Genistein potentiates the effect of 17-beta estradiol on human hepatocellular carcinoma cell line

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. This cancer may be due to a multistep process with an accumulation of epigenetic alterations in tumor suppressor genes (TSGs), leading to hypermethylation of the genes. Hypermethylation of TSGs is associated with silencing and inactivation of them. It is well-known that DNA hypomethylation is the initial epigenetic abnormality recognized in human tumors. Estrogen receptor alpha (ERα) is one of the TSGs which modulates gene transcription and its hypermethylation is because of overactivity of DNA methyltransferases. Fortunately, epigenetic changes especially hypermethylation can be reversed by pharmacological compounds such as genistein (GE) and 17-beta estradiol (E2) which involve in preventing the development of certain cancers by maintaining a protective DNA methylation. The aim of the present study was to analyze the effects of GE on ERα and DNMT1 genes expression and also apoptotic and antiproliferative effects of GE and E2 on HCC.

Materials and Methods: Cells were treated with various concentrations of GE and E2 and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay was used. Furthermore, cells were treated with a single dose of GE and E2 (25 μM) and flow cytometry assay was performed. The expression level of the genes was determined by quantitative real-time reverse transcription polymerase chain reaction.

Results: GE increased ERα and decreased DNMT1 genes expression, GE and E2 inhibited cell viability and induced apoptosis significantly.

Conclusion: GE can epigenetically increase ERα expression by inhibition of DNMT1 expression which in turn increases apoptotic effect of E2. Furthermore, a combination of GE and E2 can induce apoptosis more significantly.

Key Words: DNMT1, E2, epigenetic, estrogen receptor alpha, genistein, hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world and also is the second among cancers of digestive tract after stomach cancer. The disease has a wide geographical variation. The major risk factors of HCC are chronic viral hepatitis B and C. Other
factors include nutritional factors, toxins, and metabolic diseases.\textsuperscript{5,6} Besides, HCC may be due to a multistep process with an accumulation of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation of tumor suppressor genes (TSGs). Epigenetics refers to heritable changes that play an important role in the control of gene expression.

In contrast to genetic events, the epigenetic pathway is a reversible alteration and characterized by three main mechanisms:

- DNA hypermethylation leading to inactivation of genes,
- DNA hypomethylation causing genomic instability,
- Histone modifications affecting chromatin conformation.

It should be noted that DNA methylation does not change the genetic information. In fact, it alters the readability of the DNA and results in inactivation of genes by subsequent messenger RNA transcript repression.\textsuperscript{7,8} Epigenetic silenced TSGs genes are involved in important molecular pathways of carcinogenesis example, cell cycle regulation, apoptosis, DNA repair or cell adhesion, and hypermethylation have been widely reported in all types of tumors.\textsuperscript{9}

It is well-known that DNA hypomethylation is the initial epigenetic abnormality recognized in human tumors such as ovarian epithelial carcinomas,\textsuperscript{10,11} prostate metastatic tumors,\textsuperscript{12} HCC\textsuperscript{13} and Wilms' tumors.\textsuperscript{14} Collectively, cancer is an epigenetic disease. Hypermethylation at the CpG islands found in estrogen response element (ERE) promoters occurs in conjunction with ligand bonded alpha subunit estrogen receptor alpha (ER\textalpha) dimers wherein the ligand ER\textalpha dimer complex acts as a transcription factor and binds to the ERE promoter.\textsuperscript{15} ER\textalpha signaling plays a key role in hormonal cancer progression.\textsuperscript{16} It has been reported that hypermethylation of ER\textalpha gene is a marker for HCC.\textsuperscript{15} Furthermore, the relation between ER\textalpha and malignant disease has been discussed in a variety of tissues including breast, colon, blood, bladder, and liver.\textsuperscript{17-25}

In many cancers, hypermethylation of CpG islands results from overactivity of DNA methyltransferases (DNMTs). In humans, the primary DNMTs are DNMT1, DNMT3a, and DNMT3b. DNMT1 is the most abundant in human.

Fortunately, epigenetic changes can be reversed by pharmacological intervention. Several active compounds in the food can decrease the risk of cancers by epigenetic mechanisms. Dietary phytoestrogens like genistein (GE) have shown to possess multiple cell regulatory activities within cancer cells.\textsuperscript{26} Besides, it has been reported that phytoestrogens like GE involve in preventing the development of certain cancers such as prostate and mammary cancers by maintaining a protective DNA methylation profile.\textsuperscript{27} Some of the dietary phytoestrogens exert their chemopreventive effects by modulating various components of the epigenetic mechanism in humans and have potentially beneficial effects on DNA methylation pattern.\textsuperscript{26}

Isoflavones are structurally similar to 17-beta estradiol (E2) and have the ability to bind to ERs.\textsuperscript{28} They act as agonists or antagonists of E2\textsuperscript{29} and exert weak estrogenic activity in some tissues and antiestrogenic activity in others. They exert dual actions (both inhibitory and stimulatory effects) depending on their concentration.\textsuperscript{30-32} Because of antiproliferative effect, they protect against some cancer such as uterine, breast, prostate, lung and colon cancer.\textsuperscript{33-36}

In previous study, we indicated that E2 can inhibit proliferation and induce apoptosis in PLC/PRF5 HCC cell line\textsuperscript{28} and in this study, we investigated whether GE could alter the ER\textalpha and DNMT1 expression and also investigated apoptotic and proliferative effects of GE combined with E2 on PLC/PRF5 HCC cell line.

**MATERIALS AND METHODS**

**Materials**

Human HCC cell line (PLC/PRF5) was purchased from the National Cell Bank of Iran-Pasteur Institute. GE, E2 were purchased from Sigma (Sigma, St. Louis, MO). Total RNA extraction Kit (TRIZOL reagent), real-time polymerase chain reaction (PCR) kits (qPCR MasterMix Plus for SYBR Green I dNTP), DMEM (Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Qiagen. All other chemicals were obtained from the best available sources.

**Cell culture**

The 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\textsubscript{2}, 95% ambient air. When cells became >80% confluent, 5 x 10\textsuperscript{4} cells (PLC/PRF5) were seeded into 24-well plates (Becton, Dickinson) for 24 h in DMEM culture medium before they were incubated with certain concentrations of GE (1, 5, 10, 25, 50, 75, and 100 \mu M/L), which was dissolved in dimethyl sulfoxide (DMSO); DMSO was present at 0.01–0.3% in the medium. After 24 h, culture medium was changed with culture medium.
contains various concentrations of GE. On days 2, 3, and 4 after treatment with GE, MTT assay was done. The MTT assay for determination of IC50 value for E2 was done as done for GE with certain concentrations of E2 (1, 5, 10, 25, 50, 75, and 100 μM/L) which was dissolved in DMSO; DMSO was present at 0.01–0.3% in the medium. Photography was done for cultures before and after treatment with GE and E2 at different times using inverted microscope (Nikon, TE 2000-U, Japan).

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay for cell viability and IC50
After 24, 48, and 72 h of the treatment, the IC50 value for GE and E2 in PLC/PRF5 group were determined. Briefly, 5 x 10^5 Cells (PLC/PRF5) were counted and placed into each well of a 24-well culture plate. After 24 h of seeding, various concentrations of GE and E2 were added to the cells except in the control groups and after 24, 48, and 72 h of drug exposure, the MTT survival assay was then carried out for the evaluation of the cell viability with different drug concentration. The cells measured spectrophotometrically at 570 nm. All experiments were repeated 3 times with at least three measurements (triplicates).

Determination of apoptotic cells by flow cytometry assay
The cells were cultured in 24-well culture plates and divided into 11 groups after 24 h. Three groups received a single dose of GE at the concentration of 25 μM and also 3 groups received a single dose of E2 at the concentration of 25 μM for 24, 48, and 72 h, respectively. One group received GE (25 μM) for 24 h and followed by E2 (25 μM) for 24 h (total treatment time 48 h) and other group received same dose of GE (GE) for 48 h and followed by E2 (25 μM) for 24 h (total treatment time 72 h). Final 3 groups received DMSO as control groups. In the GE treated groups (3 groups), E2 treated groups (3 groups) after 24, 48, and 72 h and GE-E2 groups (2 groups) after 24 h of E2 treatment and also control groups, all the adherent cells were collected with 0.05% trypsin, washed with cold phosphate-buffered saline and re suspended in binding buffer (1x). After addition of Annexin V-FITC and propidium iodide (PI, Becton, Dickinson, San Diego, CA), analysis was carried out according to the manufacturer’s protocol (BMS500F1/100CE Annexin V-FITC, eBiscience, USA). 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 x 10^5 cell/ml were analyzed for each sample.

Determination of gene expression by real-time quantitative reverse transcription polymerase chain reaction
Real-time quantitative reverse transcription (RT)-PCR amplification and analysis were achieved to quantitatively estimate the expression of ERα and DNMT1 in GE (25 μM)-treated PLC/PRF5 cells at different times. Total RNA was isolated by RNeasy mini kit (Qiagen) according to the manufacturer’s protocol 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 to complementary DNA (cDNA) by 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 and DNMT1 primers were obtained from articles[37-38] which their sequences are shown in Table 1. Real-time PCR reactions were performed using the Steponeplus (BD fascicalibur StepOne plus v2.2). Thermal cycling conditions for ERα was: An initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 15 s and extension at 72°C for 15 s. Thermal cycling condition for DNMT1 was: An initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s and extension at 72°C for 20 s. Data were analyzed using the comparative Ct (ΔΔct) method, the relative expression level of ERα and DNMT1 was calculated by determining a ratio between the amount of these genes and that of endogenous control. Melting curve was used to determine melting temperature of specific amplification products and primer dimmers. These experiments were carried out in triplicate and independently repeated at least 3 times. GAPDH was used as a reference gene for internal control.

Table 1: Real-time polymerase chain reaction primers used in the study

| Gene   | Sequence (5’→3’) | Length | Temperature |
|--------|-----------------|--------|-------------|
| ERα    | Forward primer  | AGACATGAGAGCTGCCAACC | 20 | 60.04 |
|        | Reverse primer  | GCCAGGCGCATCTAGAAGG  | 20 | 58.33 |
| DNMT1  | Forward primer  | TACCTGGACGCGCCGACCCT  | 21 | 62.69 |
|        | Reverse primer  | CGTTGCGATCAAGATGGAC  | 21 | 59.19 |
RESULTS

Result of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay for cell viability and IC50

The effects of the various concentrations of GE and E2 (as mentioned) on the cell viability were assessed by MTT assay. The dose- and time-dependent antiproliferative effects were observed with IC50s for GE and E2 [Figures 1 and 2]. Reduction of cell viability by 50% (IC50) required 25 μM GE for GE-treatment groups and same dose of E2 for E2-treatment groups at different time periods (24, 48, and 72 h). Each experiment was repeated 3 times for consistency of the result. The percentage of cell viability for GE (25 μM)-treatment groups were 52% ($P < 0.001$), 48% ($P < 0.001$), and 45% ($P < 0.001$) and for E2 (25 μM)-treatment groups were 55% ($P < 0.001$), 51% ($P < 0.001$), and 48% ($P < 0.001$) at different time periods (24, 48, and 72 h), respectively [Figures 3 and 4].

Result of determination of apoptosis by flow cytometry assay

The apoptosis-inducing effect of GE and E2
was investigated by flow cytometric analysis of PLC/PRF5 cells stained with Annexin V and propidium iodide. We observed via flow cytometry that these compounds induce apoptosis in this cell line significantly. The percentage of apoptotic cells in the GE (25 μM)-treatment groups at different times (24, 48, and 72 h) were 30, 36, 42% \( (P < 0.001) \) [Figure 5] and in the E2 (25 μM)-treatment groups at different times (24, 48, and 72 h) were 22, 30, 37% \( (P < 0.001) \) [Figure 6], respectively. The percentage of apoptotic cells in the group that was treated with GE (25 μM) for 24 h and followed by E2 (25 μM) for 24 h was 44% and in the group that was treated with GE (25 μM) for 48 h and followed by E2 (25 μM) for 24 h was 60% \( (P < 0.001) \) [Figure 7]. Relative analysis between GE treatment groups and E2 treatment groups at different times indicated that GE induces apoptosis more significantly and the percentage of apoptotic cells in the groups that treated with combined compound were significantly higher than that of the experimental groups that treated with GE or E2 alone, with 44% and 60% apoptotic cells respectively as shown in the Figure 8 \( (*) P < 0.001 \). The apoptotic effect was not observed in DMSO control group. A minimum of \( 5 \times 10^5 \) cells/ml were analyzed for each sample. Results were obtained from three independent experiments and were expressed as mean ± standard error of mean.

**RESULT of determination of gene expression by real-time polymerase-chain-reaction**

Using quantitative RT-PCR, GE (25 μM) was shown to significantly increase ERα expression [Figure 9] and decrease DNMT1 expression [Figure 10] in PLC/PRF5 cell line at different time periods (24, 48, and 72 h). The relative expression of ERα was 2, 2.5, and 3.1 \( (P < 0.001) \) and that of DNMT1 were 0.33, 0.26, and 0.21 \( (P < 0.001) \) in different time periods, respectively.

**DISCUSSION**

Our study clearly demonstrated that GE (25 μM) can down-regulate the expression of DNMT1, up-regulate
the expression of ERα, inhibit cell proliferation, and induce cell apoptosis in PLC/PRF5 cell line with a dose- and time-dependent manner. Similarly, it has been indicated that dietary GE affects ERα expression via modulating epigenetic pathways such as DNMT-involved transcription regulation.

It should be noted that GE induces a maximal ERα increment at 25 μM in a time-dependent manner. Gu et al. demonstrated that GE inhibits the growth of MHCC97-H HCC cells in vitro in a concentration-dependent fashion which is more potent in the 10 μg/ml and 20 μg/ml GE-treated groups. In other cancers such as stomach cancer, breast cancer, prostate cancer, colon cancer, leukemia and melanoma, GE strongly inhibits cell proliferation and plays an important role in the prevention and inhibition of tumor. Similarly, it has been reported that GE induces and increases the apoptotic population in ovarian cancer cells (Choi et al., 2007). In rodent models, GE can protect against mammary and prostate cancers. A relationship between the intake of soy foods and reduced breast or prostate cancer has been reported in several epidemiological studies.

These findings about effect of GE appear to lend support for our current finding, but many studies have shown that GE has proliferative or biphasic effect that is not consistent with our result; Hsieh et al. reported that GE increases MCF-7 cell proliferation at a concentration of 0.01–1 μM, and maximal growth stimulation is observed at 1 μM and this level of growth is sustained up to 10 μM. At relatively low concentrations, GE is full agonists for ERα as well as for the proliferation of ER-dependent breast cancer cells.

Many mechanisms and different pathways have been reported for GE, although the exact mechanisms of GE await further elucidation; Hsieh et al. reported that GE can act via an ER-mediated mechanism. Other observations reported that GE binds to the ER with an affinity approximately 100-fold less than that of estradiol.
Kavoosi, et al.: GE, ERα, epigenetic, hepatocellular carcinoma

Figure 8: Relative analysis between genistein (GE) treatment groups, E2 treatment groups and combined compound treatment groups at different times indicated that GE induces apoptosis more significantly than E2 and also percentage of apoptotic cells in the groups that treated with combined compound were significantly higher than that of the experimental groups that treated with GE or E2 alone ($P<0.001$).

Finally, it has been shown that GE induces cell cycle arrest in the G0/G1 and G2/M phases and the number of S phase cells are decreased in a progressive way as the GE incubation time is increased.

It has previously been reported that structurally distinct phytoestrogens, including the GE, exert their estrogenic effects through direct binding and activation of the ERs (Kuiper et al., 1997; Barkhem et al., 1998). GE has biphasic effect with cytotoxic effect at concentrations >10 μM in breast cancer cell lines and cell death becomes apparent by about 72 h whereas exposure to 10 μM for only 24 h is tolerated.

Our data clearly shown that E2 has a significant inhibitory effect on the growth of liver cancer cells and induces apoptosis in this cell line with a dose- and time-dependent manner. Similar results have been obtained by other studies; Huang et al. reported that estrogen and the estrogen-like compounds (E2) induce anti-proliferative and apoptotic effects in Hep3B cells, and the E2 and the E2-like compounds mediated apoptotic effect is ER dependent. Among the

Figure 7: The apoptosis-inducing effect of genistein (GE) and E2 combination (as described in the methods) were investigated by flow cytometric analysis of PLC/PRF5 cells stained with Annexin V and propidium iodide. The combination of GE and E2 induced cell apoptosis in PLC/PRF5 cells significantly. Asterisks (*) indicate significant differences between treated cells and the control group. Results were obtained from three independent experiments and were expressed as mean ± standard error of mean $n = 3$. (a) GE 24 h/E2 24 h. (b) GE 48 h/E2 24 h. 48 h. (c) control.
estrogen-like compounds, E2 and GE show the stronger anti-tumor potential.\textsuperscript{[72]} It has been reported that 17β-estradiol at a concentration of 1 nM significantly increase apoptosis of MDA-MB-231 breast cancer cells.\textsuperscript{[73]} Besides, dietary phytoestrogens play a protective role against prostate and colon cancer and the formation of polyps\textsuperscript{[74,75]} and reduce colorectal cancer development\textsuperscript{[76]} and also decrease colorectal cancer risk.\textsuperscript{[77]} Over the past decades, epidemiological studies have indicated that consumption of diets rich in phytoestrogens is associated with low risk of breast cancer.\textsuperscript{[78]}

All reports mentioned above confirm our finding about E2, but many studies have reported that phytoestrogens have stimulatory and proliferatory effects.\textsuperscript{[79]} It has been reported that estrogen can stimulate the growth of stromal cells derived from the hyperplastic prostate (Collins \textit{et al.}, 1994). Hong \textit{et al.} (2004) found that estrogen can stimulate the growth of prostatic stromal cells and increase smooth muscle cell markers (Hong \textit{et al.}, 2004).\textsuperscript{[80]}

Furthermore, it has been shown that 17 β-estradiol administration exerts a growth-inhibitory effect on ER-positive cell lines (human gastric carcinoma-27, AGS).\textsuperscript{[81-83]}

It should be noted that phytoestrogens act through different mechanisms and pathways. They exert anticancer activity by two mechanisms including an anti-estrogenic mechanism which is due to structural similarity with estradiol and anti-aromatase activity. Many studies have reported that phytoestrogens exert antiproliferative effects by inhibition of tyrosine kinase activity, DNA topoisomerase II, and angiogenesis.\textsuperscript{[54]}

Other studies have reported that estrogen acts through four molecular pathways: Ligand-independent, ligand-dependent, cell-surface (nongenomic) signaling, and DNA binding-independent.\textsuperscript{[84]} Phytoestrogens reduce cancer risk by binding to ERs or interacting with enzymes involved in sex steroid biosynthesis and metabolism.\textsuperscript{[85]}

Our finding demonstrated that combination of GE and E2 induces apoptosis and inhibits proliferation more significant than that of these compounds alone. Other studies reported that the expression of ER and apoptosis-induction in MCF-7 cells treated with a combination of estradiol (5 μM) and GE (5 μM) are more significant than that of these compound alone.\textsuperscript{[86]} Rajah \textit{et al.} reported that 1 μM GE plus 1 nM 17 β-estradiol significantly increase apoptosis with a concomitant decrease in ERK1/2 phosphorylation. High concentrations of GE (100 μM) both in the presence and absence of 17 β-estradiol also increases apoptosis. \textsuperscript{[73]}

In summary, a combination of GE and E2 has been used synergistically in other studies but we first used these compounds separately and then used GE following by E2 (as mentioned in the material and method) and this is the advantage of our research compared to other researches using these compounds. Considering the results of our research, a combination of GE and E2 may be good candidate for HCC treatment.

We did not perform enzyme activity assays related to methylation and histone modifications and also enzyme immunoassay related to protein levels, but we will perform in the next researches and also further
researches are needed to determine the clinical applications of GE.

CONCLUSIONS

Our study clearly demonstrated that GE increases ERα expression and decreases DNMT1 expression and also inhibits proliferation and induces apoptosis in human HCC cell line through epigenetic mechanism which can provide a new strategy for HCC treatment. It should be noted that when GE (25 μM) treatment followed by E2 (25 μM) treatment, apoptosis was increased more significantly.

Acknowledgment

This article was supported by adjunctly of research of Isfahan medical university-Iran with code number 393005.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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