RESEARCH ARTICLE

Genistein and Trichostatin A Induction of Estrogen Receptor Alpha Gene Expression, Apoptosis and Cell Growth Inhibition in Hepatocellular Carcinoma HepG 2 Cells

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Abstract

Epigenetic changes such as DNA methylation and histone acetylation play important roles in determining gene expression. Hypermethylation of CpG islands of the promoter region of tumor suppressor genes can greatly influence carcinogenesis through transcriptional silencing. Acetylation of lysine in histone tails causes relaxation of chromatin, which facilitates gene transcription, while deacetylation is associated with condensed chromatin resulting in gene silencing. DNA demethylating agents such as genistein (GE) and histone deacetylase inhibitors (HDACIs) such as trichostatin A (TSA) may strongly reactivate silenced genes and exposure to these two agents in combination is reported to enhance estrogen receptor alpha (ERα) reactivation and induction of apoptosis. The present study was designed to evaluate the effect of these compounds on ERα gene expression, cell viability and apoptosis in hepatocellular carcinoma (HCC) Hep G2 cells. GE exerted biphasic effects; it stimulated cell growth at a low concentration (1 μM) but inhibitory influence was noted with high concentrations (10, 20 and 40 μM). In contrast, TSA demonstrated inhibitory effects on growth at all of concentrations tested. Furthermore, GE and GE/TSA significantly induced apoptosis at all concentrations, but TSA only after 72 h. GE induced ERα re-expression and this was maximal in combined treatment groups treated with GE/TSA for 72 h. **Discussion:** Our finding clearly indicates that GE and TSA have an inhibitory cell growth, induce apoptosis and reactivate the ERα gene expression. Conclusion: GE and TSA can significantly inhibit the growth of HCC cells and play a significant role in apoptosis and reactivation of ERα gene.

Keywords: Genistein- trichostatin A- hepatocellular carcinoma

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Introduction

In the normal mammalian cells, epigenetic changes such as DNA methylation and histone acetylation play an important role in the gene expression. Hypermethylation of CpG islands of the promoter region of tumor suppressor genes plays a major role in carcinogenesis through transcriptional silencing. Tumor suppressor gene hypermethylation is very important in the neoplastic process and carcinogenesis progress. This epigenetic process is recognized by loss of function of these genes associated with transcriptional loss without any structural changes (Bakker et al., 2002). Recently, many experimental works reported the critical role of DNA hypermethylation in human tumorigenesis. Generally, methylated CpG islands of tumor suppressor genes cannot initiate the transcriptional process. To date, several reports regarding to hypermethylation and gene silencing have been published (Rhee et al., 2002). DNA demethylating agents such as genistein (GE), daidzein and 5-azacitidine (5-Aza) can strongly reactivate silenced genes by demethylation of promoter regions (Adam et al., 2010). Soy isoflavone GE, presents in dietary plants such as soybean, has unique chemical properties with biological anticancer activity (Messina et al., 2006). Various studies indicate that GE can induce cell cycle arrest and modulate key regulator cell cycle proteins (Ramos, 2007) especially proteins involved in the G2/M checkpoint, as it has been reported in prostate cancer (Choi et al., 2000). It has been reported that GE affects the re-expression of estrogen receptor (ER) and some tumor suppressor genes in different cancers such as colon cancer (Bielecki et al., 2011; Berner et al., 2011). Previously, we reported inhibitory and apoptotic effect of GE on HCC PLC/PRF/5 (Dastjerdi et al., 2015) and also the effect of this compound on DNA methyltransfrase (DNMT1) and estrogen receptor alpha (ERα) genes expression (Kavoosi et al., 2016).

In addition to hypermethylation, histone hypoacetylation is also associated with gene silencing and cancer induction. The balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities play a crucial role in the acetylation level of histone and the regulation of gene transcription. The

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acetylation of lysine in the histone tails creates a relaxed chromatin, which facilitates gene transcription, while deacetylation of lysine is associated with condensed chromatin resulting gene silencing (Johnstone, 2002; Iizuka and Smith, 2003). One of the most important factors of tumorigenesis is HDAC activity, class I histone deacetylases are overexpressed in many cancers such as colon, stomach, prostate, esophagus, lung, breast, ovary, pancreas and thyroid cancers (Nakagawa., 2007). Histone deacetylase inhibitors (HDACIs) can inhibit cell growth and induce apoptosis. These compounds include cyclic peptides, hydroxamates, aliphatic acids, and benzamides. Trichostatin A (TSA) was the first natural hydroxamate histone deacetylase inhibitors (Milos, 2007). Apoptotic effects of the other DACIs such as sodium butyrate, suberoylanilide hydroxamic acid (SAHA), MS-27-275, FR901228 on divers cancers have been reported (Marks, 2000). Hypermethylation of the CpG islands of the promoter region of ERα has been reported in hepatocellular carcinoma HepG2, HuH2, HLE, HLF and SK-Hep1 cells (Hishida et al., 2013) and also hypoacetylation of lysine residues of core histones, particularly H3 and H4 in breast cancer (Yang et al., 2001). Therefore, hypermethylation of the CpG islands of the promoter region and hypoacetylation of lysine residues of core histones of tumor suppressor genes can silence transcription and expression of the genes resulting tumorigenesis. It should be noted that the role of ERα in cell development and differentiation has been reported. It can inhibit cancer invasion by transcriptional activation of estrogen response element which regulates target genes, such as E-cadherin (Maynadier et al., 2008). Besides, low expression of ERα in the hepatocyte cells is associated with hepatocellular carcinoma (Mitsuhiro et al., 2013). Histone deacetylase inhibitors and DNA demethylating drugs co-treatment can reactivate ERα expression and induce apoptosis. Therefore, they are attractive candidates for becoming potent new drugs in cancer therapy (Walton et al., 2008). It has been reported that 5-aza-2ˈ-deoxycytidine in combination with TSA re-activate ERα gene expression and induce apoptosis in human breast cancer MDA-MB-231 and MDA-MB-435 cell lines (Xiaowei t al., 2001). However, little is known about the effect of GE and TSA (as individual and combined) on ERα gene expression and cell growth inhibition and also apoptotic induction in hepatocellular carcinoma HepG2 cell line, therefore, this study was designed to investigative the effect of these compounds on the ERα gene expression, viability and apoptosis in this cell line.

Materials and Methods

Materials

The human hepatocellular carcinoma HepG 2 cells were obtained from the National Cell Bank of Iran-Pasteur Institute. GE, TSA, Dulbecco's modified Eagle medium (DMEM) and 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl -2H-tetrazolium bromide (MTT) were purchased from Sigma (Sigma, St. Louis, MO, USA). Total RNA extraction Kit (TRIZOL reagent) and real-time polymerase chain reaction (PCR) kits (qPCR MasterMix Plus for SYBR Green I dNTP) were obtained from Applied Biosystems (Foster, CA, USA).

Cell culture

The HepG 2 cells were cultured and grown in DMEM supplemented with 10% fetal bovine serum. The cultures were incubated at 37°C in a humidified incubator containing 5% CO_2, 95% ambient air before treatment with different concentration of GE and TSA. GE and TSA were dissolved in DMSO at a final concentration of 100 μM in order to prepare a stock solution. By diluting the stock solution, the all of other test concentrations were prepared.

Determination of cell viability by MTT assay

The experiments were performed using the HepG 2. The effect of GE and TSA (alone and combined) on the HepG 2 cell viability was assessed by MTT assay. Briefly, When the cultured cells became >80% confluent were seeded at the density of 5×10^5 cells with 1mL of medium/ well onto 96-well culture plates (Becton, Dickinson). After 24 h, DMEM culture medium were replaced by experimental media containing different concentrations of GE (1, 10, 20, 40 μM) and TSA (0.5, 1, 5, and 10 μM) dissolved in dimethyl sulfoxide (DMSO), DMSO was present at 0.01–0.3% in the medium, and finally the cells were incubated for 24, 48 and 72 h. On days 1, 2 and 3 after GE and TSA treatment, MTT assay was done. At the end of the incubation period, the culture medium was replaced with 100 μl of DMSO and the optical density of each well was determined with a spectrophotometer at 570 nm.

Determination of apoptotic cells by flow cytometry assay

The HepG 2 and control cells were cultured in 24-well culture plates and treated with GE (20 μM), TSA (1 μM) and GE (20 μM) / TSA (1 μM) for 24, 48, and 72 h. After treatment times, the cells were trypsinized and the adherent cells were collected with 0.05% trypsin, washed with cold phosphate-buffered saline and re suspended in binding buffer (1x) and analysis was carried out according to the manufacturer's protocol (BMS500F1/100CE Annexin V-FITC, eBiscience, USA) by the addition of Annexin V-FITC and propidium iodide (PI, Becton, Dickinson, San Diego, CA). Finally, the apoptotic cells were counted by FACScan flow cytometry (Becton, Dickinson, Heidelberg, Germany). All experiments were processed independently 3 times. A minimum of 5×10^5 cells/ml were analyzed for each sample.

Determination of gene expression by real-time quantitative reverse transcription polymerase chain reaction (Real-time RT-PCR)

The HepG 2 cells were cultured and treated with GE (20μM) and TSA (1 μM) as alone and combined at different time periods (24, 48 and 72 h). After treatment times, total RNA was isolated by RNeasy mini kit (Qiagen) according to the manufacturer's instructions and then treated by RNase-free DNase (Qiagen) to eliminate the genomic DNA. The RNA concentration was determined using a BioPhotometer (Biowave II Germany). Total RNA

(100 ng) was reverse transcribed into complementary DNA (cDNA) using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, K1622 for 100 reactions) according to the manufacturer's instructions. Real-time RT-PCR was performed by the Maxima™ SYBR Green/ROX qPCR Master Mix (2x1.25 ml, K0221). ERα primers were obtained using review articles (Mirza et al., 2013; Kurebayashi et al., 2000; Otsuki T et al., 2000) which their sequences are shown in the table 1. Real-time PCR reactions were performed using the Steponeplus (BD facscalibur StepOne plus v2.2). Data were analyzed using the comparative Ct (ΔΔct) method; the relative expression level of ERα was calculated by determining a ratio between the amount of this gene and that of endogenous control. GAPDH was used as a reference gene for internal control.

Results

Result of cell growth inhibition evaluated by the MTT assay

The HepG 2 cells were cultured at a density of 5×10^5 cells/well in 100 µl of medium in a 96-well culture plates. After 24 h, the cells were treated with various doses of GE and TSA as mentioned in the materials and methods, while control cells were cultured in medium with 0.05% DMSO. After treatment with these compounds (24, 48 and 72 h after addition of medium contains GE and TSA), the cell viability was determined by using the MTT assay. It was found that GE had biphasic effects; it has a proliferative effect with low concentration (1 µM) and antiproliferative effect with high concentration (10, 20 and 40 µM) of GE as shown in Figure 1 ($P< 0.05$) and that TSA showed an antiproliferative effect with all of experimental concentrations. IC50 values were obtained with approximately 20 µM of the GE and 1 µM of the TSA.

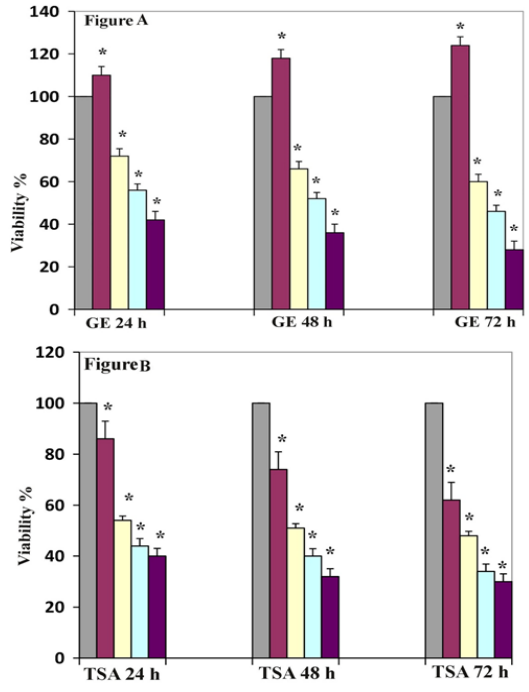

Figure 1. The Effect of GE (Figure A) and TSA (Figure B) on HepG 2 Cell Proliferation. The cells were seeded and treated with GE (1, 10, 20, 40 µM) and TSA (0.5, 1, 5, and 10 µM) for 24, 48, and 72 h. Each five columns of fig. A represents control and experimental groups (treated with 1, 10, 20, 40 µM of GE) and also in the fig. B control and experimental groups (treated with 0.5, 1, 5, and 10 µM of TSA) respectively. The cell viability was determined by the MTT assay. Data are presented as mean ± standard error of the mean from at least three different experiments. Significant differences between treated and control group are indicated by asterisks (*).

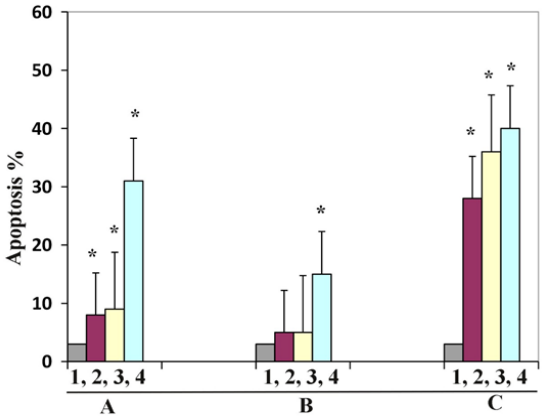

Figure 2. The Apoptosis-Inducing Effect of GE, TSA and GE/TSA Investigated by Flow Cytometric Analysis of HepG 2 Cells Stained with Annexin V and Propidium Iodide. As shown above, the significant apoptotic effect of TSA was seen after 72 h of treatment. The maximum apoptotic effect was seen after 72 h with combined treatment. A; GE treated groups, B; TSA treated groups and, C; GE/TSA treated groups (1. Control, 2. 24 h, 3. 48 h, and 4. 72 h).

Table 1. Real- Time Polymerase Chain Reaction Primers Used in the Current Study

Gene (ERα)	Primer sequence (5´→3´)	Length	Temperature
Forward primer	AGACATGAGAGCTGCCAACC	20	58.04
Reverse primer	GCCAGGCACATTCTAGAAGG	20	57.33

Result of apoptotic induction by GE and TSA

The HepG 2 cells were treated with GE (20 µM), TSA (1 µM), and GE (20 µM)/TSA (1 µM) for different time periods (24, 48 and 72 h) and then collected after

Table 2. The Percent of Apoptotic Cells after Treatment with GE, TSA and GE/TSA at Various Time Periods

Drug	Dose/µm	Duration/h	Apoptosis %	P-value
GE	20	24	8	0.001
GE	20	48	9	0.001
GE	20	72	31	0.001
TSA	1	24	5	0.144
TSA	1	48	5	0.144
TSA	1	72	15	0.001
GE/TSA	20/1	24	28	0.001
GE/TSA	20/1	48	36	0.001
GE/TSA	20/1	72	40	0.001

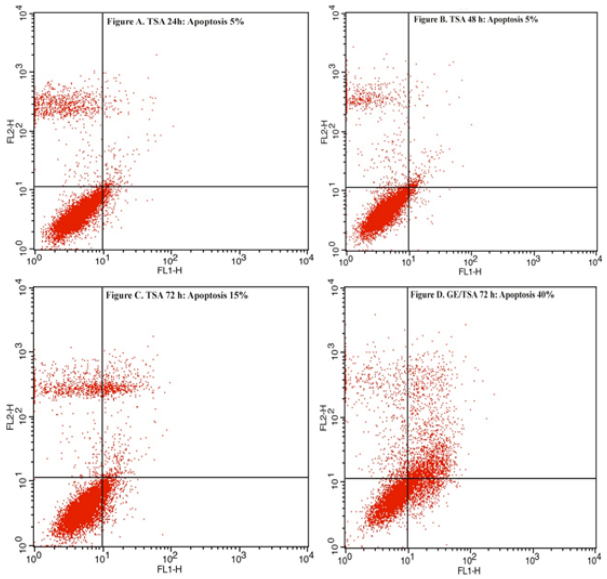

Figure 3. The Effects of GE and TSA on HepG 2 Cell Apoptosis. The cells were treated with GE (20 µM), TSA (1 µM) and GE (20 µM)/TSA (1 µM) for 24, 48 and 72 h and the apoptosis inducing effect of the agents was investigated by flow cytometric analysis. Results were obtained from three independent experiments and were expressed as mean ± standard error of the mean. It should be noted that TSA indicated a significant effect after 72 h of treatment. The apoptotic effect of GE (not shown here) was more than of TSA and maximum effect was seen with combined therapy after 72 h.

trypsinization and stained with Annexin V and propidium iodide following which investigated by flow cytometry. The result showed that GE and GE/TSA induced apoptosis significantly with all concentrations, while TSA induced significant apoptosis only after 72 h, as shown in the

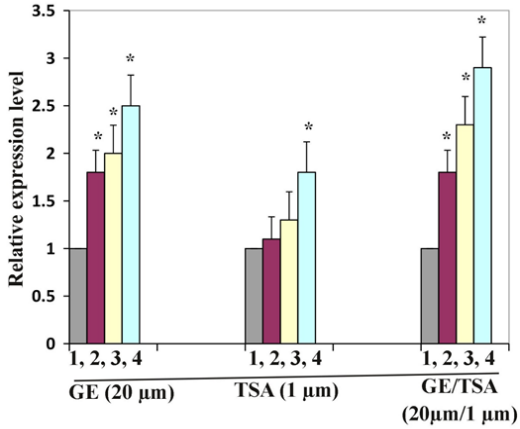

Figure 4. Relative Expression of Estrogen Receptor Alpha (ERα) in Response to GE (20 µM), TSA (1 µM) and GE (20)/ TSA (1 µM). Quantitative reverse transcription polymerase chain reaction analysis demonstrated that GE (at the 24, 48 and 72 h), TSA (only after 72 h) and combined treatment (at the 24, 48 and 72 h) increased ERα expression significantly. Effect of combined treatment was more significant than either agent alone. Results were obtained from three independent experiments and were expressed as mean ± standard error of the mean. Asterisks (*) indicate significant differences between treated and the control group, 1. Control group, 2. 24 h, 3. 48 h, and 4. 72 h

Table 3. Relative Expression of ERα after Treatment with GE, TSA and GE/TSA at Various Time Periods

Drug	Dose/µm	Duration/h	Relative expression	P-value
GE	20	24	1.8	0.032
GE	20	48	2	0.01
GE	20	72	2.5	0.001
TSA	1	24	1.1	0.941
TSA	1	48	1.3	0.382
TSA	1	72	1.8	0.009
GE/TSA	20/1	24	1.8	0.025
GE/TSA	20/1	48	2.3	0.001
GE/TSA	20-Jan	72	2.9	0.001

Figure 2. The apoptotic effect of combined treatment was more significant than either agent alone, which are shown in Table 2. As depicted in figure 3, minimum and maximum apoptosis were seen in the groups treated with TSA alone for 24 h and with GE/TSA for 72 h, respectively.

Result of ERα upregulation assessed by Real-time RT-PCR

Significant reactivation of ERα expression was observed in all treated groups (GE, TSA and GE/TSA treated groups) for different time periods except those treated with TSA for 24 and 48 h and also control groups. Result of Real-time RT-PCR showed that GE can induce ERα re-expression in hepatocellular carcinoma PLC/PRF5 cell line. In this work, as shown in Figure 4, a maximum expression of ERα gene was observed in combined treatment groups which treated with GE/TSA for 72 h. The relative gene expression is demonstrated in the Table 3.

Discussion

Epigenetic alterations such as DNA hypermethylation and histone deacetylation can silence gene expression without DNA sequence changes. These alterations in tumor suppressor genes are associated with tumorigenesis. ERα gene is one of the tumor suppressor genes that is expressed in many tissues especially in the liver tissue. The ERα promoter hypermethylation has been reported in HCC (Mitsuhiro et al., 2013). Fortunately, hypermethylated and deacetylated tumor suppressor genes are reversible by epigenetic drugs. GE, a demethylating agent, can suppress the formation and development of the certain types of tumor. It can act as a chemotherapeutic agent against various types of cancer, such as breast, prostate, liver, lung, colorectal and gastric cancers (Carmela et al., 2015). In vitro studies have reported that GE induces apoptosis in HCC HuH-7, Bel 7402, and Hep3B cell lines (Herold et al., 2002). In addition to demethylation, histone acetylation, which is controlled by a balance between histone acetyltransferases and histone deacetylases plays a key role in epigenetic regulation of gene expression. In HCC, several studies have demonstrated that histone deacetylase inhibitor TSA

induces cellular differentiation, cell growth suppression, and apoptosis induction of MH1C1, Hepa1–6 and Hep1B cell lines (Herold et al., 2002). Previously, we reported that GE increased ERα gene expression by which induced apoptosis in human HCC PLC/PRF5 cell line (Kavoosi et al., 2016). In this report, we announce that GE and TSA (alone and combined) can inhibit cell growth and induce apoptosis by re-activation of ERα gene expression in human hepatocellular carcinoma HepG 2 cells. Similar apoptotic effect for GE has been reported for other cell lines of HCC, including HuH-7, Bel 7402, and Hep3B cell lines (Herold et al., 2002) and also for other cancers such as breast, prostate, liver, lung, colorectal and gastric cancers (Carmela et al., 2015). Furthermore, in line with our result, apoptotic effect and cell growth inhibition of TSA in other HCC cell lines, such as MH1C1, Hepa1–6 and Hep1B have been reported by other researchers (Herold et al., 2002). In our experiment, cell growth inhibition, apoptotic induction and ERα gene expression were increased significantly with co-treatment of GE and TSA. In agreement with our study, it has been demonstrated that combined treatment with DNA demethylating 5-aza-dC in combination with histone deacetylase inhibitor TSA elevate the ERα gene expression more significant than either agent alone in breast cancer MDA-MB-435 and MDA-MB-231 cell lines (Xiaowei et al., 2001). In the current study, GE reactivated ERα more significant than TSA. It is most likely that GE inhibits DNA methyltransfrase activity through which increases ERα expression. In the present study, GE demonstrated biphasic effects, stimulatory and inhibitory effect. This means that GE had a stimulatory effect with low concentrations and an inhibitory effect with high concentrations. Consistent with our finding, it has been reported that GE induces a biphasic effect on estrogen-responsive breast cancer MCF-7 and MDA-MB-231 cells (Alina et al., 2016). Other researchers have reported that GE plays an important role in the prevention and inhibition of cancers such as stomach cancer, breast cancer, prostate cancer, colon cancer, leukemia and melanoma by different pathways and mechanisms (Entschladen et al., 2004; Gu et al., 2005). Recently, we reported that GE incombination with TAM induced apoptosis and cell growth inhibition in hepatocellular carcinoma HepG 2 cell line (Sanaei t al., 2017). In human prostate cancer, GE re-activates the expression of tumor suppressor genes p21 (WAF1/CIP1/KIP1) and p16 (INK4a) by which induces cell cycle arrest (Shahanaet al 2008) and also inhibits the expression of cyclin A, cdc25A, cyclin B, survivin, ki67, TGF-β, pescadillo that are involved in the cell cycle and apoptosis regulation (Yiwei and Fazlul, 2002). In the human colon cancer, GE inhibits cell growth and induces cell cycle arrest at the G2/M phase by the upregulation of GADD45α, p53/p21waf1/cip1 and downregulation of cdc2 and cdc25A (Zhiyu et al., 2013). It has been reported that GE can reactivate methylation-silenced BTG3 tumor suppressor gene by promoter demethylation and histone modification in renal cancer cell (Shahana t al., 2009). GE increases enrichment of histone acetylation chromatin markers, including acetylH3, acetyl-H4 and acetyl-H3K9 which affect ERα gene expression in MDAMB-231 breast cancer cells (Yuanyuan et al., 2013). It has been demonstrated that GE at low concentration (750 ppm) stimulates the growth of MCF-7 cells by upregulation of pS2 and c-fos genes (Allred et al., 2001).

As mentioned above, we reported that TSA increased ERα expression. One of the mechanism by which TSA increases the ERα gene expression is overexpression of p300 which induces acetylation of ERα by blocking ubiquitination (Sung-Hye et al., 2010). It induces the upregulation of mRNA and protein levels of CYLD in HCC Huh7 cell (Kotantaki and Mosialos, 2016). It has been reported that TSA reduces the expression of cyclin D1 and upregulates p21 in bladder cancer cell line by which induces cycle arrest at the G2/M and G1 phase (Wang et al., 2017).

In gallbladder carcinoma SGC-996 cells, TSA down-regulates the expression of cyclin D1, Bmi1, c-Myc and decreases the phosphorylation of AKT, S6, 4E-BP1, and mTOR p70S6K1 (Zhang et al., 2015). TSA treatment leads to the hyperacetylation of histones associated with the Suppressors of cytokine signaling (SOCS1 and SOCS3) promoters in colorectal cancer (CRC) cells. Therefore, TSA upregulates SOCS1 and SOCS3 expression resulting significant downregulation of JAK2/STAT3 signaling in colorectal cancer cells (Xiong et al., 2012). With regard to our result, ERα plays an important role in HCC and DNA demethylating agent in combination with histone deacetylase inhibitor can reactivate silenced ERα gene which provide an important clinical implication for these compounds. Additional works will be necessary to investigate the effects of these compounds on DNA methyltransfrases.

In conclusion, collectively, our result suggests a significant role of GE and TSA (as individually and specially combined) on ERα gene expression, thereby affect cell growth inhibition and apoptosis induction in HCC. Therefore, the result of the current study may provide new avenues and therapeutic strategies for cancer therapy.

Conflict of interest

The authors report no conflicts of interest in this work.

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