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*Research Paper*

# siRNA of ADAM17 gene induces apoptosis, proliferation inhibition and enhances the effects of genistein on HepG2 cells

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## Abstract

**Objective:**To investigate the effects of siRNA of ADAM17 gene and genistein on apoptosis and the inhibition of proliferation in HepG2 cells in an attempt to seek an effective therapy for hepatocellular carinoma. **Methods:**Cells were divided into control groups and experimental groups and siRNA was used to silence the ADAM17 gene, alone and in combination with genistein. Cells were harvested at several time periods and assessed for proliferation and apoptosis. Proliferation was assayed by MTT at 24, 48, 72 and 96 hours following treatment and apoptosis was assessed by flow cytometric analysis at 48 hours. **Results:**In siRNA groups, proliferation of cells was significantly inhibited compared to the control groups at 24, 48 and 72 hours($P < 0.05$), and apoptosis was significantly increased at 48 hours($P < 0.01$); In genistein groups, proliferation was inhibited at 24, 48, 72 and 96 hours, and the apoptosis ratio was significantly increased at 48 hours($P < 0.01$); while in the groups that received the combination of siRNA transfection and genistein treatment, there was a further significant decrease of proliferation and increase in apoptosis compared with either treatment alone. **Conclusion:**The ADAM17 gene could be an effective target, and genistein could be a useful agent, in the treatment of hepatocellular carcinoma. siRNA of ADAM17 gene and genistein both inhibited HepG2 cells proliferation and promoted apoptosis, and further, the combination of these treatments had a greater effect than either treatment alone.

*Keywords*: hepatocellular carcinoma; HepG2 cell; ADAM17; siRNA; genistein; proliferation; apoptosis

## INTRODUCTION

ADAM17, a member of ADAMs(a distintegrin and metalloproteinase superfamily), also known as tumor necrosis factor-α (TNF-α) converting enzyme(TACE), is involved in the shedding of other biological surface molecules, converting precursor proteins to active proteins[1]. Of these cell membrane molecules, EGFR has received considerable attention. EGFR is overexpressed in normal and transformed hepatocytes and is believed to mediate essential mitogenic and survival signals in transformed cells, including hepatocellular carcinoma cells[2-3]. Another study showed that the EGFR/TPK-I (ErbB) signaling pathway took part in the processes of cell proliferation, metastasis, differentiation and survival[4]. Unrestrained proliferation means that resistance to apoptosis has occurred. ADAM17 has been shown to play an important role in the transactivation of EGFR[5]. Based on the above information, we hypothesize that ADAM17 may change the survival of tumor cells by changing the activity of EGFR, and further regulate apoptosis and proliferation.

Genistein is a phytoestrogen derived from soybean, which was recognized by the National Cancer Institute (USA) as having an anti-cancer effect[6]. Genistein can induce programmed cell death and inhibit the growth in different cancers[7]. The processes involve the regulation of apoptosis, proliferation and signal transduction pathways. Genistein is a specific inhibitor of EGFR/TPK[8].

Hepatocellular carcinoma is one of the most common cancer-associated causes of death, with about half of the new patients occurring in China[9]. Genetic thera-

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pies have become promising in treating cancer, especially with the discovery of the siRNA(small interfering RNA) technique, which has opened an innovative avenue of therapeutics. The siRNA duplexes(the double-stranded RNA is homologous in sequence to the silenced gene), synthesized of 21~22 nucleotides(nt), when transfected into mammalian cells, can knock down the expression of target genes by specific post-transcriptional gene silencing[10]. Though various carcinomas have been studied using siRNA, there are few studies on hepatocarcinoma in which the ADAM17 gene has been targeted. In this study, we applied siRNA of the ADAM17 gene, alone and in combination with genistein, to HepG2 cells, and then investigated their effects on apoptosis and proliferation, in an attempt to find a novel therapy for hepatocellular carcinoma.

## MATERIALS AND METHODS

### Reagents

The HepG2 cell line was a gift from the laboratory of the Infection Department of the First Affiliated Hospital of Xi'an Jiaotong University(Xi'an, China). PRIM-1640, trypsin, fetal bovine serum(FBS) were purchased from Hyclone(Logan,UT,USA). Lipofectamine 2000 was purchased from Invitrogen(Carlsbad, CA,USA), and culture plates were from Costar Corporation. The Annexin V-fluorescein-isothiocyanate (FITC) kit was purchased from Jingmei Biotech (Shenzhen,China); propidium iodide(PI), dimethylsulfoxide(DMSO) and 3(4, 5- dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium-bromide(MTT) were from Sigma Chemical Co. (St. Louis, MO, USA). Genistein was purchased from Biyuntian Biotech Corporation (Haimen City, Jiangsu Province, China).

The siRNA targeting to ADAM17 and negative siRNA(siRNA-NC) and FAM-labeled siRNA-NC were chemically synthesized by Shanghai Jima Biological Technique Corporation(SJBTC, China). The sequence of siRNA was as follows:sense 5′-GAGGAAGC AUCUAAAGUUUUU-3′; antisense 5′-AGAGUC AGUGAUCUUGUACTT-3′, selected from the article of Ali and Knäuper[11]; Sequece of siRNA-NC was a random sequence as follows: sense 5′-UUCUCC GAACGUGUCACGUTT-3′; antisense 5′-ACG UGACACGUUCGGAGAATT-3′, designed by SJBTC, which targets none of the mammalian genes.

### Cell culture and cell groups

Cells were cultured in PRIM-1640 medium(pH 7.3) supplemented with 10% FBS, grown at 37℃ under a 5% $CO_2$ atmosphere. Culture media were changed every 2 days. When cells reached confluence, they were dissociated with 0.05% trypsin-0.02% ethylenediamine tetraacetic acid(EDTA) and replated at a 1:3~1:4 dilution.

Before being transfected with siRNA or treated with genistein, cells were divided into groups as following: ① blank group; ② mock transfection group (mock); ③ siRNA negative transfection group(siRNA-NC); ④ siRNA of TACE transfection group(siRNA); ⑤ genistein group; ⑥ genistein + siRNA transfection group. The former three groups were control groups and the latter three were experimental groups. Each group was sampled and measured at four time points: 24, 48, 72 and 96 hours.

### Interfered with siRNA and genistein

#### Evaluation of transfection efficiency

Transfection with siRNA was carried out using lipofectamine 2000 following the manufacturer's instructions. In order to choose the optimal transfection efficiency, FAM-labeled siRNA-NC was transfected into HepG2 cells at serial concentrations. The successfully transfected cells were assessed by fluorescent microscopy. When the optimal transfection efficiency was identified, we adopted the same parameters for all subsequent experiments.

#### siRNA preparation and transfection

The HepG2 cells were cultured in PRIM-1640 medium with 10% FBS, then the appropriate density of cells was chosen to transfect siRNA. Before the transfection, medium was changed, and new PRIM-1640 medium without FBS and antibiotics was added. The optimal transfection method was the same as described above. Six hours after transfection, the medium was changed to new medium with 10% FBS. The medium was changed every 2 days thereafter.

#### Usage of genistein

Genistein was dissolved in 0.1%(v/v) DMSO to make a 25 mmol/L genistein stock solution from which dilutions were made for each experiment. Genistein has been shown to inhibit proliferation and induce apoptosis in HepG2 cells at a concentration of 16 μmol/L[12]. Therefore, we used a concentration 16 μmol/L in our experiment.

#### MTT assay

The proliferation inhibition of HepG2 cells was determined using the MTT assay, which assessed the number of cells based on the reduction of MTT by the mitochondrial dehydrogenases present in viable cells. First, cells were seeded in 96-well culture plates at a density about $1\sim2\times10^3$ cells/well in 200 μl medium. Ten wells of each group were used in repeated experiments. After 24 h post-attachment, cells were transfected and treated with genistein. At the appropriate times, 20 μl of MTT(2 mg/ml) was added to each

well and the cells were incubated at 37℃ for 4 hours. The supernatants were removed and the formazan crystals were dissolved by adding 150 μL DMSO. The plates were then checked on a microplate reader at 490 nm. The inhibiting ratio=(1-$A_{\text{value of experiment group}}/A_{\text{value of control group}}$) × 100%.

### Detection of apoptosis

Apoptosis of HepG2 cells was detected by the dual-color flow cytometric(FCM) technique following the manufacturer's instruction. Cells were first seeded at $1.5 \times 10^5$ per well in 6-well plates and transfected as decribed previously. At 48 hours cells were harvested with trypsin, washed twice with cold PBS, and resuspended in 0.5 ml binding buffer. Then, cells were labeled with Annexin-V and PI for 15 min at room temperature away from light, and another 400 μl binding buffer was added to the cell suspension. Finally, cells were analysed on the FACSCalibur flow cytometer (Becton Dickinson Corporation, USA).

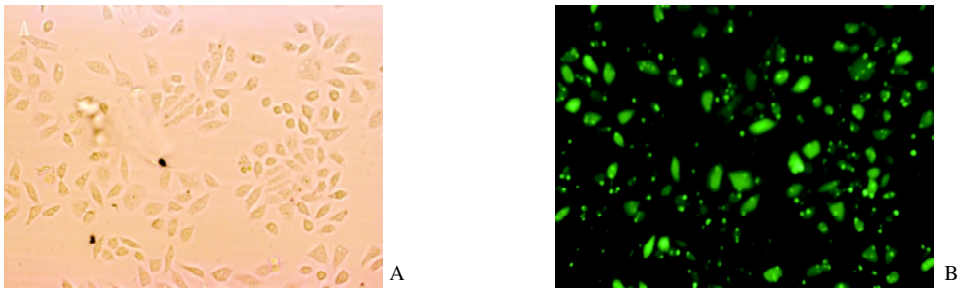
### Statistical analysis

Data are presented as Means ± SD from three independent experiments, except where indicated, and analysed by ANOVA. *P* values of less than 0.05 were taken to be statistical significant. All analyses were performed using SPSS 15.0 statistical software(SPSS, Chicago, IL, USA).

## RESULTS

### Identification of the optimal transfection efficiency with siRNA in HepG2 cells

FAM-labeled siRNA-NC was transfected at serial concentrations after 6 hours, and cells were observed by fluorescent microscopy. The successfully transfected cells were stained with FAM and the transfection efficiency was analysed by the ratio of stained cells to total cells. The optimal transfection efficiencies obtained were near 100%(***Fig. 1***). The optimal concentration of FAM-labeled siRNA-NC was 64 nmol/L, and this transfected concentration and method were used in the following experiments.



A B

The left panel(A) shows the field of view under transmitted light, while the right panel(B) shows the fluorescent image of the same field

***Fig. 1*** Assessment of optimal transfection efficiency

### Inhibition of proliferation

The MTT assay indicated that the siRNA significantly inhibited proliferation at 48 and 72 hours compared to control groups($P < 0.05$), but the inhibiting ratio decreased and the number of viable cells increased rapidly at 96 hours. Genistein significantly inhibited proliferation from 24 to 96 hours($P < 0.05$), and the inhibition of proliferation was greater in the siRNA and genistein groups compared to control groups($P < 0.01$) at all time periods, and differed from the siRNA-interfered group or the genistein-treated group($P < 0.05$) at 48 to 72 hours(***Fig. 2***).

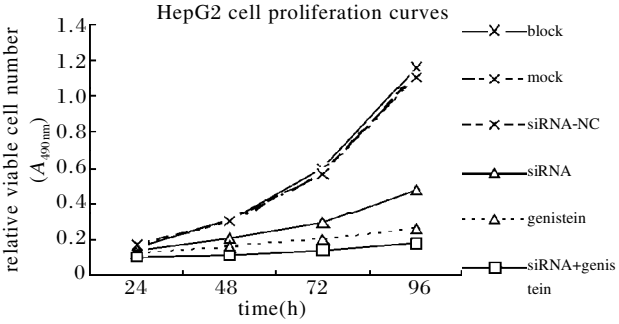
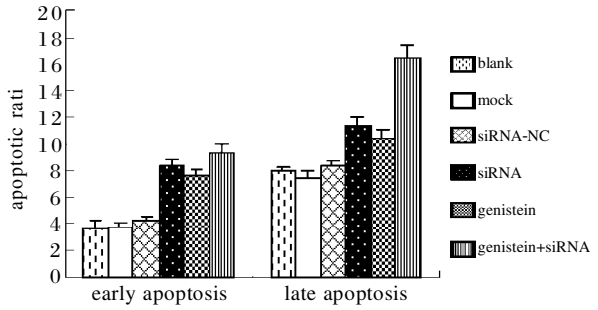



***Fig. 2*** Cell proliferation curves of HepG2 cells

### Induction of apoptosis

Flow cytometric analysis was performed to determine the induction of apoptosis and assess the number of apoptotic cells at 48 hours after intervention by siRNA and genistein. The apoptotic ratios induced by siRNA or genistein were significantly different from control groups($P < 0.01$). Furthermore, both early apoptotic ratios(Annexin V positive and PI negative cells) and late apoptotic ratios(Annexin V positive and PI positive cells) were significantly different($P < 0.01$). Results indicated that the effects of both siRNA and genistein were statistically significant($P < 0.01$) and the combination of siRNA with genistein had a greater effect than either treatment alone(early apoptosis, $P < 0.05$; late apoptosis, $P < 0.01$). (***Fig. 3*** includes the statistical results; ***Fig. 4*** shows representative dot plots of different groups).



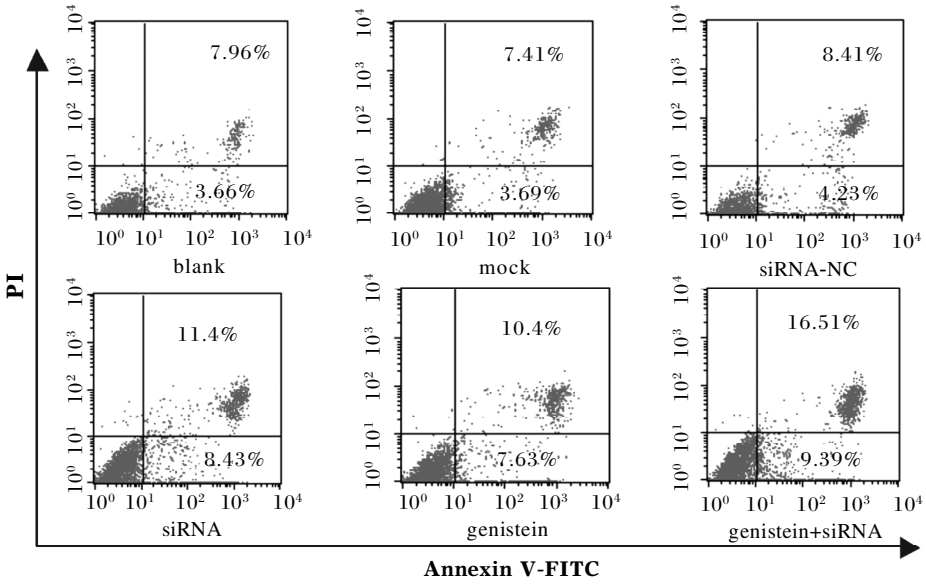

Experimental groups exhibited greater number of apoptotic cells than control groups($P < 0.01$) on both early apoptosis and late apoptosis. Differences between the siRNA group, the genistein group and the combined treatment group were statistically significant in both early and late apoptosis.

***Fig. 3*** Apoptosis in different groups of HepG2 cells at 48 hours after intervention

## DISCUSSION

Unrestrained proliferation of malignant tumor cells involves many aspects of a tumor's characteristics, including cells proliferation, differentiation, remodeling of the extracellular matrix, a reduction of cell adhesion, and an increase of cell motility, even an ability to promote angiogenesis. However, resistance to apoptosis may be an underlying basis for tumorigenesis and tumor progression[13]. Intervention at each step of these aspects or interfering with the function of critical genes closely related to these aspects may change the status of tumor cell proliferation, apoptosis, and therefore tumorigenesis.

ADAM17 is an important gene and its protein can promote the shedding of many precursor proteins, such




Representative dot plots show early apotosis(LR, Annexin $V^+/PI^-$), late apoptosis(UR, Annexin $V^+/PI^+$), necrotic cells(UL, Annexin $V^-/PI^+$), and normal cells(LL, Annexin $V^-/PI^-$) by flow cytometric analysis.

***Fig. 4*** Induction of apoptosis in HepG2 cells at 48 hours after interference by siRNA and genistein, alone and in combination

as growth factors and growth factor receptors on the cell surface[1]. As a result of the intercellular communications mediated by ADAM17, the motility of signaling pathway change in many different ways through interaction between ligands and receptors, such as hepatocyte growth factor receptor Met[14], and colony stimulating factor-1 receptor[15]. Some research has shown that ADAM17 is overexpressed in most malignant tumors and some chronic diseases, including hepatocarcinomas[16]. Accordingly, silencing ADAM17 inhibited several features in human renal carcinoma cell lines, including growth autonomy, tumor inflammation, and tissue invasion[17]. Because of its extensive roles, ADAM17 is considered as a critical gene in invasion and metastasis of malignant tumors and as a novel therapeutic target in cancer[18]. In the present study, siRNA of ADAM17 induced apoptosis and inhibited proliferation in HepG2 cells. At 48 hours post-treatment, these effects of siRNA were statistically significant. The results hinted at the possibility that ADAM17 might be a genetic target or molecular target to treat hepatocellular carcinomas.

Genistein, is a major constituent of soybean-based health foods, and a blocking agent of TPK at high concentrations(>10 μmol/L)[8]. It is also a promising chemopreventive or chemotherapeutic agent[12]. In a study of the effects of genistein on human uterine leiomyoma(UtLM) cells showed that low concentrations(≤1 μg/ml) stimulated proliferation while higher concentrations(≥10 μg/ml) inhibited proliferation and induced apoptosis[19]. In our experiment, we adopted the concentration 16 μmol/L(about 4.3 μg/L) of

genistein, which both induced apoptosis and inhibited proliferation of HepG2 cells. Our results therefore supported the findings of others, and suggested that genistein at a concentration of 16 μmol/L might be generally effective in inhibiting proliferation and inducing apoptosis in tumor cells.

Apoptosis involves several different signaling pathways and different check points in the cell cycle, and cells will arrest at different phases of the cell cycle after intervention by corresponding stimulating factors[20]. Our study confirmed once more that the TPK signaling pathway is closely related to the apoptotic signaling pathways. However the details of its mechanism of action are yet to be clearly elucidated.

Although both siRNA of ADAM17 and genistein were effective in inducing apoptosis and inhibiting proliferation in HepG2 cells, the use of genistein, especially at high concentrations, may have some side effects. One example may be that genistein can inhibit the function of thyroid peroxidase, although no obvious reduction of thyroid hormone levels occurred when rats were fed genistein[21]. Combining the siRNA method used here with genistein could enhance the effectiveness of genistein without increasing its side effects. Our study showed that siRNA silencing of ADAM17 could enhance the effects of genistein on the induction of apoptosis and the inhibition of proliferation. Two possible reasons could explain this enhancement. One is that the siRNA of ADAM17 reduces the TPK signal in the EGFR/TPK pathway in coordination with TPK signal inhibition by genistein, and the other is that it attenuates the proliferation roles of other receptors, other than the EGFR/TPK pathway.

In conclusion, silencing ADAM17 by siRNA can significantly induce apoptosis and inhibit proliferation in HepG2 cells, as does genistein. The ADAM17 gene is therefore a potential target for genetic therapy. Combining siRNA of ADAM17 with genistein treatment can enhance the effects of genistein, perhaps without increasing the side effects of the latter. Further *in vivo* and clinical studies are required to confirm the safety and efficacy of this combination of treatments in hepatocellular and other carcinomas.

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