Emodin promotes the arrest of human lymphoma Raji cell proliferation through the UHRF1-DNMT3A-ΔNp73 pathways

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Abstract. Emodin is an active constituent found in the roots and rhizomes of numerous Chinese medicinal herbs. It exerts antitumor activity against Dalton’s lymphoma in vivo, although the detailed mechanisms by which emodin induces apoptosis remains to be elucidated. The present study aimed to analyze the mechanisms underlying the response to emodin treatment. Using lymphoma Raji cells, an emodin-induced cell proliferating inhibition model was first established, then flow cytometry, western blotting, reverse transcription-quantitative polymerase chain reaction and luciferase reporter assay were performed. It was found that emodin decreased the percentage of Raji cell viability, induced apoptosis, and increased the activation of caspase 3, caspase 9 and poly (ADP-ribose) polymerase through the downregulation of ubiquitin-like protein containing PHD and RING domains 1 (UHRF1). The emodin-induced downregulation of UHRF1 led to an increase in the level of DNA methyltransferase 3A, which in turn inhibited the activity of p73 promoter 2 and decreased the levels of NH2-terminally truncated dominant-negative p73. The treatment of Raji cells with emodin combined with doxorubicin led increased cell death of Raji cells, indicating that emodin may sensitize Raji cells to doxorubicin-induced apoptosis.

Introduction

Emodin (C₁₅H₁₀O₆; Fig. 1A), as an active constituent isolated from Chinese traditional herbs, has long been used as a constituent of several prescriptions in traditional Chinese medicine to treat and cure various diseases (1). Emodin has been shown to possess several pharmacological effects, including antibacterial, antiviral and anti-inflammatory effects (2). Emodin has also been shown to inhibit cell growth in different types of tumor cell, including human hepatocellular carcinoma cells, human promyeloleukemic HL-60 cells, human cervical cancer cells and Dalton’s lymphoma in mice (3). The potency of emodin in inhibiting cancer cell growth has been reported to be attributed primarily to the induction of cell apoptosis (1). However, the detailed mechanism by which Emodin exerts its anticancer effects remains to be fully elucidated.

As a stress inducible effector, the tumor suppressor gene p53 exerts important effects on the regulation of cellular processes, including cell cycle arrest, apoptosis, autophagy, necroptosis, cell metabolism and senescence (4,5). Under cellular stresses, p53 is stabilized and binds to the promoters of target genes, which results in the transcriptional activation of downstream effectors (6,7). However, p53 is the most frequently mutated tumor suppressor gene in human cancer, and clinical studies have demonstrated that ~50% of tumor cells lack functional p53 (8). P73, a p53 family member, shares significant homology with p53; it is localized to chromosome lp36.2-3, a region frequently deleted in several tumor types in humans (9,10). The p73 gene encodes two isoforms: Transcriptionally active full-length p73 (TAp73) and NH2-terminally truncated dominant-negative p73 (ΔNp73), which are expressed from two different promoters, p73-promoter-1 and p73-promoter-2 (11,12). TAp73 shares significant similarity with p53, including binding with p53 DNA target sites, transactivation of p53-target genes, and induction of cell cycle arrest and apoptosis; however, the ΔNp73 isoform has opposite effects, as it can inhibit the functions of TAp73 and p53 by competing for binding to the p53/TAp73 DNA target sequence or by forming oligomers with p53/TAp73 (13). Compared with p53, p73 gene mutations are rarely found in human tumors, suggesting the existence of other molecular mechanisms, which may be responsible for the regulation of p73 during oncogenesis.

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Abbreviations: TAp73, transcriptionally active full-length p73; ΔNp73, NH2-terminally truncated dominant-negative p73; UHRF1, ubiquitin-like protein containing PHD and RING domains 1; DNMT3A, DNA methyltransferase 3A

Key words: emodin, NH2-terminally truncated dominant-negative p73, ubiquitin-like protein containing PHD and RING domains 1, p52, apoptosis
The proto-oncoproteins MDM2 and MDMX are negative regulators of p53 family members (14). These two proteins contain p53 binding domains, which can bind with p53, export it from the nucleus to the cytosol, and target it for degradation on the 26S proteasome or repress p53 transcriptional targets (15). Although MDM2 and MDMX can also bind with p73 and inhibit P73-transcriptional activity, they do not ubiquitinate p73 (16,17). Therefore, the overexpression of MDM2 or MDMX can lead to suppression of p53/p73 tumor suppressor activities. Additionally, the inhibition of MDM2/MDMX through small interfering (si)RNA or specific inhibitors sensitizes cancer cells to radiotherapy and chemotherapy (18,19), suggesting the MDM2 and MDMX may be therapeutic targets for use in cancer therapies.

In addition to MDM2 family members, other ubiquitin E3 ligases, including ubiquitin-like protein containing PHD and RING domains 1 (UHRF1), can also target p53 and p73 ubiquitination, and suppress p53-dependent transactivation and cell apoptosis in response to DNA damage signals (20,21). Conversely, p53 and p73 can regulate the expression of UHRF1 (22,23). UHRF1 contains several domains, including the N-terminal ubiquitin-like domain (NIRF), plant home-domain, Set and Ring Associated domain (also known as the YGD domain), and C-terminal RING finger. It can form complexes with histones and non-histone proteins, including promyelocytic leukemia and DNA methyltransferase (DNMT) 1, and exert its E3 ligase activities (24,25). In addition to UHRF1, the UHRF family contains three other members, NIRM, ICBP55 (also known as UHRF3) and ICBP87 (also known as UHRF3), all of which have similar domains to those of UHRF1 (26), suggesting they can also perform UHRF1-like biological functions. However, the activities of NIRM, ICBP55 and ICBP87 remain to be elucidated.

Earlier studies have reported that emodin has antitumor activity against Dalton’s lymphoma in vivo (27), however, the detailed mechanisms by which emodin induces apoptosis remain to be elucidated. The present study aimed to analyze the mechanisms underlying the response to emodin treatment. Using lymphoma Raji cells, an emodin-induced cell proliferating inhibition model was established and the possible underlying mechanisms were investigated. It was found that emodin downregulated the expression of UHRF1, which in turn suppressed p73 promoter 2 activity through the upregulation of DNMT3A, and increased TAp73ΔNp73 and cell apoptosis.

Materials and methods

Chemicals. Emodin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). RPMI-1640, Lipofectamine™ 2000, Opti-MEM I medium, TRIzol RNA purification kit and SYBR green I mix, for quantitative polymerase chain reaction (qPCR) analysis, were obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Fetal bovine serum (FBS) was from Hyclone; GE Healthcare Life Sciences (Logan, UT, USA). Protein isolation and BCA protein quantification kits were supplied by Beyotime Institute of Biotechnology (Nanjing, China). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was obtained from MultiSciences Biotechnology (Hangzhou, China). The siRNA sequence targeting UHRF1 (cat. no. sc-270551) was a product of Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). P73-Luc-1, p73-Luc-2 reporter constructs, and the negative control plasmid were obtained from GeneChem Co., Ltd. (Shanghai, China). All specific primers for specific genes and the PrimeScript RT Reagent kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China).

Antibodies. Primary antibodies against active-caspase 3 (cat. no. 9661L), active-caspase 9 (cat. no. BS7070), active-poly (ADP-ribose) polymerase (PARP; cat. no. AB3620), p53 (cat. no. AP0104), β-actin (cat. no. BS2237) and UHRF1 (cat. no. MB0055) were purchased from BioWorld Technology, Inc. (St. Louis Park, MN, USA). Primary antibodies against ICBP55 (cat. no. BM1924), ICBP 87 (cat. no. BM2090) and NIRM (cat. no. BM2989) were supplied by Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Primary antibodies against MDM2 (cat. no. sc-812), MDM4 (cat. no. sc-14740), TAp73 (cat. no. sc-9651), ΔNp73 (cat. no. sc-70966), DNMT1 (cat. no. sc-271729), DNMT2 (cat. no. sc-271513), DNMT3A (cat. no. sc-10232), DNMT3B (cat. no. sc-393845) and DNMT3L (cat. no. sc-10239) were all products of Santa Cruz Biotechnology, Inc. IRDye-conjugated anti-rabbit secondary antibody (cat. 611-744-127), IRDye-conjugated anti-mouse secondary antibody (cat. 610-145-121) and IRDye-conjugated anti-goat secondary antibody (cat.605-744-002) were all supplied by Rockland Immunochemicals Inc. (Limerick, PA, USA).

Cell culture and treatment. The Raji cells were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). The Raji cells were maintained in DMEM supplemented with 10% (v/v) FBS and grown at 37°C with a humidified 5% CO2 atmosphere.

Cell proliferation assay. The Raji cells were plated at 1x10^4 cells per well in a 96-well plate. Following culture for ~24 h, the Raji cells were treated with or without different concentrations of Emodin including 6.25, 12.5, 25 and 50 µM for 4, 8, 12, 24 and 48 h, and cultured at 37°C with a humidified 5% CO2 atmosphere. At the end of the experiment, medium was replaced with 100 µl (500 µg/ml) MTT solution and incubated for 4 h at 37°C. The MTT solution was then removed, and 150 µl DMSO was added to dissolve the formazan crystals. The absorbance at 570 nm for each well was read on a microtiter plate reader (BioTek Instruments, Inc., Winooski, VT, USA). The results were determined as the percentage of the control group.

Flow cytometry. The Raji cells were plated at 2x10^5 cells per well in a 6-well plate. After 24-48 h, the Raji cells were treated with or without emodin at concentrations of 6.25, 12.5, 25 and 50 µM for 24 h. At the end of the exposure, the Raji cells were collected and washed twice in PBS, then suspended in 0.5 ml binding buffer containing 5 µl annexin V-FITC and 10 µl PI, and incubated in the dark at room temperature for 30 min. The samples were then analyzed using a FACScan flow cytometer (BD Biosciences, Hercules, CA, USA), and 1x10^4 events were collected, recorded on a dot plot, and analyzed using ModFit software version 2.0 (Verity Software House, Inc., Topsham, ME, USA).
Western blot analysis. The Raji cells were treated with or without emodin at concentrations of 6.25, 12.5, 25 and 50 µM for 24 h and, at the end of exposure, the Raji cells were harvested for protein extraction. The whole-cell lysate was extracted using a protein isolation kit and protein content was quantified using a BCA protein quantification kit. The proteins were separated on a 10% SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked with TBS containing 5% BSA at 4°C for 1 h, and then exposed to the specific primary antibodies at 4°C overnight. This was followed by incubation with the corresponding IRDye-conjugated secondary antibodies (1:5,000 dilution for all) at room temperature for 1 h. The proteins were visualized using the Odyssey Infrared Imaging system with Odyssey v1.2 (LI-COR Biosciences, Lincoln, NE, USA). The relative expression levels of target proteins were normalized to the intensities of β-actin.

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The Raji cells were treated with or without emodin at concentrations of 6.25, 12.5, 25 and 50 µM for 24 h. At the end of the experiment, the Raji cells were harvested for RNA extraction using the TRIzol RNA purification kit. First-strand cDNA synthesis was performed using the PrimeScript RT Reagent kit according to the manufacturer's protocol. Amplification was performed using a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), using the SYBR Green RT-PCR kit, according to the manufacturer's protocol [95°C for 10 sec and 56°C for 30 sec (45 cycles)]. Relative mRNA expressions were calculated using the 2-ΔΔCq method and normalized to the internal reference gene β-actin (28).

Luciferase reporter assay. The p73 promoter 1 and promoter 2 sequences, synthesized by TransheepBio-Tech Co., Ltd. (Shanghai, China), were cloned into the pGL3-Luc vector (cat. no. 1741; Promega Corporation, Madison, WI, USA) using a Universal GenomeWalker kit supplied by Seebio Biotech (Shanghai) Co., Ltd. (cat. no. 638904; Shanghai, China), according to the manufacturer’s protocol. The Raji cells were transfected with the pGL3-Luc vector or a negative vector (pRL-TK plasmid) using Lipofectamine™ 2000 transfection reagent according to the manufacturer's protocol. Following transfection for 24 h, the Raji cells were treated with or without emodin at concentrations of 6.25, 12.5, 25 and 50 µM for 24 h. At the end of the exposure, the Raji cells were harvested for mRNA isolation, following which cDNA amplification was performed at 95°C for 10 sec and 56°C for 30 sec (40 cycles), and RT-qPCR analysis were performed using a two-step method.

UHRF1 siRNA transfection. The Raji cells were plated into 6-well plates at a density of 5x10^4 cells per well. Following incubation for 24 h, the cells were harvested and diluted to a density of 8x10^3/ml with DMEM. The Lipofectamine™ 2000 transfection reagent and the UHRF1 siRNA sequence were diluted with Opti-MEM I medium, the final concentrations were 5% and 50 nM, respectively. The above reagents were incubated at 37°C for 10 min. The two reagents were then mixed and incubated at room temperature for an additional 20 min. Finally, the transfection mixture was added to the 6-well plates. The cell suspensions were overlaid onto the transfection mixture. Following incubation for 4 h, the medium was removed and the cells were cultured with fresh DMEM containing 10% FBS.

**Results**

**Emodin decreases the viability of Raji cells.** The inhibitory effects of emodin (0, 6.25, 12.5, 25 and 50 µM) on Raji cell viability were evaluated at 4, 8, 12, 24 and 48 h post-treatment. As shown in Fig. 1B, compared with the control groups (untreated groups), significant inhibition of cell viabilities were induced by 6.25-50 µM emodin at 24 and 48 h (P<0.05), whereas 25 and 50 µM emodin significantly decreased Raji cell viabilities at 4, 8 and 12 h. However, no significant cytotoxic effects were found in the 6.25 and 12.5 µM emodin-treated groups of Raji cells over 4-12 h (P>0.05).

**Emodin induces the apoptosis of Raji cells.** To investigate whether the emodin-induced decrease in cell viability in Raji cells was attribute to apoptosis, the cell death was detected using flow cytometry. As shown in Fig. 2A, compared with the control group, 6.25-50 µM emodin treatment for 24 h significantly increased the apoptosis of Raji cells (P<0.05); these results were further confirmed by the activation of apoptotic effectors caspase 3, PARP and caspase 9, compared with the control group, as shown in Fig. 2B. Treatment with 6.25-50 µM
Emodin for 24 h significantly increased the protein levels of active-caspase 3, active-PARP and active-caspase 9 (P<0.05).

Emodin induces an increase of TAp73/ΔNp73 in Raji cells through the downregulation of ΔNp73. To investigate whether the emodin-induced apoptosis was associated with p53 or p73, the mRNA and protein expression levels of p53 and p73 in Raji cells were examined using RT-qPCR and western blot analyses. As shown in Fig. 3A, compared with the control group, 6.25-50 µM emodin treatment for 24 h significantly decreased the mRNA levels of p73 (P<0.05), however, no change in the mRNA level of p53 was observed in the emodin-treated groups (P>0.05). Analysis of protein levels, as shown in Fig. 3B, showed that, compared with the control group, the protein levels of ΔNp73, but not of p53 or TAp73, were significantly reduced in the 6.25-50 µM emodin-treated groups (P<0.05). The relative ratio of TAp73 and ΔNp73 (TAp73/ΔNp73) was calculated and is summarized in Fig. 3C. Treatment with 6.25-50 µM emodin for 24 h increased the TAp73/ΔNp73 ratio, compared with that in the control group, and these changes were significant (P<0.05).

Emodin induces decreased protein levels of UHRF1 in Raji cells. To investigate whether the emodin-induced changes in the expression of ΔNp73 were associated with negative regulators of p53 family members, the mRNA and protein expression levels
of MDM2, MDM4, ICBP55, ICBP87, NIRF and UHRF1 were examined using RT-qPCR and western blot analyses. As shown in Fig. 4A, compared with the control group, no significant changes were found in the mRNA levels of the target genes in the 6.25-50 μM emodin-treated groups (P>0.05). By contrast, as shown in Fig. 4B, the protein levels of UHRF1 in the 6.25-50 μM emodin-treated groups were decreased, compared with those in the control group, and these changes were significant (P<0.05).

Emodin decreases p73 promoter 2 activity through the inhibition of UHRF1. To investigate the associations of UHRF1 and ΔNp73 in this emodin-treated cell model, the activities of p73 promoter 1 (p73-Luc-1) and p73 promoter 2 (p73-Luc-2) in the Raji cells, and the UHRF1 siRNA-transfected Raji cells were examined using RT-qPCR analysis. As shown in Fig. 5A, compared with the control group, the activities of p73-Luc-2 in the 6.25-50 μM emodin-treated groups were significantly decreased (P<0.05). These emodin-decreased p73-Luc-2 activities were attenuated by UHRF1 siRNA transfection, as shown in Fig. 5B.

UHRF1 inhibits p73-Luc-2 activity through the upregulation of DNMT3A. It is well known that p73 promoter activities can be directly regulated by methylases, therefore, the present study examined the mRNA and protein levels of DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L...
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using RT-qPCR and western blot analyses. As shown in Fig. 6A, no significant change in the mRNA levels of the target genes were found in the 6.25-50 µM emodin-treated groups, compared with the control group (P>0.05); however, as shown in Fig. 6B, treatment with 6.25-50 µM emodin significantly increased the protein levels of DNMT3A compared with that in the control group (P<0.05). The associations between UHRF1 and DNMT3A were further investigated, as shown in Fig. 6C and D, and it was found that the emodin-induced upregulation of DNMT3A and decrease of Raji cell viability were attenuated by the knockdown of UHRF1.

Emodin sensitizes doxorubicin-induced Raji cell apoptosis. To further investigate whether emodin exerted a possible synergistic effect in doxorubicin-induced Raji cell apoptosis. The inhibitory effects of emodin (6.25, 12.5 and 25 µM) and doxorubicin (1 µg/ml), alone or in combination were examined. As shown in Fig. 7, compared with the control group (untreated group), both emodin and doxorubicin significantly decreased Raji cell viabilities (P<0.01); when the concentrations of emodin and doxorubicin were added in combination, the decrease in cell viability was more marked (P<0.01).
**Discussion**

Lymphoma is a blood cell tumor, which develops from lymphatic cells. It is characterized by enlarged lymph nodes, fever, drenching sweats, unintended weight loss, itching and tiredness (29,30). Although lymphoma can be cured as it is usually localized, it can be life threatening, with the majority of victims being children or young adults. Current treatments for lymphoma consist primarily of chemotherapy and radiation therapy; however, their lack of specificity and the existence of toxicity to normal cells are limitations of these methods. Therefore, the identification of more effective anticancer agents to improve tumor specificity and decrease toxicity to normal cells is urgently required. Emodin is a natural compound derived from Chinese traditional herbs, which has been found to induce apoptosis in several human tumor cell lines *in vitro* (3). In agreement with these findings, the present study found that emodin at concentrations of 6.25, 12.5, 25 and 50 µM markedly decreased the viability of Raji cells at 24 h post-treatment. This was further confirmed using flow cytometry and western blot analysis, which demonstrated that emodin concentrations of 6.25, 12.5, 25 and 50 µM induced apoptosis of the Raji cells, and increased the activation of caspase 3, caspase 9 and PARP following 24 h exposure.

p73 is a p53 family member and, unlike the p53 gene which is expressed from a single transcript encoding a single protein, p73 transcribes multiple isoforms (TAp73 and ∆Np73) due to the utilization of two promoters. The overexpression of TAp73 can induce the transactivation of p53-target genes, and inhibit cell proliferation in a p53-like manner through cell apoptosis (9). The overexpression of ∆Np73 has been found in various human cancer cell lines and tumor tissues (10,11). It has been suggested that the ratio between the levels of TAp73 and ∆Np73 (TAp73/∆Np73) is important in regulating cell fate in response to anticancer agents during chemotherapy (31,32). In the present study, the results confirmed the effects of TAp73/∆Np73 on cell growth, which was demonstrated by the concurrence of cell apoptosis and increase in TAp73/∆Np73, the latter attributed to the downregulation of mRNA levels of ∆Np73.

Abnormal DNA hypermethylation catalyzed by DNMTs is critical in regulating gene transcription, gene imprinting and X chromosome inactivation (33,34). The transcription of p73 can be mediated by its two promoters, which locate at CpG islands (35,36). The methylation of cytosine residues of the CpG region results in silencing of the expression of p73 (36,37). A previous study reported that p73 was aberrantly methylated in ~30% of cases of primary acute lymphoblastic leukemia and Burkitt's lymphomas, however, no p73 methylation was detected in normal tissues (38,39). In the present study, a significant association was found between the decreased mRNA expression of ∆Np73 and the downregulation in the activity of p73 promoter 2, suggesting the modification of p73 promoter 2.

The DNMT superfamily contains five members, including DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (40). DNMT1 is primarily responsible for maintaining the methylation status of the genome; DNMT2 encodes a protein responsible for the methylation of tRNA; and DNMT3A, DNMT3B and DNMT3L catalyze the addition of methyl groups to cytosine residues of CpG nucleotides (40). It was previously reported that DNMT1 can regulate p73 promoter methylation in the overexpression of rTCS in Caski cells (41). In the cell model used in the present study, the emodin-induced decrease in the activity of p73 promoter 2 concurred with an increase in the levels of DNMT3A, suggesting an association between p73 promoter methylation and DNMT3A. However, the detailed mechanism for the DNMT3A methylation of p73 requires further investigation.

UHRF1, as an oncogenic factor, is expressed at high levels in various types of cancer (26). It can transmit DNA methylation information from parental cells to daughter cells by discriminating hemimethylated DNA, recruiting DNMT1, and replicating cell nuclear antigen to the correct location to methylate newly synthesized DNA sequences (24,42). UHRF1 can also bind with histone H3, which is tri-methylated at lysine 4 and 9, and maintain the later heterochromatin status (42-44). Therefore, UHRF1 is able to recognize DNA methylation and histone methylation, physically linking these two epigenetic markers. Of note, the present study found that emodin induced the downregulation of UHRF1 and upregulation of DNMT3A, rather than DNMT1. To determine the contribution of the downregulation of UHRF1 to the upregulation of DNMT3A, the present study inhibited the expression of UHRF1 using siRNA in Raji cells. It was found that the UHFF1 siRNAs effectively knocked down the expression of UHRF1, and significantly attenuated the emodin-induced upregulation of DNMT3A. This suggested the existence of associations between UHFF1 and DNMT3A. However, the detailed mechanism underlying the upregulation of DNMT3A remains to be fully elucidated.

In conclusion, the present study demonstrated that emodin treatment resulted in Raji cell proliferation arrest and apoptosis through an increase in the UHRF1-DNMT3A-TAp73/∆Np73 pathways. These results suggested that emodin may be used as an anticancer agent to treat the overexpression of UHRF1 in tumors. Further investigations are required to confirm the biological functions of emodin in tumor therapy using animal models.

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