Research Article

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Effects of glabridin on cell proliferation and long non-coding RNA expression in HEC-1B cells

HEC-1B hücrelerinde glabridinin hücre proliferasyonu ve uzun kodlamayan RNA ekspresyonu üzerindeki etkileri

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Abstract

Purpose: Endometrial cancer is one of the most common gynecological cancers in the world. Glabridin is a main isoflavone in Glycyrrhiza glabra (licorice) root. It has several therapeutic effects such as anti-proliferative and anti-inflammatory. Long non-coding RNAs (LncRNAs) play a role in a variety of cellular processes, and their abnormal expression may contribute to tumor development and progression. In this study, the effects of glabridin on LncRNAs gene expression and viability of HEC-1B human endometrial cancer cell lines have been investigated.

Materials and methods: Glabridin was applied to HEC-1B cells in concentrations of 1 μM, 10 μM, 20 μM, 40 μM, 60 μM, and 80 μM. Glabridin's effect on HEC-1B cell proliferation was also evaluated using MTS assay. Expression profiles of LncRNAs such as H19, RNU43, LNC-MYC-3:1 and ABCC5-AS1:1 were determined by real-time PCR.

Results: Glabridin reduced the viability of HEC-1B cells in a time- and dose-dependent manner. The half maximal inhibitory concentration (IC50) dose in HEC-1B cells was detected to be 21.32 μM and 13.5 μM at the 24th and 48 hours, respectively. Glabridin has been observed to cause a significant decrease in the expression of H19 and RNU43 while increasing in the expression of LNC-MYC-3:1 and ABCC5-AS1:1.

Conclusion: Glabridin could induce HEC-1B cell death by regulating LncRNAs expression. As a result, glabridin is a potential candidate for a more effective therapeutic agent against human endometrial cancer.

Key words: Glabridin, endometrial cancer, long non-coding RNA, HEC-1B.

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Öz

Amaç: Endometrium kanseri dünyada en sık görülen jinekolojik kanserlerden biridir. Glabridin, Glycyrrhiza glabra (meyan kökü) kökündeki ana izoflavonoidlerden biridir. Glabridin anti-proliferatif ve anti-inflamatuar gibi çeşitli farmakolojik etkilere sahiptir. Uzun kodlamayan RNA'lar (LncRNAs) çeşitli hücresel mekanizmalarda rol oynar ve bunların değişen ekspresyonu, tümör gelişimine ve ilerlemesine katkıda bulunabilir. Bu çalışmada, glabridinin LncRNAs gen ekspresyonlarına etkileri ve HEC-1B insan endometriyal kanser hücre hattının canlılığı üzerindeki etkileri araştırılmıştır.

Gereç ve yöntem: Glabridin HEC-1B hücrelerine 1 uM, 10 uM, 20 uM, 40 uM, 60 uM ve 80 uM konsantrasyonlarda uygulandı. Glabridin'in HEC-1B hücre canlılığı üzerindeki etkisi MTS testi kullanılarak değerlendirildi. Çalışmamızda araştırdığımız LncRNAs olan H19, RNU43, LNC-MYC-3:1 ve ABCC5-AS1:1 ekspresyon profilleri Real time-PCR ile yöntemi ile belirlendi.

Bulgular: Glabridin, zamana ve doza bağlı bir şekilde HEC-1B hücrelerinin canlılığını azalttı. Glabridinin HEC-1B hücrelerinde maksimum yarı inhibisyon konsantrasyon (IC50) dozu 24. ve 48. saatlerde sırasıyla 21,32 μM ve 13,5 μM olarak tespit edildi. Glabridin'in LNC-MYC-3:1 ve ABCC5-AS1:1 ekspresyonunu arttırırken H19 ve RNU43 ekspresyonunda önemli bir azalmaya neden olduğu tespit edilmiştir.

Sonuç: Glabridin muhtemelen LncRNAs ekspresyonlarını düzenleyerek HEC-1B kanser hücrelerinin ölümünü sağlamıştır. Sonuç olarak, glabridin insan endometriyal kanserine karşı ileride kullanılabilecek etkili bir terapötik ajan olarak önemli bir potansiyel taşımaktadır.

Anahtar kelimeler: Glabridin, endometriyal kanser, uzun kodlamayan RNA, HEC-1B.

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Introduction

Endometrial cancer (EC) is the fourth most common cancer in women worldwide after breast, lung, and colorectal cancers, and its incidence is rapidly increasing [1]. North America and Europe have ten times the incidence of less developed countries [2]. Cancer invasion and metastasis are multi-step processes that cause the majority of cancer deaths [3]. Understanding the molecular mechanisms that drive tumor cell proliferation and metastasis, as well as developing a treatment strategy, is thus critical for EC. Tumor cell proliferation, invasion, metastasis, and apoptosis are all associated with the dysregulation of multiple signaling pathways [4].

Long non-coding RNAs (LncRNAs) are longer than 200 nucleotides in length and regulate cell growth by activating or inhibiting specific signaling pathways [4]. Previous study has revealed that LncRNAs have the potential to be biomarkers and therapeutic targets in cancer diagnosis and treatment [5]. Ren et al. [6] reported that H19 expressed by carcinoma-associated fibroblasts of the colorectal tumor stroma contributes to tumor development and chemoresistance. Nevertheless, the roles of LncRNAs in controlling cell proliferation in many cancer types, including EC, are not fully understood.

Licorice root (*Glycyrrhiza glabra*) has been used widely in traditional medicine of different countries [7], and glabridin is the main flavonoid constituent of licorice root [8]. Glabridin has many pharmacological properties such as anti-proliferative [9], anti-inflammatory [10], anti-asthmatic [11]. It has been shown that glabridin can inhibit human hepatoma cells by inhibiting the JNK1/2 signaling pathway [12]. Glabridin can inhibit human breast adenocarcinoma cells by inhibiting the focal adhesion kinase signaling pathway [13]. Furthermore, Huang et al. [14] reported that glabridin induces human promyelocytic leukemia cells apoptosis through p38 MAPK and JNK1/2 pathways and could serve as a potential additional chemotherapeutic agent for treating acute myeloid leukemia.

In this study, we investigated the effects of glabridin on long non-coding RNA gene expression and viability of the cultured HEC-1B human endometrial cancer cell.

Materials and methods

Chemicals

Glabridin, ≥98% (HPLC), powder was purchased from Sigma Chemical Co. (St. Louis, MO, USA, CAS no: 59870-68-7). Glabridin was dissolved in dimethylsulfoxide (DMSO).

Cell culture

HEC-1B cancer cell line (ATCC/ HTB-113) was used in this study. HEC-1B cells were grown in DMEM medium supplemented with 2 mM L-glutamine, penicilin (20 units/ml), streptomycin (20 µg/ml), and 10% (vol/vol) heat-inactivated fetal bovine serum at 37°C in a saturated humidity atmosphere containing 5% CO_2. HEC-1B cells were seeded in 96 well plates at 5000 cells per well. After 24 hours, cancer cells were applied with glabridin at different concentrations as 1 µM, 10 µM, 20 µM, 40 µM, 60 µM and 80 µM.

Determination of cell viability: MTS assay

Cell viability was determined by the MTS method (CellTiter 96® AQueous MTS Reagent Powder and phenazine methosulfate) according to the manufacturer's instructions. The absorbance values from the negative controls were calculated and the results were accepted as 100% cell viability. IC_{50} concentrations were calculated in the GraphPad Prism (GraphPad Software, Version 7) program using the cell viability results.

RNA isolation and Real Time-PCR

Glabridin were applied at IC50 concentrations to HEC-1B cells seeded in 6-well plates. At the end of the 48h incubation time, total RNA isolation, cDNA synthesis and RT-PCR analysis were performed for gene expression analysis, respectively. Only cell medium was added to the control group used to compare the results. Total RNA isolation from cells was performed using One Step RNA Reagent (BioBasic) according to the manufacturer's protocol. According to the protocol, One Step RNA Reagent was added at 1 ml per well and the cells were removed with a scraper. It was centrifuged by adding 200 µl of chloroform and 500 µl of isopropyl alcohol, respectively. The cell pellet obtained was washed with 1 ml of ethanol and then dissolved with 30 µl of RNase-DNase free water. The concentration and purity of the isolated total RNAs were

measured with a Nanodrop spectrophotometer. As a result of the measurements, the expected purity value of 260/280 for RNA was accepted as approximately 2.0.

cDNA synthesis was performed according to the manufacturer's protocol Total-Reveal Comprehensive cDNA Synthesis kit (abm, Cat No: G904, Canada). The expression analysis of H19, RNU43, LNC-MYC-3:1, and ABCC5-AS1:1 was performed by Real Time RT-PCR (SteoPne Plus RT-PCR, Applied Biosystem) according to the ABT 2x-X qPCR SYBEr-Green Master Mix (Cat No. Q03-02-05, Turkey) protocol. The RT-PCR conditions for the genes were: pre-denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s and 60°C for 60 s. GAPDH was used as housekeeping gene for normalization of the PCR data. The primer sequences of long non-coding RNAs and GAPDH used in the quantification of RT-PCR were given in Table1.

Statistical analysis

The values represent the means ± standard deviation, and the experiments were repeated three times (n=3). In the analysis of RT-PCR data, quantitation was performed using the 2-ΔΔCT method. For RT-PCR analysis RT² Profiler™ PCR Array Data Analysis program on the Internet-based Gene Globe platform was used.

Results

Cytotoxic assay and cell viability by MTS

Glabridin decreased HEC-1B cell viability in a time- and dose-dependent manner. The IC50 doses of glabridin in our study were calculated to be 21.32 μM at the 24th hour and 13.50 μM at the 48th hour (Figure 1, 2).

Gene expression levels by Real time-PCR

RT-PCR analysis revealed that, in comparison to the control group, dose group cells displayed reduced levels of H19, RNU43 and enhanced levels of LNC-MYC-3:1, and ABCC5-AS1:1 expression. The expressions of statistically significant and non-significant genes are shown in Table 2 (p<0.05).

Table 1. Primer sequences of the genes were used in this study

Gene	Primer sequences	
GAPDH	F	GTCTCCTCTGACTTCAACAGCG
	R	ACCACCCTGTTGCTGTAGCCAA
LNC-MYC-3:1	F	CGACTACTCATGGCTGGTTT
	R	AAGAAGCAGAACGTCCAAGT
ABCC5-AS1:1	F	CGACTCACAGGGTACTCAAAG
	R	CCAGCTTGTGAGAGTAGAGTTG
RNU43	F	ACTTATTGACGGGCGGACA
	R	AATCAGAACGTGACAATCAGCAC
H19	F	CGTGACAAGCAGGACATGA
	R	TCCGTGGAGGAAGTAAAGAAAC

Table 2. Effects of glabridin on fold change of the LncRNAs according to control group

Gene Name	Fold change	*p* value
LNC-MYC-3:1	5.21	0.32
ABCC5-AS1:1	2.14	0.18
H19	0.29	0.29
RNU43	0.22	0.003

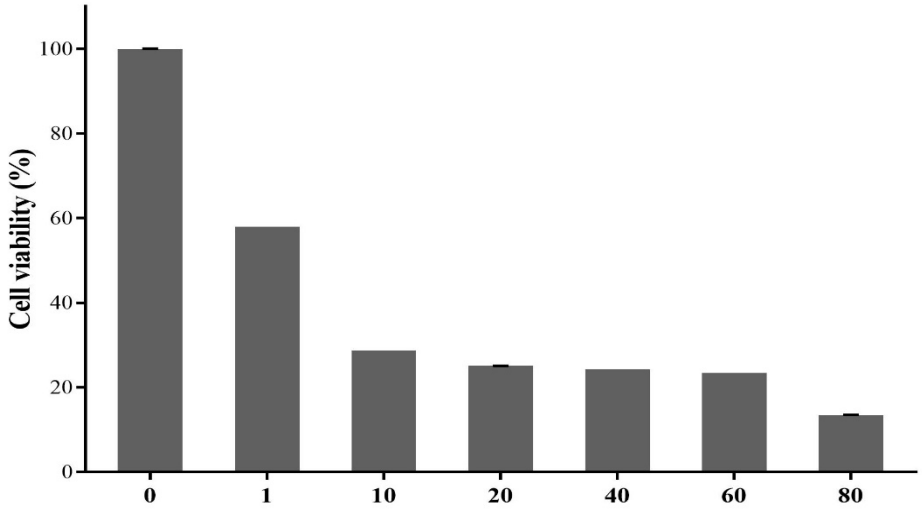

Figure 1. Cytotoxic effect of glabridin in HEC1-B cells. HEC1-B cells cultured in presence of glabridin for 24 by MTS assay
Data represent mean of 3 determinations per condition repeated 3 times. Results are shown as mean ± SD. IC50 dose of glabridin in HEC-1B cells was detected 21.32 μM at 24th hour

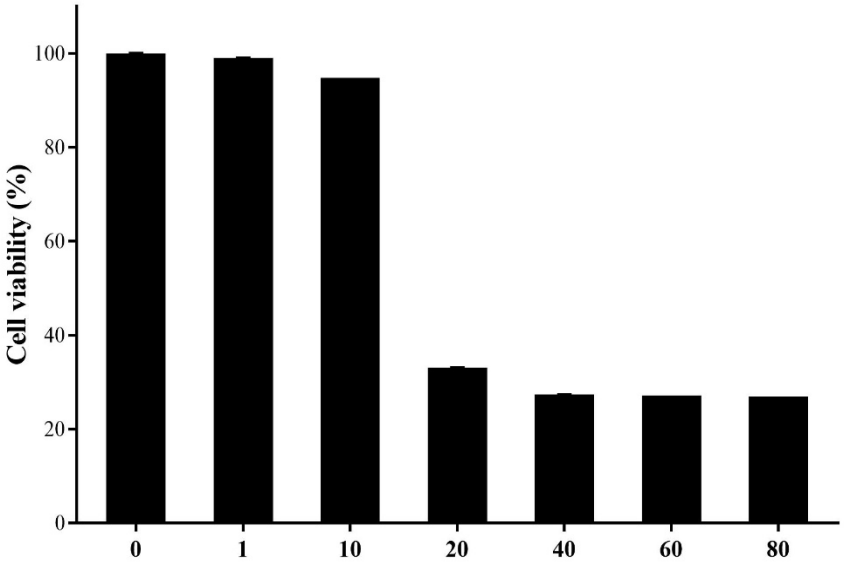

Figure 2. Cytotoxic effect of glabridin in HEC1-B cells. HEC1-B cells cultured in presence of glabridin for 48 by MTS assay
Data represent mean of 3 determinations per condition repeated 3 times. Results are shown as mean ± SD. IC50 dose of glabridin in HEC-1B cells was detected 13.5 μM at 48th hour

Discussion

EC is the fourth most common malignancy in women and the most common gynecologic cancer in developed countries [15]. LncRNA is a relatively new discovery in the RNA world that plays an important role in EC development and progression [16]. LncRNAs may be important components of the molecular mechanisms that shape the genome's three-dimensional structure [17]. Lnc-RNAs are emerging as critical regulators of cellular and disease processes. They may also play a role by competing for gene loci of miRNAs to indirectly regulate mRNA expression [18]. It has been reported that LncRNAs has a significant impact on the proliferation, migration, and invasion of cancer cells [19]. Increasing evidence suggests that LncRNAs can serve as potential therapeutic targets in cancer [20]. In addition, the special functional significance of LncRNAs suggests that they could be used as promising biomarkers in clinical cancer therapy [16]. Glabridin has been shown in previous studies to inhibit cancer cell proliferation via various mechanisms, including the miR-148a/SMAd2 signaling pathway in breast cancer cells [21] and the braf/MEK signaling pathway in hepatocellular carcinoma cells [22]. Here, we examined the effects of glabridin on viability and LncRNAs gene expression of the cultured HEC-1B cells.

LncRNAs could be tumor suppressor and oncogene genes that bind directly to RNA, DNA, or protein to perform biological functions such as cell proliferation, differentiation, apoptosis, and migration [23]. In our study, changes in gene expressions of H19, RNU43, LNC-MYC-3:1 and ABCC5-AS1:1 were analyzed. Zhao et al. [24] suggested that H19 contributed to the aggressiveness of EC by modulating the epithelial-mesenchymal transition process. Furthermore, H19 has been shown to promote the migration, invasion, and metastasis of colorectal cancer cells in vitro and *in vivo* [25]. RNU43 has also been identified as an intronic small nucleolar RNA within cancer-related genes [26]. Our findings showed that glabridin reduced mRNA expression of H19 and RNU43 compared to control cells, implying that glabridin may play a role in inhibiting HEC-1B cell proliferation by decreasing expression of these genes. The overexpression of ABCC5 in hepatocellular and breast carcinoma is associated with multidrug resistance, which can induce tumor resistance [27, 28]. Zhang et al. [29] discovered that ABCC5 expression was increased in colorectal cancer cells and reported that overexpression of antisense LncRNAs played an important role in ABCC5 regulation. Similarly, in our study, glabridin increased the expression of ABCC5-AS1:1, an antisense LncRNA, in HEC-1B. MYC family genes are frequently activated in human cancer, and their activation results in increased expression of their protein products [30]. In the present study, ABCC5-AS1:1 and LNC-MYC-3:1 mRNA expression was not significantly increased in the glabridin-treated cell.

As a result, glabridin exerted its influence by upregulating or downregulating the expression of LncRNAs. It has been reported that glabridin at concentrations higher than 10 μM was toxic to Ishikawa cells and decreased cell proliferation [31]. The IC50 of glabridin was found to be 21.32 μM at 24 hours and 13.50 μM at 48 hours in this study. Collectively, we discovered that glabridin changes the expression of LncRNAs in endometrial cancer cells. Glabridin inhibited EC cell proliferation, possibly via LncRNAs effects.

In conclusion, LncRNAs have been identified as critical regulators in the tumor formation and cancer development processes. Thus, LncRNAs may be an important therapeutic target for cancer treatment. In the present study, glabridin is thought to act as an anti-cancer agent in HEC-1B cells by regulating the expression of LncRNAs. Further *in vitro* and *in vivo* research is needed to demonstrate glabridin's anticancer effect via this mechanism.

Conflict of interest: No conflict of interest was declared by the authors.

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Ethics committee approval: The study is a cell culture study that does not require ethics committee approval.

Authors' contributions to the article

M.F.D., M.S. and Y.S. studied experimental processes and collected data. M.F.D. interpreted the data and prepared the draft text. M.S. and Y.S. prepared tables and graphs. M.S. and O.C. performed the statistical analysis. M.F.D. and O.C. critically reviewed the final version of the article. All authors approved the final version of the article.