RESEARCH ARTICLE

Diosgenin Inhibits hTERT Gene Expression in the A549 Lung Cancer Cell Line

Rahmati Yamchi Mohammad[1,2,4]*, Ghareghomi Somayyeh[3], Haddadchi Gholamreza[3], Mobasseri Majid[4], Rasmi Yousef[5]

Abstract

Background: Diosgenin, a steroidal saponin from a therapeutic herb, fenugreek (*Trigonellafoenum-graceum* L.), has been recognized to have anticancer properties. Telomerase activity is not detected in typical healthy cells, while in cancer cell telomerase expression is reactivated, therefore providing a promising cancer therapeutic target. Materials and Methods: We studied the inhibitory effect of diosgenin on human telomerase reverse transcriptase gene (hTERT) expression which is critical for telomerase activity. MTT- assays and qRT-PCR analysis were conducted to assess cytotoxicity and hTERT gene expression inhibition effects, respectively. Results: MTT results showed that IC_{50} values for 24, 48 and 72h after treatment were 47, 44 and 43μM, respectively. Culturing cells with diosgenin treatment caused down-regulation of hTERT expression. Discussion: These results show that diosgenin inhibits telomerase activity by down-regulation of hTERT gene expression in the A549 lung cancer cell line

Keywords: Lung cancer - hTERT - diosgenin - telomerase - qRT-PCR - MTT assay

Introduction

Human telomerase is a ribonucleoprotein that adds TTAGGG repeats to telomere ends of linear chromosomes in eukaryotes (Sato et al., 2012). Telomerase activity is not evident in normal cells, with elimination of germ cells and regeneration tissues (Buseman et al., 2012). Though, it is reactive currently in almost 80-90% of human cancer. Telomerase includes three main constituents including hTR (human telomerase RNA component), tp1 (telomerase associate protein) and hTERT (human telomerase reverse transcriptase) (Noel and Wellinger, 2012). Between these three constituents, hTERT plays a vital role in telomerase activation. Telomerase inhibitors can be divided into two groups (A sprouse et al., 2012). One group binds to telomerase and blocks its activity, although others are suppressors of gene expression of hTERT (Hsin et al., 2010). Complementary and alternative treatment use is common among cancer patients. In many studies, herbal medicines are among the most normally used group of treatments. Herbal medicines are believed by the general public to be safe, cause less side effects and less likely to cause dependency (Olaku and White, 2011). Diosgenin is a main bioactive constituent of fenugreek (Trigonellafoenum-graecum Linn) that is structurally similar to cholesterol and other steroids (Nagore et al.,

2012). Basically, diosgenin [(25R)-spirost-5-en-3b-ol] is a spirostanolsaponin containing of a hydrophilic sugar moiety connected to a hydrophobic steroid aglycone (Figure 1) (Miyoshi et al., 2011). Study effect of diosgenin on human hepatocellular carcinoma cells was showed that diosgenin can induce the expression of Src homology 2 phosphatase 2 (SH-PTP2) that correlated with down-regulation of constitutive STAT3 activation. Diosgenin also down-regulatedthe expression of various STAT3-regulated gene products, inhibited proliferation and potentiated the apoptotic effects of paclitaxel and doxorubicin (Li et al., 2010). Diosgenin induced apoptosis in HT-29 cells at least in part by inhibition of Bcl-2 and by induction of caspase-3 protein expression (Raju et al., 2004). diosgenin, a steroidal saponin, inhibits migration and invasion of human prostate cancer PC-3 cells by reducing matrix metalloproteinases expression (Chen et al., 2011). Lung cancer is the most widespread type
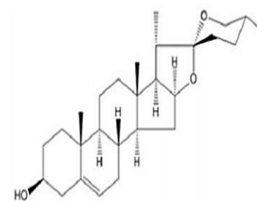

Figure 1. Structure of Diosgenin

[1]Drug Applied Research Center, Tabriz University of Medical Sciences, [2]Department of Clinical Biochemistry, Faculty of Medicine, Tabriz University of Medical Sciences, [4]Bone Health Research Center, Tabriz University of Medical Sciences, Tabriz, [3]Department of Biology, Faculty of Sciences, Golestan University, [5]Department of Biochemistry, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran *For correspondence: rahmati_bio@yahoo.com

Table 1. Analysis of RNA Purity and Integrity and Primer Sequences. This Table Shows an Example of RNA Purity and Integrity Analysis Using Spectrophotometry. The OD260/280 Ratio Measured for All Samples on the Nanodrop Spectrophotometer is between 1.7 and 2.1 Indicating that the Samples are Empty of Protein Infection

Sample	A260/280	Concentration (ng/μl)	Primer name	Primer length	Sequence (5' to 3')	Product size (bp)
Control	1.94	1503	hTERT Forward primer	19	CCGCCTGAGCTGTACTTTGT	198
Pure Dg* (24h)	1.98	1091	hTERT Reverse primer	19	CAGGTGAGCCACGAACTGT	
Pure Dg (48h)	1.94	1938.5	β-actin Forward primer	20	ACCGTGAAAAGATGACCCAG	131
Pure Dg (72h)	1.94	414	β-actin Reverse primer	20	CCATACCCAAGAAGGAAGGC	

*Dg: Diosgenin, Generally accepted ratios (A260/280) for good quality RNA are 1.7-2.1
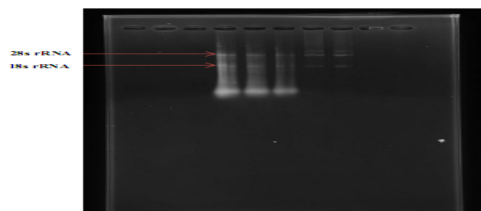
Figure 2. Gel Image Generated by Electrophoresis Agarose Gel 1.5% at Different Times After Treatment (24, 48 and 72h) A549 Cell Line with 5mM Pure Diosgenin Dissolved in Ethanol

of cancer and is also one of the major causes of death in the world (Athey et al., 2012). Lung cancer between many types of cancer such as breast cancer, lung cancer, ovarian cancer, colon cancer and stomach cancer, is the leading cause of cancer deaths in the world. The two major forms of lung cancer are non-small-cell lung cancer (about 85% of all lung cancers) and small-cell lung cancer (about 15%). Despite advances in early detection and standard treatment, non–small-cell lung cancer (NSCLC) is often diagnosed at an advanced stage and has a poor prognosis. This type of lung cancer can be divided into three major histologic subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer (Herbst et al., 2008). There are many molecular targets for prevention or treatment of none-small cell lung cancer (Hamilton et al., 2005). From these molecular targets, telomerase is the best because it is active in almost 85% of cancers including non-small cell lung cancer. Therefore, it is an appropriate molecular target. So the goal of current study is to define the effect of diosgenin on gene expression regulation of hTERT gene in A549 lung cancer cell line.

Materials and Methods

Pure diosgenin obtained from Sigma co, A549 lung cancer cell lines prepared from Cell Bank of Pasteur Institute of Iran (Code: C137) were cultured in RPMI-1640 medium (Invitrogen, UK) include 10% of Heat-inactivated Fetal Bovine Serum (FBS) (Gibco, Invitrogen, Uk) with Penicillin G (Serva Co, Germany) and Streptomycin (Merck Co, Germany).

MTT-assay

Cytotoxicity of diosgenin on A549 lung cancer cell line evaluates by MTT assay which is based on the reduction of MTT (catalogue number M2128, sigma Co, Germany) to MTT-formazan by mitochondrial enzymes. Cells were harvested and resuspended in growth media and counted by Trypan blue 0.4% solution, to make a stock cell suspension containing cells/ml. 200µl of this stock cell suspension was added to the each wells of a 96-well plate with blank. After incubation, cells were treated diosgenin in ethanol (0, 10, 20, 30, 40, 50 µM), then cells in cancer) for 24, 48 and 72h. except growing media, 50 µl MTT solution (2 mg/ was added to each well. After an incubation in dark at 37°C, medium and MTT assay and 200µl DMSO 5µl formazan crystal were added to each wells, and the absorbance was measured by an ELISA reader on plate reader (STAT FAX 2100) at 570nm wave length with 620nm wavelength as a reference value. The cell viability was calculated by the subsequent formula. *Inhibitory ratio (%)=(OD control–OD treated)/OD control×100%*. Cytotoxicity was expressed as the concentration of diosgenin inhibiting cell growth by 50% (IC$_{50}$ value).

Cell treatment

After determination of IC$_{50}$ 1×10^{6} cells were treated with 5µM of Diosgenin solution. The ethanol without diosgenin solution was added to flask of control cells. Then, culture flasks were incubated in 37°C containing 5% CO$_2$ with humidified atmosphere incubator for 24, 48 and 72h.

RNA extraction and cDNA synthesis

Total RNA was isolated using the Total RNA isolation TRIZOL reagent protocol (Cinnagene, Iran). Since sample purity and integrity are not associated, both should be evaluated to determine that the RNA sample meets minimal acceptance standards for the downstream workflow. Purity of spectrophotometrically by measuring the OD260/280 ratio in nanodrop ND1000. An OD260/280 of 1.7-2.1 was acceptable (Table 1). The sample with respect to protein contaminant and it's concentration can be measured. However, no RNA integrity information can be obtained from a spectrophotometric reading. RNA integrity can be assessed using gel electrophoresis on a formaldehyde agarose gel in the presence of a fluorescent dye such as ethidium bromide. Observation of two sharp bands for the large (28S rRNA) and the small (18S rRNA) subunits of ribosomal RNAs (rRNA) with the intensity of the larger band being about twice that of the smaller band is indicative of intact RNA (Figure 2).

Complementary DNA (cDNA) was synthesised using reverse transcriptase (RT) using the First Strand cDNA Synthesis Kit (Fermentase, K1622). For cDNA synthesis
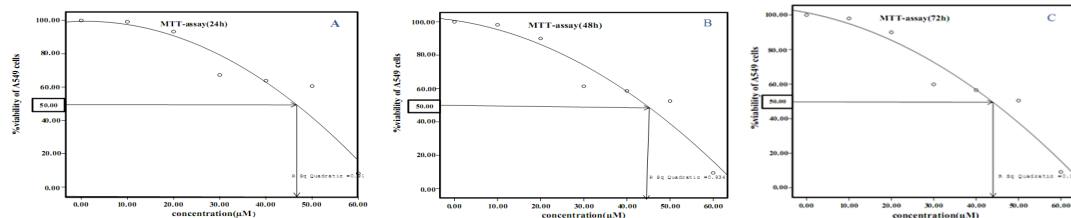
Figure 3. Result of MTT-Assay for A549 Cell Line in Different Times (24, 48, 72h). A) A549 Treated with Pure Diosgenin for 24h (IC$_{50}$=47μM); **B)** For 48h (IC$_{50}$=44μM); and **C)** For 72h(IC$_{50}$=43μM)
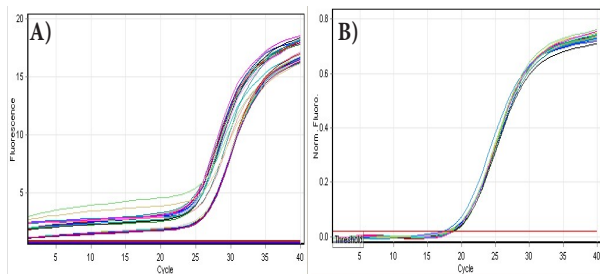
Figure 4. A) Real-Time PCR Quantification Curve of hTERT Gene; and B) Real-Time PCR Quantification Curve of β-Actin Gene
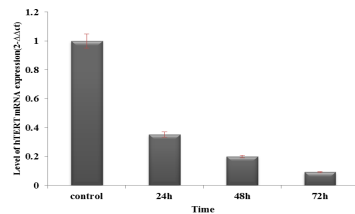
Figure 5. Level of hTERT mRNA Expression (2$^{-\Delta\Delta ct}$) at Different Times (24, 48 and 72 h)

were used 2μg of pure RNA as template.

Quantitative Real-Time PCR (qRT-PCR)

For real-time PCR, according to previous study (Kazemi et al., 2011), hTERT primers (Genbank accession: NM_ 198255) and beta actin primers (Genbank accession: NM_001101) were used (Table 2). According to manufacture, the total volume of PCR reaction was 20μl that contained: 5 pmole of the forward and reverse PCR primers of hTERT or for beta actin, 2× PCR Master Mix Syber Green I, and 2μl of the cDNA was used. The hTERT and beta-actin gene were amplified by real-time PCR as triplicate. Beta actin and hTERT for each sample at the same run was done, 20μl of PCR reaction mixture contained 10μl of SYBER Ex Taq II (2×) PCR master mix, 2μl template cDNA, 6.4μl dH$_2$O water per reaction. Negative controls were prepared each time with 2μl ddH$_2$O instead of the cDNA template. Real time PCR amplification was performed using a Corbett (Rortor Gene 6000) system with the following setting as manufacture protocol. The reaction mixture was incubated under the following conditions: 95˚C, 2 minutes, 1 cycle (Holding step); 65˚C, 20 seconds, 45 cycles (Annealing); 72˚C, 20 seconds, 40 cycles (Extension);72˚C, 5min, 1cycle and 75-95˚C, 1 cycle (Melting).

Data analysis

Changes in the hTERT gene expression levels between the control and treated A549 cells were calculated by the 2$^{-\Delta\Delta Ct}$ method. The formulas for the calculation were as follows: *ΔCT=CT$_{(target)}$-CT$_{(reference)}$* ; *ΔΔCT=ΔCT$_{(test\ sample)}$ −ΔCT$_{(control\ sample)}$*

The fold for target, relative to a calibrator sample, is calculated by: 2$^{-\Delta\Delta Ct}$.

Results

The MTT results for different concentrations of diosgenin at different times for the treatment of A549 cell line showed that IC$_{50}$ for diosgenin were 47, 44 and 43 μM after 24, 48, and 72 hours of treatment, respectively (Figure 3). RNA extracts quantity and qualify by nanodrop and agarose gel electrophoresis. The MTT results showed that diosgenin effect on A549 cells has time-dependent and dose-dependent manner, and it's best effect was in 72h after treatment.

Analysis of real- time PCR for evaluation of hTERT gene expression inhibition with 2$^{-\Delta\Delta Ct}$ values showed that diosgenin inhibited hTERT gene expression and decrease hTERT mRNA level in comparison with control group (Figure 4A, B). Fold of decreasing were 0.352, 0.200, 0.092 for 24, 48 and 72h, respectively (Figure 5).

Discussion

Since most cancer cells hold telomerase activity, one possible gain of telomerase targeted therapy would be its specificity on telomerase positive tumor cells, because most human somatic tissues are telomerase negative. On the base of these comments various types of telomerase inhibitors have been discovered and advanced. Modulators of mRNA expression of telomerase components (hTERT) have been regarded as one of type of anti telomeral agents. These include all transretonoic acid (Xiao et al., 2005), 5, 6-trans-16 -ene vitamin D3 (Holysz et al., 2013) ceramide (Goldkom et al., 2013) and curcumin (Kazemi et al., 2011). These complexes act as inhibitors of hTERT mRNA expression which results in decreased telomerase activity. According to the prior studies there is a strong association among the expression of hTERT mRNA and telomerase activity in extract after culture cells and tissues (Wojtyla et al., 2011). Modulators of hTERTgene expression are regarded as anti telomeral agents. In recent years, various dietary components that can potentially be used for the prevention and treatment of cancer have been identified. One of the most compounds that founded in fenugreek is Diosgenin with anticancer activity.The study effect of diosgenin on osteosarcoma cells were showed that diosgenin treatment caused an

inhibition of 1547 cell growth with a cycle arrest in G1 phase and apoptosis induction and moreover exist a correlation between p53, p21 mRNA expression and nuclear factor-kappaB activation (Moalic et al., 2001). Diosgenin induces cell cycle arrest and apoptosis in HEL cells with increase in intracellular calcium level, activation of cPLA2 and COX-2 overexpression (Leger et al.,2004). Another study about diosgenin effect on colon cancer cells showed that diosgenin induces death receptor-5 through activation of p38 pathway and promotes TRAIL-induced apoptosis (Lepage et al., 2011). *In vitro* and *in vivo* effect of steroid saponins of Paris polyphylla var. yunnanensis against lung adenocarcinoma cell line were showed that this compounds have anticancer activity and their effect was dependent on structure of compounds in a certain grade (Yan et al., 2009). Also, another study by Shishodia and Aggarwal (2006) showed that diosgenin could inhibits cmyc gene expression that cmyc acts as a upregulator for hTERT gene expression. Therefore, based on these studies were showed this fact that diosgenin have anticancer activity and exert this property by several mechanisms probably with downregulation of cmyc gene expression. Since the relationship between cancer and high expression of hTERT gene, our results are especially interesting in demonstrating that Diosgenin downregulates hTERT gene expression in A549 cell line. In this study, for the first time, we studied the effect of diosgenin on hTERT gene expression. Result of Real-time PCR indicated that diosgenin cause decreased hTERT gene expression and inhibits growth of A549 cell line. Therefore, dioggenin could be a good antocancer candidate for cancer therapy in the future.

In conclusion, according to the results, diosgenin by antioxidant activity affect the growth of A549 lung cancer cell line and these cytotoxic effects were time-dependent manner, and diosgenin could inhibit hTERT gene expression as time dependent manner. The results showed that diosgenin could be a candidate subject for lung cancer therapy because natural products like genistein, curcumin, and retinoic acid are proved as chemopreventive agents.

Acknowledgements

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