SPANDIDOS PUBLICATIONS

Inhibitory effects of *Gleditsia sinensis* fruit extract on telomerase activity and oncogenic expression in human esophageal squamous cell carcinoma

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Abstract. Previous studies have shown that the anomalous fruit extract of *Gleditsia sinensis* (GSE) exhibited apoptotic properties in various solid and non-solid tumors *in vitro*. However, the inhibitory actions of GSE on oncogenic expression and telomerase activity in esophageal squamous cell carcinoma (ESCC) have not been studied before. In the present study, the anti-cancer effects of GSE were demonstrated in three ESCC cell lines (HKESC-1, HKESC-2 and SLMT-1) by MTS and anchorage-independent clongenicity assays, expression studies on oncogenes at 11q13 (*CCND1*, *INT2*, *FGF4* and *EMS1*) and real-time quantitative telomeric repeat amplification protocol assay to show the inhibitory effect of GSE on telomerase in ESCC. The means of MTS_{50} of GSE for the ESCC cell lines and non-tumor NIH 3T3 cells were 21 and 163 μg/ml respectively. The anchorage-independent clongenicity assay showed that SLMT-1 cells lost their colony-forming potential which was dose-dependent to GSE. Moreover, GSE demonstrated dose-dependent suppression on the expression of *INT2*, *EMS1* and *FGF4*, and inhibition of telomerase activity in the ESCC cell lines. Our overall results thus provide the first evidence that the anti-cancer effects of GSE on ESCC involve the suppression of oncogenic expression and inhibition of telomerase activity. Our findings also offer a new opportunity for the future development of GSE as a novel anti-cancer agent for ESCC and possibly for other cancers.

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Introduction

Gleditsia sinensis (*GS*) is a traditional Chinese medicine that is widely distributed in Chinese mainland (1). The anomalous fruit of *GS* produced by old or injured plants is rich in saponin and has promising therapeutic actions (2). The *Gleditsia sinensis* extract (GSE) was also demonstrated by our group to have anti-cancer properties on both solid and non-solid tumors *in vitro* (1,3) acting through different mechanisms (4-8). Thus, a further understanding of the anti-cancer actions of GSE on specific targets will yield great benefits for exploring it as a novel anti-cancer agent.

Moreover, previous results of comparative genomic hybridization (CGH) analysis showed that a high level of gene amplification at chromosome 11q13 was frequently found in ESCC cases (9). Within this amplified region, there are 4 candidate oncogenes, namely *CCND1*, *INT2*, *FGF4* and *EMS1*. Both *CCND1* and *EMS1* were shown to be over-expressed in various tumors (10). It was well documented that the targeted suppression of expression of these oncogenes could yield great benefits as anti-cancer mechanisms that yield new approaches for the development of anti-cancer drugs. Examples include a downregulation of the expression of *CCND1* in human Burkitt's lymphoma Daudi cells *in vitro* after exposure to deguelin, which is an isolated natural plant product and used as a lung cancer chemopreventive agent (11).

Telomerase is a ribonucleoprotein enzyme complex that adds telomeric DNA repeats (TTAGGG) to chromosome ends to compensate for the losses that occur with each round of DNA replication (12). Telomerase activity is not detected in most human somatic tissues (13). However, a high level of telomerase is shown in 85-90% of human cancers (14), including tumors of esophageal squamous cells as well as in their preneoplasia lesions (15). Telomerase is thought to be involved in the long-term proliferation and immortalization of tumors, and the by-passing of apoptosis (16). The aim of the present study was to further investigate the anti-cancer effects of GSE, in terms of suppressing the expression of the ESCC-related oncogenes located at the 11q13 region and the telomerase activity on the selected ESCC cell lines of Hong Kong Chinese origin. Our findings will provide a better understanding of the targeted anti-cancer mechanisms of GSE on ESCC and possibly on other cancers in the future.

Materials and methods

ESCC cell lines. Four ESCC cell lines of Hong Kong Chinese origin, including SLMT-1 (17), HKESC-1 (18), HKESC-2 and HKESC-3 (19), were kindly provided by Professor Gopesh Srivastava, Department of Pathology, The University of Hong Kong and maintained as described (20). The mouse fibroblast cell line NIH 3T3 was purchased from the American Type Culture Collection (ATCC) and cultured as described (20). NE1 cells which are non-tumor esophageal epithelial cells (21) were kindly provided by Professor George S.W. Tsao, Department of Anatomy, The University of Hong Kong and cultured as described (20).

RT-PCR analysis. The extraction of total RNA and reverse transcription were performed as previously described by our group (20). The expression levels of *CCND1*, *INT2*, *EMS1* and *FGF4* in the NE1 cells, and the ESCC cell lines with or without GSE or CDDP treatments were analyzed by multiplex RT-PCR as previously described (20). Two μg of cDNA produced by reverse transcription from the RNA was amplified as previously described by using the specific PCR primers of *CCND1*, *INT2*, *EMS1* and *FGF4* and specific *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene primers acting as an internal control for normalizing the cDNA quantity. The primers for *CCND1* (22) were: *CCND1*-F 5'-CTG CTC CTG GTG AAC AAG CTC-3' and *CCND1*-R 5'-CTC TGG AGA GGA AGC GTG TG-3'; the primers for *INT2* (23) were: *INT2*-F 5'-CAG AAG CAG AGC CCG GAT AA-3' and *INT2*-R 5'-ACG CCA AGA TGT CGC CAG GA-3'; the primers for *EMS1* (24) were: *EMS1*-F 5'-TCC CCT GAT GCC CAG GTC-3' and *EMS1*-R 5'-TCC CAA TCC AGA GAC CCG-3'; and the primers for *FGF4* (25) were: *FGF4*-F 5'-ACC TTG GTG CAC TTT CTT CG-3' and *FGF4*-R 5'-CTC CAC TGT TGC ACC AGA AA-3' and the primers of *GAPDH* were: *GAPDH-1* 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3' and *GAPDH-2* 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3' (Clontech). The PCR products were then electrophoresed in a 2% agarose gel and visualized under UV. The intensities of the PCR products were measured by the densitometric analysis using the Quantity One program (Bio-Rad). The intensities of the target PCR product were normalized against the *GAPDH* expression in each sample. The relative expression level of the target gene was expressed by the formula: (target gene/GAPDH, for ESCC cell line with or without drug treatment)/(target gene/GAPDH, for NE1 or untreated control) (26). The level was regarded as overexpression when the ratio was >1.2; a ratio between 0.8 and 1.2 was regarded as no change; and a ratio <0.8 was regarded as underexpression of the gene (27).

Preparation of GSE. The dried fruit of *Gleditsia sinensis* was ground into powder in a mortar and the GSE was prepared as previously described by our group using absolute ethanol (6).

MTS cytotoxicity and soft agar assays. The cytotoxic effects of GSE and CDDP on the 3 ESCC cell lines and NIH 3T3 cells were investigated by using the MTS activity assay as previously described (5) using CellTiter96® AQueous One Solution cell proliferation assay (Promega). The soft agar assay for the SLMT-1 cells was performed as previously described with the bottom layer containing different concentrations of GSE, ranging from 0, 6.25, 12.5, 25, 50 and 100 μg/ml (1).

Real-time quantitative telomeric repeat amplification protocol (Q-TRAP) assay. The Q-TRAP assay was performed by using Quantitative Telomerase Detection (QTD) kit (US Biomax, Inc), in which about $2.5x10^4$ cells of each pellet treated or untreated with GSE or cisplatin (CDDP, Sigma) were lysed according to the manual's instructions. Three μg of total protein in the cell extracts was added to the SYBR-Green PCR master mix and the PCR qualified water to give a final volume of 25 μl, and then programmed in the real-time PCR detection system using real-time thermal cycler (SmartCycler® Technology, Cepheid). The reactions were performed at 25˚C for 20 min, followed by incubation at 95˚C for 10 min, and were amplified for 38 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. Four controls were included in the assays; 3 sets of telomerase negative controls: a) lysis buffer; b) heat-inactivated cell extracts; and c) 5 μM of telomerase inhibitor (MST-312, Calbiochem) (13), as well as d) a telomerase-positive control of non-inactivated HKESC-1 cell extract (18). The statistical significance of the difference in the means of relative telomerase activity after the treatments of different GSE concentrations was determined by the ANOVA test (single factor) and Paired t-test (one-tailed), requiring $P<0.05$ (95% confidence level) for statistical significance.

Results

MTS growth inhibition assay. Results of MTS assay revealed that the means of MTS_{50} of GSE for the ESCC cell lines (SLMT-1, HKESC-1 and HKESC-2) and non-tumor NIH 3T3 cells were 21 and 163 μg/ml respectively after 48 h of GSE treatment (Fig. 1). This suggested that GSE may be useful for treating ESCC while causing low cytotoxicity on non-tumor cells. The mean of MTS_{50} of CDDP for the ESCC cell lines was 12.3 μg/ml and that for the NIH 3T3 cells was 12 μg/ml

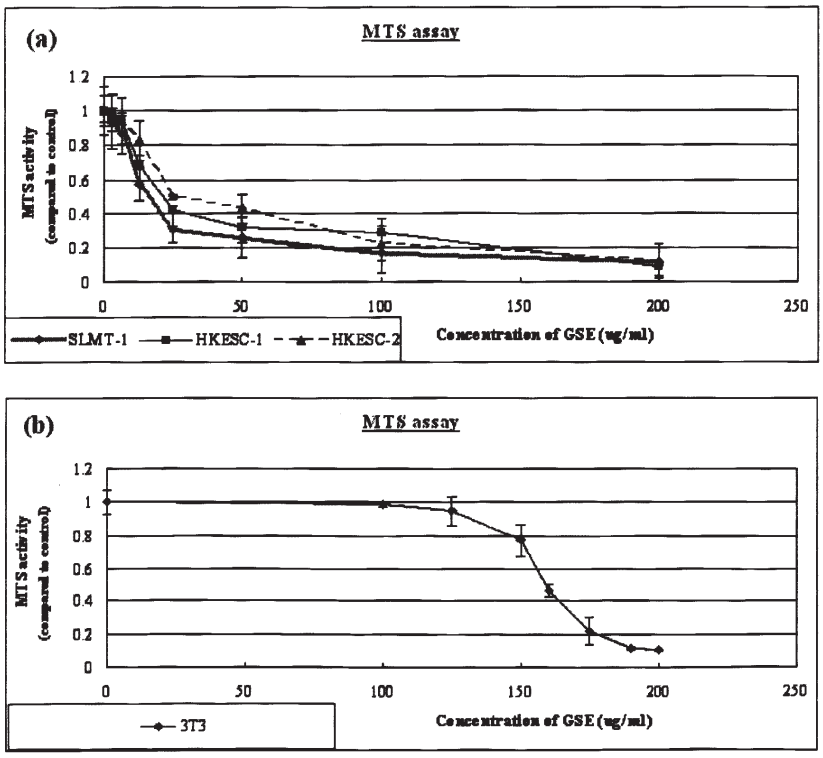

Figure 1. MTS activity assay showing the anti-cancer actions of GSE at the concentrations of 0, 3.125, 6.25, 12.5, 25, 50, 100 and 200 μg/ml on (a) ESCC cell lines and (b) NIH 3T3 cells after 48 h of GSE incubation. Results are representative of three independent experiments.

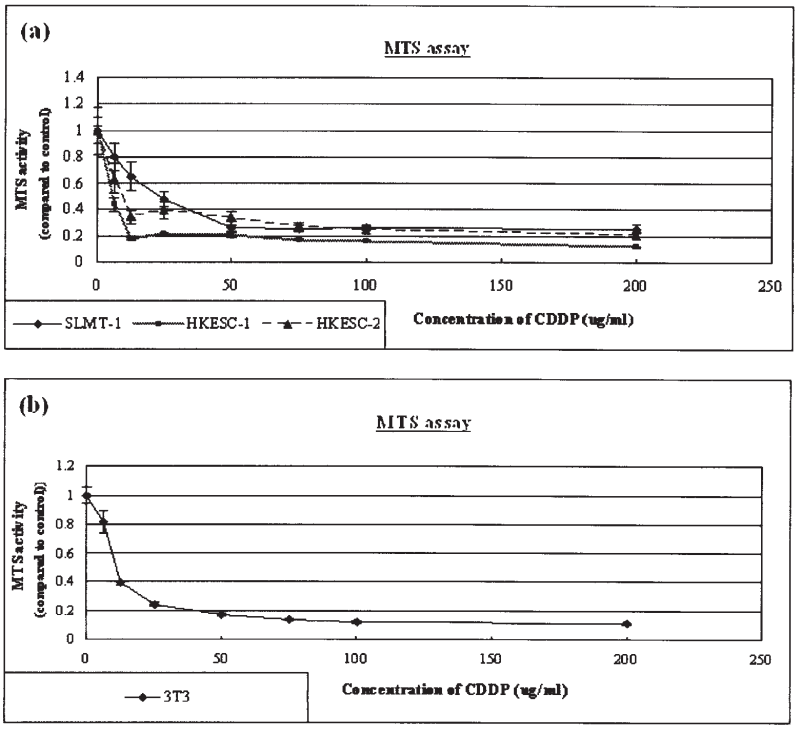

Figure 2. MTS activity assay showing the anti-cancer actions of CDDP at the concentrations of 0, 6.25, 12.5, 25, 50, 100 and 200 μg/ml on (a) ESCC cell lines and (b) NIH 3T3 cells after 48 h of CDDP incubation. Results are representative of three independent experiments.

(Fig. 2). The overall MTS assay showed that both GSE and CDDP contributed dose- and time-dependent growth inhibitory effects on ESCC and non-tumor NIH 3T3 cell lines.

Soft agar assay. Among the 3 ESCC cell lines, only SLMT-1 cells could form colonies on soft agar. Thus, SLMT-1 was chosen for this part of the study. The anchorage-independent clongenicity assay showed that SLMT-1 cells lost their

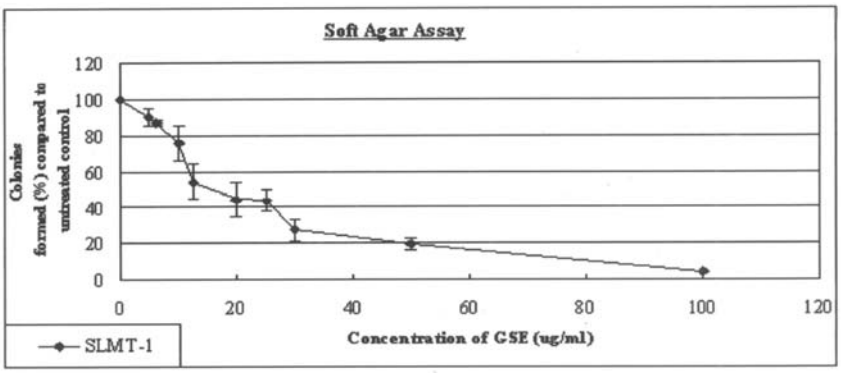

Figure 3. Inhibition of colony formation of SLMT-1 cells after GSE action. The assay was performed to establish the action of GSE at the concentrations of 0, 5, 6.25, 10, 15, 20, 25, 30, 50 and 100 μg/ml on the inhibition of colony formation (%) of SLMT-1 cells. Results are representative of three independent experiments.

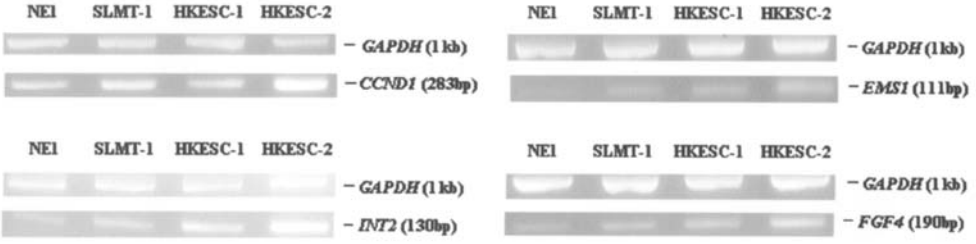

Figure 4. Studies of 11q13 oncogene expression in three ESCC cell lines. Without GSE and CDDP treatments, the expression levels of *EMS1*, *INT2* and *FGF4* were elevated in all ESCC cell lines (HKESC-1, HKESC-2 and SLMT-1) when compared with the non-tumor epithelial cell line (NE1). However, only both SLMT-1 and HKESC-2 cells overexpressed *CCND1*, while HKESC-1 cells showed no change in *CCND1* expression. *GAPDH* expression was used as an internal control.

colony-forming potential which was dose-dependent to GSE (Fig. 3). The colonies were reduced by 50% when the dose of GSE was increased to 20 μg/ml, and <5% of colonies formed once it reached 100 μg/ml.

Effects of GSE on oncogenic expression in ESCC studied by multiplex RT-PCR analysis. The effects of GSE on the expression of 4 oncogenes in 11q13 (*CCND1*, *EMS1*, *INT2* and *FGF4*) were studied in the 3 ESCC cell lines (SLMT-1, HKESC-1 and HKESC-2) when compared with NE1 cells. Without GSE and CDDP treatments, the expression levels of *EMS1*, *INT2* and *FGF4* were elevated in all (3/3) ESCC cell lines (Fig. 4). However, only both SLMT-1 and HKESC-2 cells overexpressed *CCND1*, while HKESC-1 cells showed no change in *CCND1* expression (Fig. 4). After GSE treatment, the *CCND1* level was not changed significantly with an increased GSE dose in all 3 ESCC cell lines (HKESC-1, HKESC-2 and SLMT-1). In contrast, they all showed under-expression of *FGF4* when the GSE dose was increased to the range of 25-100 μg/ml (Fig. 5). Moreover, *INT2* and *EMS1* expression levels were reduced in SLMT-1 and HKESC-2 respectively once the GSE dose reached 100 μg/ml (Fig. 5). The effect of CDDP on these 4 oncogenes was also included and regarded as a control in the expression study, since 15 μg/ml of CDDP induced a 50% reduction in MTS activity in all 3 ESCC cell lines; hence, this concentration of CDDP was used to treated the ESCC cells prior to oncogenic expression studies. Approximately 15 μg/ml of CDDP was found to cause underexpression of *EMS1* in all ESCC cell lines (3/3); it also led to underexpression of *INT2* and *FGF4* in both SLMT-1 and HKESC-2 cells as well. CDDP-treated HKESC-1 cells showed an overexpression of *CCND1*, in contrast to the results of the CDDP-treated SLMT-1 and HKESC-2 cells which showed no change in expression and underexpression of *CCND1,* respectively (Fig. 5). Since the GSE was extracted by absolute ethanol, its effect on the oncogenic expression could also be examined in the assay, which showed that absolute ethanol did not change the expression of the 4 selected oncogenes in 11q13 in all of the 3 ESCC cell lines (Fig. 5). The overall results of the expression study demonstrated that GSE exerted a dose-dependent inhibitory effect on the expression of the oncogenes in 11q13, including *INT2*, *EMS1* and *FGF4*, while CDDP downregulated all the selected oncogenes located on 11q13 in the ESCC cell lines.

Effects of GSE on telomerase activities in ESCC. The results of the Q-TRAP assay indicated that telomerase activities were detectable in all of the extracts of the ESCC cell lines without drug treatment (Table I and Fig. 6). Dose-dependent telomerase inhibition was detected after 48 h of treatment using GSE and CDDP. Relative telomerase activity (RTA) was reduced by ~96, 50 and 76% in the cells of SLMT-1, HKESC-1 and HKESC-2, respectively when 12.5 μg/ml of GSE was applied to the cultured cells prior to the Q-TRAP assay, and the RTA was low or even undetectable (repressed

Figure 5. Selected results of the oncogenic expression in the three ESCC cell lines. The alterations in the expression level of (A) *CCND1*, (B) *INT2*, (C) *EMS1* and (D) *FGF4* in ESCC cell lines (HKESC-1, HKESC-2 and SLMT-1) after GSE or CDDP treatments were compared to the untreated controls. GSE downregulated the expression of *INT2*, *EMS1* and *FGF4*, while CDDP downregulated all of the selected oncogenes located on 11q13 in the ESCC cell lines. NT, untreated control; Ab, absolute ethanol treated ESCC cells. *GAPDH* expression was used as an internal control.

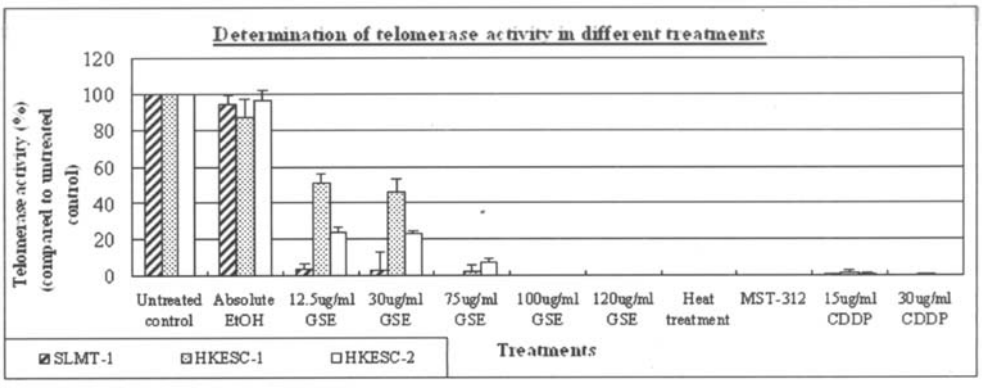

Figure 6. Determination of telomerase activity in different treatments by the Q-TRAP assay. Without any drug treatment, the telomerase activities were detectable in all ESCC cell extracts (SLMT-1, HKESC-1 and HKESC-2). There was no significant difference in the mean of RTA between the cells treated with absolute ethanol and the untreated control (t-test, P=0.0569). Telomerase activity was undetected in SLMT-1 cells when ≥75 μg/ml of GSE was added, while ~100 μg/ml or more of GSE was needed to give a similar response in both HKESC-1 and HKESC-2 cells. The RTA values were approximately zero in both the heat-inactivated and telomerase inhibitor (MST-312)-supplemented samples. Results were expressed with mean ± SD from triplicate experiments. RTA, relative telomerase activity; untreated control, ESCC cell extracts (SLMT-1, HKESC-1 and HKESC-2).

by 98%) when CDDP reached ≥15 μg/ml reducing the MTS_{50} activity by >50% in the ESCC cell lines. The telomerase activity was undetectable in SLMT-1 cells when ≥75 μg/ml of GSE was added, while ~100 μg/ml or more of GSE was required to give a similar response in both the HKESC-1 and HKESC-2 cells. Since the RTA values were approximately zero in the negative controls, including both heat-inactivated and telomerase inhibitor MST-312-supplemented samples, this indicated that the specificity of product formation in the Q-TRAP assay was attributed to the heat-sensitive telomerase activity. Since the GSE was extracted by absolute ethanol, the inhibitory effect of absolute ethanol on telomerase activities was examined in the assay showing no significant difference in the means of RTA between the cells

Table I. Studies of GSE and CDDP actions on telomerase activities in different drug-treated samples compared with untreated control.[a]

Sample treatments	ESCC cell lines					
	SLMT-1	HKESC-1	HKESC-2	SLMT-1	HKESC-1	HKESC-2
	Mean number of TSR molecules (molecules/reaction)			Telomerase activity compared to untreated control (RTA) (%) RTA = $(M_S - M_H)/(M_C - M_H)$ x 100%		
Untreated control	58468±6303	1505±49	1244±69	100±10.78	100±9.90	100±5.55
Absolute ethanol	55227±4044	1317±228	1198±149	94.45±6.92	87.51±15.15	96.30±11.98
GSE 12.5 μg/ml	2119±448	766±190	291±55	3.62±0.77	50.90±12.62	23.39±4.42
GSE 30 μg/ml	1719±555	690±97	282±70	2.94±0.95	45.86±6.45	22.67±5.63
GSE 75 μg/ml	0±0	36±5	89±19	0±0	2.39±0.33	7.15±1.53
GSE 100 μg/ml	0±0	0±0	0±0	0±0	0±0	0±0
Heat treatment	0±0	0±0	0±0	0±0	0±0	0±0
MST-312 inhibitor	0±0	0±0	0±0	0±0	0±0	0±0
CDDP 15 μg/ml	335±44	22±3	6±0	0.57±0.08	1.46±0.20	0.48±0
CDDP 30 μg/ml	27±3	6±1	0±0	0.05±0.01	0.40±0.07	0±0

[a]Results were expressed with mean ± SD from triplicate experiments. Relative telomerase activity (RTA) was expressed by the formula of RTA = $(M_S - M_H)/(M_C - M_H)$ x 100%; M_S, mean number of TSR molecules of sample; M_C, mean number of TSR molecules of the untreated control; M_H, mean of TSR concentration of the heat-treated sample.

treated with absolute ethanol and the untreated control (t-test, P=0.0569). The overall results of the Q-TRAP assay showed that highly significant differences in the means of RTA were observed in the three ESCC cell lines, with reduction in telomerase activity when the concentration of GSE was increased, compared with the untreated control (ANOVA test, $P=1.08x10^{-5}$).

Discussion

The anti-cancer effects of GSE on the ESCC cell lines were first evaluated by MTS assay, and the results showed that a 48-h incubation was the minimal time required for GSE to bring the cytotoxic effects to the ESCC cell lines. The mean GSE concentration of 21 μg/ml was required to inhibit 50% of MTS activity in the ESCC cell lines (SLMT-1, HKESC-1 and HKESC-2) after 48 h of GSE incubation, while that of the positive control CDDP was 12.3 μg/ml. This suggested that GSE, similar to a widely used anti-cancer drug, CDDP, could exhibit inhibitory effects on the growth of ESCC cells *in vitro*. Most importantly, the non-tumor NIH 3T3 mouse fibroblasts demonstrated much more resistance to GSE (mean MTS_{50}=163 μg/ml) than CDDP (mean MTS_{50}=12 μg/ml), together with the previous observation that the normal peripheral blood lymphocytes could withstand a relatively high toxicity concentration, which was ~38-43 μg/ml of GSE (1). This implies that GSE may have a selective anti-cancer action on cancer cells and it may be a potential chemotherapeutic agent for ESCC.

Recent studies have indicated that telomerase expression is associated with cell immortalization and tumorigenesis (28) which suggests that telomerase is an attractive target for cancer therapy. The possibility of GSE or CDDP in inhibiting telomerase activity was investigated by the Q-TRAP assay. Our results indicated that GSE demonstrated a dose-dependent repression on telomerase activity in SLMT-1, HKESC-1 and HKESC-2 cells, and telomerase activity in ESCC cells was undetected once GSE reached 100 μg/ml when compared to the untreated controls after 48 h of GSE incubation. Similarly, CDDP also exhibited a dose-dependent inhibition on telomere lengthening in the ESCC cell lines, and the level of telomerase was repressed by ~98% at 15 μg/ml of CDDP relative to the cells without treatment in the Q-TRAP assay. Hence, our results suggested that both CDDP and GSE may exert anti-cancer effects on the ESCC cell lines by repressing telomerase activity. The action of CDDP on telomerase inhibition was suggested by Ishibashi and Lippard (12), who found that a substantial reduction in telomere length followed by apoptosis was shown in HeLa cells after CDDP treatment (12). In the present study, ~10-20 μg/ml of CDDP induced cytotoxic effects as well as suppressed telomerase activity in the telomerase-positive ESCC cell lines (SLMT-1, HKESC-1 and HKESC-2). Telomerase activity is a biomarker of cell proliferation since it is present in highly proliferative normal tissue *in vivo*, including hemopoietic cells and the oral mucosa, as well as in the immortalized cell lines and in most tumors, including ESCC, because of their high proliferative ability (29). Thus, any repression of telomerase activity would be unfavorable to cell proliferation and might contribute to apoptosis. In the present study, the results of the Q-TRAP assay suggested that GSE may be a potential telomerase inhibitor to human ESCC exhibiting a concentration-dependency to GSE which exerts cytotoxicity on human ESCC cell lines in MTS assay. Hence,

s proposed that GSE may cause growth inhibition GSE-treated ESCC cells, and this may correlate to substantial telomere reduction resulting from telomerase suppression.

Soft agar assay can be used to measure the sensitivity of human tumors to anti-cancer agents (3). Results of this assay revealed that SLMT-1 cells were sensitive to GSE which reduced their ability to grow as colonies in semi-solid media. Hence, this implicated that GSE might alter the transformed phenotype and abnormal growth characteristic as well as reduce the selectable growth advantage of SLMT-1 cells by suppressing their potential for anchorage-independent growth. Our present results of soft agar assay indicated that GSE might be a potential anti-cancer agent as it could inhibit the anchorage-independent growth of ESCC cells which was a key feature of ESCC tumor cells.

DNA amplification is a common mechanism for oncogene overexpression since it is assumed that the amplified DNA may include some critical genes whose overexpression provides a selective force for tumor development (30). An amplification at 11q13 is considered to have a relation with metastasis to the lymph nodes and may have an important role to play during the malignant progression in tumors (31). Four oncogenes located in 11q13, were found to be associated with various human tumors; for instance, *CCND1* and *EMS1* were shown to be overexpressed in all carcinomas with 11q13 amplification (32). *Cyclin D1* (*CCND1*) encodes a cell-cycle regulatory protein of the G1/S phase checkpoint (33). Amplification and overexpression of *CCND1* were reported as prognostic markers associated with metastasis to the lymph nodes and distal organs of ESCC (33). The protein of *EMS1* was proposed to function as a signal transmitter between cell-matrix contact sites (34). An overexpression of *EMS1* might affect the adhesive properties and metastasis of human carcinomas (34). Both *INT2* (35) and *FGF4* (32) belong to the basic fibroblast growth factor (FGF) gene family (30).

In the present study, the multiplex RT-PCR analysis demonstrated that GSE suppressed the expression of the oncogenes *EMS1*, *INT2* and *FGF4* at 11q13 which may be important for neoplastic transformation and tumor progression. A similar example includes epigallocatechin-3-gallate (EGCG) which is one of the green tea polyphenolic compounds that has demonstrated anti-carcinogenic activities in various human cancers, including tumors of the stomach, esophagus, colon and lung (36). The anti-tumor effects of EGCG have been reported to be mediated by apoptosis, in which proteins of *bcl-*X_L and *proliferating cell nuclear antigen* (*PCNA*) are important in regulating EGCG-dependent apoptosis (36). A significant downregulation of *bcl-*X_L and *PCNA* resulting from 48-h post-EGCG treatments were involved in inducing apoptosis in ovarian cancer cells (36). These findings suggest that both EGCG and the existing anti-tumor drugs, including vinblastine, etoposide, Ara-C and nocodazole, function through modulation of the expression levels of oncogenes, tumor-suppressor genes, and the genes related to metastasis and apoptosis.

Our overall results showed that GSE having a cytotoxic effect on the human ESCC cell lines exhibits dose-dependent inhibitory effects on telomerase activity, the expression of *INT2*, *FGF4* and *EMS1*, and the colony-forming potential of SLMT-1 cells. Our data implies that GSE exerts its anti-cancer effects through modulating the oncogenic expression and telomerase activity. Further effort is ongoing to elucidate the molecular mechanisms of GSE and the actions of its active components for use in cancer therapy.

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