Effect of Genistein on DNMT1 Gene Expression and Cell Proliferation of Hepatocellular Carcinoma HepG2 Cell Line

Masumeh Sanaei ¹, Fraidoon Kavoosi *¹, Abazar Roustazadeh ¹, Fatemeh Golestan ²

1. Research Center for Non-communicable Diseases, Jahrom University of Medical sciences, Jahrom, Iran
2. Student of Research Committee, Jahrom University of Medical Sciences, Jahrom, Iran
* Corresponding Author: E-mail: Kavoosifraidoon@gmail.com

ARTICLE INFO

Keywords:
Genistein
DNMT1
Proliferation
Hepatocellular carcinoma

ABSTRACT

Hepatocellular (HCC) carcinoma is one of the most common types of cancer and the major form of primary liver cancer. Genetic and epigenetic changes play a significant role in tumourigenesis. Deregulation of epigenetic patterns leads to cancer in different tissues. Epigenetic alterations are reversible by epi-drugs. DNA methyltransferases (DNMTs) catalyze DNA methylation and directly responsible for hypermethylation of tumor-suppressor genes. Genistein (GE) has been shown to decreases methylation of DNA promoter of tumor suppressor genes by reduction of DNMT1 activity which suppress the growth of various cancers. The aim of the present study was to analyze the effect of GE on cell growth and DNMT1 gene expression in the hepatocellular carcinoma HepG2 cell line. Materials and Methods: MTT assay and Real-Time Quantitative RT-PCR were used to evaluate proliferative effect and DNMT1 gene expression. Results: GE inhibited the growth of HepG2 cells and decreased DNMT1 gene expression significantly with a time- and dose-dependent manner. Discussion: Our finding clearly indicated that GE has a significant inhibitory effect and decreases DNMT1 gene expression significantly. Conclusion: GE can significantly inhibit the growth of HepG2 cells and plays a significant role in DNMT1 gene expression.

© 2014 Global Journal of Medicine Researches and Studies. All rights reserved for Academic Journals Center.

INTRODUCTION

Hepatocellular (HCC) carcinoma is one of the most common types of cancer and the major form of primary liver cancer. This cancer is the sixth most prevalent cancer in the world and the third leading cause of cancer related mortality [1]. It is a main complication of cirrhosis and typically starts with a liver disease caused by infection with hepatitis C virus (HCV), hepatitis B virus (HBV) or alcohol consumption [2]. It is associated with a poor prognosis due to a delay in diagnosis and complex pathogenesis because of involving different molecular pathways in this cancer. Carcinoma arises from both, genetic and epigenetic alterations [3] that change gene expression and cell function [4]. It is well-known that an important cause of the cancer is genetic and epigenetic changes. [5]. These changes led to a general classification of cancer genes as either tumour suppressors or oncogenes [6]. Genetic and epigenetic changes play a significant role in tumourigenesis [7]. Deregulation of epigenetic patterns leads to cancer in different tissues [8]. The hallmark of cancer is the deregulation of gene expression profiles and disruption of molecular networks [9]. Epigenetic alterations are reversible by epi-drugs which are drugs targeting epigenetic mechanisms can reverse epigenetic alternation [10-12]. Aberrant epigenetic changes are consistently associated with different cancer types, including lung cancer, colorectal cancer and HCC. DNA methyltransferases (DNMTs), a family of enzymes that is encoded by DNMT genes in the human genome, catalyze DNA methylation. There are four types of DNMT genes contain DNMT1, DNMT2, DNMT3A, and DNMT3B (13) that DNMT1 is the most abundant DNA methyltransferase and considered to be the key maintenance methyltransferase in the mammals. The enzymes directly responsible for CpG island hypermethylation of tumor-suppressor genes are the DNA methyltransferases (DNMTs). DNMT1 exhibits a 5- to 30-fold preference for hemimethylated substrates. This property led to the identification of DNMT1 as the enzyme responsible for maintaining the methylation patterns following DNA replication.
Tumor suppressor genes are normally involved in regulating cell growth but may become cancer-causing when damaged. Tumour suppressor genes encode for proteins that are involved in inhibiting the proliferation of cells, which is crucial to normal cell development and differentiation. Because of this ability, tumour suppressor genes can also act to stem the uncontrolled growth of cancer cells. Genetic damage to tumor suppressor genes contributes to the development of a cancerous tumour.

Estrogen receptor tumor suppressor gene is one of the genes that has recently attracted the attention of researchers and its role in various cancers has been demonstrated. This gene has two types include ESR1 and ESR2 genes that located on chromosomes 6q25.1 and 14q22-24 and code ERα and ERß respectively [14]. These receptors are very important choices for endocrine therapy.

Genistein (GE), a major isoflavone constituent of soybeans and soy products, has been shown to suppress the growth of various cancers such as ovarian, oesophagus, breast, lung and colon cancers through modulation of various pathways, in particular, reactivation of ERα. GE has multiple molecular targets including receptors, enzymes, and signaling pathways but often acts via ER [15]. This compound significantly decreases methylation of DNA promoter by reduction of DNMT1 activity.

In this study, we investigated whether GE could alter the DNMT1 expression and also investigated proliferative effect of GE on HepG2 hepatocellular carcinoma cell line.

MATERIALS AND METHODS

MATERIALS

Human hepatocellular carcinoma HepG2 cell line was purchased from the National Cell Bank of Iran Pasteur Institute. GE, Total RNA extraction Kit (TRIZOL reagent), Real-time PCR kits (qPCR MasterMix Plus for SYBR Green I dNTP, DMEM (Dulbecco’s modified Eagle’s medium Nutrient Mixture F12 Ham) and MTT (3-4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) were purchased from Sigma (Sigma, St. Louis, MO). All other chemicals were obtained from the best available sources.

Cell culture and Determination of IC50 Value by MTT Assay

The HepG2 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% CO2, 95% ambient air. When cells became >80% confluent, 5 × 10^5 cells 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, 20, 40, 80, and 100 μM/lit) , which was dissolved in dimethyl sulfoxide (DMSO); DMSO was present at 0.01–0.3% in the medium. After 24 h, attached cells were treated with various concentrations of GE (Sigma, St. Louis, MO). The medium with GE was not replaced every 24 h for the duration of the experiment. Control cells received equal amounts of DMSO (Sigma) in the Medium. After 24, 48 and 72 hours, the cells were washed twice with PBS, and a fresh medium containing MTT (0.5 mg/mL) was added. After 4-hour incubation, the formazan crystals were dissolved in acidic isopropanol and the absorbance was measured at 570 nm. Photography was done for cultures before and after treatment with GE at different times using inverted microscope (Nikon, TE 2000-U, Japan). All experiments were repeated three times, with at least three measurements (triplicates).

Determination of Cell Viability by MTT Assay

For determination of Cell Viability, cells were treated with an optimal concentration (20 μM) of GE based on our IC50 assay results. Cell viability was estimated by a colorimetric assay based on the conversion of tetrazolium dye (MTT) to a blue formazan product. The effect of GE on cellular proliferation was assessed by MTT assay, according to standard protocols. To determine the effects of GE on cell viability, aliquots of 5 × 10^5 HepG2 cells were seeded in triplicate in 24-well plates and treated with GE at concentration of 20 μM in different period times (24, 48 and 72 h). The MTT assay was commonly used to assess cell proliferation and viability by measuring the reduction of yellow MTT by mitochondrial dehydrogenases in viable cells. This yields purple formazan crystals that detected colorimetrically at 570 nm. The absorbance of the cell lysates in DMSO solution was read at 570 nm by a microplate reader (Bio-Rad Hercules, CA).

Determination of Gene Expression by Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR amplification and analysis were achieved to quantitatively estimate the expression of DNMT1 in GE (20μM)-treated HepG2 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 (Eppendorf). Total RNA (100 ng) was reversetranscribed to cDNA by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s instructions. Real-time RT-PCR was performed by the Maxima SYBR Green RoxqPCR master mix kit (Fermentas). DNMT1 primers were used as referenced articles [16-18] which their sequences are shown in table1. Real-time PCR reactions were performed using the Steponeplus (Applied Biosystem). Thermal cycling conditions for DNMT1 was: an initial denaturation at 95°C for 10 minute followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds and extension at 72°C for 20 seconds. The relative changes of expression were calculated using the following formula: Fold change in gene expression, 2−ΔΔCt=2−[ΔCt
Effect of Genistein on DNMT1 Gene Expression and Cell Proliferation …

Global Journal of Medicine Researches and Studies, 4(1) 2017

(natural compound treated samples)-ΔCt (untreated control)], where ΔCt=Ct (detected genes)-Ct (18S rRNA) and Ct represent the threshold cycle number. 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 three times. GAPDH was used as a reference gene for internal control.

**Table 1. Real time polymerase chain reaction primers used in the study**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>5′- TAC CTG GAC GAC CCT GAC CTC - 3′</td>
</tr>
<tr>
<td>Forward</td>
<td>5′- CGT TGG CAT CAA AGA TGG ACA - 3′</td>
</tr>
</tbody>
</table>

**Result of Determination of IC50 Value by MTT Assay**

The effects of the GE on the HepG2 cell viability after exposure with various concentrations (as mentioned) were assessed by MTT assay. Dose- and time-dependent antiproliferative effect was observed with IC50s for GE. Reduction of cell viability by 50% (IC50) required 20 μm GE. Each experiment was repeated three times for consistency of the result. The Percentage of cell viability with various dose of GE at different time periods (24, 48 and 72h) are shown in fig1 (P<0.001).

![Figure 1. Effect of GE on viability of hepatocellular carcinoma HepG2 cell line determined by MTT assay. Data are presented as mean ± standard error of the mean from at least three different experiments.](image)

**Result of Determination of Cell Viability by MTT Assay**

The cell vitality in the cells which treated with concentration of 20 μM in different time periods were analysed by using the MTT assay. The amounts of reduced MTT in the all groups treated with GE were significantly lower than that of the control group (P < 0.001). The Percentage of cell viability for GE (20μM)-treated groups was 54%, 49 % and 47 % at different times (24, 48 and 72h) respectively (fig2).
Effect of Genistein on DNMT1 Gene Expression and Cell Proliferation …

Global Journal of Medicine Researches and Studies, 4(1) 2017

Figure 2. The cell vitality in the cells which treated with GE at concentration of 20 μM in different time periods were analysed by using the MTT assay. Data are presented as mean ± standard error of the mean from at least three different experiments. Asterisks (*) indicate significant differences between treated cells and the control group.

Result of Determination of Gene Expression by Real-Time Quantitative RT-PCR

To characterize the effect of GE on HepG2 mRNA expression, time-course experiments were performed (24, 48 and 72 h). Using quantitative RT-PCR, GE was shown to significantly decrease DNMT1 gene expression at different time periods. The relative expression of DNMT1 gene was 0.33, 0.24 and 0.16 (P < 0.001) in different times respectively. In conclusion, GE decreased DNMT1 expression significantly as shown in fig.3. Data are presented as means ± S.E.M. n=3.
Figure 3. Relative expression level of DNMT1. GE decreases DNMT1 expression significantly. Data are presented as mean ± standard error of the mean from at least three different experiments. Asterisks (*) indicate significant differences between treated cells and the control group.

Discussion

Dietary compounds have been demonstrated to have a great potential to regulate disrupted tumor suppressor genes and reprogram them during carcinogenesis. It has been noted that DNA methylation patterns have been used as a crucial marker for the study of cancer-related epigenetics. GE, one of the soy-derived bioactive isoflavones and one of the epi-genetic drugs, affects tumorigenesis through epigenetic regulations. This compound activates tumor suppressor genes histone modifications and DNA methylation [19]. It has been known that overall increase in the enzymatic DNA methyltransferase activity occurs in tumors tissues by which CpG islands are hypermethylated [20]. GE demethylates hypermethylated promoter of tumor suppressor genes by inhibition of DNA methyltransferase (DNMT) activity [21]. In the present report, we studied the effect of GE on proliferation of HepG2 cell and expression of DNMT1 gene. We report that GE inhibits proliferation of HepG2 cell and decreases of DNMT1 gene in this cell line. Our previous work had reported that GE can inhibit the growth of PLC/PRF5 hepatocellular carcinoma cells significantly with a time- and dose-dependent manner. Similarly, inhibitory effects of GE on human prostate cancer, HT-29, colo320 cell, HCT 116 cells, ADA/MB231, MCF-7 and HBL-100, have been reported by other studies [22-25]. Opposite with our findings, many studies have reported proliferative effects of GE. It has been reported that GE has biphasic effect in the LNCap cell line [26]. Similar studies also have shown that GE has a proliferative effect on human intestinal, IEC18 [27] and MCF-7 breast carcinoma cells [28]. Our study clearly demonstrated that GE (20 μM) can down regulate the expression of DNMT1 in HepG2 cell line with a time-dependent manner. This result is consistent with other works that have shown that GE decreases DNMT1 gene expression in other cancers [29]. Considering the result of our research, GE may be a good candidate for HCC treatment. This compound can change DNMT1 expression in vivo experiments by which modulates DNA methylation.

CONCLUSION

Our work clearly demonstrated that GE increase decreases DNMT1 expression and also inhibited proliferation in human hepatocellular carcinoma HepG2 cell line through epigenetic mechanism which can provide a new strategy for hepatocellular carcinoma treatment.

ACKNOWLEDGEMENTS

This article was supported by adjutancy of research of Jahrom medical University-Iran. This article has been extracted from Ms Fatemeh Golestan’s thesis.
REFERENCES