Cancer cell research 5 (2015) 105-110

www.cancercellresearch.org Article

A Traditional Chinese Medicine Huaier Triggers G1 Cell Cycle Arrest and Apoptosis Through Cyclins-cdks-ckis Machinery in GIST-T1 Cells

Yonghong Zhang, Yongxiang Li*

General Surgery, Anhui Medical University affiliated First Hospital, Hefei, 230000, China

Abstrac: **It is well believed that cancer is a cell cycle dysfunction disease, and most of chemotherapeutic reagents are targeting proliferating cancer cells. Huaier, a traditional Chinese medicine, has been extensively used as a kind of anti-cancer drug in clinic for many years; however, the effect and mechanism of its treatment on human cancer cells are still unknown. In the current study, GIST-T1 cells treated with Huaier were analyzed by flow cytometry to detect the cell cycle distribution and cell apoptosis. In addition, expression levels of cell-cycle regulators and apoptotic proteins in response to DNA damage were examined by immunoblotting. Our data showed that Huaier decreased the viability of GIST-T1 by inducing G1 cell cycle arrest and induced apoptosis in a dose- and time-dependent manner. In GIST-T1 cells treated with Huaier, expression of cyclinD3/cyclinE and Cdk2/Cdk4/Cdk6 proteins significantly decreased; in contrast, expression of p16/p21/p27 proteins increased. Bax protein also increased and Bcl-2 decreased after Huaier treatment. Taken together, we firstly tested the biological effect of Huaier on human cancer cells in vitro and further probed its potential molecular targets, which provided the direct evidence for its clinical application in cancer patients.**

Keywords: Apoptosis; G1 arrest; Cyclin-Cdk- CKI; Huaier; Chinese medicine

Received 28 September 2014, Revised 20 December 2014, Accepted 25 December 2014

* Corresponding Author: Yongxiang Li, 379847410@qq.com

1. Introduction

The normal cell cycle core machinery is a family complex of catalytic components called cyclin-dependent protein kinases (Cdks) and regulatory subunits named cyclins [1]. Distinct cyclin–Cdk complexes sequentially phosphorylate their respective substrates, thereby driving the cell through different phases of the cell cycle. Three essential classes of cyclin–Cdk complexes include the D-type cyclins (cyclins D1, D2 and D3), which activate Cdk4 and Cdk6 to execute critical events in G1 phase, the E-type and A-type cyclins, which activate Cdk2 to drive the cell through S phase, and the A-type and B-type cyclins, which activate Cdk1 to direct structural and regulatory events in mitosis [2, 3, 4]. Two families of Cdk inhibitors (CKIs) are known as p16INK4A family (i.e. p16INK4A, p15INK4B, p18INK4C, p19INK4D) and the p21CIP1 family (i.e. p21CIP1, p27KIP1 and p57KIP2) [4].

One hallmark of cancer is uncontrolled cell proliferation and/or insufficient apoptosis [5]. To inhibit cancer cell proliferation and induce apoptosis, most anti-tumor drugs were developed for targeting different cell cycle and apoptosis regulators [6, 7].

Huaier, which come from an important traditional Chinese medicinal herb fungi growing on the Chinese scholar tree, has a long history as an effective anti-cancer drug in China. Being extracted from the trametes robiniophila murr, the major active ingredient of Huaier is a polysaccharide protein (PS-T) which consists of 6 kinds of monosaccharides and 18 kinds of amino acids [8].

In this study, we planned to investigate the biological function and molecular targets of Huaier in human cancer cells.

2. Methods

2.1 Preparation of Huaier Dissolution

Huaier (PS-T) was provided by Gaitianli Pharmaceutical Co. Ltd. (Jiangsu, China). 5mg powder was dissolved in 1ml water, and thus its initial concentration is 5mg/ml (each culture well containing 1 ml RPMI 1640 medium).

2.2 Cell Culture

The acute lymphocytic leukemia cell line GIST-T1 (ATCC, Manassas,VA,USA) was cultured in a six-well plate with DMEM medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All media, supplements, and sera were purchased from GIBCO (Grand Island, NY, USA). Cells were incubated in a 5% CO2 humidified incubator at 370C. The culture was diluted and re-plated every 3 days to keep them in an asynchronous and exponential phase of growth.

Huaier solution was added into 4 wells with 0 μl, 1 μl, 5 μl and 10 μl, respectively. After the cells grew for 24 hours, they were then rinsed with phosphate buffered saline (PBS), centrifugated and harvested, while Huaier solution was also added to another 4 wells with 5 μl. After cells were incubated for 0 hr, 12 hrs, 24 hrs and 36 hrs and then rinsed with PBS, centrifugated and harvested.

Copyright@2015 by Cancer Cell Research.

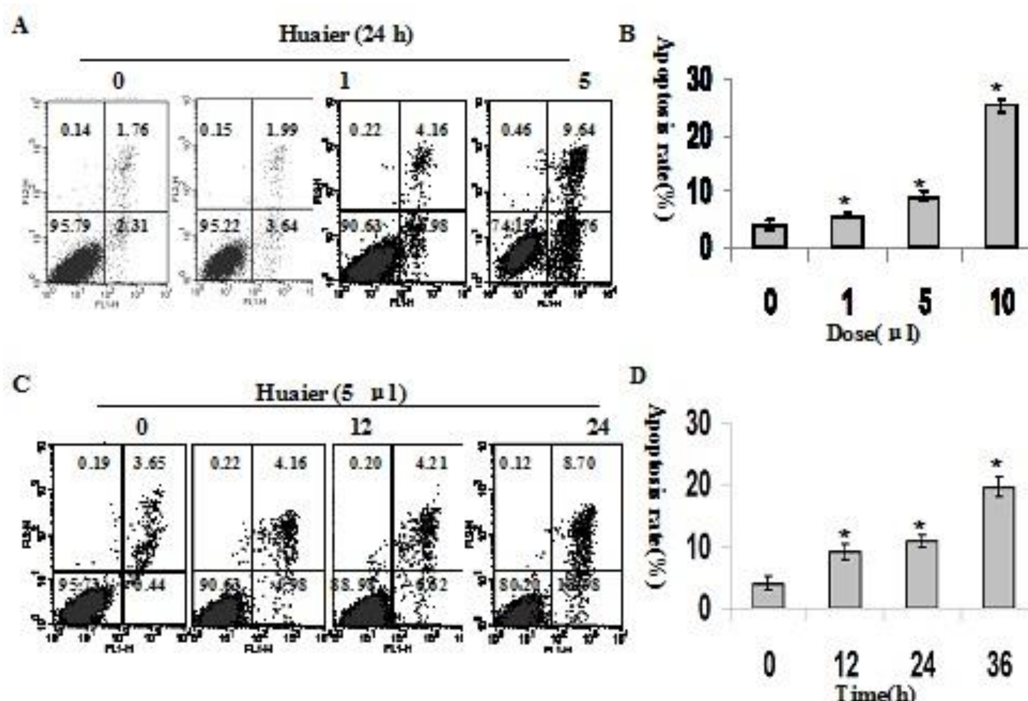

Figure 1. Effect of Huaier on GIST-T1 cells apoptosis with different doses or at different times. (A) and (C) show Annexin V/PI assay was performed to detect apoptosis with different doses or at different times; (B) and (D) display histogram was used to show an increase of apoptosis rates as doses rised or time went by. (* $p<0.05$).

2.3 Flow Cytometric Analysis

Annexin V/PI assay for apoptosis. 5 μl FITC-Annexin V and 10 μl PI were added to harvested fresh cells that had been resuspended in 100 μl cold binding buffer at a density of 106 cells/ml. The cells were then incubated at room temperature in the dark for 30 min, and finally detected by flow cytometry and analyzed with Cellquest software (FACSVantage, Becton Dickinson, USA).

PI assay for cell cycle analysis based on DNA content and distribution. Havested cells were re-suspended in 1ml of 0.1% sodium citrate containing 0.3% NP-40, 0.0002 mg/ml RNase and 50 μg/ml propidium iodide, and then were incubated on ice in the dark for 30min. Cell cycle were analyzed on a FACSort with Cellquest (Becton Dickinson) and ModFit software (Verity Software House, Topsham, ME, USA).

γH2AX for DNA damage. Harvested cells were fixed by 1% formaldehyde in PBS without methanol on ice for 15 min, and rinsed, then centrifugated. The collected cells were fixed by ice-cold 80% ethanol at -200C for at least 24 hrs. These cells were washed with PBS twice and permeated with 0.5% Triton X-100 in PBS on ice for 5 min. After centrifugation, they were incubated overnight at 40C in the presence of primary antibody γH2AX (BD PharMingen; diluted in PBS containing 1% bovine serum albumin (BSA)). The next day cells were rinsed and incubated with the secondary FITC-conjugated antibody (DAKO, Glostrup, Denmark; diluted in PBS containing 1% BSA) for 30 min. Finally, cells were rinsed and resuspended in propidium iodide solution (50 μg/ml PI) and incubated at room temperature for 30 min. Cell fluorescence was measured by a FACSVantage flow cytometry (Becton Dickinson).

2.4 Western Blotting Detection

Harvested cells were re-suspended in lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 2 mM ethyleneglycoltetraacetic acid, 2 mM ethylenediamine-tetraacedtic acid, 1 mM NaF, 0.1 mM vanadate, 0.1 mM phenylmethylsulphonyl fluoride, 2.5 mg/ml leupeptin and 1 mM dithiothreitol) and lysed on ice for 30 min. After centrifugation the collected supernatant protein ran on sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS- PAGE) and were then transferred to nitrocellulose membranes. The membranes were first blocked in 10% dry skim milk (dissolved in PBS plus 0.1% Tween-20) for 1 hr at room temperature, and were then incubated with primary antibodies (diluted to 1:1000 in 10% dry skim milk). Monoclonal and polyclonal primary antibodies: cyclin D3, cyclin E, CDK2, CDK4, CDK6, p16, p21, p27, Bcl-2, Bax and Beta-actin antibodies (BD PharMingen)) overnight at 40C. Next day the membranes were rinsed twice and incubated with the horseradish peroxidase-coupled secondary antibody (diluted to 1:5000 in 10% dry skim milk. Wuhan Boster Biological Technology Ltd., China) at room temperature for 2 hrs. Detection was performed by ECL system (Amersham Pharmacia).

Copyright@2015 by Cancer Cell Research.

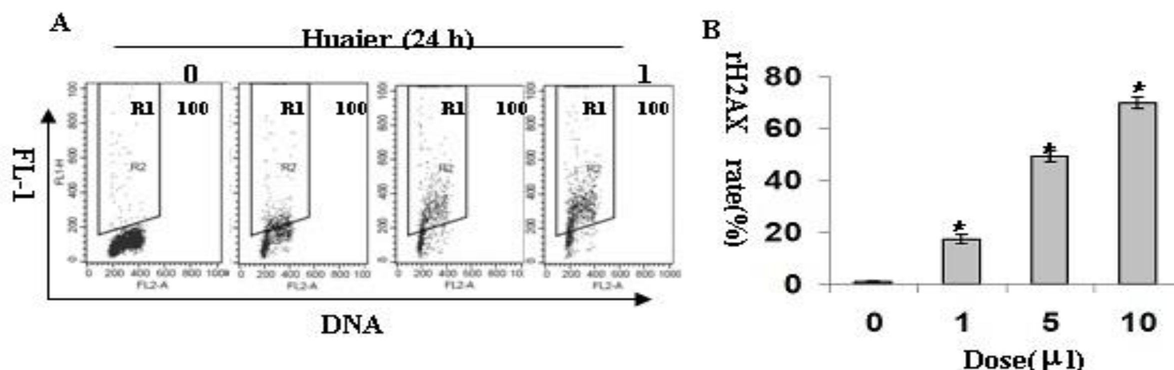

Figure 2. Effect of different doses Huaier on GIST-T1 cell cycle distribution after 24 hrs. (A) PI assay was performed to detect every percentage of G1,S and G2/M. (B) histogram was used to show an increase of G1 percentage and a decrease of G2/M percentage (＊p<0.05).

2.5 Statistical Analysis

The study was repeated thrice. The statistical significance of all results was evaluated by paired Student's t-test. Data are presented with P <0.05 accepted as significance.

3. Results

3.1 Huaier Treatment Induces A Dose- and Time-dependent Apoptosis in GIST-T1

To examine its apoptotic effect on GIST-T1 cells, different dosages of Huaier (0 μl, 1 μl, 5 μl, 10 μl) were treated for 24 hours, and cell apoptosis was determined by Annexin V/PI assay. With the increased dosage of Huaier, the percentage of cell apoptosis also increased from 4.07% to 25.40% (Fig. 1A and B). In addition, apoptosis at different time courses (0 hr, 12 hrs, 24 hrs, 36 hrs) was detected. Similarly, as the time went by, the apoptosis rate increased from 4.09% to 19.68% (Fig. 1C and D).

3.2 Huaier Treatment Induces G1 Cell Cycle Arrest in GIST-T1 Cells

To examine the effect of Huaier on cell proliferation, we analyzed cell cycle distribution of GIST-T1 cells treated at different dosages (0 μl , 1 μl, 5 μl ,10 μl) for 24 hrs. Flow cytometry data showed that cell cycle was arrested in G1 phase, which was indicated by increasing in percentage G1 phase from 52.37% to 58.51% with a dose-dependent manner and concomitant decreasing in the percentage of G2/M cells from 8.22% to 1.50%, whereas the S phase did not change significantly (Fig. 2A &B).

3.3 Detection of DNA Damage by Measuring γH2AX in GIST-T1 Cells Treated by Huaier

To examine the DNA damage induced by Huaier, we measured the expression of γH2AX protein by multiparameter flow cytometric analysis. After GIST-T1 cells were treated with Huaier (0 μl, 1 μl, 5 μl, 10 μl) for 24 hrs, the expression of γH2AX protein increased significantly compared to control treatment (from 0.96% to 69.71%, Fig. 3A and B).

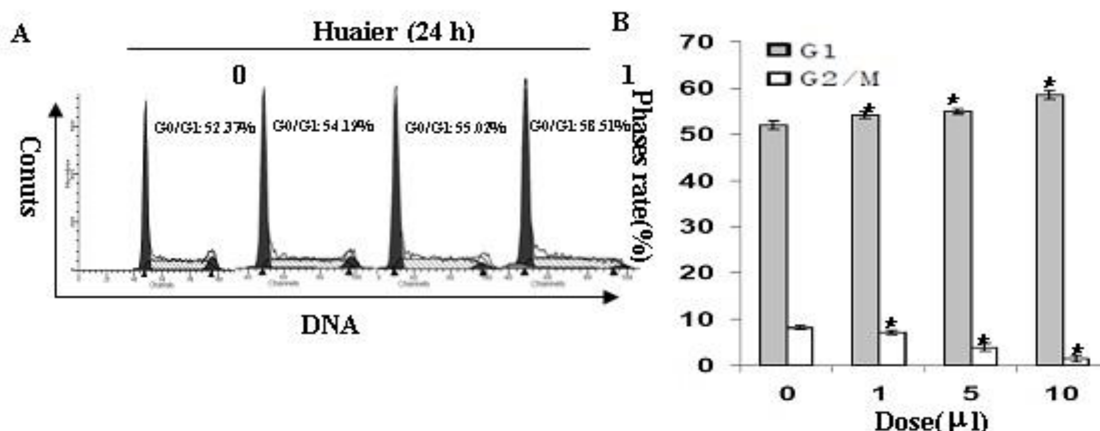

Figure 3. Effect of different doses Huaier on GIST-T1 cells DNA damage after 24 hrs. (A)γH2AX/DNA multiparameter assay was performed to detect the expression of γH2AX foci. (B) histogram was used to show an aggravation of DNA damage degree as doses increased .(＊p<0.05).

Copyright@2015 by Cancer Cell Research.

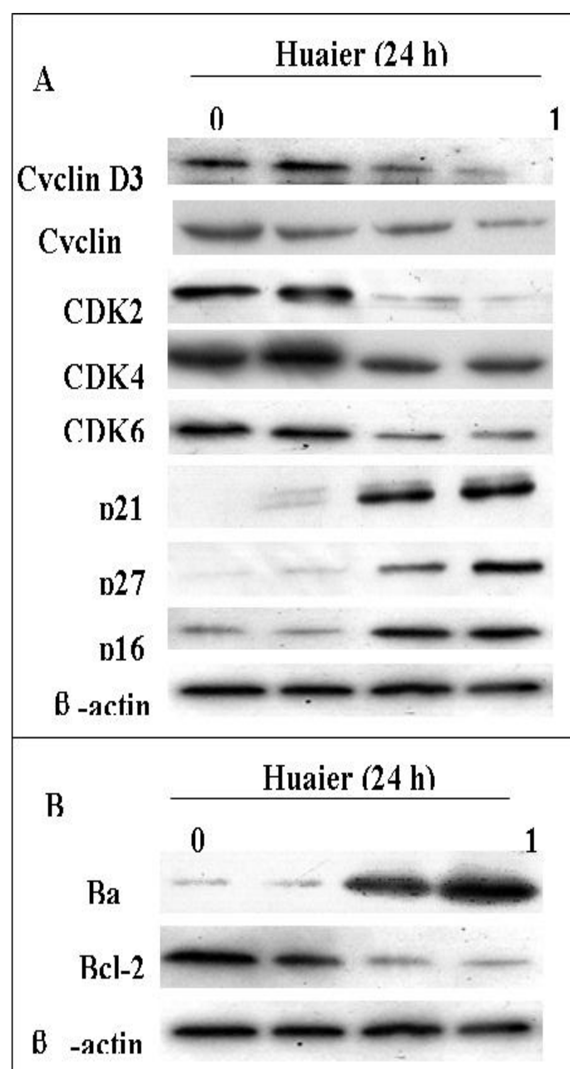

Figure 4. Effect of different doses Huaier on the expression of G1- and apoptosis-associated proteins after 24 hrs (Fig4.A&B).Western blot analysis was performed to detect expression of these proteins.

3.4 Expression of G1-phase and Apoptosis-associated Proteins in GIST-T1 Cells Treated by Huaier

We further determined the molecular mechanism of Huaier in inducing G1 arrest and apoptosis. Expression of G1- and apoptosis-associated proteins in GIST-T1 cells treated with Huaier was detected by Western blotting. Our data showed that Huaier induced a dramatic upregulation of p16, p21 and p27 and downregulation of cyclin D3, cyclin E, CDK2, CDK4 and CDK 6 in a dose-dependent manner (Fig. 4A). The apoptosis-associated Bcl-2 family includes anti-apoptotic (such as Bcl-2) and pro-apoptotic proteins (such as Bax) [9]. Western blotting analysis showed that the expression of Bax protein was significantly increased, whereas the expression of Bcl-2 protein was markedly decreased in the cells treated with Huaier (Fig. 4B). The ratio of Bax/Bcl-2 showed a significant increase, which is believed to induce apoptosis in GIST-T1 cells [10].

4. Discussion

Huaier used to be extracted for the treatment of many kinds of carcinomas in the practice of Chinese traditional medicine (CTM), such as liver cancer [11], breast cancer[12], lung cancer[13]. Besides, we found it have also the same effect on colorectal cell line SW480 and human leukemic cell line HL-60 (data not shown), but better effect on GIST-T1 cells. It is reported that Huaier can induce cells to secrete IFN-α and IFN-γ, then activate NK cells, and finally in turn kill cancer cells[14].

In our study, we showed that Huaier could inhibit cancer cell proliferation by triggering G1 cell cycle arrest and induce cell apoptosis, which provided the *in vitro* evidence for its anti-tumor effect. Huaier-induced DNA Stranded Break (DSB) indicated by γH2AX upregulation was measured by flow cytometry (Fig. 3). It can activate DNA checkpoints including Ataxia telangiectasia mutated (ATM) and/or ATM and Rad 3-related (ATR) [15-17], which inhibits CDKs to arrest cell cycle progression, allowing cells to repair the damaged DNA or removing those cells by inducing apoptosis whose DNA damage can not be repaired. [18-20]. ATM and ATR were shown to activate Chk1 and Chk2 respectively [21-23], and the latter can cause ubiquitin-dependent degradation of protein phosphatase Cdc25A, which inhibits CDK2 [24,25], CDK4 and CDK6 (Fig.5). It was reported that CDK2 phosphorylates FOXO1 at ser^{249} in vivo and inhibit FOXO1 function [26, 27]. FOXO1, as a transcription factor, on the one hand up-regulates p21 [28], p27 [29, 30] and p16; on the other hand, it represses cyclin D3 expression [31, 32]. Our data showed that in Huaier-treated cells, CDK2 was down-regulated; p21, p27, and p16 were up-regulated, as well as cyclin D3 was down-regulated, which is consistent with previous studies. Activated FOXO1 can also trigger a complicated network of mitochondria apoptotic signaling, such as Bax/Bcl-2 pathway [11] activated indirectly by p21, p27, and p16 [28-30,33,34] (Fig.4), and/or death receptor apoptotic signaling, such as FasL/Bim/TRAIL pathway activated directly by FOXO1 [26,27]. Both apoptotic signaling can activate common downstream caspase-3 to trigger apoptosis [Fig. 5].

5. Conclusion

In summary, we have shown that Huaier can effectively inhibit cell proliferation by inducing G1 arrest through the down-regulation of CDK2/CDK4/CDK6, and cyclin D3/cyclin E, together with the up-regulation of CKIs including p16/p21/p27. Apoptosis was accompanied by the G1 cell cycle arrest. To our knowledge, this is the first study that shows the involvement of the cyclins–CDKs–CKIs machinery

Copyright@2015 by Cancer Cell Research.

during the G1 arrest and apoptosis of GIST-T1 cellstreated with Huaier. These findings indicate that Huaier could be a potential therapeutic anti-cancer agent. In our future study, we will further purify the Huaier's monosomy effective ingredient and elucidate the mechanism of Huaier-triggered both mitochondria- and death receptor-mediated apoptotic pathways.

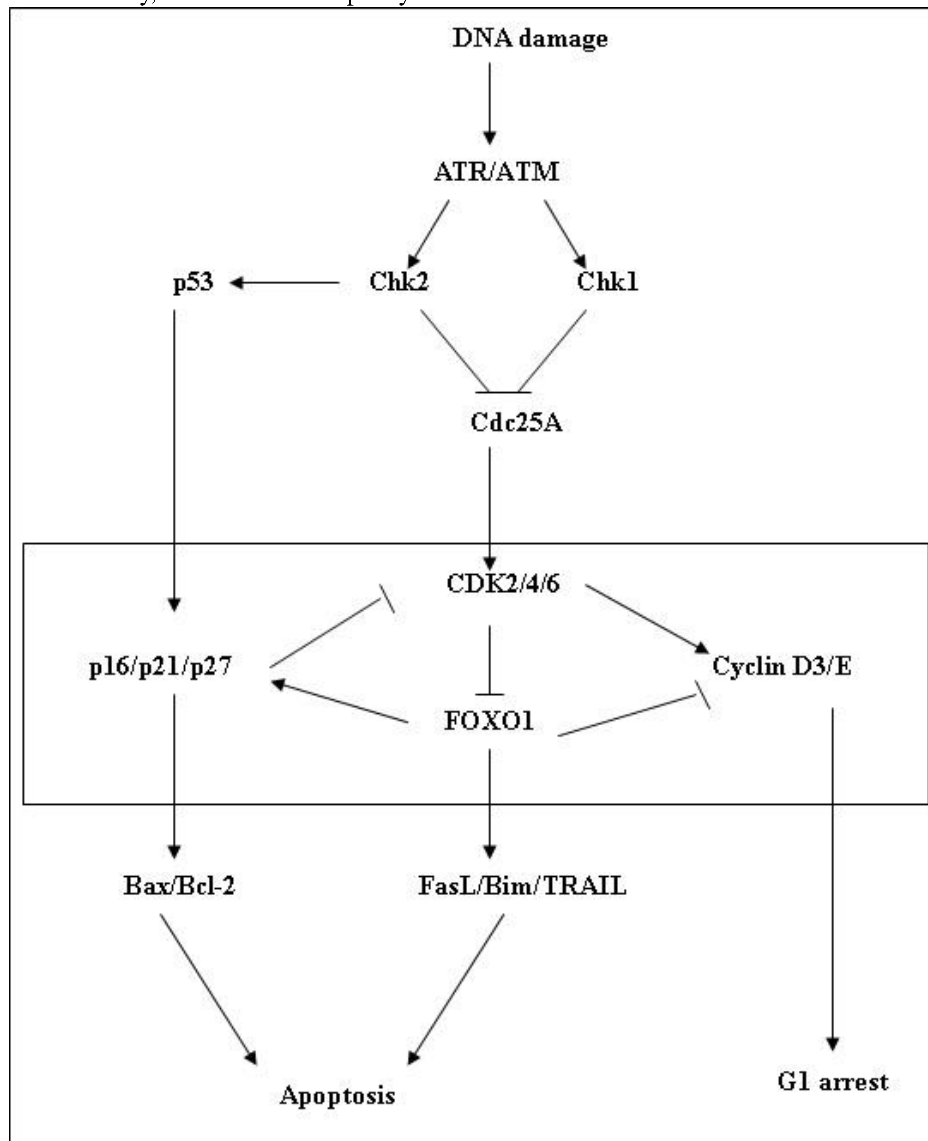

Figure 5. Model of the role for Cyclin-CDK-CKI in the apoptotic response to DNA damage in GIST-T1 cells induced by Huaier.

Conflict of interest

The authors state that they have no conflict of interest about the article.

References

[1] Murray A, Hunt T, The cell cycle: an introduction. In: Murray A ,ed. The cell cycle: an introduction. New York (USA):Oxford University Press: 1993 1-244.
[2] Nurse P, Universal control mechanism regulating onset of M-phase. Nature, 344: 1990 503-508.
[3] Nurse P, A long twentieth century of cell cycle and beyond. Cell, 100: 2000 71-78.
[4] Sherr CJ, and Roberts JM, CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev, 13: 1999 1501-1512.
[5] Hanahan D and Weinberg RA ,The hallmark of cancer. Cell, 100: 2000 57-70.
[6] Sherr CJ. Cancer cell cycle. Science, 274(5293): 1996 1672-1677.
[7] Kamb A. Cell-cycle regulators and cancer. Trends genet, 11(4): 1995 136-140.
[8] YueWei Guo, PeiYuan Cheng, YuJun Chen, et al. Studies on the constituents of polysaceharide from the hyphae of trametes robiniophila (II) identification of polysaccharide from the hyphae of trametes robiniophila and determination of its molar ratio. J China Pharma Univ, 23(3): 1992

Copyright@2015 by Cancer Cell Research.

155-157.
[9] Korsmeyer SJ,Bcl-2 gene family and the regulation of programmed cell death. Cancer Res, 8: 1999 2666-2674.
[10] Cheng EH, Wei MC, Weiler S, et al.Bcl-2, Bcl-X(L) sequester BH3 domain-only molecules preventing Bax- and Bak-mediated mitochondrial apoptosis. Mol Cell, 8: 2001 705-711.
[11] Ren J, Zheng C, Feng G, et al. Inhibitory effect of extract of fungi of Huaier on hepatocellular carcinoma cells. J Huazhong Univ Sci Technolog Med Sci, 29(2): 2009 198-201.
[12] Xu GL, Jia WD, Ma JL, et al. Experimental study of extract of funji of huaier on angiogenesis in vitro. Chin Pharmacol Bull (Chinese), 19(12): 2003 1410-1412.
[13] Huang T, Kong QZ, Lu HD, et al. Experimental study of extract of huaier inducing apoptosis of the human adenocarcinoma of lung A549 cells. Chin J Tuberc Respir Dis (Chinese), 24(8): 2001 487-488.
[14] Chen SB, Ding RN. The effect of huaier fungi on the immune function of mice. Acta Edulis Fungi ,2(1): 1995 21-25.
[15] Huang X, Okafuji M, Traganos F, et al. Assessment of histone H2AX phosphorylation induced by DNA topoisomerase I and II inhibitors topotecan and mitoxantrone and by DNA crosslinking agent cisplatin. Cytometry Part A, 58A: 2004 99-110.
[16] Olive PL. Detection of DNA damage in individual cells by analysis of histone H2AX phosphorylation. Methods cell Biol, 75: 2004 355-373.
[17] Kataoka Y, Bindokas VP, Duggan RC,et al. Flow cytometric analysis of phosphorylated histone H2AX following exposure to ionizing radiation in human microvascular endothelial cells. J Radiat Res, 47: 2006 245-257.
[18] Susumu Noda. Seeking the ultimate nanolaser. Science, 314 (5797): 2006 260-261.
[19] Trenz K, Smith E, Smith S, et al. ATM and ATR promote Mrel 1 dependent restart of collapsed replication forks and prevent accumulation of DNA breaks. EMBO J, 25: 2006 1764-1774.
[20] Jazayeri A, Falck J, Lukas C, et al. ATM- and cell cycle-dependent regulation of ATR in response to double-strand breaks. Nature cell Biol, 8: 2006 37-45.
[21] Shuhei Matsuoka, Mingxia Huang, Stephen J. Elledge. Linkage of ATM to Cell Cycle Regulation by the Chk2 Protein Kinase. Science ,282(5395): 1998 1893-1897.
[22] Cuadrado M, Martinez-pastor B, Fernandez-Capetillo O. ATR activation in response to ionizing radiation: Still ATM territory. Cell Division, 1: 2006 7.
[23] Ward IM, Minn K, Chen J. UV-induced ataxia-telangiectasia-mutated and Rad3-related (ATR) activation requires replication stress. J Biol Chem, 279: 2004 9677-9680.
[24] Mailand N, Falck J, Lukas C, et al. Rapid destruction of human Cdc25A in response to DNA damage. Science, 288: 2000 1425-1429.
[25] Falck J, Mailand N, Syliuasen RG, et al. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresisitant DNA synthesis. Nature, 410: 2001 842-847.
[26] Haojie Huang, Kevin MR, Zhenkun Lou, et al. CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. Science, 314: 2006 294-297.
[27] Haojie Huang, Donald J. Tindall. CDK2 and FOXO1, a fork in the road for cell fate decisions. Cell cycle, 6(8): 2007 902-906.
[28] Seoane J, Le HV, Shen L, et al. Integration of Smad and Forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell, 117: 2004 211-223.
[29] Medema RH, Kops GJ, Bos JL, et al. AFX-like forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through P27kip1 . Nature, 404: 2000 782-787.
[30] Nakamura N, Ramaswamy S, Vazquez F, et al. Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. Mol Cell Biol, 20: 2000 8969-8982.
[31] Ramaswamy S, Nakamura N, Sansal I, et al. A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. Cancer cell, 2: 2002 81-91.
[32] Schmidt M, Fernandez de mattos S, Van der Horst A, et al. Cell cycle inhibition by FOXO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol, 22: 2002 7842-7852.
[33] StahI M, Dijkers PF, Kops GJPL, et al. The forkhead transcription factor FoxO regulates transcription of P27 Kip1 and Bim in response to IL-2. J Immuno, 168: 2002 5024-5031.
[34] Gil-Gomez G, Berns A, Brady HJ. A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis. The EMBO J, 17(24): 1998 7209-7218.

Copyright@2015 by Cancer Cell Research.