Quercetin induces the apoptosis of human ovarian carcinoma cells by upregulating the expression of microRNA-145

JUNBO ZHOU[1], JIAN GONG[1], CHUN DING[2] and GUIQIN CHEN[3]

[1]College of Life Sciences, Huzhou Teachers College, Huzhou, Zhejiang 313000;
[2]College of Fisheries, Henan Normal University, Xinxiang, Henan 453007; [3]Department of Gynaecology, The Medical Group of Zhengzhou First People's Hospital, Zhengzhou, Henan 450004, P.R. China

Received April 8, 2014; Accepted January 30, 2015

DOI: 10.3892/mmr.2015.3679

Abstract. Ovarian cancer is one of the most malignant types of cancer of the female human reproductive track, posing a severe threat to the health of the female population. Numerous previous studies have demonstrated that microRNA (miR)-145 is downregulated in ovarian cancer, and that quercetin can inhibit the growth of cancer cells via regulating the expression of miRs. Therefore, the present study investigated the effect of quercetin on the expression of miR-145 in SKOV-3 and A2780 human ovarian cancer cell lines. The results revealed that the expression levels of cleaved caspase-3 in the SKOV-3 and A2780 cells were significantly increased following treatment to induce overexpression of miR-145 compared with treatment with quercetin alone ($P<0.01$). However, the expression of cleaved caspase-3 in the anti-miR-145 (miR-145 inhibitor) group of cells was markedly decreased compared with that in the miR-145 overexpression group ($P<0.01$). Taken together, the results suggested that treatment with quercetin induced the apoptosis of human ovarian carcinoma cells through activation of the extrinsic death receptor mediated and intrinsic mitochondrial apoptotic pathways.

Introduction

Ovarian cancer, also termed epithelial ovarian cancer, is the sixth most common type of cancer affecting female individuals worldwide, with a mortality rate of ~125,000 annually (1). Ovarian cancer occurs as four major histological subtypes, serous, mucinous, endometrioid and clear cell, with serous being the most common (2). According to previous studies, 5-year survival is observed in only 30% of patients with advanced-stage ovarian cancer, however, only 19% of all cases of ovarian cancer are diagnosed at an early stage (2,3), therefore, additional therapeutics strategies for ovarian cancer are required. Quercetin is one of the most abundant flavonoids in plants, fruits and vegetables, and possess several pharmacological properties that are closely associated with those of existing therapeutic agents, including cardioprotective, antiviral, anti-inflammatory and anti-aging properties, and capacities to extend lifespan (4). Previous studies have demonstrated that quercetin not only induces tumor cell apoptosis, but also acts as a chemosensitizer in anticancer therapy via a different cell signaling pathway (5-7). Furthermore, quercetin can inhibit the growth of OVCAR-3 human ovarian cancer cells, associated with expression of vascular endothelial growth factor (VEGF) (8). However, no data is available regarding the antitumoral activity of quercetin on the SKOV-3 and A2780 human ovarian cancer cell lines.

MicroRNAs (miRNAs/miR) are a novel class of endogenous, non-coding RNAs involved in post-transcriptional gene regulation by binding to a target site in the 3'-untranslated region of target mRNAs (9,10). miRNAs can function as regulatory molecules, which act as tumor suppressors or oncogenes and are involved in the development of human cancer (11,12). miR-145 is located on chromosome 5q32-33 within a 4.09 kb region (13). miR-145 has frequently been reported to be downregulated in certain types of cancer, including bladder (13) and colon cancer, and acts as a tumor suppressive miRNA, which inhibits the growth, invasion and migration of cancer cells (14). In a previous study, miR-145 was downregulated in ovarian cancer cells compared with normal groups of cells (15,16). Notably, the overexpression of miR-145 has been observed to suppress MCF-7 cell growth and induce apoptosis *in vitro* (17). Another study suggested that dietary quercetin supplementation can increase the concentrations of hepatic miR-122 and miR-125b, which contribute to the gene-regulatory activity of quercetin *in vivo*, suggesting that quercetin regulates the expression of miRNAs (18). However, the exact mechanisms underlying the action of miR-145 in ovarian cancer remain to be elucidated and require further examination. The aim of the present study was to clarify the role of miR-145 in human ovarian cancer and elucidate whether quercetin induces the apoptosis of human ovarian cancer cell lines (SKOV-3 and A2780) via miR-145.

Correspondence to: Dr Guiqin Chen, Department of Gynaecology, The Medical Group of Zhengzhou First People's Hospital, 1 East Road of Jian She, Zhengzhou, Henan 450004, P.R. China
E-mail: gqchenguiqin@163.com

Key words: quercetin, microRNA-145, ovarian carcinoma, caspase-3

Materials and methods

Cell culture. The SKOV-3 and A2780 human ovarian cancer cell lines were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Cell Resource Center (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) with 10% fetal bovine growth serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin (BD Pharmingen, San Jose, CA, USA). The cells were maintained at 37˚C in humidified conditions containing 5% CO_2.

MTT assay. The cell viability was determined using an MTT assay. Briefly, the cells were seeded in 96-well plates at $1x10^4$ cells/well and treated without (control) or with different concentrations of quercetin (25, 50 or 100 μm/ml; Sigma-Aldrich, St. Louis, MO, USA) for 12, 24, 48 or 72 h at 37˚C. Subsequently, 10 ml MTT (Sigma-Aldrich) was added to each well, followed by incubation for 4 h at 37˚C. The medium was then removed and 150 ml dimethyl sulfoxide (Sigma-Aldrich) was added to solubilise the formazan produced. The optical densities of the cells were read at 490 nm using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

miRNA-145 extraction and purification. For extraction and purification, $5x10^6$ cells were treated without (control) or with quercetin for 24 h at 37˚C. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine the expression levels of miR-145 in the cells with a Roche Lightcycler 480 (Roche Diagnostics, Mannheim, Germany). Initially, total RNAs were extracted from the cultured cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The RT-qPCR analysis of the levels of miR-145 was performed using a TaqMan Reverse Transcription kit and TaqMan microRNA Assay kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturers' instructions. Primers were purchased from Invitrogen Life Technologies and the sequences were as follows: miR145 forward, 5'-ACA CTCCAGCTGGGCAGGTCAAAAGGGTCC-3' and reverse, 5'-TGTGAGGTCGACCCGTCCAGTTTTCCCAGG-3'. and U6 forward, 5'-TGCGGGTGCTCGCTTCGGCAGC-3', which could ensure the specificity of the PCR products and reverse 5'-GGTGTCGTGGAGTCG-3', which was universal. The PCR cycling conditions were set as follows: Initial denaturation at 95˚C for 5 min, followed by 30 cycles at 94˚C for 0.5 min, 56˚C for 1 min and 72˚C for 0.5 min, and a final extension at 72˚C for 10 min. The expression levels of miR-145 were calculated by calculating the threshold cycle (Ct) values and using the $2^{-\Delta\Delta Ct}$ method, with U6 as an internal control (14-16).

Transfection of anti-miR-145. The human ovarian cancer cell lines SKOV-3 and A2780 were transfected with 100 nmol/l of the miR inhibitor, anti-miR-199a (Ambion, Austin, TX, USA). At 24 h after transfection, the cells were treated with or without quercetin.

Western blotting. Following harvesting, the cells were washed in phosphate-buffered saline (PBS) and lysed in 1X sodium dodecylsulfate (SDS) loading buffer (Sigma-Aldrich). The lysates were then clarified via centrifugation at 15,000 x g for 10 min at 4˚C and collected. The protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent quantities of protein (20-50 μg) were separated using SDS-PAGE. Briefly, the proteins were resolved using 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with PBS containing 0.05% Tween 20 (Abcam, Cambridge, MA, USA) and 5% non-fat dry milk to block non-specific binding. The membranes were then incubated with the appropriate rabbit monoclonal antibodies against cleaved caspase-3 (cat. no. 9661) and β-actin (cat. no. 8457) (1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. Subsequently, the blots were washed with iced PBS and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) at 4˚C for 2 h, washed again, and immunoreactivity was detected by chemiluminescence. For all immunoblots, β-actin immunoreactivity was used as a loading control. All antibodies were used at a dilution of 1:2,000. Western blot analyses were performed in three independent experiments. The blots were then exposed to radiographic film (China Lucky Film Corp., Baoding, China) to visualize the immunoreactive signals. Signals were quantified using Multi Gauge Image Analysis version 3.0 software (FujiFilm, Tokyo, Japan).

Statistical analysis. The results are expressed as the mean ± standard error of the mean. Statistical analysis was performed using commercially available SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Comparisons among groups were performed using a Student's t-test, as appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibitory effect of quercetin on SKOV-3 and A2780 cell growth. Following treatment with quercetin at different concentrations, the growth of the SKOV-3 and A2780 cells were inhibited in a dose- and time-dependent manner, as determined by the MTT assay (Fig. 1A and B). Furthermore, the half maximal inhibitory concentration (IC_{50}) value of the two cell lines at 48 h was 50 μm/ml.

Quercetin stimulates the expression of miR-145. To determine whether quercetin induces the expression of miR-145 in the SKOV-3 and A2780 cells, RT-qPCR was performed. Following quercetin treatment at different concentrations, the expression levels of miR-145 in the SKOV-3 and A2780 cells increased in a dose-dependent manner compared with the untreated control group, at 24 h (Fig. 2). Therefore, this change suggested that miR-145 may be important in the inhibitory effect of quercetin on SKOV-3 and A2780 cell growth.

Anti-miR-145 reverses the effect of quercetin. To confirm the role of miR-145 in the growth inhibition of SKOV-3 and A2780 human ovarian cancer cell lines by quercetin, miR-145 was either overexpressed or the cells were transfected with anti-miR-145 to inhibit its expression. As shown in Fig. 3A

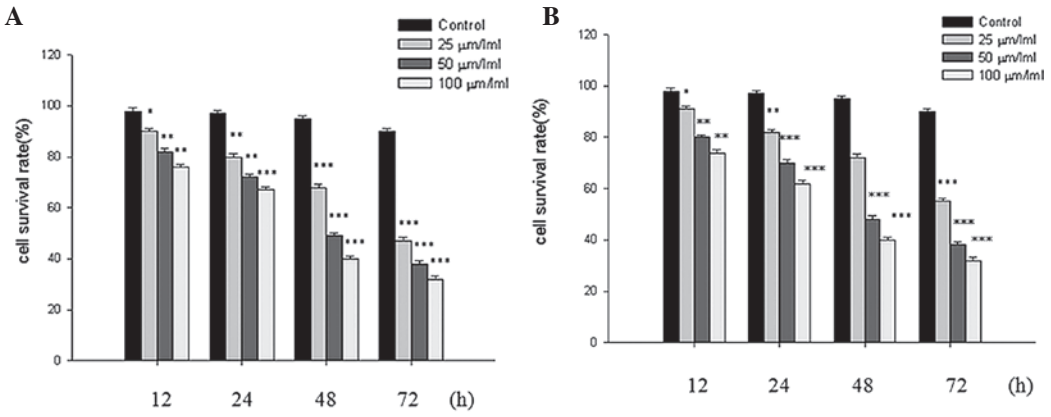

Figure 1. Inhibitory effect of quercetin on the growth of the SKOV-3 and A2780 human ovarian cancer cell lines in a dose- and time-dependent manner. (A) Inhibitory effect of quercetin on the growth of SKOV-3 cells. (B) Inhibitory effect of quercetin on the growth of A2780 cells. *P<0.05, **P<0.01 and ***P<0.001, compared with the respective control groups (0 μm/ml).

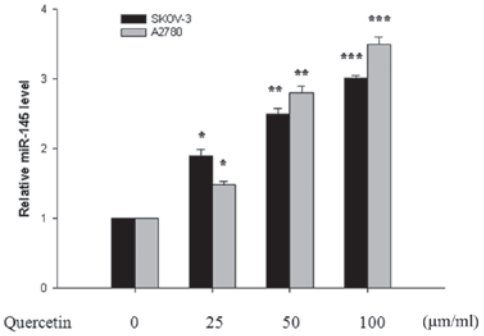

Figure 2. Quercetin stimulates the expression of miR-145 in the SKOV-3 and A2780 human ovarian cancer cell lines. *P<0.05, **P<0.01 and ***P<0.001, compared with the respective control groups (0 μm/ml). miR, microRNA.

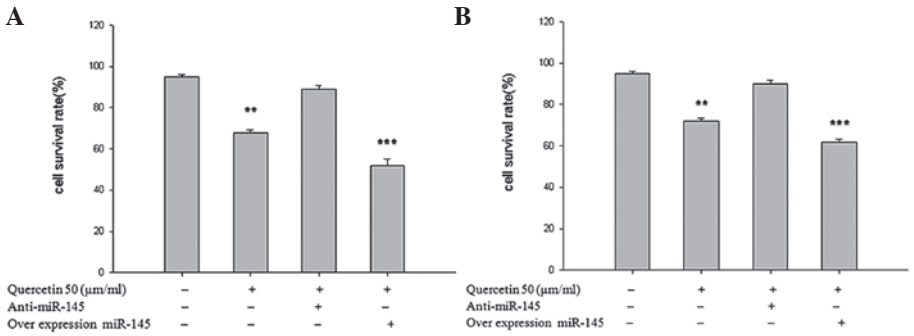

Figure 3. Anti-miR-145 reverses the effect of quercetin in the SKOV-3 and A2780 human ovarian cancer cell lines. (A) Anti-miR-145 reversed the effect of quercetin in SKOV-3 cells. (B) Anti-miR-145 reversed the effect of quercetin in A2780 cells. *P<0.05, **P<0.01 and ***P<0.001, compared with the respective control groups. miR, microRNA.

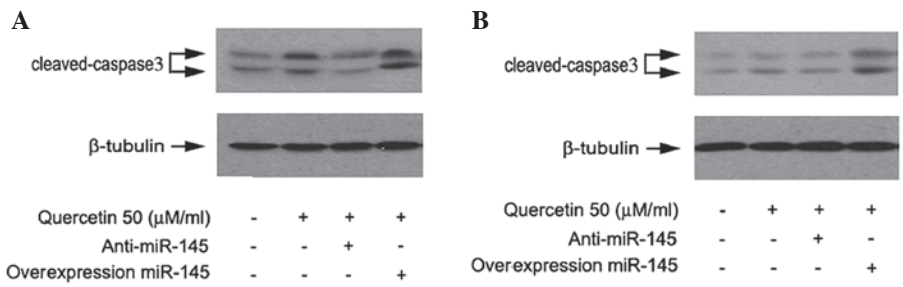

Figure 4. Expression of caspase-3 via the upregulatiion of the expression of miR-145 by quercetin. (A) Expression of cleaved caspase-3 in the SKOV-3 cells. (B) Expression of cleaved caspase-3 in the A2780 cells. miR, microRNA.

and B, the overexpression of miR-145 reduced SKOV-3 and A2780 proliferation at 24 h compared with the untreated cells and the cells treated with anti-miR-145, demonstrating that anti-miR-145 entered the SKOV-3 and A2780 cells and knocked down miR-145.

Quercetin stimulates the expression of caspase-3 via upregulating the expression of miR-145. To confirm the mechanism underlying the upregulated expression of miR-145 by quercetin on the growth of the SKOV-3 and A2780 human ovarian cancer cell lines, the expression of cleaved caspase-3, a cell apoptosis marker, was examined. This was assessed in the SKOV-3 and A2780 human ovarian cancer cell lines, with or without the overexpression of miR-145 or following transfection with anti-miR-145 and with 50 μm/ml quercetin for 24 h. As shown in Fig 4A and B, the expression levels of cleaved caspase-3 in the SKOV-3 and A2780 cells were significantly increased when miR-145 was overexpressed compared with those treated with quercitin alone ($P<0.01$). However, the expression of cleaved caspase-3 in the anti-miR-145 group was markedly decreased compared with the overexpressed miR-145 group ($P<0.01$). Therefore, these results suggested that the extrinsic death receptor-mediated and intrinsic mitochondrial pathways were involved in quercetin-induced apoptosis.

Discussion

The present study is the first, to the best of our knowledge, to demonstrate that quercetin induced apoptosis in the SKOV-3 and A2780 human ovarian carcinoma cell lines, via upregulation of the expression of miR-145, which significantly induced the activity of caspase-8 and -9, and the expression of cleaved caspase-3. These results suggested that quercetin, a common constituent in food, may be used as an antitumoral therapy in human ovarian carcinoma by inducing apoptosis.

Quercetin is present in certain fruits and vegetables and has been observed to possess antitumoral properties in cancer, including lung cancer (6), leukemia (19) and breast cancer (20). Furthermore, quercetin inhibits the growth of OVCAR-3 human ovarian cancer cells, associated with the expression of VEGF (8). In the present study, quercetin was administered at different doses resulting in the inhibition of SKOV-3 and A2780 cell growth in a dose- and time-dependent manner (Fig. 1A and B), and the IC_{50} value of the two cell lines at 48 h was 50 μm/ml. These results suggested that quercetin may inhibit the growth of SKOV-3 and A2780 cells.

miRNAs may function as regulatory molecules, which act as tumor suppressors or oncogenes and are involved in the development of human cancer (11,12). Several studies have reported that the expression of miR-145 is downregulated in human ovarian cancer (15,16). By contrast, dietary quercetin supplementation may increase the hepatic expression levels of miR-122 and miR-125b, which contribute to the gene-regulatory activity of quercetin *in vivo,* and suggest that quercetin regulates the expression of miRNA (18). The exact mechanisms underlying the effect of miR-145 in ovarian cancer have not been previously reported and, therefore required further examination. The present study demonstrated that quercetin increased the expression levels of miR-145 in the SKOV-3 and A2780 cells in a dose- and time-dependent manner compared with the control group at 24 h (Fig. 2), and these changes were reversed by anti-miR-145 (Fig. 3A and B). Furthermore, the expression of cleaved caspase-3, a cell apoptosis marker, which induces cell death (21,22) was examined. Following overexpression of miR-145 or transfection with anti-miR-145 prior to treatment with 50 μm/ml quercetin for 24 h, the expression levels of cleaved caspase-3 in the SKOV-3 and A2780 cells were significantly increased when treated with overexpressed miR-145 compared with quercetin treatment alone ($P<0.01$). However, the expression of cleaved caspase-3 in the anti-miR-145 group was markedly decreased compared with the overexpressed miR-145 group ($P<0.01$). Therefore, these results suggested the involvement of the extrinsic death receptor-mediated and intrinsic mitochondrial pathways in quercetin-induced apoptosis. In conclusion, the present study indicated that miR-145 may be important in quercetin-induced apoptosis in ovarian cancer cells. However, the role of quercetin in the modulation of miR-145 requires further clarification.

Acknowledgements

The present study was supported by grants from the Scientific Research Fund of Zhejiang Provincial Education Department (grant no. Y201224795).

References

1. Maier-Lenz H, Hauns B, Haering B, *et al:* Phase I study of paclitaxel administered as a 1-hour infusion: toxicity and pharmacokinetics. Semin Oncol 24: 16-19, 1997.
2. Cannistra SA: Cancer of the ovary. N Engl J Med 351: 2519-2529, 2004.
3. Greenlee RT, Hill-Harmon MB, Murray T and Thun M: Cancer statistics. CA Cancer J Clin 51: 15-36, 2001.
4. Ramos C, Coin-de-Carvalho JE and Corrêa-Mangiacavalli MA: Impact and (im)mobilization: a study of cancer prevention campaigns. Cien Saude Colet 12: 1387-1396, 2007 (In Portuguese).
5. Lin CW, Hou WC, Shen SC, *et al:* Quercetin inhibition of tumor invasion via suppressing PKC delta/ERK/AP-1-dependent matrix metalloproteinase-9 activation in breast carcinoma cells. Carcinogenesis 29: 1807-1815, 2008.
6. Murakami A, Ashida H and Terao J: Multitargeted cancer prevention by quercetin. Cancer Lett 269: 315-325, 2008.
7. Sun ZJ, Chen G, Hu X, *et al:* Activation of PI3K/Akt/IKK-alpha/NF-kappaB signaling pathway is required for the apoptosis-evasion in human salivary adenoid cystic carcinoma: its inhibition by quercetin. Apoptosis 15: 850-863, 2010.
8. Luo H, Jiang BH, King SM and Chen YC: Inhibition of cell growth and VEGF expression in ovarian cancer cells by flavonoids. Nutr Cancer 60: 800-809, 2008.
9. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
10. Li YJ, Zhang ZY, Mao YY, *et al:* A genetic variant in miR-146a modifies digestive system cancer risk: a meta-analysis. Asian Pac J Cancer Prev 15: 145-150, 2014.
11. Ji T, Zheng ZG, Wang FM, *et al:* Differential microRNA expression by Solexa sequencing in the sera of ovarian cancer patients. Asian Pac J Cancer Prev 15: 1739-1743, 2014.
12. Winter J, Jung S, Keller S, Gregory RI and Diederichs S: Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 11: 228-234, 2009.
13. Chiyomaru T, Enokida H, Tatarano, *et al:* miR-145 and miR-133a function as tumour suppressors and directly regulate FSCN1 expression in bladder cancer. Br J Cancer 102: 883-891, 2010.
14. Arndt GM, Dossey L, Cullen LM, *et al:* Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer. BMC Cancer 9: 374, 2009.
15. Iorio MV, Visone R, Di-Leva G, *et al:* MicroRNA signatures in human ovarian cancer. Cancer Res 67: 8699-8707, 2007.

16. Xu Q, Liu LZ, Qian X, *et al:* MiR-145 directly targets p70S6K1 in cancer cells to inhibit tumor growth and angiogenesis. Nucleic Acids Res 40: 761-774, 2012.
17. Wang S, Bian C, Yang Z, *et al:* miR-145 inhibits breast cancer cell growth through RTKN. Int J Oncol 34: 1461-1466, 2009.
18. Boesch-Saadatmandi C, Wagner AE, Wolffram S and Rimbach G: Effect of quercetin on inflammatory gene expression in mice liver in vivo-role of redox factor 1, miRNA-122 and miRNA-125b. Pharmacol Res 65: 523-530, 2012.
19. Spagnuolo C, Russo M, Bilotto S, Tedesco I, Laratta B and Russo GL: Dietary polyphenols in cancer prevention: the example of the flavonoid quercetin in leukemia. Ann NY Acad Sci 1259: 95-103, 2012.
20. Duo J, Ying GG, Wang GW and Zhang L: Quercetin inhibits human breast cancer cell proliferation and induces apoptosis via Bcl-2 and Bax regulation. Mol Med Rep 5: 1453-1456, 2012.
21. Yang MY, Wang CJ, Chen NF, Ho WH, Lu FJ and Tseng TH: Luteolin enhances paclitaxel-induced apoptosis in human breast cancer MDA-MB-231 cells by blocking STAT3. Chem Biol Interact 213: 60-68, 2014.
22. Smith MA and Schnellmann RG: Calpains, mitochondria, and apoptosis. Cardiovasc Res 96: 32-37, 2012.