Original Article
miR-211 and miR-429 are involved in emodin’s anti-proliferative effects on lung cancer

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Abstract: Aim: It was proved that emodin exerted its anti-cancer activity against lung cancer by inducing cell apoptosis. Though several possible mechanisms were indicated, the facts are still in vague and incomplete. This study was aimed to further investigate the possible mechanisms of emodin’s anti-cancer activity in apoptosis. Methods: Emodin at serial concentrations was used to incubate with human lung cancer A549 cells. Cell proliferation was detected by MTT assay. Microarray of microRNAs was used to identify the differential expression of microRNAs before and after emodin in A549 cells. TUNEL staining was used to evaluate the cell apoptosis. Relative expression levels of microRNAs were investigated by real-time PCR; western blotting was used to assess the protein expression levels. Results: Emodin inhibited the cell proliferation of A549 cells by inducing cell apoptosis in a concentration-dependent manner. By microarray, there were 14 miRNAs were up-regulated while 13 were down-regulated >2 folds (P<0.05) among 2498413 miRNAs which were found differentially expressed before and after emodin incubation in A549 cells. MiR-211 and miR-429 were selected and their differential expressions were testified by real-time PCR. Furthermore, the expressions of miR-211 and miR-429 were found increased after emodin incubation in a concentration-dependent manner; accordingly, expressions of their targeted proteins, SATB2 and CRKL were decreased after emodin incubation. Both antimiR-211 and antimiR-429 transfection impaired emodin’s apoptosis-inducing activity by preventing decrease of SATB2 and CRKL in emodin-incubated A549 cells. Conclusions: Emodin altered the expression profile of microRNAs in A549 cells. Several microRNAs, such as miR-211 and miR-429, participated critically in emodin’s cytotoxic activity against lung cancer cells via affecting transcription of their targeted proteins.

Keywords: Emodin, microRNA, apoptosis, microarray

Introduction

In worldwide, as one of the most frequency malignant tumor, lung cancer takes responsibility for over 1 million death annually which is regard as leading cause of cancer-related death currently [1]. Primary lung cancer could be divided into mainly two types: the small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The proportion of NSCLC of lung cancer globally is 85%-87% according to previous reports. The prognosis of NSCLC is poor with the 5-year survival rate of only 10%-15% [2]. Though NSCLC is relatively sensitive to several chemotherapeutic agents, the side effects limited their clinical application. Since recent two decades, natural plant-original agents such as emodin, circumin and matrine attracted our attentions due to their potent anti-cancer activities [3].

Emodin, also referred as 1,3,8-thrihydroxy-6-methyl-anthraquione, is one of the constituent with bio-activities extracted from the root of Rheum Palmatum L [4]. It has been believed that emodin exerts multiple biological activities under different pathological conditions. The anti-bacterial, anti-viral, anti-fibrotic, anti-inflammatory, anti-proliferative, vasorelaxant and anti-cancer activities of emodin have been described by previous studies [5-8]. It was proved that emodin showed anti-cancer activities against many human cancers including lung cancer [9], but the specific mechanism are still unclear.

MicroRNA (miR) is defined as a group of small, endogenous and non-coding RNAs. By binding to the 3'-untranslated region (3'-UTR) of mRNA, the miR could regulate the gene expression negatively via repressing the post-transcription-
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Al process or degradating the binded mRNA [10]. Thus, miRs are involved in many cellular biological processes including apoptosis, proliferation metabolism, migration and so on [11, 12]. Accumulating studies suggest that the uncontrolled miR generation and regulation play critical roles in the occurrence and development of many human malignant cancers including breast cancer, lung cancer, bladder cancer, et al [13-15]. It is reasonable to speculate that maybe the anti-cancer agent inhibits human cancer by targeting and regulating miR expressions.

In this study, human NSCLC cell line A549 was used to investigate whether emodin incubation could affect the miR expressions by miR microarray analysis. Furthermore, a specific miR regulated apoptotic signaling pathway was selected for further investigation of mechanism of emodin’s anti-cancer activity. Therefore, we believe the better knowledge concerning the association between emodin and miR could be acquired from this study. These information would be benefit in further understanding the molecular mechanism of NSCLC and the pharmacology of emodin.

Materials and methods

Cell line and treatment

Human lung cancer cell A549 was acquired from American Type Culture Collection (ATCC, Rockville, USA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), antibiotic mixture (100 μg/ml streptomycin and 100 U/ml penicillin, Sigma-Alderich, USA) and 2.5 mmol/L L-glutamine (Invitrogen, USA). The cells were cultured in a humidified incubator with 5% CO₂ and 95% fresh air at 37°C. According to previous studies, emodin at 60 μmol/L was recognized as the concentration inducing distinct cytotoxicity. Cells were incubated with emodin at 60 μmol/L for 48 hours and the cytotoxic effect was confirmed by trypan blue dye exclusion method. This assay was used to determine the viable cells. Cells were suspended in PBS with trypan blue dye (Sigma-Aldrich, USA) at final concentration of 0.4%. Under microscopy observation, cells could exclude dye were recognized as viable ones. Then, further experiments were implemented.

Cell apoptosis assay

In this study, the cell apoptosis was determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. Briefly, cells were treated fixed in paraformaldehyde (2%, Solarbio, China) at room temperature for 30 minutes and then permeabilized by Triton X-100 solution (0.1%, Solarbio, China) at room temperature for another 30 minutes. After PBS washing, TUNEL assay was performed by using TUNEL assay kit (Roche, Holland) per manufacturer’s instructions. Then a fluorescence microscope was used to observe the labeled cells. Cells stained with green fluorescence were identified as TUNEL-positive cells.

Total RNA extraction and miR expression profile determination

When cell growth reached above 80% confluence, the cells were washed by PBS and then incubated with TRIzol (Invitrogen, USA) to extract RNA according manufacturer’s instructions. The samples were sent for miRNA differential expression profile scanning by using Affymetrix GeneChip miRNA 3.0 Arrays (Agilent Technologies). Data acquired from this assay was normalized and further analyzed by College of Bioinformatics and Epidemiology of Zhejiang University. The differential expression of miRs were confirmed by Real-time PCR.

Transfection of antimiR-211

The antimiR-211 oligonucleotide (KeyGen Biotechnology, Nanjing, China) and its antimiR negative control (KeyGen Biotechnology, Nanjing, China) were transfected into cells by using HiPerFect agent (Qiagen, USA) according to the manufacturer’s instructions. Briefly, anti-miR and negative control at final concentration of 20 mmol/L and 12 μL transfection agent were mixed in every 100 μL RPMI 1640 medium which was used to incubate the cells at room temperature for 10 minutes. Subsequent experiments were carried out 48 hours after the transfection.

Real-time PCR

The miRs were extracted from cells by using miRNeasy mini kit (Qiagen, USA) per manufacturer’s instructions. Specific primers were used in real-time PCR detection. SYBR Premix Ex
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Taq™ II (TaKaRa, Japan) was used to accomplish the detection following the manufacturer’s instructions with SetpOne Plus Real-time PCR system (Applied Biosystems, USA). The results were analyzed by 2-ΔΔCt method when U6 was used as the internal reference. Primers for miR-429 forward was 5’-ACACTCCAGCTGGGTAATCCTGCTGGTAA-3’; for miR-211 forward was: 5’-UUCCCUUUGUCAUCCU-3’; for U6 forward was: 5’-CTCGCTTCGGCAGCACA-3’.

Western blotting

The protein was extracted from harvested A549 cells by using Protein Extraction kit (Beyotime, China) after the cells were lysed with RIPA lysis buffer system (Santa Cruz). A BCA protein assay kit was used to determine the protein concentrations. The protein was loaded into SDS-PAGE gels and then separated by electrophoresis and then transferred to electronically to poly vinylidene difluoride (PVDF) membranes. Specific antibodies against CT10 regulator of kinase like (CRKL, CST, USA), special AT-rich sequence-binding protein-2 (SATB2, CST, USA), cleaved caspase-3 (Abcam, USA), Bax (Abcam, USA) and GAPDH (Santa Cruz, USA) were used to incubate the membranes. The final bands were detected by ECL system.

Figure 1. Emodin inhibited proliferation of A549 cells by inducing apoptosis. Chart on (A) demonstrated the proliferation inhibition rate of A549 cells after incubated with emodin solutions at concentrations at 0, 20, 40, 60 and 80 μmol/L. On the left part of (B), the upper panel showed the captured images of TUNEL staining of A549 cells, while the lower panel demonstrated the immunobots of cleaved caspase-3 of A549 cells incubated with emodin solutions at concentrations at 0, 20, 40, 60 and 80 μmol/L. The data of apoptotic rate and relative expression levels of cleaved caspase-3 were presented as columns on the right part of (B) in a (mean ± SD) manner. [*values are significantly different from previous concentration (P<0.05)].
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(Bio-Rad, USA) using SuperSignal West Pico kit (Peirce). The band intensities were detected and analyzed by a software Image Lab (Bio-Rad, USA).

Statistics

The data acquired in this study was processed by software SPSS (v16.0). The comparisons of

Figure 2. Several differential expressed miRs detected by microRNA microarray. The left part showed some of the differential expressed miRs detected by microarray in A549 cells before and after emodin incubation (60 μmol/L). miR-429 and miR-211 were boxed. The right part of this figure showed the relative expression levels of miR-211 and miR-429 detected by real-time PCR. [*values are significantly different from control (P<0.05)].
differences were carried out by student t-tests and ANOVA. P<0.05 was considered statistically significant.

Results

Emodin inhibited proliferation of A549 cells by inducing apoptosis

As demonstrated in Figure 1A, detected by MTT assay, emodin solution incubation significantly inhibited the proliferation of A549 cells in a concentration-dependent manner. Furthermore, emodin solution induced the apoptosis of A549 cells also in a concentration-dependent manner, which is proved by TUNEL staining and cleaved caspase-3 expression (Figure 1B). The apoptosis is considered as one of the most critical cause of inhibiting proliferation.

Differentially expressed miRNAs in emodin incubated A549 cells

RNA samples from A549 cells were prepared and analyzed by by Affymetrix GeneChip miRNA 3.0 Array. Total amount of 2498413 miRNAs were found differentially expressed before and after emodin incubation in A549 cells. Among them, 14 miRNAs were up-regulated while 13 were down-regulated >2 folds (P<0.05) in A549 cells after emodin incubation. This result was shown in Figure 2A. In addition, after reviewing scientific literatures, miR-211 and miR-429 were selected and their differential expressions were verified by real-time PCR assay. The results were demonstrated in Figure 2B.

Emodin incubation altered the expressions of miR-211 and miR-429 and their targeted proteins in A549 cells

After incubated with serially diluted emodin solutions, by real-time PCR, the expressions of both miR-211 and miR-429 were increased significantly in a concentration-dependent manner (Figure 3A). According to the results from previous studies, SATB2 was recognized as the targeted protein of miR-211 while miR-429 targeted CRKL. Demonstrated in Figure 3B, as the expression levels of miR-429 and miR-211
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A

B

SATB2

CRKL

GAPDH

GAPDH

C

PI

TUNEL

Merge

0μmol/L  60μmol/L  60μmol/L + Neg  60μmol/L + Ant

0μmol/L  60μmol/L  60μmol/L + Neg  60μmol/L + Ant

0μmol/L  60μmol/L  60μmol/L + Neg  60μmol/L + Ant

0μmol/L  60μmol/L  60μmol/L + Neg  60μmol/L + Ant

0μmol/L  60μmol/L  60μmol/L + Neg  60μmol/L + Ant

Apoptotic Percentage (%)
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Figure 4. Effect of antimiRs of miR-211 and miR-429. (A) Showed the relative expression levels of antimiR-211 (left) and antimiR-429 (right) after the antimiRs were transfected into A549 cells. (“Neg” refers to “negative control”; “Ant” refers to “antimiRs”). On (B), immunoblots of SATB2, CRKL and GAPDH were demonstrated. The relative expression levels of SATB2 and CRKL were demonstrated as columns on the lower panel of (B). (C) Demonstrated the captured images of TUNEL staining of A549 cells. The apoptotic rate of A549 cells were showed as columns on the lower part. [a]differences were significant when compared with “0 μmol/L” (P<0.05); [b] differences were significant when compared with “60 μmol/L” (P<0.05); [c] differences were significant when compared with “60 μmol/L + Neg” (P<0.05).

were elevated as the concentration of emodin increasing, the expression levels of SATB2 and CRKL were decreased.

AntimiR-211 and antimiR-429 impaired emodin’s apoptosis-inducing activity by regulating miR-211 and miR-429 and their targeted proteins

The synthesized antimiR-211 and antimiR-429 were transfected into A549 cells which were further incubated with emodin. Figure 4A demonstrated antimiR-211 and antimiR-429 were successfully transfected into A549 cells which was evidenced by real-time PCR. After incubated with emodin, expression levels of the targeted protein of miR-211 and miR-429, namely SATB2 and CRKL, were significantly higher than cells transfected with negative control miR (Figure 4B). As a result, the apoptosis of emodin-induced apoptosis of A549 cells was also inhibited (Figure 4B).

Discussion

In the present study, we demonstrated that emodin could significantly inhibit the cell proliferation of lung cancer A549 cells by inducing apoptosis. Though the mechanism of inducing apoptosis by emodin were described in many previous studies, such as affecting membrane stability of mitochondria, triggering reactive oxygen species generation, inducing calcium overloading and so on, the mechanism is still not specific and comprehensive. MicroRNAs have been considered playing important roles in sophisticated regulating network of numerous biological processes. In this study, we focused on the affection of emodin on miRNAs expression profile to identify possible miRs involved in the anti-proliferative activity of emodin on lung cancer cells. We believe that these results would be helpful not only in broadening our knowledge of the pharmacology of emodin, but also in providing theoretical basis for clinical application of emodin or emodin-contained compounds in lung cancer treatments.

Emodin is one of the bio-active components extracted from the roots or rhizomes of numerous herbal plants such as Rheum officinale. This herb was applied in Traditional Chinese Medicine from the ancient times in treatment of many diseases such as chronic inflammation, hepatitis, infection, skin burns and osteomyelitis [16]. The anti-cancer activity of emodin has been reported and established. Emodin shows anti-proliferative by inducing apoptosis in many human malignant tumors including hepatic cancer, pancreatic cancer, ovarian cancer and lung cancer [4, 17, 18]. In this study, we found that the proliferation of lung cancer A549 cells was dramatically inhibited by emodin in a concentration-dependent manner. Furthermore, evidenced by TUNEL staining and caspase-3 cleavage, the apoptosis of A549 cells was found elevated after emodin incubation in a concentration-dependent manner. This result is in accordance with several previous studies concerning emodin’s anti-proliferative activity on lung cancer cells.

Generally accepted, the gene expression is highly regulated by an independent class of RNAs which is referred as microRNAs. MiRNAs are a set of small, non-coding and evolutionarily conserved RNAs that regulate gene expression by translational repression or degradation of targeted mRNAs [19]. Till now, approximately 600 human miRs are identified which target over 30% of human genes [20]. The specificity of miRs is guaranteed by 5’ proximal “seed” region. Every miR could target about 200 mRNAs. The technology of microarray has been used to screen the differential expression profile of miRNAs to help in identify tumor markers, molecular targets and to understand pharmacological mechanisms of certain drugs. In the present study, we profiled differential miR expression in A549 cells before and after emodin incubation, attempting to acquire new knowledge about the mechanisms of emodin’s anti-cancer activity. 14 miRNAs were up-regulated while 35 were down-regulated >2 folds (P<0.05) in A549 cells after emodin incubation.
Two of these miRs, namely miR-211 and miR-429 were highly up-regulated after in emodin-incubated A549 cells.

Previously, it was illustrated that miR-211 was associated with the occurrence and development of several human cancers [21]. The expression of miR-211 was down-regulated in human hepatic cancer tissue compared with adjacent normal hepatic tissue [22]. Furthermore, overexpression of miR-211 was found as a tumor-suppressor by inhibiting proliferation [21]. In this study, emodin incubation dramatically increased miR-211 expression in A549 cells, leading to apoptotic events. According to bio-informatic analysis, SATB2 was believed the direct down-stream target of miR-211 [22]. SATB2 is an AT-rich DNA binding protein which could alter the chromatin structure. It was also found that up-regulation of SATB2 was associated with poor prognosis of colorectal and breast cancer [23, 24]. In a recent study, SATB2 was proved playing a crucial role in preventing apoptosis [25]. In our study, the up-regulated miR-211 depressed the expression of SATB2 after emodin which induced apoptosis of A549 cells. Furthermore, transfection of antimiR-211 prevented the down-regulation of SATB2 in emodin-incubated A549 cells. As a result, the emodin-induced apoptosis was also suppressed.

After emodin incubation, another highly up-regulated miR, miR-429 also attracted our attention. As one of the members of miR-200 family, miR-429 was found to participate in regulating malignancy of various types of tumors [26]. It was reported that the overexpression of miR-429 suppressed invasion and metastasis of colorectal carcinoma and ovarian cancer [27, 28]. Revealed by bioinformatic analysis, CRKL was predicted the target down-stream protein of miR-429 [28]. Indeed, in this study, emodin-induced up-regulation of miR-429 abated the expression of CRKL. Previous literature suggested the cell death protecting role of CRKL that inhibited caspase-8 mediated apoptosis. In this study, antimiR-429 avoided the affection of emodin-induced miR-429 on expression level of CRKL. As a result, antimiR-429 impaired the apoptotic inducing activity of emodin in A549 cells.

In summary, with the accordance with other studies, emodin exerts strong proliferative inhibiting activity by inducing apoptosis in lung cancer cells. Our research showed that the miR expression profile in lung cancer cells was affected by emodin treatment. According to the results of our study, two miRs, miR-211 and miR-429 were involved in emodin’s anti-proliferative activity by prohibiting the expressions of their target protein, SATB2 and CRKL, in lung cancer cells.

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Disclosure of conflict of interest

None.

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