Synergistic Growth Inhibitory Effects of Chrysin and Metformin Combination on Breast Cancer Cells through hTERT and Cyclin D1 Suppression

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Abstract

Objective: To explore the possibility of a novel chemopreventive strategy for improving breast cancer treatment, the anticancer effects of a combination two natural compounds, Chrysin and Metformin, against T47D breast cancer cells were investigated. Materials and Methods: After treatment of T47D cells with Metformin, Chrysin and the two drugs in combination, toxicity to cancer cells was evaluated by MTT assay. Real time PCR was then used to determine the expression levels of hTERT and cyclin D1 genes. Results: The MTT test findings showed that the combination of metformin and chrysin had high synergistic effects in killing cancer cells. In addition PCR demonstrated a significant decrease in cyclin D1 and hTERT gene expression in the T47D breast cancer cell line. Conclusion: The combination of metformin and chrysin suppressing hTERT and cyclin D1 gene expression might offer an appropriate approach for breast cancer therapy.

Keywords: Breast cancer- Cyclin D1- hTERT- Metformin- Silibinin

Introduction

Nowadays, cancer is one of the most usual threatening diseases in world, and Breast cancer is one of the most diagnosed cancer among women (Sarhadi et al., 2017). Cancer is the second cause of death after cardiovascular disease (Stratton et al., 2009). There are various strategies to block and delay the steps of carcinogenesis (Nejati-Koshki et al., 2014; Montazeri et al., 2017). Among them, combinational chemotherapy has been approved in clinics as an initial cancer therapy regimen. Several works revealed that applying numerous chemotherapeutic agents with different molecular targets can upgrade the genetic hindrances that require to be overwhelmed for cancer cell mutations, thus delaying the cancer adaptation process (Montgomery et al., 2016; Lotfi-Attari et al., 2017). Besides, it has been described that several drugs targeting the same cellular pathways could act synergistically for higher therapeutic effectiveness and higher target selectivity (Valencia et al., 2013; Maasomi et al., 2017).

Chrysin, 5,7-dihydroxyflavone, is a natural flavone found in propolis, honey, honeycomb, the passion flowers, Passiflora caerulea and Passiflora incarnata, and in Oroxylum indicum (Mohammadian et al., 2016a; Mohammadian et al., 2016b). Currently, Chrysin has become the notable candidate displaying health profits, due to its numerous bioactivities such as antibacterial, anti-inflammatory, antioxidant, anti-diabetic, anti-allergic and antitumor properties (Deldar et al., 2017a; Deldar et al., 2017b; Mohammadian et al., 2017b). In vitro and in vivo studies have revealed that Chrysin suppresses cancer cell proliferation via apoptosis induction, cell cycle alteration, microRNA modulation and inhibition of invasion, metastasis and angiogenesis without triggering undesirable side effects and toxicity to normal cells (Mohammadian et al., 2015; Mohammadian et al., 2017a). Chrysin exhibits these effects via selective modulation of several cell signaling pathways that are related to survival, growth, angiogenesis, inflammation, invasion and metastasis of cancer cells.

Metformin is one of the most extensively prescribed oral anti-diabetic drugs. In spite of the introduction of many novel therapeutic agents to treat type 2 diabetes, Metformin remains as an appropriate first-line therapy for type 2 diabetes mellitus (Farajzadeh et al., 2017a). Recently, numerous investigations have introduced evidence implying a potential role for metformin in cancer treatment (Javidfar et al., 2017). Numerous molecular mechanisms for anticancer effects of Metformin including cytotoxic properties, mTOR inhibition and immunomodulation have been revealed in preclinical studies (Chae et al., 2016). Epidemiologic data have displayed reduced cancer occurrence and mortality in the cases taking Metformin (Zhang et al., 2013). Also, some
clinical trials, focused on the assessment of Metformin as an anti-cancer agent are currently ongoing (Chae et al., 2016).

Cyclin D1 is classified to the family of three closely associated D-type cyclins, named cyclin D1, D2 and D3. CKD1 is a candidate proto-oncogene involved in pathogenesis of various human tumor types that lead to shift the cells from G1 phase to S phase and high expressed in many cancers such as breast cancer. D-cyclins concordantly regulate cell-cycle process by activating their cyclin-dependent kinase such as CDK4 and CDK6 (Hydbring et al., 2016). Several of evidence indicate the significant role for cyclin D1 in breast cancer progression. The cyclin D1 gene is expressed in up to 20% of human breast cancers (Park et al., 2001) while cyclin D1 protein is high-expressed in over 50% of human mammary tumors (Arnold and Papamikolou, 2005).

The maintenance of telomere length is essential for prolonged cell proliferation, and ~85–90% of human cancers indicate high activity of telomerase (Amirsadat et al., 2017). Three major subunits composing telomerase complex included hTR (human telomerase RNA), TP1 (telomerase-associated protein 1), and hTERT (human telomerase reverse transcriptase) (Pirmoradi et al., 2017). The hTERT gene is the most important regulator of telomerase activity. hTERT is highly expressed in all tissues regardless of telomerase activity, but in cancer cells generally have fivefold-higher expression (Amirsadat et al., 2017). Therefore, hTERT targeting may be promising step in breast cancer treatment.

The aim of this study was to evaluate the hypothesis that Silibinin and Metformin will work in synergism and inhibit the growth of T47D breast cancer cells. Thus, Silibinin, Metformin and their combination were used to determine the cytotoxicity and expression levels of hTERT and Cyclin D1 in T47D cells.

Materials and Methods

Cell culture

The human breast cancer cell line, T47D was purchased from Pasteur Institute cell bank of Iran (code: C137) and cultured under standard cell culture conditions in RPMI-1640 media (Gibco, Invitrogen, UK) supplemented with 10% FBS and 1% penicillin/streptomycin at a temperature of 37 °C in a humidified atmosphere.

Reagents

RPMI1640 and Fetal Bovine Serum (FBS) were supplied from Gibco (Invitrogen, UK). Penicillin G, Streptomycin, DL-Lactide (LA), glycolide (GA), Polyethylene glycol (PEG, molecular weight: 4000) Stannous octoate (SnOct2), polyvinyl alcohol (PVA), dichloromethane (DCM), MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide), and dimethyl sulfoxide were provided by Sigma-Aldrich (St Louis, MO, USA). Ferric chloride hexahydrate (FeCl3·6H2O), ferrous chloride tetrahydrate (FeCl2·4H2O), and ammonium hydroxide (25 wt%) were purchased from Fluka (Buchs, Switzerland). RNX-plus kit were obtained from CinnaGen, Iran. First Strand cDNA synthesis Kit and SYBR Green PCR Master Mix were purchased from Fermentas (Vilnius, Lithuania).

Cell viability assay

To measure the effect of Metformin and Chrysin on cell growth, MTT assay was used with breast cancer (T47D) cell lines. For this assay, 6.5 x 104 cells/well were seeded in a 96-well plates and cultured. The cells were treated with different concentrations (0-120 µM) of free Metformin and free Chrysin after 24 h and equivalent doses of mixed Metformin-Chrysin for different time intervals (24–72 h). The cells grown in media containing equivalent amount of ethanol without Metformin and Chrysin served as control. After the treatment, media containing Metformin, Chrysin and mixed Metformin-Chrysin were removed carefully and 50 µL of 2 mg/mL MTT dissolved in phosphate buffered solution was added to each well, and the plate was coated with aluminum foil and incubated for 4 h. Then, contents of all wells were removed and 200 µL of pure DMSO were added to the wells followed by adding 25 µL Sorensen’s glycine buffer to each well. All experiments were set up in triplicates. Finally, the optical density (OD) was measured at 570 nm ELISA-reader with a reference wavelength of 630 nm. Results were expressed as mean ± S.D. IC50 (inhibitory concentration at 50%) was evaluated by Graph Pad 6 (Prism) software. Then, 5 x 104 cells were treated with serial concentrations of pure Metformin and Chrysin and mixed Metformin-Chrysin (10, 20, 30, 40, and 50 µM). The same volume of 10% DMSO without Metformin and Chrysin was added to the flask containing control cells. The cells were then incubated at a temperature of 37 °C in a humidified incubator with a 5% CO2 atmosphere for a 24 h.

Real-time PCR Assay

After 24 h, total RNA was extracted from T47D cell line that was treated with different concentrations of pure Metformin and Chrysin and mixed Metformin-Chrysin using trizol reagent kit (Invitrogen, USA). Then, purity and concentration of total RNA were estimated by nanodrop ND1000 and at 260-280 nm purity of RNAs were assessed. The quality of total RNA was confirmed by electrophoresis of the individual samples on a 1% agarose gel.

Complementary DNA (cDNA) was produced using first Strand cDNA synthesis Kit (thermos fisher) according to protocol. For cDNA synthesis, 2 µg of pure RNA were used as template.

Levels of gene expression was determined in total volume of 14 µL per reaction using a MIC (Rortor Gene 6000) system. Real time PCR reaction mixture included 1 µL template cDNA (2000 ng), 1 µL (4 picomolar) of primers mixture (Table 1), 7 µL of SYBR green master mix, 5 µL nuclease-free water.

The following sequences of the sense and antisense primers of hTERT and cyclin D1 (Genbank accession: NM_001193376, NM_053056) were used: forward 5’-CCCATTTTCATCGAAGATGTTGG-3’, reverse 5’-CTTGCTTTTTCAGGATGAGTA-3’ for hTERT and forward 5’-CCACTCTCAGCATCGCTACTA-3’, reverse 5’-CCAGCATCTCATAACAGGTCA-3’ for
inhibitory effect of Metformin, Chrysin and mixed form against T47D cells were in a dose and time dependent manner. The IC50s for Metformin, Chrysin and combined form on T47D cells after 24 and 48 h exposure have been shown in Table 2. According to the findings, IC50 value of mixed of Metformin-Chrysin had a reduction about 50% inhibitory effect of Metformin, Chrysin and mixed form against T47D cells were in a dose and time dependent manner. The IC50s for Metformin, Chrysin and combined form on T47D cells after 24 and 48 h exposure have been shown in Table 2. According to the findings, IC50 value of mixed of Metformin-Chrysin had a reduction about 50%

Statistical analysis

SPSS software version 22.0 (SPSS, Chicago, IL, USA) was used For Statistical analysis. The differences in mRNA levels of hTERT between control and treated cells were evaluated by ANOVA and Tukey’s method and p-value <0.05 was considered as significant difference. Results were expressed as mean±S.D.

Results

In vitro cytotoxicity

Cell viability was evaluated by MTT assay using different concentrations of Metformin and Chrysin individually and mixed of Metformin-Chrysin on breast cancer cell line (T47D) during 24 and 48h (Figure 1). Cells without treatment used as control groups. According to the diagrams of this analysis displayed in Figure 2, growth

Table 1. Length and Sequence of Primers and Product Size

| Primer          | Primer length | Sequence (5’ to 3’)       | Product size (bp) |
|-----------------|---------------|---------------------------|-------------------|
| hTERT Forward   | 22            | CCCATTTCATCAGCAAGTTTGG    | 94                |
| Reverse         | 22            | CTTGGCTTTCAGGATGGAGTAG    |                   |
| β-actin Forward | 18            | GGTGAAGGTGACAGCAGT        | 154               |
| Reverse         | 18            | TGGGGTGGCTTTTAGGAT        |                   |
| Cyclin D1 Forward | 20          | TGCCCTCTGTGCCACAGATG      | 148               |
| Reverse         | 21            | TCTGGAGAGGAAGCCTGTGA      |                   |

Figure 1. The Inhibitory Effect of Chrysin, Metformin and Mixed of Chrysin-Metformin on T47D Breast Cancer Cells Viability. Cells were treated with a range of concentrations (20:6,000, 40:12,000, 60:18,000, 80:24,000, 100:3,000, 120:36,000) for 48h, then MTT assay was performed.

Figure 2. Result of MTT- Assay for T47D Cell Line in Different Times (24, 48). A) T47D treated with pure Chrysin for 24h, 48h (IC50=62.70 µM, IC50=44.78 µm respectively); B) T47D treated with pure Metformin for 24h, 48h (IC50=18.08µM, IC50=15.54 µm respectively)

Figure 3. Result of hTERT and CKD1 Gene Expressions Evaluate by Real- Time PCR. Effect of different concentrations of Chrysin, Metformin and mixed of Chrysin-Metformin on the levels of genes expressions in T47D breast cancer cell line. Concentrations are 20 (A), 40 (B) and 60 (C) in Chrysin and 600 (A), 6,000 (B) and 6,000 (C) in Metformin and 20:600 (A), 40:6,000 (B) and 60:12,000 (C) in mixed of Chrysin-Metformin.
Table 2. IC50 Values Obtained from Chrysin and Metformin

| Drug       | 24 h  | 48 h  |
|------------|-------|-------|
| Chrysin    | 62.70 µm | 44.78 µm |
| Metformin  | 18.08 mM | 15.54 mM |

as compared the IC50 value of Metformin and Chrysin individually, demonstrating mixed of Metformin-Chrysin were more effective in preventing cell growth than each one of them.

Gene expression findings

Effect of Chrysin, Metformin and mixed of Chrysin-Metformin on the levels of hTERT and CKD1 genes expressions were determined by real-time PCR. Analysis real-time PCR data showed that the hTERT and CKD1 gene expressions are reduced with increasing concentrations of Chrysin and Metformin, and the changes in hTERT and CKD1 gene expressions in T47D breast cancer cell line treated with mixed of Chrysin-Metformin were more intense (Figure 3). It can be expected that downregulation of the hTERT and CKD1 and inhibition of telomerase activity triggers telomere shortening and the cell reaching a critical circumstance, finally cell death by apoptosis.

Discussion

The incidence and prevalence of breast cancer has increased worldwide (Wolf et al., 2005). In order to reduce breast cancer deaths, development of new treatment approach to treat or prevent the cancer are very vital (Parrella et al., 2004). In this regard, herbal extracts and compounds of natural origin used in traditional medicine have attracted attention in treatment of various cancers (Alibakhshi et al., 2016; Montazeri et al., 2016; Dadashpour et al., 2017a). Therefore, using of alternative treatments that have no side effects or much more effective, it seems essential for the treatment of breast cancer. Various reports have shown that the phytochemical inhibits cell proliferation and induces apoptosis in different types of cancer cell especially breast cancer cells. Due to the significant effects of Metformin and Chrysin in cancer inhibition, this study tried to determine the effects of mixed form of the agents in breast cancer suppression. the results of this study indicated that different concentrations of Chrysin and Metformin strikingly prevent growth and proliferation in T47D cells at a dose-dependent manner. Our results displayed that Chrysin and Metformin has impressive cytotoxic activity against human breast cancer cells. Also, The results of qPCR showed that Chrysin and Metformin can significantly inhibit hTERT and CKD1 gene expressions (70%) and also mixed of Chrysin-Metformin has more inhibitory effect than free drug (up to 98%) in T47D cell line. Numerous other studies have shown that Chrysin and Metformin have anti-cancer effects by stimulating apoptosis and inhibits the growth of cancer cells.

In a study, Chrysin displayed the cytotoxic and antiproliferative effects on prostate cancer which is in confirmation with our results. Their results indicated that Chrysin demonstrated regulatory properties on angiogenesis in mouse model, the critical phase of tumor growth and metastasis (Fu et al., 2007). Hong et al., (2010) described the anticancer effect of Chrysin on breast cancer cells. In their research, the effect of Chrysin on cells was assessed 24 and 48 h after treatment. 24 h after treatment no important effect was detected, but after 48 h treatment with concentrations of 20 and 40 µM respectively 73.9 and 73.1 viability were detected. Thus, 20 µM Chrysin for 48 h was reported as the effective concentration (Fu et al., 2007). Also, previous studies on Chrysin showed that this combination is caused to stop growth of skin cancer cells in G1 phase and decreased expression of cyclin D1 and cdk2 genes and increased expression of P21 gene as well as treatment of ATC cells with Chrysin inhibits expression of cyclin D1 and induces apoptosis in this cells (Fu et al., 2007).

Anti-growth functions of Metformin effect in cell survival, proliferation and cell death were exerted through inhibition of cell cycle, induction of apoptosis and changing of signaling proteins (Schuld et al., 2014). Several studies displayed that Metformin blocks cell cycle in G0/G1 phase with a significant reduction in expression of cyclin G1 (including cyclin D1) and no change in P53 status (Fu et al., 2004). However, some of the other studies have shown that the inhibitory effect of Metformin on the growth of cancer cells was associated with P53 activity (Sharpless and DePinho, 2004). Also, Metformin can cause apoptosis by inducing the expression of P53 in some of the cell lines such as MCF-7 and MDA-MB-231 (Queiroz et al., 2014).

In this study, the alteration in expression levels of Cyclin D1 and hTERT genes were surveyed to describe the synergistic inhibitory effects of Chrysin and Metformin. Though, further works are needed to provide exact insight into the mechanisms involved in the elicited anticancer effects of the combination treatment of Chrysin and Metformin. Also, several reports displayed some unfavorable properties of phytochemicals such as poor water solubility and low cellular uptake that can restrict the efficacy of the therapeutic agents (Badrzadeh et al., 2014; Dadashpour et al., 2017b; Farajzadeh et al., 2017b). So, using appropriate nano drug delivery systems may improve bioavailability of the natural therapeutic agents and cause strong synergistic effects on various target cells (Zeighamian et al., 2016; Nejati-Koshki et al., 2017; Zarghami et al., 2017).

In conclusion, as one of novel molecular effect of the combination treatment, it has been displayed that the combined treatment of Chrysin and Metformin exhibits more inhibitory effect on hTERT and Cyclin D1 genes than either drug alone. Also, results of this study showed that Metformin and Chrysin have a cytotoxic effect substantially on T47D cell line and it can be concluded based on this study and previous research that Metformin and Chrysin have anticancer strong effects on breast cancer cells and mixed of Chrysin-Metformin have synergistic effects that derivatives of these compounds can play an important role in the treatment of cancer in future.
Conflict of Interest
The authors declare that they have no competing interests.

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