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Gypenosides Causes DNA Damage and Inhibits Expression of DNA Repair Genes of Human Oral Cancer SAS Cells

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Abstract. *Gypenosides (Gyp) are the major components of Gynostemma pentaphyllum Makino, a Chinese medical plant. Recently, Gyp has been shown to induce cell cycle arrest and apoptosis in many human cancer cell lines. However, there is no available information to address the effects of Gyp on DNA damage and DNA repair-associated gene expression in human oral cancer cells. Therefore, we investigated whether Gyp induced DNA damage and DNA repair gene expression in human oral cancer SAS cells. The results from flow cytometric assay indicated that Gyp-induced cytotoxic effects led to a decrease in the percentage of viable SAS cells. The results from comet assay revealed that the incubation of SAS cells with Gyp led to a longer DNA migration smear (comet tail) when compared with control and this effect was dose-dependent. The results from real-time PCR analysis indicated that treatment of SAS cells with 180 μg/ml of Gyp for 24 h led to a decrease in 14-3-3σ, DNA-dependent serine/threonine protein kinase (DNAPK), p53, ataxia telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR) and breast cancer gene 1 (BRCA1) mRNA expression. These observations may explain the cell death caused by Gyp in SAS cells. Taken together, Gyp induced DNA damage and inhibited DNA repair-associated gene expressions in human oral cancer SAS cells in vitro.*

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In Chinese populations, *Gynostemma pentaphyllum* Makino (family Cucurbitaceae) has been used as a folk medicine for centuries. Gypenosides (Gyp) are compounds found in the crude extracts from *G. pentaphyllum* Makino and they have been shown to exert various biological effects such as anti-inflammatory and anti-oxidative (1), antihyperlipidemic, anticardiovascular (2, 3) and anticancer (4-6). Our previous studies have shown that Gyp induced apoptosis in human colon cancer colo 205 cells (7) and human tongue cancer SCC-4 cells through endoplasmic reticulum stress and mitochondria-dependent pathways (8).

It is well documented that many carcinogens and chemicals can induce DNA damage in normal or cancer cells. The repair of damaged DNA is important in the cell maintaining the genome before its replication. It is well known that DNA repair for eliminating spontaneous and carcinogen-induced DNA damage is an important cellular defense mechanism against mutagenesis and carcinogenesis (9, 10). DNA damage is also involved in apoptosis of cancer cells (11).

Although Gyp has been shown to induce cell cycle arrest and apoptosis in several human cancer cell lines, there is no available information to address whether Gyp induces DNA damage or affects DNA repair genes in SAS human oral cancer cells. Therefore, in the present study, we investigated the effects of Gyp on DNA damage and DNA repair genes in SAS cells.

Materials and Methods

Cell culture. SAS human oral cancer cell line was obtained from Dr. Pei-Jung Lu (Graduate Institute of Clinical Medicine, National Cheng Kung University, Tainan, Taiwan). SAS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin in 75 cm^2 tissue culture flasks at 37°C under a humidified 5% CO_2 and 95% air atmosphere as previously reported (8).

Table I. *DNA sequences were evaluated using Primer Express software.*

Primer name	Primer sequences
Human *14-3-3σ*-F	GCCATGGACATCAGCAAGAA
Human *14-3-3σ*-R	GGCTGTTGGCGATCTCGTA
Human *DNAPK*-F	CCAGCTCTCACGCTCTGATATG
Human *DNAPK*-R	CAAACGCATGCCCAAAGTC
Human *p53*-F	GGGTTAGTTTACAATCAGCCACATT
Human *p53*-R	GGGCCTTGAAGTTAGAGAAAATTCA
Human *ATM*-F	TTTACCTAACTGTGAGCTGTCTCCAT
Human *ATM*-R	ACTTCCGTAAGGCATCGTAACAC
Human *ATR*-F	GGGAATCACGACTCGCTGAA
Human *ATR*-R	CTAGTAGCATAGCTCGACCATGGA
Human *BRCA1*-F	CCAGGGAGTTGGTCTGAGTGA
Human *BRCA1*-R	ACTTCCGTAAGGCATCGTAACAC
Human *GAPDH*-F	ACACCCACTCCTCCACCTTT
Human *GAPDH*-R	TAGCCAAATTCGTTGTCATACC

Each assay was conducted at least twice to ensure reproducibility.

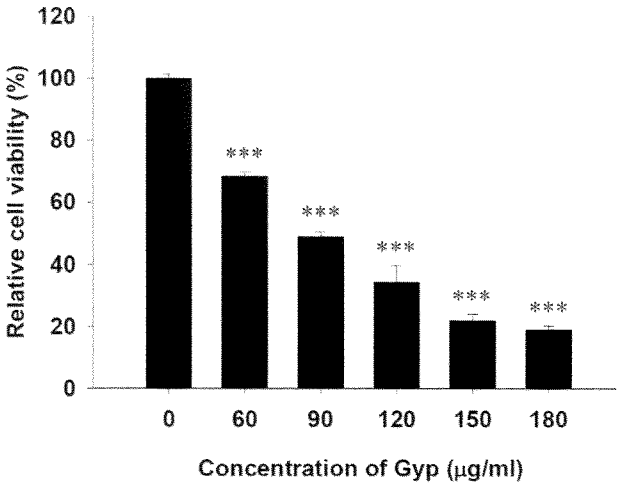

Figure 1. *Gyp reduced the percentage of viable SAS cells as examined by flow cytometric assay. About 2×10^5 cells/well of SAS cells in 12-well plates were incubated with different concentrations of Gyp for 24 h. The cells were collected, stained by PI, and then the percentages of viable cells were determined by flow cytometric assay as described in the Materials and Methods. Data represent the mean±S.D. of three experiments. ***$p<0.001$ Compared to untreated SAS cells.*

Flow cytometric assay for viability of SAS cells after exposure to Gyp. Approximately 2×10^5 SAS cells/well were cultured in 12-well plates at 37°C for 24 h, and each well was individually treated with 0, 60, 90, 120, 150, and 180 μg/ml Gyp for 24 h. Dimethyl sulfoxide (DMSO, solvent for Gyp) was used for the control regimen. For cell viability determination, the cells were harvested by centrifugation from each treatment, stained by propidium iodine (PI), and then were analyzed by a flow cytometric protocol as previously described (8, 12).

Comet assay for examining the DNA damage in SAS cells after Gyp treatment. Approximately 2×10^5 SAS cells/well in 12-well plates were incubated with Gyp at final concentrations of 0, 60, 90, 120, 150, and 180 μg/ml, vehicle (1% DMSO) and 5 μM of H_2O_2 (positive control), and grown in 5% CO_2 and 95% air at 37°C. Cell debris was removed and cells remaining in the plates from each treatment were harvested by centrifugation and then used for the examination of DNA damage using the comet assay as described previously (13-14). Comet tail length was calculated, quantified and expressed (fold of control) in mean±S.D (n=3) by using the TriTek CometScoreTM software image analysis system (Tritek Corp, Sumerduck, VA, USA).

Real-time PCR of 14-3-3σ, DNA-PK, p53, ATM, ATR and BRCA1 in SAS cells after Gyp treatment. SAS cells (5×10^5 cells/well) in 6-well plates were indicated with 180 μg/ml of Gyp for 24 h. The cells from each treatment were harvested by centrifugation and the total RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as described previously (12, 15). Each RNA sample was reverse-transcribed for 30 min at 42°C with High Capacity cDNA Reverse Transcription Kit according to the standard protocol of the supplier (Applied Biosystems, Foster City,CA, USA). Quantitative PCR from each RNA sample was performed under the followed condition: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C, 1 min at 60°C using 1 μl of the cDNA reverse-transcribed as described above, 2X SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers as showing in Table I. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicates and expression fold-changes were derived using the comparative C_T method (12, 15).

Statistical analysis. Student's *t*-test was used to analyze differences between Gyp-treated and control groups and significance presented as *$p<0.05$, **$p<0.01$* and ***$p<0.001$.

Results

Gyp-reduced the percentage of viable SAS cells examined by flow cytometry. As shown Figure 1, there were fewer viable cells in the treated groups as the concentration increased when compared to control groups and this effect was dose dependent ($p<0.001$).

Gyp-induced DNA damage in SAS cells was examined by comet assay. Previous studies had shown that Gyp induced cytotoxic effects on SAS cells. In the present study, we investigated whether Gyp induced DNA damage in SAS cells. The results from comet assay are shown in Figure 2A and B and indicate that Gyp induced DNA damage in SAS cells in a dose-dependent manner.

Gyp affected DNA damage and repair gene expression in SAS cells as shown by real-time PCR. Based on the results from comet assay showing that Gyp induced DNA damage in SAS cells, it was investigated whether or not Gyp affected

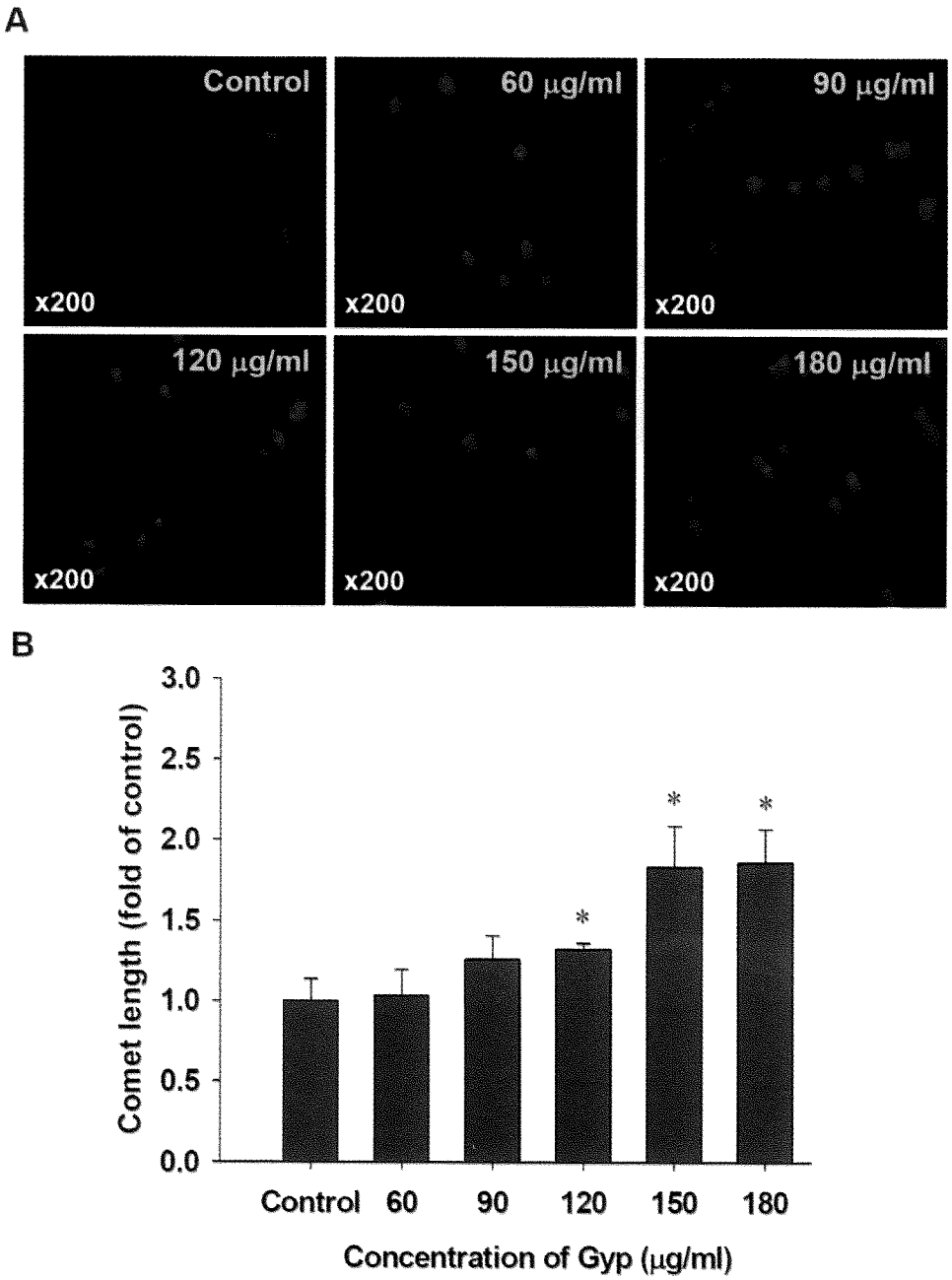

Figure 2. *Gyp induced DNA damage in human oral cancer SAS cells as examined by comet assay. About 2×10^5 cells/well of SAS cells in 12-well plates were incubated with different concentrations of Gyp for 24 h. The cells were collected and DNA damage was determined by comet assay as described in the Materials and Methods. A: Representative profile of comet assay (×200); B: the ratio of comet tail length relative to the control. *p<0.05 Compared to untreated SAS cells.*

expression of DNA damage and repair genes. Expression levels of *14-3-3σ*, *DNAPK*, *p53*, *ATM*, *ATR* and *BRCA1* mRNA are shown in Figure 3A and B and the results indicate that Gyp reduced all examined DNA repair gene expressions in SAS cells and these effects occurred in a time-dependent manner.

Discussion

Although several reports have shown that Gyp induced cell cycle arrest and apoptosis in human cancer cell lines, there is no information to show Gyp inhibited DNA repair gene expression in SAS human oral cancer cells. Herein, we also

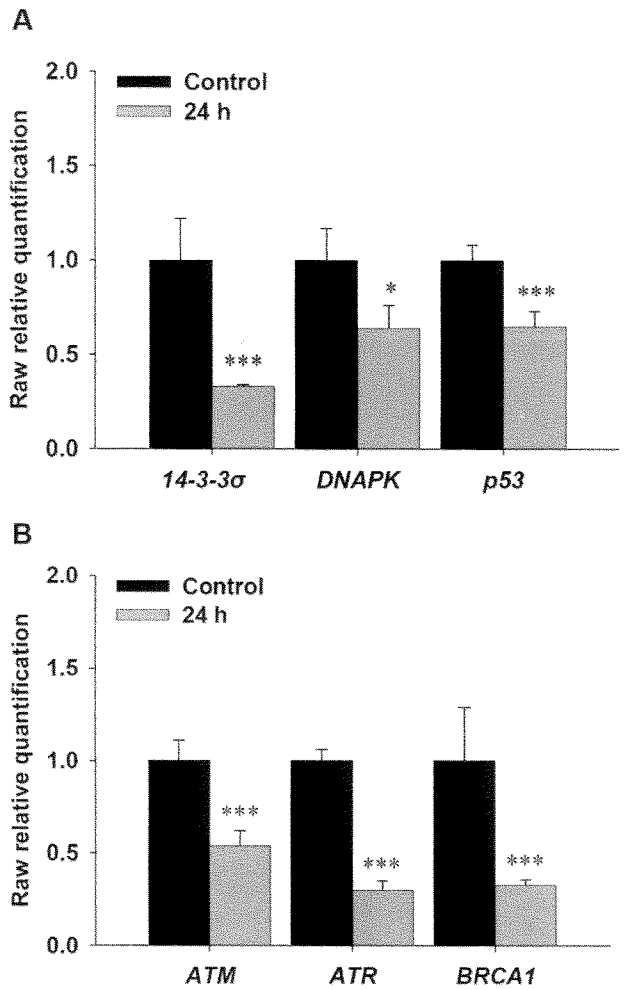

Figure 3. *Expression of DNA damage and repair genes in SAS human oral cancer cells on treatment with Gyp as examined by real-time PCR. About 5×10^5 SAS cells/well in 6-well plates were incubated with 180 μg/ml Gyp for 0 and 24 h. The total RNA from each treatment was extracted and RNA samples were reverse-transcribed for cDNA then for real-time PCR as described in the Materials and Methods. The ratio of A: 14-3-3σ, DNAPK and p53 and B: ATM, ATR and BRCA1 mRNA to GAPDH expression are presented. Data represent the mean±S.D. of three experiments. *p<0.05 and ***p<0.001 Compared to untreated SAS cells.*

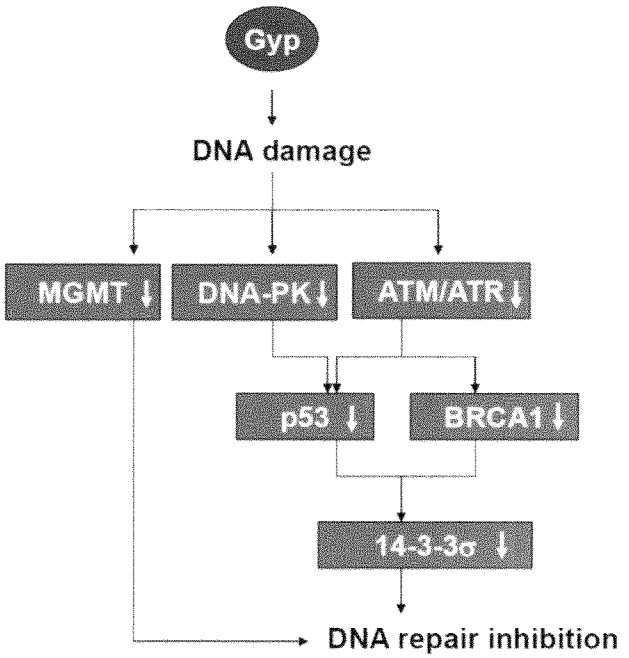

Figure 4. *The possible signaling pathway for Gyp inhibition of expression of DNA damage and DNA repair gene in SAS human oral cancer cells.*

confirmed that Gyp reduced the percentage of viable SAS cells and this effect occurred in a dose-dependent manner (Figure 1A). The results from the comet assay (single cell gel electrophoresis) indicated that Gyp induced DNA damage in SAS cells and led to a significant increase in the tail moment of the comets of SAS cells (Figure 2A). These effects were dose dependent (Figure 2B). It is well documented that comet assay is highly sensitive technique for DNA damage examination (16-18). In the present study, we also used an inducer of DNA damage (H_2O_2) as positive control (19, 20), which showed significant tail movement in SAS cells. Other reports also showed that strand-break formation during the process of excision repair may cause DNA migration measurable in the comet assay (21-22). It is well known that DNA repair can reduce DNA damage development through eliminating DNA lesions. DNA repair genes including *ATM*, *ATR*, *BRCA1*, *14-3-3σ*, *DNAPK* and *p53* are involved in DNA repair during DNA damage in cells. In the present study, the results showed that Gyp inhibited expression of DNA repair genes including *14-3-3σ*, *DNA-PK*, *p53*, *ATM*, *ATR* and *BRCA1* in the examined SAS cells.

In the preliminary experiments, Gyp induced cytotoxic effects (reduced the percentage of viable SAS cells) and triggered apoptosis in a dose-dependent manner (data not shown). We also showed the role of reactive oxygen species (ROS) in A549 human lung cancer cells after Gyp treatment (23), and these observations were also made in SAS cells (data not shown). ROS play an important role in Gyp-induced apoptosis and DNA damage (8, 23). Further studies are needed to establish the role of the interaction of Gyp with DNA in carcinogenesis.

In conclusion, the results from the comet assay clearly indicated that Gyp induced DNA damage and inhibited expression of DNA repair genes in SAS human oral cancer cells and these effects appear to have led to cell death. The proposed signaling pathway for Gyp-induced gene expression of DNA damage and DNA repair can be seen in Figure 4.

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