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Correction of malignant behavior of tumor cells by traditional Chinese herb medicine through a restoration of p53

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Abstract

We have previously demonstrated that a UVC-induced tumorigenic HeLa x skin fibroblast cell line could be induced to form a more normal phenotypic state ('reversion'), including loss of IAP expression. We have now used the loss of IAP expression to monitor the enhancement of this reversion in the cervical cancer cell line, HeLa, by a traditional Chinese herb medicine (TCM), Yigan Kang (YGK). IAP level decreased, and the reversion frequency increased, in a dose-dependent manner at concentrations of YGK of more than 10 mg. YGK significantly repressed E6/E7 oncogenes at the transcriptional level, with subsequent reactivation of p53 and p21 expression ($P<0.01$). YGK had little effect on the cell cycle of HeLa cells and slightly increased the apoptotic cell death between 20 and 40 mg. In vivo, tumorigenicity studies were performed using six different animal experimental protocols, which demonstrated that YGK was effective at inducing reversion of the tumorigenic phenotype, with YGK-treated HeLa cells showing much less aggressive tumor growth than untreated cells. YGK may raise the possibility of the continuing management of some cancers as a chronic condition in which the malignant behavior of the tumor cells is constrained.

Keywords: Cancer reversion; TCM; IAP; p53; HPV; Yigan Kang

1. Introduction

Neoplastic transformation of normal cells can occur spontaneously or can be induced by viruses, chemical carcinogens, or irradiation. Such transformed cells, however, can also spontaneously, or be

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induced to, lose some of their tumorigenic characteristics and revert back to a more normal phenotypic state through correction of malignant behavior by microenvironmental cues [1–5]. It has been suggested that the frequency of cell reversion to the non-tumorigenic phenotype is much higher than re-reversion to the tumorigenic phenotype [3], and that the reversion is paralleled by a regulation in malignant behavior and tumorigenicity [6]. Such reversion may be a consequence of both genetic and epigenetic processes. One of the most challenging goals of cancer therapy is to exploit the potential of such reversion. Meeting this goal requires the identification of agent(s) with the ability to arrest tumor growth while having minimal effect on normal tissue.

The globalization of TCM has resulted in its practice, in one form or another, by more than 300,000 practitioners in over 140 countries [7]. They have demonstrated potent anti-cancer properties [8], inhibited virus activity [9,10], and functioned as a biological response modifiers [11]. Most studies of TCM to date have emphasized endpoints such as cell cycle arrest and cell-killing, including apoptosis [8,12]. The ability to reverse transformed cells to more normal phenotypic state by TCM is largely unknown. Yigan Kang (YGK), which consists of 15 crude ingredients extracted from herbs, has been used for liver diseases and chronic hepatitis in Taiwan and China and reported to inhibit transmissible gastroenteritis virus (TGEV) infectivity [13]. It can also directly inhibit HBsAg production by Hep3B and HepA2 cells in a dose-dependent manner. Recently, YGK was approved by the US Food and Drug Administration (FDA) phase II clinical trials for the treatment of hepatitis B and non-small cell lung cancer [14].

The expression of the HeLa tumor-associated marker, intestinal alkaline phosphatase (IAP), has been shown to be necessary but not sufficient to confer the tumorigenic phenotype on HeLa x skin fibroblast human hybrid cells [15–19]. We have previously used the loss of IAP activity and expression to investigate the reversion of a UVC-induced tumorigenic HeLa x skin fibroblast human hybrid cell line to the non-tumorigenic phenotype [4]. In this paper, we describe for the first time an interesting series of observations on the reversion of tumorigenic HeLa cervical carcinoma cells by YGK to a much less aggressive tumorigenic phenotype. This reversion was associated with the down-regulation of E6 and E7 oncoproteins at the transcriptional level and subsequent reactivation of p53.

2. Materials and methods

2.1. Drugs

The major ingredients of YGK herbal composition are diffuse hedyotis, bistort rhizome, giant knotweed rhizome, Asiatic moonseed rhizome, baical skullcap root, bovine biliary powder, milkvetch root, barbary wolfberry fruit, sanqi, red ginseng, figwort root, Chinese magnoliavine fruit, turmeric root-tuber, hawthorn fruit, and Chinese angelica [13]. The herbal solution contains 0.4 g of crude herbs in each milliliter ddH_2O. Final concentrations used in these experiments were obtained by diluting the stock solution in culture medium.

2.2. Cell cultures

HeLa 0400 is a highly tumorigenic subclone derived from a HeLa cervical carcinoma cell line. CGL1, used in all experiments as a control for detailed quantitative studies of reversion, is a non-tumorigenic hybrid cell line and expresses IAP at very low levels [4,20]. Despite expressing the E6 oncogene, CGL1's p53 gene was still functional [21,22]. No antibiotics were used in routine cultures and in all experiments.

2.3. Cell viability assay

Inhibition concentration 50% (IC50) for cell viability of YGK was measured by MTT assay based on the ability of live cells to convert tetrazolium salt into purple formazan [8]. Additional clonogenic survival assays were performed for cytotoxicity induced by the concentrations used in treating the cells prior to injecting them for tumor growth studies. Cells were seeded into triplicate 6 cm dishes with medium containing YGK at concentrations ranging from 10 to 60 mg/ml for 24 h. After 10–14 days, the colonies were fixed and stained with crystal violet.

2.4. Intestinal alkaline phosphatase activity assay

Intestinal alkaline phosphatase (IAP) activity was quantitated by a standard colorimetric assay kit (Sigma, St Louis, USA) previously described [4,17,23]. In brief, confluent cells were pretreated with various concentrations of YGK for 24 h. The pretreated cells were seeded in 24-well plates, incubated for 4 h, and then washed twice with PBS. After adding 0.6 ml of *p*-nitrophenyl phosphate (16 mM) (Sigma, St Louis, USA) to each well, 100 μl of supernatant was sampled for optical density measured at 405 nm at time intervals of 10, 20, 30, 40, 50, and 60 min.

2.5. Reversion frequency assay

Clonogenic survival and IAP activity were detected by crystal violet (CV, Fisher Scientific, USA) and western blue (WB, Promega, Madison, USA) staining. A monolayer of cells pretreated with various doses of YGK for 24 h was harvested. Five-hundred pretreated cells were seeded in triplicate. After incubation for 10 days, these dishes were washed twice with PBS, fixed with 2% paraformaldehyde for 5 min, and stained with 5 ml of WB for 3 min. The blue-stained colonies were counted as IAP positive and thus tumorigenic. After wash, the dishes were then re-stained with 3 ml of CV for 3 min and the purple-stained colonies were counted as total number of colonies. The frequency of reversion to the non-tumorigenic phenotype is thus expressed by:

$$\text{Reversion frequency} = \frac{\text{CV stained colonies} - \text{WB stained colonies}}{\text{CV stained colonies}} \times 100\%$$

2.6. Semi-quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted using Trizol (GIBCO BRL, CA, USA), after which the first strand complementary DNA (cDNA) was synthesized using Super script III reverse transcriptase (Invitrogen, CA, USA). 2.5 μl of each cDNA product was amplified with the following primers. For E6 oncoprotein: sense, 5′-ATggCgCgCTTTgAggATCC; anti-sense, 5′-TTATACTTgTgTTTCTCTgC. The thermal cycle was set at 94 °C for 5 min; then 30 cycles of 94 °C 1 min, 45 °C 45 s and 72 °C 1 min; followed by 72 °C for 5 min. For E7 oncoprotein: sense, 5′-ATgCATggACCTAAggCAAC; anti-sense, 5′-TTACTgCTgggATgCACACC. The thermal cycle was set at 94 °C for 5 min; then 30 cycles of 94 °C 1 min, 52 °C 45 s and 72 °C 1 min; followed by 72 °C for 5 min.

2.7. Western blot

HeLa cells were incubated for 24, 48, or 72 h with or without the YGK. The immunoblots were developed using ECL (Amersham, NJ, USA) following the manufacture's instructions. The primary Abs used were specific for E6 (clone C1P5, Santa Cruz, CA, USA), E7 (clone N-19, Santa Cruz, CA, USA), p53 (clone DO-1, Santa Cruz, CA, USA), pRB (clone G3-245, Pharmingen, CA, USA), p21 (clone Ab-1, Oncogene Research, CA, USA) and actin (clone C-11, Santa Cruz, CA, USA). The densitometric analysis of the bands was performed using TotalLab software.

2.8. Flow cytometry

HeLa 0400 cells in exponential growth were treated with YGK at the indicated doses for 24, 48, or 72 h and stained with propidium iodide (PI) solution (15 μg/ml PI + 2.5 μg/ml RNase A; Sigma, CA, USA). The appearance of phosphatidylserine in the extracellular membrane was evaluated with annexin-V derivatized with fluorescein (Roche, GmbH, Germany). Stained cells were determined by FACScan flow cytometer (Becton Dickinson, CA, USA) and analyzed by ModFit software (Becton Dickinson, CA, USA).

2.9. Tumorigenicity testing

The Institutional Animal Care and Use Committee of Taipei Medical University approved the animal experiment protocol. The tumorigenicity of cells was tested by injecting 2.5×10^6 cells in 0.25 ml per site subcutaneously (s.c.) into the right flank of NOD-SCID mice. Six mice were injected for each of six approaches to determine the efficiency of YGK on the induction of cancer reversion (Fig. 1). In approach

Approach 1 : Inject HeLa0400 as positive control.
Approach 2 : Inject HeLa0400 cell pretreated with YGK for 24hr.
Approach 3 : Inject YGK to the tumors with size 0.5cm^3 by intratumor injection.
Approach 4 : Inject YGK and HeLa0400 simultaneously.

YGK

1, the highly tumorigenic HeLa 0400 were injected as a positive control for tumor formation; and in approach 6, the non-tumorigenic CGL1 were injected as a negative control for tumor reversion. In approach 2, HeLa 0400 cells were pretreated in vitro with YGK in two doses of 10 and 40 mg, respectively, for 24 h. After wash with PBS, the cells were s.c. injected into NOD-SCID mice (YGK $IC_{50}=60$ mg/ml). As for approach 3, HeLa 0400 cells were first s.c. injected into NOD-SCID mice. When the tumor size reached 0.5 cm^3, three doses of YGK (5, 10, and 20 mg) were administrated by intratumor injection (i.t.) (YGK $LD_{50}=22.5$ g/kg). For approach 4, three doses of YGK (5, 10, and 20 mg) were s.c. injected simultaneously with HeLa 0400 into the same site of NOD-SCID mice. In approach 5, to mimic the practical condition of routine administration, two doses of YGK (10 and 20 mg) were s.c. injected into NOD-SCID mice and 24 h later, HeLa 0400 were then injected into the same site. Within the following 4 weeks, the respective dosages of YGK were routinely administered on a weekly basis. The volume of tumor was examined weekly. The volumes were then calculated using the equation: $(1/2\times\text{length}\times\text{width}^2)$ [3]. Growth inhibition (GI), expressed as a percentage, was then calculated by the following equation which takes into account both the tumor volume of treated compared to untreated cells as well as the fraction of injected sites that grew tumors:

$$\%\text{GI} = \left(1 - \frac{\text{MTV}\times F}{\text{MTV}^{\text{P}}\times F^{\text{P}}}\right)\times 100\%$$

MTV mean tumor volume
F fraction of injected sites with tumors
MTV^{P} mean tumor volume of positive controls
F^{P} fraction of injected sites with tumors in positive controls

2.10. Statistical analysis

Quantitative differences between groups were statistically examined by one-way analysis of variance. The means of groups with significant differences were compared by the Poisson distribution method. Correlation coefficients were further examined with the Student's t-test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of YGK on cell proliferation

The growth-inhibiting effect of YGK on HeLa 0400 cells as determined by the MTT assay was dose-dependent (Fig. 2A). IC_{50} values at 24, 48 and 72 h exposure times were all around 60 mg/ml. At lower doses of 5 and 10 mg YGK treatment, a minimal growth induction effect was seen, even at 48 h exposure time. These results were supported by clonogenic survival data, where the colony forming efficiency (CFE) % of HeLa0400 following treatment with 60 mg/ml YGK for 24 h was $55.01\pm0.4\%$ ($n=3$; Fig. 2B).

3.2. Intestinal alkaline phosphatase activity assay

The effects of YGK at low dosages of 5, 10, and 20 mg on IAP activity in HeLa 0400 cells followed a similar trend to that of the control group, prior to 50 min. However, an increase in IAP activity was observed after 50 min for dosage levels of 5 and 10 mg (Fig. 3A). IAP activities of HeLa 0400, when treated with 40 and 80 mg of YGK, decreased to 50% in 30 min and continued decreasing to 30% by 60 min, which was five times greater than that of CGL1 (5.95%).

3.3. Reversion frequency of HeLa 0400 by YGK in vitro

The increased reversion frequency of HeLa 0400 by YGK in vitro was dose-dependent (Fig. 3B). The curve increased rapidly in reversion frequency at lower doses. At 40 mg YGK treatment, the reversion frequency of HeLa 0400 reached a plateau at almost 90% of the possible maximum. CGL1, as a non-tumorigenic control, was also tested with and without YGK treatment and the colonies were all WB-negative and CV positive. This is consistent with CGL1 having a low IAP activity and high genomic stability.

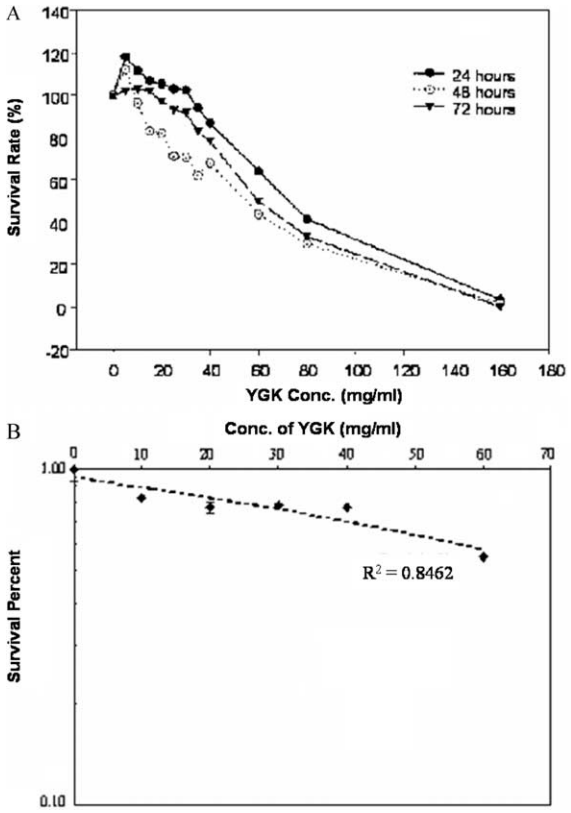

Fig. 2. Dose-dependent loss of cell viability effect of YGK in HeLa 0400. (A) Cells were cultured in medium in the absence or presence of indicated doses of YGK for 24 h (●), 48 h (⊙) 72 h (▼) and followed by MTT assay. The IC_{50} was 60 mg/ml for YGK in HeLa 0400. (B) Survival curve for HeLa 0400 following the treatment of various doses of YGK for 24 h. Little effect of YGK on HeLa 0400 cells was shown in colony formation.

3.4. YGK suppressed HPV-18 E6 and E7 oncogenes

In HeLa cells, mRNAs of HPV-18 E6 and E7 were down-regulated by YGK (Fig. 4A). HPV E6 transcripts were reduced by 40, 18 and 38% of that of untreated HeLa cells when exposed to YGK (40 mg) for 24, 48 and 72 h, respectively (Fig. 4B). In addition, similar reductions of 40 and 33% in HPV E7 expression were observed in HeLa cells at 48 and 72 h (Fig. 4C). This suppression of E6 and E7 was significant ($P<0.01$). In terms of protein level, the amount of E6 protein after YGK (40 mg) in HeLa cells decreased by 30% at 48 h, respectively (Fig. 4D–F). In addition, similar reduction in HPV E7 expression was observed in HeLa cells by 15% at 48 h.

3.5. YGK induction of p53

When high-risk HPV E6 binds to normal p53 protein, p53 degrades through the ubiquitin pathway, thus abrogating its function. After YGK treatment for various times, p53 was induced in a dose-dependent manner and persisted for at least 72 h (Fig. 5A and C). For further evidence of p53-mediated transactivation, the expression of p53 targeted Cdk inhibitor, $p21^{WAF1/CIP1}$, was markedly increased by 24 h YGK treatment (Fig. 5B and D). High-risk HPV E7 binds to Rb to mediate carcinogenesis, however, in this study the expression of total Rb did not show any significant increase in comparison with control cells after YGK treatment for 24–72 h (data not shown).

3.6. Effects of YGK on the cell cycle progression and apoptosis

Since, p53 is an important cell cycle checkpoint regulator, we examined whether the up- regulation in p53 expression by YGK could alter the ability of cells to progress through the cell cycle. When increasing YGK dose, the cell cycle distribution from 2 to 4 N DNA content indicated that YGK had little effect on the cell cycle of HeLa 0400 cells most notably at 40 mg/ml (Figs. 6A and 7). Furthermore, the lack of accumulation of cells in the sub-G1 phase indicated a lack of apoptotic DNA fragmentation. The flow cytometric analysis did not show apoptotic evidence induced by YGK either (Fig. 6B). Around 10% of necrosis or late apoptosis was observed after 40 mg of YGK treatment for 24 h. Under these conditions, the frequency of reversal to IAP negative cells was 90% (Fig. 3B). These results strongly suggest that the YGK-mediated apoptosis is unlikely to play a significant role in cancer growth inhibition by YGK (see below).

3.7. Reversion frequency of HeLa 0400 by YGK: tumor growth inhibition in vivo

The paragraphs below describe the results observed over a 30-day period using six different approaches to analyze the efficiency of YGK

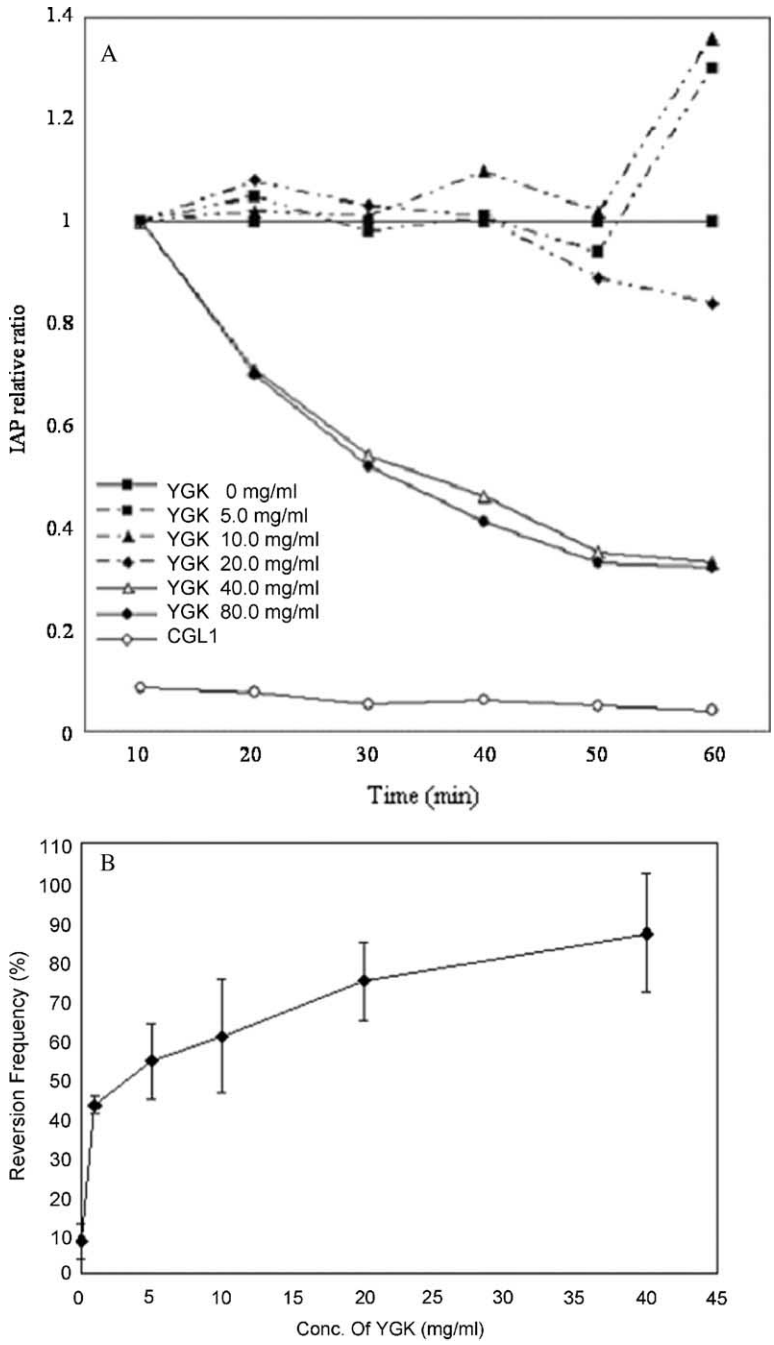

Fig. 3. The effect of YGK on IAP activity and reversion frequency. (A) IAP activity effects of HeLa 0400 and CGL1 by YGK. Individual wells with cell counts of 10^4 were pretreated with various concentrations of YGK. At different time intervals, 100 μl of supernatant was sampled and measured at a wavelength of 405 nm by colorimetric determination for IAP activity detection [5]. IAP activity relative to the control as a function YGK exposure time at the indicated doses. (B) Reversion frequency effects of HeLa 0400 by YGK. CV-stained colonies showed the total colonies and W-B stained colonies showed the revertant cells. The reversion frequency was the percentage of (C-V stained colonies − W-B stained colonies)/CV-stained colonies.

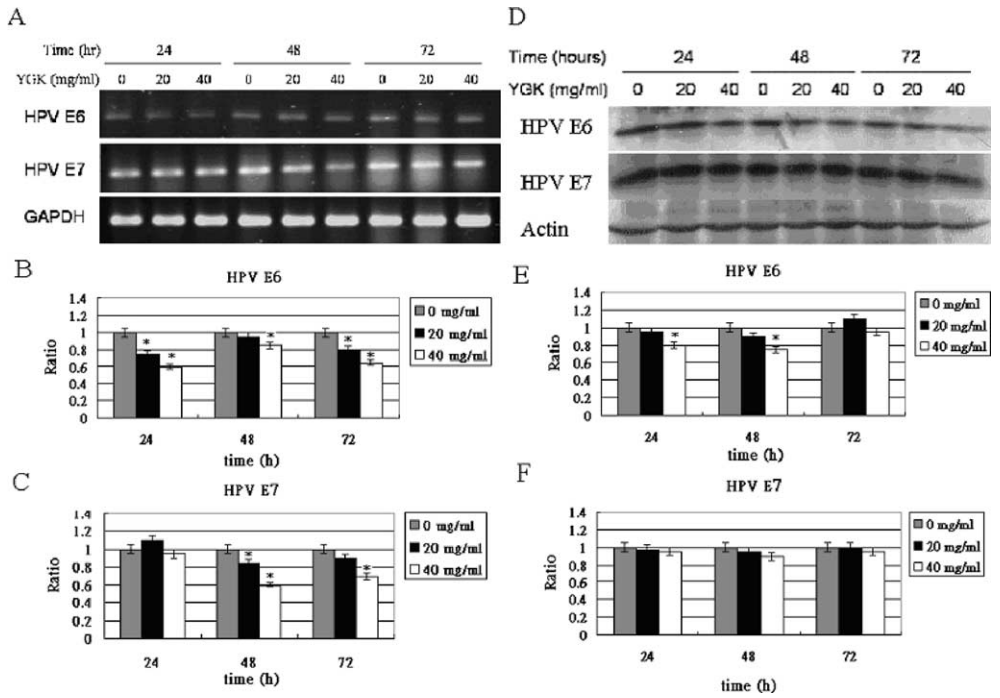

Fig. 4. YGK suppressed HPV-18 E6 and E7 oncogenes. (A–C) RT-PCR analysis of E6 and E7 transcripts in HeLa cells before and after YGK treatment (0, 20, 40 mg) for 24, 48, and 72 h. (D–F) Western blot analysis of E6 and E7 viral oncoproteins in HeLa cells before and after YGK treatment (0, 20, 40 mg) for 24, 48, and 72 h. Blots were incubated with monoclonal antibodies to E6 or E7 and detected with ECL reagent. Results are expressed as means (columns) ± SE (bars) of three independent experiments. $*P < 0.05$; significant difference from control.

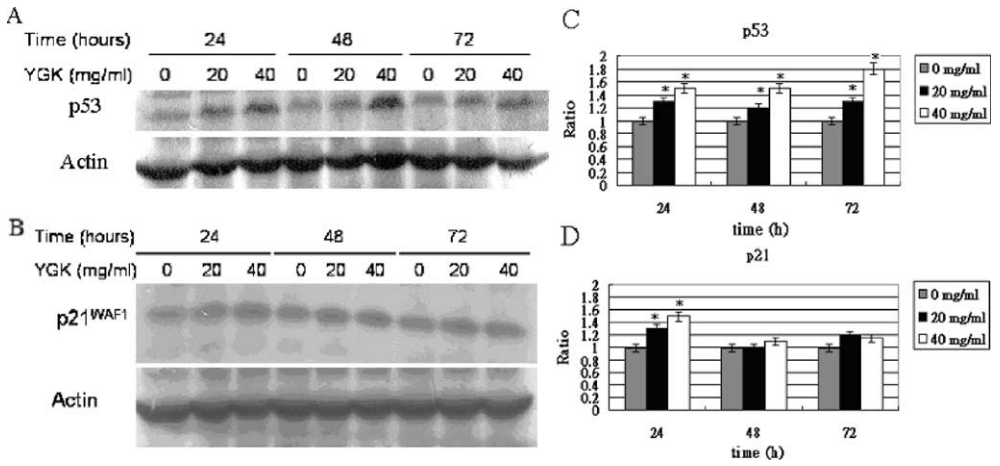

Fig. 5. YGK activated the p53 tumor suppressor pathway. (A) and (C) Treatment with YGK induces expression of cellular p53 protein. Western blot analysis of p53 protein in HeLa cells before and after YGK treatment (0, 20, 40 mg) for 24, 48, and 72 h. (B) and (D) Treatment with YGK induces activation of cellular $p21^{WAF1}$ protein. Western blot analysis of $p21^{WAF1}$ protein in HeLa cells before and after YGK treatment (0, 20, 40 mg) for 24, 48, and 72 h. Results are expressed as means (columns) ± SE (bars) of three independent experiments. $*P < 0.05$; significant difference from control.

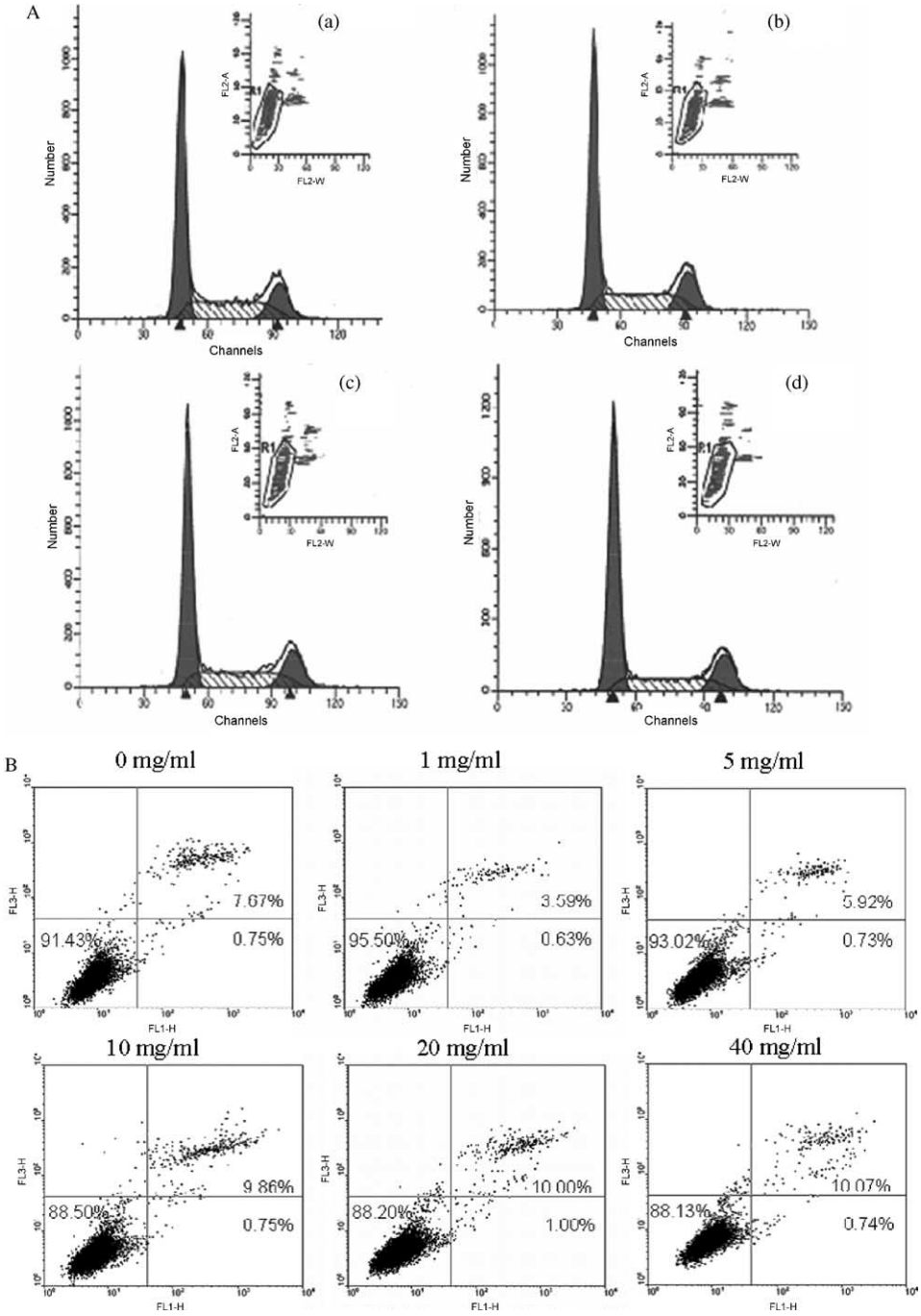

Fig. 6. Flow cytometric analysis of cell cycle and apoptosis. (A) Cell cycle analysis. HeLa 0400 cells were pretreated with (a) 0, (b) 10, (c) 20 (d) 40 mg/ml YGK for 24 h. The values represent the number of cells in the various phases of cell cycle as a likely percentage of total cells. (B) HeLa cells were exposed to YGK at 24 h and quantificated of apoptotic cells by flow cytometric analysis of YGK-treated HeLa cells labeled with annexin V and PI. Intact cells, early apoptotic cells, and late apoptotic and necrotic cells are located in the lower left, lower right, and upper right quadrants of the cytograms, respectively.

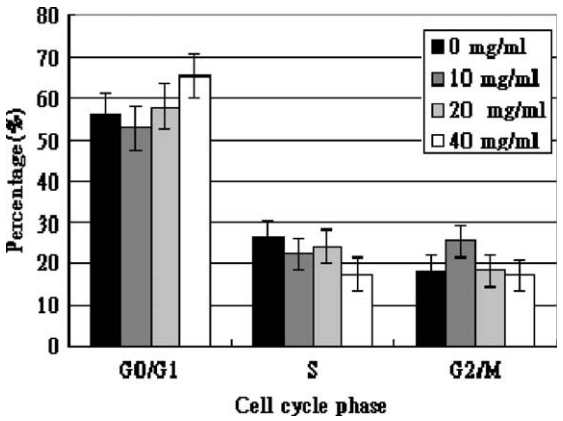

Fig. 7. Quantification analysis of G_0/G_1, S, and G_2/M cell cycle from FLOW cytometry. Cell cycle percentages were derived from FLOW cytometric analysis in Fig. 6A. HeLa cells treated with YGK at 40 mg/ml showed an approximate 10% increase in G_0/G_1 cell cycle arrest and a 9% percent decrease in S phase DNA synthesis.

as a tumor reversion agent in an animal model. The approaches are outlined in Fig. 1. The % growth inhibition (GI) in approach 2 revealed that 10 mg (Table 1, A-2-1) inhibited tumor growth by 69% and 40 mg (A-2-2) inhibited growth by 92% at 30 days, post-treatment. Importantly, with 40 mg YGK pre-treatment, only two of six sites injected grew tumors and these tumors were significantly smaller than in controls ($P<0.01$). This potent effect at 40 mg cannot be a consequence of the small amount of cell-killing (<25%) induced by this dose of YGK.

Furthermore, approach 3 in Table 1 illustrates whether YGK could inhibit the growing tumor directly. YGK was administrated by intratumor injection when the tumor reached a volume of 0.5 cm^3. The GI was almost 50% for 10 (A-3-2) and 20 mg (A-3–3). When YGK and HeLa 0400 were both simultaneously injected into NOD-SCID mice in approach 4, tumor growth was remarkably inhibited. The GI was close to 100% in 10 (A-4-2) and 20 mg (A-4-3) treatments. In order to mimic the practical condition of routine administration of YGK to prevent the tumorigenesis, YGK was injected into NOD-SCID mice 1 day prior to inoculation with HeLa 0400 cells and thereafter at weekly intervals in approach 5. The results showed a GI of 76% at 10 mg (A-5-1) and 91% at 20 mg (A-5-2) YGK treatment.

Table 1
Tumor growth inhibitory ratio (GI%) of HeLa 0400 in different approaches

Approach[a]	No. sites injected	YGK conc. (mg)	Tumors per site injected[b]	Injected YGK (day)[c]	MTV (cm^3)[d]	GI (%)[e]	MW (g)[f]
A-1	6	0	6/6	–	2.01±0.29	0	17.18±3.0
A-2-1	6	10	5/6	–	0.74±0.45	69.32	17.98±0.54
A-2-2	6	40	2/6	–	0.46±0.22	92.37	17.51±2.27
A-3-1	6	5	3/6	20th	1.80±0.19	10.44	18.50±3.29
A-3-2	6	10	6/6	22nd	1.00±0.34	50.25	19.27±1.56
A-3-3	6	20	4/6	22nd	0.92±0.12	54.23	17.29±1.80
A-4-1	6	5	6/6	–	0.40±0.27	80.1	19.09±1.40
A-4-2	6	10	6/6	–	0.073±0.017	96.37	18.67±1.45
A-4-3	6	20	4/6	–	0.053±0.015	98.24	18.07±2.26
A-5-1	6	10	5/6	–	0.57±0.2	76.37	18.61±0.67
A-5-2	6	20	5/6	–	0.25±0.09	91.17	19.49±1.97
A-6	6	0	0/6	–	0	–	17.84±3.49

[a] A-1 is positive control (HeLa 0400 cells) and A-6 is negative control (CGL1). A-2 HeLa 0400 cells were pretreated in vitro with different doses of YGK, and then subcutaneously (s.c.). injected into the SCID mice. A-3 HeLa 0400 cells were s.c. injected into SCID mice to form 0.5 cm^3 tumors. Different doses of YGK were then administrated by intratumor injection. A-4 Different doses of YGK were injected simultaneously with HeLa 0400 cells into the same site. A-5 Different doses of YGK were s.c. injected into SCID mice for pretreatment overnight; HeLa 0400 cells were then injected into the same site. Additional doses of YGK were then administered weekly.

[b] Tumors/site injected.

[c] The time of YGK injection after 0.5 cm^3 tumor developed.

[d] Mean tumor volume, measured 30 days after YGK injection was statistically significant, different from control ($P<0.01$).

[e] Growth inhibition was statistically significantly, different from control ($P<0.01$).

[f] Mean weight.

4. Discussion

We have demonstrated the ability of the traditional Chinese herb medicine, Yigan Kang (YGK), to revert HeLa cells to a much less aggressive tumorigenic phenotype. Mechanistic studies indicate that this reversion is likely to be through the suppression of E6 oncogene, and subsequent restoration of normal p53 function. Reversion of tumorigenic cells to a near-normal and much less aggressive tumorigenic phenotype has previously been observed to occur spontaneously under epigenetic control in several tumorigenic mouse cell lines [3] and in X-ray-induced aggressive tumorigenic phenotype C3H10T1/2 cells [2]. In addition, we have previously found that UVC-induced tumorigenic HeLa x skin fibroblast cell line could revert in vitro to a more normal phenotypic state under optimal growth conditions [4]. This was associated with decreased activity of the HeLa tumor-associated antigen, intestinal alkaline phosphatase and reduction in tumorigenicity as assessed by number of injected sites that developed tumors and by an increase in tumor latency period. We have now used the same IAP activity assay to identify reversion enhanced by YGK and its ability to suppress the proliferation of HeLa cells in vivo. Unlike the case for many other anti-cancer drugs, this inhibition of proliferation in vivo is not through the induction of apoptosis and/or arrest at the G0/G1 phase of the cell cycle [24–27].

The YGK ingredients include 15 ingredients shown in Materials and Methods [13]. Among the herbs used in the composition, *diffuse hedyotis*, *bistort rhizome*, *giant knotweed rhizome*, and *Chinese magnoliavine fruit* are the necessary ingredients that provide for the efficacy of the composition. *Asiatic moonseed rhizome*, *baical skullcap root*, *bovine biliary powder*, *tumeric root-tuber*, *hawthorn fruit*, and *sanqi* are used mainly to improve or enhance the flavor, toning, and medicinal effects of, and to balance the excessive effects cause by *diffuse hedyotis*, *bistort rhizome*, *giant knotweed rhizome*, and *Chinese magnoliavine fruit*. In addition, *barbary wolfberry fruit*, *red ginseng*, *figwort root*, *Chinese angelica* and *milkvetch root* can be added to the composition to provide further nutrition to the liver during the recovery stage. *Asiatic moonseed rhizome* induced apoptosis in human promyelocytic leukemia cells. *Tumeric root-tuber* contributed significantly to anti-oxidant activity. *Hawthorn fruit* was used primarily for various cardiovascular conditions resulting in positive inotropic activity, ability to increase the integrity of the blood vessel wall and improve coronary blood flow, and positive effects on oxygen utilization. *Red ginseng* inhibited exercise-induced 5-hydroxytryptamine synthesis and tryptophan hydroxylase expression. *Chinese angelica* possessed anti-tumor effects on experimental tumor models in vivo and inhibitory effects on invasion and metastasis of hepatocellular carcinoma cells in vitro. *Milkvetch* inhibited the ruminal cellulose fermentation. Basically, YGK may play a multi-function role in this study and presume that there could be synergistic or additive effects of various ingredients to induce cancer reversion.

Fig. 2A showed growth-inhibiting effect of YGK on HeLa 0400 cells as determined by MTT assay to indicate IC50 values, which actually reflected a decrease in cell proliferation and a growth inhibition but not a cell death. The IC50 at 24, 48 and 72 h exposure times were all around 60 mg/ml, and at lower doses of YGK treatment a minimal growth induction effect (not statistically significant) was seen, even at 48 h exposure time. Comparing data from Table 1 (A-2) and Fig. 3, it is observed that under a high concentration of YGK (40 mg/ml) there is a correlation between decrease in IAP activity and expression (Fig. 3A and B) and decrease in tumorigenicity (Table 1, A-2-2). This is consistent with the known correlation between IAP expression and tumorigenicity in HeLa x human skin fibroblast hybrids [17]. At low levels of YGK (<20 mg) the IAP activity remained unaffected (Fig. 3A), yet the ratio of reversion to IAP negative colonies reached 50–70% (Fig. 3B). This apparent discrepancy can be explained by the fact that for IAP activity measurements the assay was performed 3 h after replating cells that had been treated for 24 h, while the reversion assay was scored 10 days after drug treatment.

In order to simulate human supplemental exposure to cancer drug therapy, approach A-5 (Table 1) was used where mice were subjected to pretreatment with YGK followed by weekly treatments following the injection of HeLa cells. The results show that weekly exposure to YGK led to a dose dependent growth

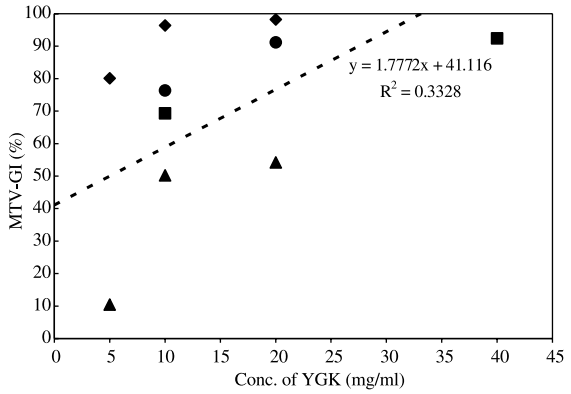

Fig. 8. Responsive effects of growth inhibition during cancer reversion from pooling together the four approaches in Table 1. The growth inhibition (GI %) during cancer reversion is as a function of YGK dose. The correlation is $y=1.7772x+41.116$, $R_2=0.3328$. ■: A-2-1, A-2-2; ▲: A-3-1, A-3-2, A3-3; ♦: A-4-1, A-4-2, A-4-3; ●: A-5-1, A-5-2.

inhibition. Pooling together the four approaches (A-2 to A-5) from Table 1, the growth inhibition during cancer reversion is as a function of YGK dose (Fig. 8). The correlation is $y=1.7772x+41.116$, $R_2=0.3328$.

This study has demonstrated that YGK-induced reduction in expression of the HeLa tumor-associated antigen, IAP, results in reversion of the cells to a much less aggressive tumorigenic phenotype. Since, IAP expression has been shown to be necessary but not sufficient for the tumorigenic phenotype [18], YGK must be having some other effect that results in reversion of the tumorigenic phenotype. In order to explain the mechanism underlying these observations, and to gain new insights into the molecular pathways underlying YGK activity, we examined the effect of YGK on E6/E7 expression, and consequential results on p53 tumor suppressor pathways. The results showed that the stability of p53 was increased in YGK-treated HeLa cells, an effect presumably due to the loss of the HPV-18 E6 protein, which otherwise promotes accelerated, ubiquitin-mediated degradation of p53. Furthermore, in these YGK-treated cells, expression of p53-responsive p21 gene increased. In Fig. 5 we determined the dependence for p53 and p21 and found that only in 24 h the dependence for p53 and p21 is consistent. It is possible that the human $p21^{WAF1/CIP1}$ locus produces three different transcripts utilizing two different promoters, in which the transcript-1 is highly dependent on p53 and the other two are not necessary [28]. This clearly needs further study.

Although YGK showed increased levels of p53, it was unable to produce a significant G1/S arrest. This indicates that the mere increase of p53 levels is not enough to produce complete G1/S arrest in YGK-treated HeLa cells. Interestingly, most of the YGK-treated cells that died had characteristics of necrotic, as opposed to apoptotic, cell death.

In conclusion, we have provided initial evidence showing that YGK may restore a normal p53 tumor suppressor pathway. The induction of p53 expression by YGK has been correlated with repression of the E6 oncogene. YGK also suppresses E7 oncogene. Therefore, YGK is a potential therapeutic candidate for restoring normal tumor suppressor function in HPV-positive cervical cancer cells. In addition, it should be noted that the down-regulation of viral E6/E7 genes might be directly responsible for inhibition of invasiveness of cervical cancer cells as reported [29]. YGK may be useful in the long-term management of treatment resistant and recurrent tumors through the constraint of their malignant behavior.

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