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Journal of Nutritional Biochemistry 21 (2010) 140–146
Journal of Nutritional Biochemistry

Epigallocatechin gallate up-regulation of miR-16 and induction of apoptosis in human cancer cells

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Received 27 May 2008; received in revised form 28 November 2008; accepted 2 December 2008

Abstract

Epigallocatechin gallate (EGCG) is a major type of green tea polyphenols and is known to have cancer prevention effect. MicroRNAs (miRNAs) are 19 to 25 nucleotides and are believed to be important in gene regulation. In the present study, the influence of EGCG on the expressions of miRNAs in human cancer cells was investigated as this has not yet been reported. By miRNA microarray analysis, EGCG treatment was found to modify the expressions of some of the miRNAs in human hepatocellular carcinoma HepG2 cells, 13 were up-regulated and 48 were down-regulated. miR-16 is one of the miRNAs up-regulated by EGCG and one of its target genes is confirmed to be the anti-apoptotic protein Bcl-2. EGCG treatment induced apoptosis and down-regulated Bcl-2 in HepG2 cells. Transfection with anti-miR-16 inhibitor suppressed miR-16 expression and counteracted the EGCG effects on Bcl-2 down-regulation and also induction of apoptosis in cells. Results from the present study confirm the role of miR-16 in mediating the apoptotic effect of EGCG and also support the importance of miRNAs in the regulation of the biological activity of EGCG.
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Keywords: EGCG; Tea polyphenols; miRNA; Bcl-2; Apoptosis

1. Introduction

MicroRNAs (miRNAs) are 19 to 25 nucleotides long and are a new class of small noncoding RNAs found in both animals and plants [1,2]. They are phylogenetically conserved and are important in the regulation of many fundamental cellular processes, such as development, cellular proliferation and cell death [3–5]. Currently, about 723 human miRNAs are annotated in the miRNA registry (miRBase version 11.0), and the total number is predicted to be more than 1000 [6,7]. miRNAs bind to the complementary sequences in the 3′ untranslated region (3′UTR) of the protein coding genes and induce mRNA degradation or translational repression of the target genes [8]. A single miRNA is capable of regulating multiple target genes. Therefore, the miRNAs could regulate the expressions of thousands of the protein-coding genes.

Recent studies have shown that miRNAs not only play a fundamental role in maintaining cellular functions but also are involved in cancer development. By genome-wide studies, miRNA genes are frequently located at genomic regions showing loss of heterozygosity, amplification or breakpoints in cancers [3,5,9]. On the other hand, some miRNAs are down-regulated in human cancers, indicating that miRNAs may also function as tumor suppressors. For instance, let-7, which targets the oncogene Ras, is shown to be down-regulated in lung cancers [10]; miR-122a, which targets cyclin G1, is frequently down-regulated in human hepatocellular carcinoma [11]. Moreover, the expression pattern of miRNAs in cancer cells may also be influenced by cancer therapeutic agents [12]. These prompt us to speculate that a specific or a cluster of miRNAs in cancer cells may also serve as the target for the anticancer agents.

Green tea is a popular beverage and has shown to protect against many cancer types. The tea extract obtained from *Camellia sinensis* leaves and its major component catechin

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doi:10.1016/j.jnutbio.2008.12.003

(−)-epigallocatechin-3-gallate (EGCG) were shown to have antiangiogenic, antiproliferative and apoptotic effects on various tumors [13–20]. To date, catechins from tea extracts are considered as potential chemotherapeutic drugs as well as cancer prevention agents. Extensive investigations have shown that green tea catechins (GTEs) through their influences on apoptotic proteins, cell cycle regulatory proteins, EGFR, Ras/Raf/MAPK, PI3-K/AKt, etc., exert their cancer chemoprevention effects [15,17,19,20]. miRNAs are known to be important in the regulation of many fundamental cellular processes including cancer development. Therefore, miRNA may likely act as a new layer of gene regulation to mediate the cancer prevention effect of GTEs. By miRNA microarray, the present study is the first to demonstrate that EGCG is able to modify the miRNA expression in human cancer cells (human hepatocellular carcinoma HepG2 cells). Furthermore, miR-16, which is one of the miRNAs up-regulated by EGCG treatment, is confirmed to mediate the EGCG induction of apoptosis in HepG2 cells by targeting Bcl-2.

2. Materials and methods

2.1. Cell lines and reagents

The human hepatocellular carcinoma HepG2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in a 37°C humidified incubator with 10% CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 2 mM of L-glutamine (Invitrogen, Carlsbad, CA). EGCG was purchased from Sigma (St. Louis, MO, USA), dissolved in DMSO and stored at −20°C.

2.2. miRNA microarray analysis

HepG2 cells were seeded in 100-mm culture dishes for 24 h and then incubated with 100 μM of EGCG for 24 h. Total RNA was isolated by using the mirVana RNA isolation kit (Ambion, Austin, TX, USA). Fifty micrograms of total RNA was enriched for small RNA species, followed by 3′ amine-tailed modification using the mirVana miRNA labeling kit

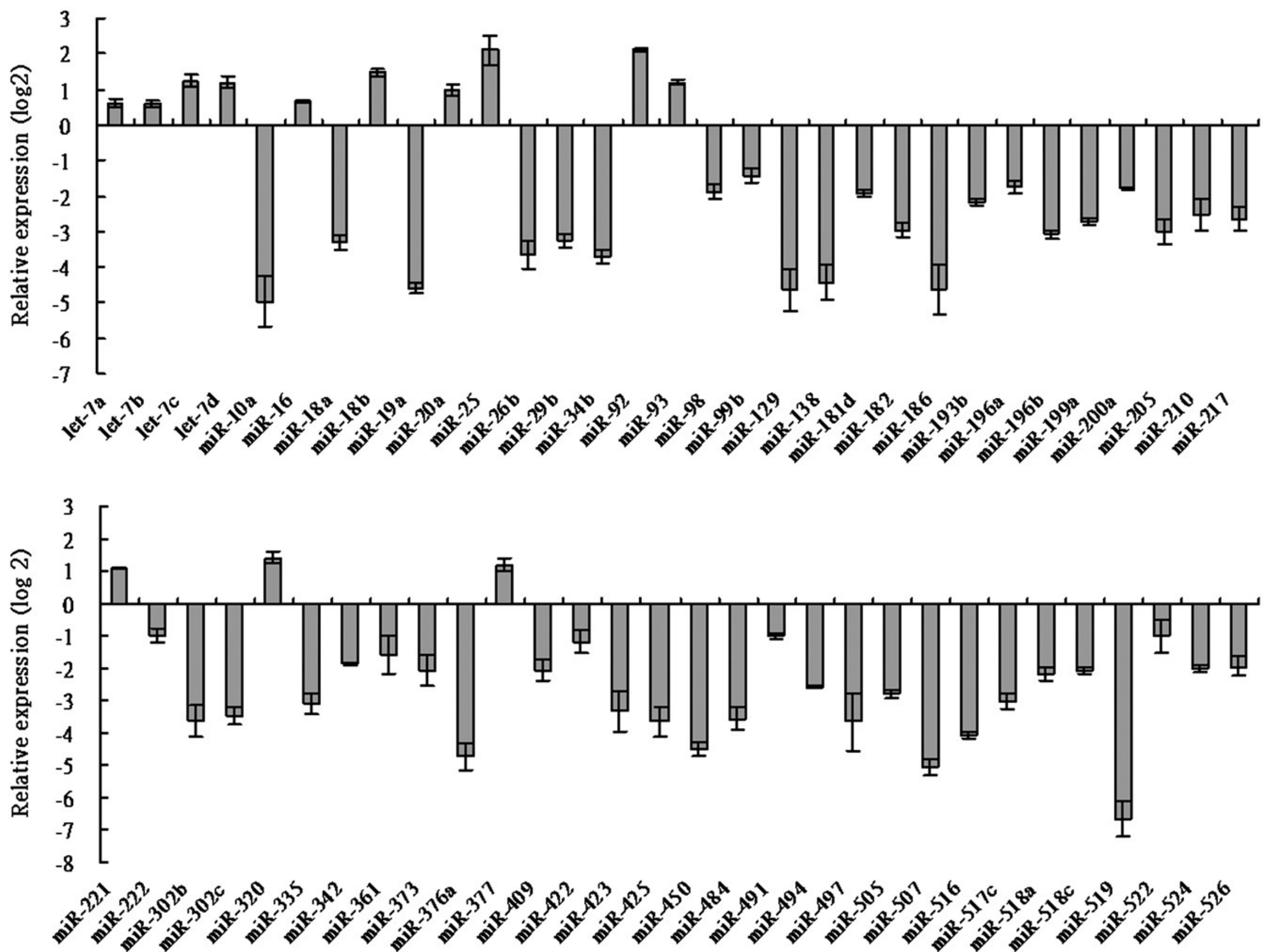

Fig. 1. The expression profile of miRNAs in HepG2 cells upon treatment with EGCG. The cells were treated with 100 μM of EGCG for 24 h. The data are presented in terms of mean±S.E.M. for the fold change in miRNA expression in log 2 scale between EGCG-treated and untreated cells obtained from four separate experiments. The miRNAs with differential expression were identified as instructed by the manufacturer's protocol using a filter based on a fold change of 1.5 plus statistical significance ($P<.05$) as calculated by Student's t test (SPSS 12.0 software).

Table 1
Expression profiling of miRNAs upon EGCG treatment in HepG2 cells

microRNA	Map[a]	Normalized mean ratio EGCG/control[b]	P value[c]	Up-/down-regulation
Let-7a	9q22.2	1.52	.0199	Up
Let-7b	22q13.3	1.50	.0210	Up
Let-7c	21q11.2	2.37	.0345	Up
Let-7d	9q22.2	2.30	.0057	Up
miR-10a	17q21.32	0.03	.0088	Down
miR-16	8q24.22	1.57	.0205	Up
miR-18a	13q31.3	0.10	.0210	Down
miR-18b	Xq26.2	2.81	.0243	Up
miR-19a	13q31.3	0.04	.0007	Down
miR-20a	13q31.3	2.00	.0186	Up
miR-25	7q22.1	4.26	.0499	Up
miR-26b	2q35	0.08	.0050	Down
miR-29b	7q32.3	0.11	.0070	Down
miR-34b	11q23.1	0.08	.0033	Down
miR-92	13q31	4.33	.0100	Up
miR-93	7q22.1	2.28	.0135	Up
miR-98	Xp11.22	0.27	.0050	Down
miR-99b	19q13.4	0.37	.0180	Down
miR-129	7q32.1	0.04	.0056	Down
miR-138	3p21.33	0.05	.0210	Down
miR-181d	19p13.12	0.26	.0050	Down
miR-182	7q32.2	0.13	.0083	Down
miR-186	1p31.1	0.04	.0080	Down
miR-193b	16q13.12	0.22	.0020	Down
miR-196a	17q21.32	0.30	.0330	Down
miR-196b	7p15.2	0.12	.0101	Down
miR-199a	19p13.2	0.15	.0090	Down
miR-200a	1p36.33	0.29	.0025	Down
miR-205	1q32.2	0.12	.0035	Down
miR-210	11p15.5	0.17	.0100	Down
miR-217	2p16.1	0.16	.0059	Down
miR-221	Xp11.3	2.11	.0155	Up
miR-222	Xp11.22	0.50	.0089	Down
miR-302b	4q25	0.08	.0090	Down
miR-302c	4q25	0.09	.0050	Down
miR-320	8p21.3	2.64	.0258	Up
miR-335	7q32.2	0.12	.0051	Down
miR-342	14q32.2	0.27	.0080	Down
miR-361	Xp21.2	0.33	.0049	Down
miR-373	19q13.42	0.24	.0060	Down
miR-376a	14q32.31	0.04	.0160	Down
miR-377	14q32.31	2.29	.0395	Up
miR-409	14q32.31	0.24	.0092	Down
miR-422	15q22.31	0.44	.0180	Down
miR-423	17q11.2	0.10	.0055	Down
miR-425	3p21.31	0.08	.0098	Down
miR-450	Xq26.3	0.05	.0040	Down
miR-484	16p13.11	0.09	.0381	Down
miR-491	9p21.3	0.50	.0201	Down
miR-494	14q32.31	0.17	.0058	Down
miR-497	17p13.1	0.08	.0240	Down
miR-505	Xq27.1	0.14	.0088	Down
miR-507	Xq27.3	0.03	.0050	Down
miR-516	19q13.42	0.06	.0241	Down
miR-517c	19q13.42	0.12	.0050	Down
miR-518a	19q13.42	0.22	.0101	Down
miR-518c	19q13.42	0.24	.0234	Down
miR-519	19q13.42	0.01	.0330	Down
miR-522	19q13.42	0.48	.0052	Down
miR-524	19q13.42	0.25	.0039	Down
miR-526	19q13.42	0.26	.0148	Down

(Ambion) and fluorescent labeling with amine-reactive Cy3 (cells with no treatment) or Cy5 dyes (EGCG-treated cells) (GE Healthcare, Singapore). Thereafter, fluorescent-labeled RNAs were co-hybridized for 14 h at 42°C on mirVana miRNA Bioarray (Ambion). The microarray was washed as recommended by the manufacturer, and the fluorescence signals were scanned by using the GenePix scanner.

2.3. Quantitative real-time PCR for miRNA expression

To verify the miRNA levels obtained from the microarray analysis, total RNA from cells was extracted by Trizol reagent. Three micrograms of total RNA was subjected to RT reaction using the QuantMir RT Kit (System Biosciences, Mountain View, CA, USA). Quantitative real-time PCR was performed by using the SYBR-green PCR Master Mix in Fast Real-time PCR 9700 System (Applied Biosystems, Foster City, CA, USA). In each miRNA detection, the mature miRNA DNA sequence was used as the forward primer and the 3′ universal primer provided with the QuantiMir RT Kit was used as the reverse primer. The reactions were performed in a 96-well optical plate (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 50°C for 1 min. ΔC_t was calculated by subtracting the C_t of *U6* small nuclear RNA from the C_t of the miRNA of interest. $\Delta\Delta C_t$ was then calculated by subtracting the ΔC_t of the untreated control from the ΔC_t of the EGCG-treated sample. Fold change of miRNA in log 2 scale was calculated by the equation $2^{-\Delta\Delta C_t}$.

2.4. Western blot analysis

HepG2 cells were lyzed in Lammeli's lysis buffer containing 1% Triton X-100 and scraped by a cell lifter. Thirty micrograms of protein was resolved in 12% SDS-PAGE minigel and immunoblotted to Immobilon-P membrane (Millipore, Billerica, MA, USA). Membranes were probed with primary antibody against Bcl-2 (Oncogene, Boston, MA, USA) at room temperature for 2 h, washed extensively with 0.1% Tween-20 in PBS and incubated with secondary antibody conjugated with horse-radish peroxidase at 1:10,000 dilution. The signals were visualized with enhanced chemiluminescence (Amersham Life Science, Inc., Buckinghamshire, UK).

2.5. Luciferase reporter assay

A 3′UTR segment of Bcl-2 mRNA containing the intact miR-16 recognition sequence was amplified and subcloned into *Nhe*I and *Hin*dIII sites of pGL3 vector immediately

Notes to Table 1

[a] Chromosome localization of microRNAs as referred in miRBase Sequences (http://microrna.sanger.ac.uk).

[b] The mean value was obtained from four sets of microRNA arrayed on the slide. The mean ratio of miRNA expression after EGCG treatment with the respective untreated control was normalized with the background fluorescent intensity and also the positive control miRNAs on the arrayed slide.

[c] *P* value was calculated by SPSS 12.0 software.

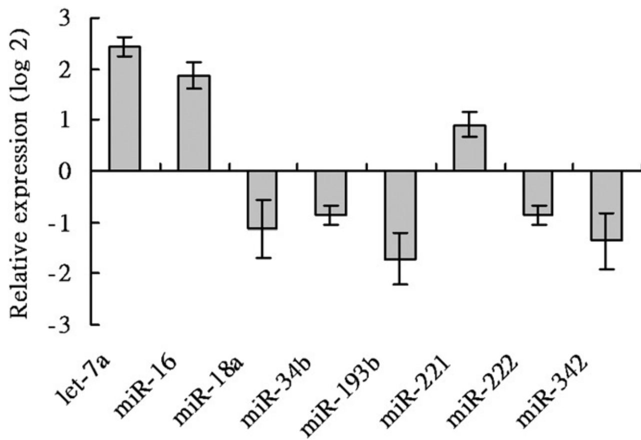

Fig. 2. Validation of miRNAs expression by quantitative real-time PCR as described in Materials and Methods. Data are expressed as mean±S.D. of three independent experiments. Fold change of miRNA expression was presented in log 2 scale.

downstream of the luciferase gene to form the pGL3-bcl2 construct. The primer sequences for PCR amplification were described elsewhere [21]. HepG2 cells were seeded in 24-well plates for 24 h and then co-transfected with 600 ng of pGL3-bcl2 construct with or without 50 nM of miR-16 precursor or anti-miR-16 inhibitor for 24 h. To monitor the transfection efficiency, the samples were also co-transfected with 0.05 μg pRL-CMV plasmid that expressed Renilla luciferase (Promega, Madison, WI, USA). At 24 h posttransfection, the activity of firefly luciferase was measured by using dual-luciferase reporter assay system as described by the manufacturer (Promega). Relative luciferase activity of the samples was normalized with Renilla luciferase activity and then compared with that transfected with the pGL3 control.

2.6. miR-16 precursor and inhibitor transfection

miR-16 expression in HepG2 cells was up-regulated or knocked down by transfection with miR-16 precursor or anti-miR-16 inhibitor, respectively (Ambion). Cells were plated in 60-mm culture dishes for 24 h and then transfected with 25 nM of the respective precursor or inhibitor with lipofectamine 2000 (Invitrogen) for 24 h. Commercially available precursor/inhibitor control (Ambion) was transfected in parallel. Thereafter, cells were subjected to further drug treatment or RNA/protein extraction.

2.7. DNA Fragmentation assay

HepG2 cells were transfected with miR-16 precursor or anti-miR-16 inhibitor for 24 h. Thereafter, the cells were treated with EGCG for 72 h and lysed in lysis buffer (5 mM Tris–HCl, 100 mM EDTA, 1% SDS, 0.4 μg/ml proteinase K) for 1 h at 45°C. Genomic DNA was isolated by phenol/chloroform extraction and then precipitated with absolute ethanol. RNA was removed by incubation of the DNA pellet in Tris-EDTA buffer (pH 8.0) with 0.4 μg/ml of RNase A overnight. Thirty micrograms of DNA was run in 1.5% agarose gel.

3. Results

3.1. EGCG on miRNA expressions in HepG2 cells

EGCG suppressed the growth of HepG2 cells and the suppression is dose dependent; the % inhibition (by MTT assay) at 50, 75 and 100 μM of EGCG for 72 h was 45%, 70% and 80%, respectively. Similarly, EGCG also induced apoptosis in HepG2 cells in a dose-dependent manner (data not shown). To examine whether miRNAs could be involved in the response of cells to the treatment with EGCG, HepG2 cells were incubated with 100 μM of EGCG for 24 h and total RNA was then extracted for miRNA microarray analysis. miRNAs with differential expression between the EGCG-treated cells and control cells were identified by using a filter based on a fold change of 1.5 plus statistical significance ($P<.05$) as calculated by Student's t test [11]. The results are shown in Fig. 1 and summarized in Table 1. Among the 328 human miRNAs in the microarray, 13 miRNAs were

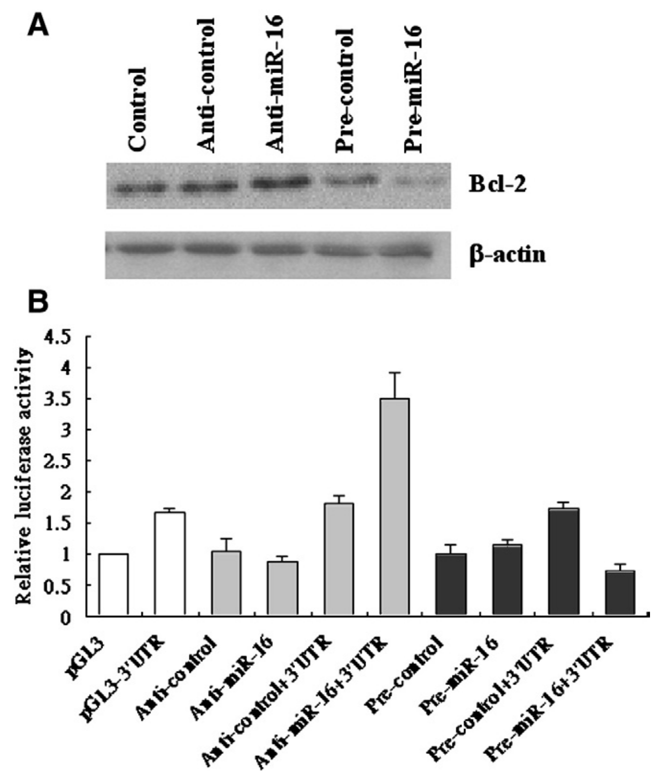

Fig. 3. miR-16 targets Bcl-2 in HepG2 cells. (A) The effect of miR-16 transfection on Bcl-2 expression as assessed by Western blot analysis. β-Actin was used as a loading control. Experiments were repeated at least three times with similar results. The one shown is the representative one. (B) The cells were seeded and then co-transfected with pGL3-bcl2 reporter construct and miR-16 precursor or anti-miR-16 inhibitor for 24 h. Relative luciferase activity was compared with those transfected with the pGL3 vector. Data are expressed as mean±S.D. of three independent experiments.

up-regulated and 48 were down-regulated in cells with EGCG treatment. To further validate the results obtained from the microarray study, eight of the differentially expressed miRNAs (up-regulated: let-7a, miR-16 and miR-221; down-regulated: miR-18a, miR-34b, miR-193b, miR-222 and miR-342) were assayed by quantitative real-time PCR (Fig. 2). The relative expressions of these miRNAs between the EGCG-treated cells and control cells were found to be comparable with those indicated from the microarray analysis.

3.2. miR-16 mediated EGCG induction of apoptosis

miRNAs are believed to serve as an additional layer of gene regulation in cells. The expressions of some of the miRNAs were altered by EGCG treatment in HepG2 cells, and these miRNAs are therefore believed to be involved in the regulation of the biological activity of EGCG. One of the miRNAs up-regulated by EGCG in HepG2 cells is miR-16 (Figs. 1 and 2), and the miRNA is known to target the anti-apoptotic protein Bcl-2 [21]. It is therefore hypothesized that EGCG may induce apoptosis in HepG2 cells through silencing of Bcl-2 by up-regulation of miR-16. By Western blot analysis, transfection with miR-16 precursor was found to suppress, while transfection with anti-miR-16 inhibitor increased the Bcl-2 expression in HepG2 cells. Furthermore, transfection with anti-miR-16 inhibitor increased, while transfection with miR-16 precursor suppressed the luciferase reporter activity of pGL3-bcl2 construct in cells (Fig. 3). Both results support that Bcl-2 is the target of miR-16 in HepG2 cells. EGCG suppressed the Bcl-2 level in HepG2 cells, while transfection with anti-miR-16 inhibitor counteracted the EGCG suppression on Bcl-2 (Fig. 4A and B). At the same time, by DNA fragmentation assay, EGCG induced apoptosis in HepG2 cells, while anti-miR-16 inhibitor reduced the induction (Fig. 4C). On the other hand, transfection with miR-16 precursor further increased the EGCG suppression on Bcl-2 and also enhanced the EGCG

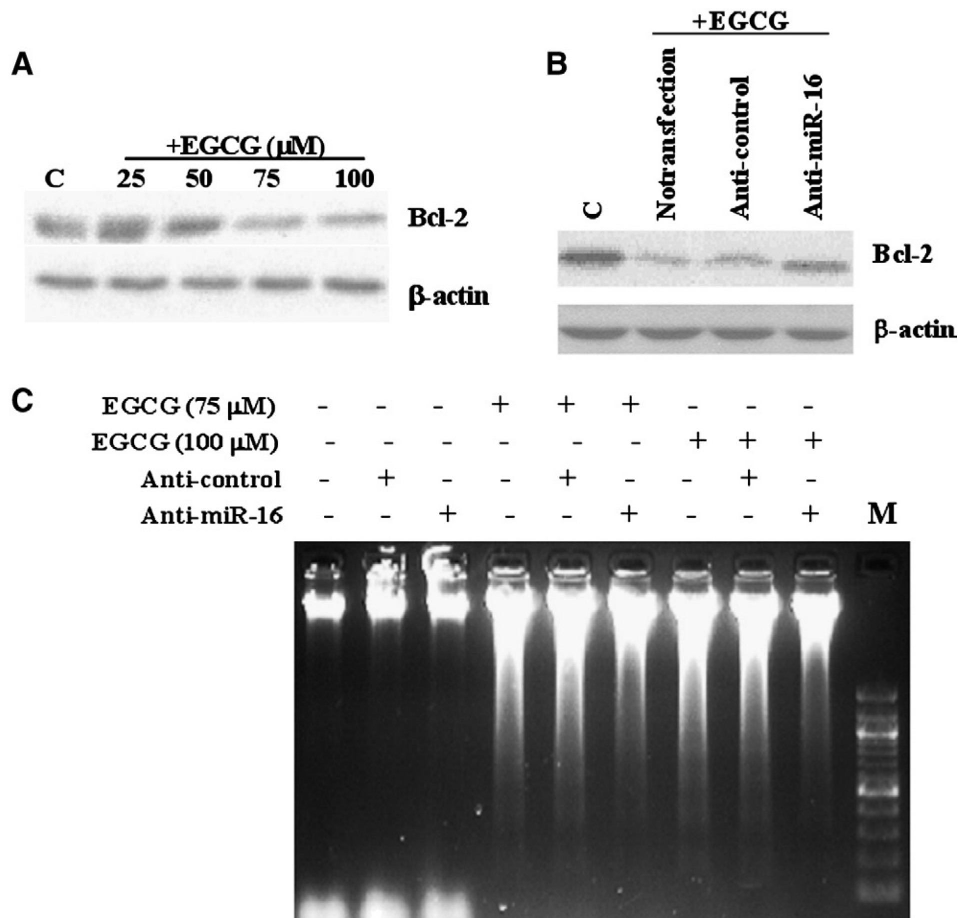

Fig. 4. miR-16 modulates EGCG effect on Bcl-2 expression and apoptosis in HepG2 cells. (A) Effect of EGCG treatment on Bcl-2 expression as detected by Western blot analysis. The cells were treated with various concentrations of EGCG for 48 h. β-Actin was used as a loading control. (B) miR-16 effect on Bcl-2 expression. HepG2 cells were transfected with anti-miR-16 inhibitor for 24 h and then with EGCG treatment for 48 h. Thereafter, the cells were lyzed for protein extraction. (C) miR-16 regulates EGCG-induced apoptosis in HepG2 cells. The cells were transfected with anti-miR-16 inhibitor for 24 h, followed by the exposure to 75 or 100 μM EGCG for 48 h. Genomic DNA was lyzed for DNA fragmentation assay. M: 100 bp. DNA marker. Experiments were repeated at least three times with similar results. The one shown is the representative one.

induction of apoptosis (data not shown). The data therefore support that miR-16 by targeting Bcl-2 may mediate the EGCG effect on apoptosis induction.

4. Discussion

The expression of a specific spectrum of microRNAs is altered in HepG2 cells upon treatment with EGCG as revealed by microarray-based miRNAs expression profiling analysis. Polyphenols such as EGCG from green tea have anticancer and antioxidant properties through which the polyphenols may exhibit antiangiogenic, antiproliferative and apoptotic effects on cancer cells [13–20]. miRNA is known to be important in the regulation of many cellular events including cancer development [3–5,9,10,22,23]. Modification of miRNAs expression by EGCG may indicate the possible role of miRNA in the regulation of cancer prevention effect of EGCG. Among the miRNAs that are induced by EGCG, some of their target genes are known to have oncogenic activity, e.g., members in the let-7 family are known to suppress Ras [10], miR-16 for Bcl-2 [21], miR-20a for E2F and TGFBR2 [9,24], and miR-221 for c-Kit [25]. Furthermore, the inhibitor of miR-18b repressed cardiomyocyte hypertrophy, although the target is not yet identified [26]. Among those miRNAs down-regulated by EGCG, their target genes, however, show diversified function: miR-196 and miR-10a target some of the HOX family proteins [27,28]; miRNA-19a for PTEN [29]; miRNA-26 for SMAD [29]; miR-29b for MCL1 [30]; and miR-376a for SLC16A1, TTK, PRPS1, ZNF513 and SNX19 [31]. Results from the miRNA profiling suggest that EGCG may exert their biological functions through up- or down-regulation of multiple miRNAs.

miR-16 may mediate the EGCG induction of apoptosis by targeting Bcl-2. EGCG was shown to induce apoptosis and also suppress Bcl-2 in different cell lines [19,20,32,33]. In addition to apoptosis induction, treatment of HepG2 cells with EGCG induced miR-16 and also down-regulated the expression of its target gene Bcl-2. By the use of miR-16 precursor and inhibitor, it is indicated that EGCG may act through the miR-16–Bcl-2 pathway to regulate apoptosis in HepG2 cells. The results therefore support that miRNA may have a critical role in the regulation of the biological activity of EGCG.

In conclusion, the present study illustrates, for the first time, that polyphenol EGCG exerts their biological functions by modulating the miRNA expression profile in human cancer cells. Further study to investigate the interaction between EGCG and miRNAs may provide new perspectives on understanding the chemoprevention effect of EGCG.

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