Therefore, the regulation of *IL15* expression by quercetin is expected to be used for the treatment of autoimmune diseases.

Moreover, the current data support the idea that quercetin plays an important role in the immune regulation of cancer through epigenetic modifications. It has been found that quercetin may exert anti-tumor activity through the demethylation of the *p16INK4a* promoter[57]. Therefore, we suggest that quercetin might somehow target methylation modifications of different functional DNA regions in cells and that this novel mechanism of action has important clinical implications. Many substances that promote methylation modifications, especially nucleoside analogs such as 5-azacytidine or 5-deoxycytidine, are known

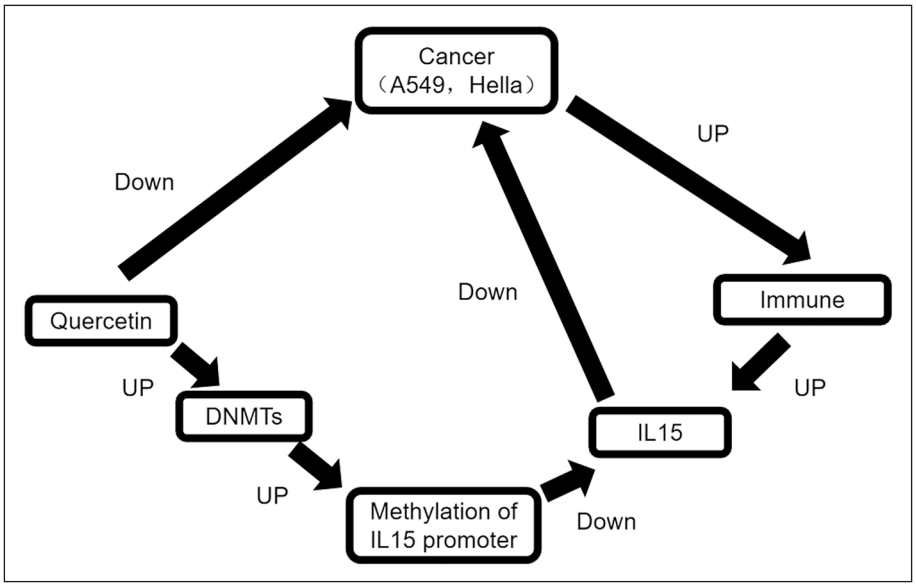

Figure 5. The relationship between quercetin and *IL15* in cancer. Up: Enhanced or up-regulated; Down: Suppress or down-regulated; Immune: immune reaction *IL15*: Interleukin 15; DNMTs: DNA methyltransferases.

to activate the expression of some tumor-specific, methylation-suppressed genes in cancer cells[58]. However, their clinical application is limited due to the cytotoxic and non-specific effects on normal and cancer cells. Therefore, there is a need to develop novel, specific, and less toxic or even non-toxic methylation modifiers in cancer therapy, which requires further investigation to validate the mechanisms of quercetin-mediated changes in the degree of DNA methylation and the selection characteristics of the target genes for methylation modifications.

Combining the results of previous studies[16,21,22,30,59,60] with that of the present study, we were able to propose a schematic network diagram as shown in Figure 5. The development of cancer induces an immune response from the body's immune system, which results in the production of multiple cytokines, including *IL15*, to inhibit the proliferation of cancer cells. Although the proliferation of cancer cells can be inhibited with quercetin, quercetin promotes the expression of methyltransferase in the cells. This increases the methylation of the *IL15* promoter site, ultimately leading to the downregulation of *IL15* expression. The medicinal function of quercetin in humans is complex, and a better understanding of its principles of action will make it more effective in clinical practice.

Conclusions

The present study demonstrated that quercetin could downregulate *IL15* expression in cancer cells by regulating the degree of gene methylation. This result promotes the development of quercetin pharmacology and provides new insights into the treatment of autoimmune diseases. As such, quercetin has the potential to be a new drug to target cancer *via* epigenetic modification. We also need to further understand the epigenetic impact of other genes to provide more information for its clinical use.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

Mengzhou Zhang: Cell culture and treatment with quercetin, CCK8 assays, RNA extractions, DNA extractions, data compilation, and article writing. Anrui Lu: qRT-PCR, Bisulfite DNA sequencing, and the design and guidance of all experiments. Hongxia Wang and Jun Yang: article design and revision. All authors have no objection to the article and the byline.

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Data Availability

The data that support the findings of this study are available from the corresponding authors, H. Wang and J. Yang, upon reasonable request.

Ethics Approval

This study does not involve human or animal experiments, and all experiments are in accordance with the ethics requirements.

Informed Consent

Not applicable.

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