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Triptolide: Regulator of Cellular Metabolism of Significant Fraction of Small Cell Lung Cancer via lncRNAs

Triptolit: Küçük Hücreli Akciğer Kanserinin Önemli Fraksiyonunun lncRNA'lar aracılı Hücresel Metabolizmasının Düzenleyicisi

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ABSTRACT

Objective: Small cell lung cancer accounts for 15% of all lung cancer cases. Myc family is frequently amplified and/or overexpressed in 20% of small cell lung cancers and can promote carcinogenesis. Triptolide, a Chinese medicinal herb, is an anticancer reagent isolated from Tripterygium wilfordii Hook f. This study aimed to investigate Triptolide's possible effect on Myc overexpressed/ amplified SCLC cells.

Materials and methods: To determine the expression of Myc and Myc-dependent metabolic genes, H209, H209myc, N417, and Lu135 cells were treated with Triptolide, and expression levels of Myc and associated lncRNAs were measured by qRT-PCR. Western blot analysis was performed to evaluate Myc, p21, cyclin D1 expressions. Besides, glucose uptake activity was assessed in the triptolide-treated cells.

Results: We have observed that Triptolide inhibited the proliferation of SCLC cells by inhibiting MYC expression. Triptolide has been found to repress cellular proliferation, glucose metabolism, and glucose uptake, resulting in decreased Glut1, Glut4, HK2, LDHA, and Eno1. Triptolide changed the expressions of cellular proliferation and metabolism-related lncRNAs (ANRIL, PVT1, PTENP1, H19, and lincRNA-p21).

Conclusion: The results have strongly indicated that triptolide treatment reduces cellular proliferation and glucose metabolism by regulating lncRNA expressions. Triptolide treatment would be a promising therapeutic strategy for SCLC.

Keywords: Triptolide, Small cell lung cancer, MYC, lncRNA, Metabolism

ÖZ

Amaç: Küçük hücreli akciğer kanseri (KHAK), tüm akciğer kanseri vakalarının %15'ini oluşturmaktadır. Myc ailesi, küçük hücreli akciğer kanserlerinin %20'sinde sıklıkla amplifiye/ aşırı eksprese edilir ve karsinogenezi indüklemektedir. Bir Çin şifalı bitkisi olan Triptolit, Tripterygium wilfordii Hook f'den izole edilen bir antikanser moleküldür. Bu çalışma, Triptolit'in Myc aşırı eksprese/amplifiye KHAK hücreleri üzerindeki olası etkisini araştırmayı amaçlamıştır.

Materyal ve metod: Myc ve Myc'ye bağlı metabolik genlerin ekspresyonunu belirlemek için H209, H209myc, N417 ve Lu135 hücreleri Triptolit ile muamele edildi ve Myc ve ilişkili lncRNA'ların ekspresyon seviyeleri qRT-PCR ile analiz edildi. Myc, p21, siklin D1 protein ifadelerini değerlendirmek için Western blot analizi yapıldı. Ayrıca, triptolit ile muamele edilmiş hücrelerde glikoz alınım aktivitesi ölçüldü.

Bulgular: Triptolit'in MYC ekspresyonunu inhibe ederek KHAK hücrelerinin proliferasyonunu baskıladığını gözlemledik. Triptolitin hücresel proliferasyonu, glikoz metabolizmasını ve glikoz alımını inhibe ettiği ve Glut1, Glut4, HK2, LDHA ve Eno1 ifadelerinde azalmaya neden olduğu saptandı. Triptolit'in hücresel proliferasyon ve metabolizma ile ilişkili lncRNA'ların (ANRIL, PVT1, PTENP1, H19 ve lincRNA-p21) ifadelerini düzenlediği gözlemlendi.

Sonuç: Sonuçlar, triptolit tedavisinin lncRNA ekspresyonlarını düzenleyerek hücresel proliferasyonu ve glikoz metabolizmasını baskıladığını güçlü bir şekilde göstermiştir. Triptolit tedavisinin KHAK için umut verici bir terapötik strateji olacağını düşünmekteyiz.

Anahtar Kelimeler: Triptolit, Küçük hücreli akciğer kanseri, MYC, lncRNA, Metabolizma

INTRODUCTION

Small cell lung cancer (SCLC) is one of the subtypes of neuroendocrine tumors and accounts for 15% of all lung cancer cases (1). Disruption of essential tumor suppressor genes such as RB1, Tp53, and PTEN, rapid doubling time and development of chemoresistance, metastasis to distant cities, and poor prognosis are the characteristic features of SCLC (1,2). SCLC differs from other lung cancer types in morphological, histological, and genetic characteristics (3). SCLC is classified into two subtypes as limited and extensive-stages, and a majority of patients with SCLC are diagnosed with extensive-stage and metastatic findings in diagnosis. One-year survival and five-year survival rates are 40% and 5%, respectively, and most patients have smoking histories (4). Therapeutic options are determined according to the disease stage, but traditional therapies comprising combination platinum-based chemotherapy are insufficient (3,5).

In vitro and in vivo studies revealed that Ascl1, NeuroD1, SOX2, and Myc family members are driver transcription factors that conduct the genetic composition of cells that constitute tumoral structure in the SCLC (6). Aberrant transcriptional activity of these transcription factors can regulate cellular processes, including proliferation, apoptosis, cell cycle regulation, chemoresistance, differentiation, survival changes. Recent molecular studies revealed that at least one member of the Myc family is frequently amplified and/or overexpressed in 20% of SCLCs. Genomic profiling studies reported that Myc regulates 15% of the whole genome roughly (7). Myc is the transcription factor that can promote carcinogenesis as a result of oncogenic activation by regulating the expression of specific genes that play essential roles in cell growth (8), cell cycle (9), apoptosis (10,11), energy metabolism (12), DNA replication (8), etc. Therefore, Myc is defined as one of the SCLC targets, and any drugs developed against Myc can block tumor progression (13).

Malignant cells are characterized by abnormal metabolic programming and over-energy production. Cellular metabolism and proliferation are closely linked to cellular processes. Myc activates metabolism-related genes by binding to their conserved E-box sites. Accumulative pieces of evidence have demonstrated that Myc directly regulates the expressions of exclusive glycolytic genes involving *SLC2A1, HK2, ENO1, LDHA* (14,15). On the other hand, Myc can indirectly regulate cancer metabolism through non-coding RNAs such as lncRNAs (16). Myc-regulated lncRNAs play roles in various metabolic pathways like glucose uptake and glycolysis/ gluconeogenesis (17). Accordingly, Myc is a crucial target as a therapeutic target because of regulating different aspects of cancer cell metabolism. In various levels as transcription, mRNA maturation and stability, translation, post-translational modification, and protein-protein interaction, Myc is targetable by different molecules to treat SCLC

(17,18). Kato et al. showed that JQ1 inhibits SCLC cell growth by repressing Myc expression (19). Moreover, Fiorentino et al. demonstrated that Omomyc could reduce MYC's ability to bind MYC target genes' promoters by disturbing interaction with Max (20). Although multiple molecularly targeted agents have been assessed in SCLC with limited success in patient clinical outcomes, recently, Myc targeted therapy strategies could be successful.

Triptolide is the anticancer reagent isolated from Tripterygium wilfordii Hook f (TWHf), a Chinese medicinal herb (21,22). It has crucial properties as immunomodulatory and antitumor effects as well as pharmacological activities. On the other hand, Triptolide does regulate not only oncogenes but also tumor suppressor genes. It increases the expression of p21, leading to cell cycle arrest, and induces DNA damage despite reducing cyclin family members' expression, including cyclin A, B, C, D (23,24). In the literature, it has been reported that Triptolide suppresses the cellular proliferation of pancreatic cancer by regulating c-Myc expression. However, there is no report showing the effect and mechanism of Triptolide on SCLC in the literature. For this reason, in this study, we aimed to investigate Triptolide's possible effect on Myc overexpressed/ amplified SCLC cells.

MATERIALS AND METHODS

Cell Culture

Human small cell lung cancer cell lines (H209, N417, and Lu135) were obtained from Dr. Jun Yokota. Myc status of the cell lines used in the study; H82 and N417 cell lines carrying MYC amplification and H209 cells with very low expression of MYC (13). Cell lines were cultured in RPMI-1640 medium, including 10% fetal bovine serum (FBS)(Capricorn, South America), 1% penicillin/ streptomycin (Gibco by Life Technologies, USA) at 37^{0}C with 5% CO_2.

Generation of Myc overexpression by lentiviral Myc expression vector

H209 cells are characterized by growing in floating aggregates, expressing neuroendocrine markers and not expressing c-Myc (25). Therefore, for the overexpression of Myc, H209 cells were infected with the pCDH-Myc vector which is generated by ourselves. pCDH-Myc vector has the strong CMV promoter upstream of an MCS and provides stable Myc expression. We named the infected H209 cells H209myc and H209, respectively. H209 cells were cultured in T25 flasks with 5 ml of medium. Cells were infected with a combination of lentivirus and Polybrene (Sigma Aldrich, Germany). 24hrs after infection, cells were washed with PBS twice and cultured with RPMI140 medium supplemented with FBS (Sigma, USA). H209 cells were grown for approximately five days. To select the stably infected cells, the cells were then treated with G418 (400 μg/ml) (Sigma, USA) for at least two passages to maintain optimal plasmid integration. After the infection step, Myc expression status was evaluated by Western blot analysis.

Cell Viability Assay

Triptolide was purchased from Aktin Laboratories (cat: SPC-305, PRC.). H209, H209myc, N417, and Lu135 cells were seeded in a 96-well plate (Corning) at a concentration of $1{,}5\times10^4$ cell/well. Cells were incubated at 37°C in a 5% CO2 incubator. After 24hrs, cells were treated with Triptolide which was added to the medium in different concentrations (0,1,5,10,25,50,100 ng/ml) for 24hrs. Its control groups were not treated with Triptolide. At the end of incubation periods, Abbkine cell counting kit-8 (China) was used to determine cell viability. The protocol was performed according to the manufacturer's instructions.

RNA Isolations, cDNA synthesis, and qRT-PCR Assay

To determine the expression of Myc and Myc-dependent metabolic genes in SCLC cell lines, N417 and Lu135 cells (non-treated, controls groups) and its counterpart treated with Triptolide were isolated using TRIzol LS Reagent (Invitrogen, USA) and RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. To determine quality, RNA samples were measured by means of OD260/280 using a NanoDrop ND-1000 instrument. cDNAs from total RNA samples were synthesized using a Reverse Transcription Kit (miScript II RT kit, Qıagen). Expression levels of Myc and associated genes (ANRIL, PVT1, H19, PTENP1, and LINCRNAp21) were measured by qRT-PCR using a QuantiTect SYBR Green PCR Kit. GAPDH was used as the internal control, and the transcription levels of relative genes were calculated using the 2^-DDCt method.

Western blotting analysis

Cells were lysed with ice-cold RIPA buffer(50 mM TRIS, 0.5% sodium deoxycholate, 1.0% NP-40, 0.1% SDS, 150 mM NaCl, 2 mM EDTA) including phosphatase and protease inhibitors (Roche). Equal amounts (30 μg) of protein lysates were run to separate sodium dodecyl sulfate-polyacrylamide gels. After separation, nonspecific bindings were blocked in BSA solution for two h. Following, protein samples were probed with primer antibody anti-human c-Myc (sc-40, Santa Cruz, USA), (p21, Cyclin D1) (Cell Signaling Technology, Danvers, MA, USA), and α-tubulin (CP06, CalBiochemicals, USA). Membranes were then incubated with a peroxidase-conjugated secondary antibody at room temperature. The bands were scanned with Odyssey® Fc Imaging System (LI-COR Biosciences). The band intensities were quantified by densitometric analysis using Image Studio Lite Ver 5.0. For each sample, the values of the

band were firstly normalized with band values of its α-tubulin. Subsequently, fold change calculated by its control group. The band intensities were quantified by using the ImageJ gel analysis program.

Glucose Uptake Assay

A Glucose Uptake-Glo™ Assay kit (Promega, USA) was used to measure glucose uptake activity in the cells. Human SCLC cells, N417 and Lu135, were seeded into a 6-well plate (1×10^5 cells/well). Briefly, cells were treated with triptolide 24hrs after incubation. 2 days later, the medium was removed, and cells were then washed twice in PBS following the manufacturer's instructions. To initiate glucose uptake, 50µl of the 1mM 2DG in PBS was added per well and incubated for 10 minutes at room temperature. The uptake reaction was stopped by adding 25µl of Stop Buffer. The samples were processed as a regular protocol by adding 25µl of Neutralization Buffer, and 100µl 2DG6P Detection Reagent followed by incubation for 5 hours at room temperature. Luciferase activity was determined using a Promega GloMax® instrument. The non-treated counterpart of cells was used as the control group when statistically analyzed.

Statistical analysis

All experiments were performed in triplicates and repeated independently to confirm the results. Data analysis of gene expression was performed using Excel-based PCR Array Data Analysis Software provided by the Qiagen company. Significance was determined with p values of ≤0.05.

RESULTS

Expression of Myc in pCDH-Myc Vectors Infected in H209 Cells

To validate if selected colonies express Myc, the lentiviral infection was performed in H209, MYC non-amplified cell line. Moreover, the baseline expression of c-Myc was much lower than L-Myc in H209 cells. Two passages after G418 selection, c-Myc expression was observed in H209 cells (Figure 1a). Besides, we obtained that the overexpression of Myc

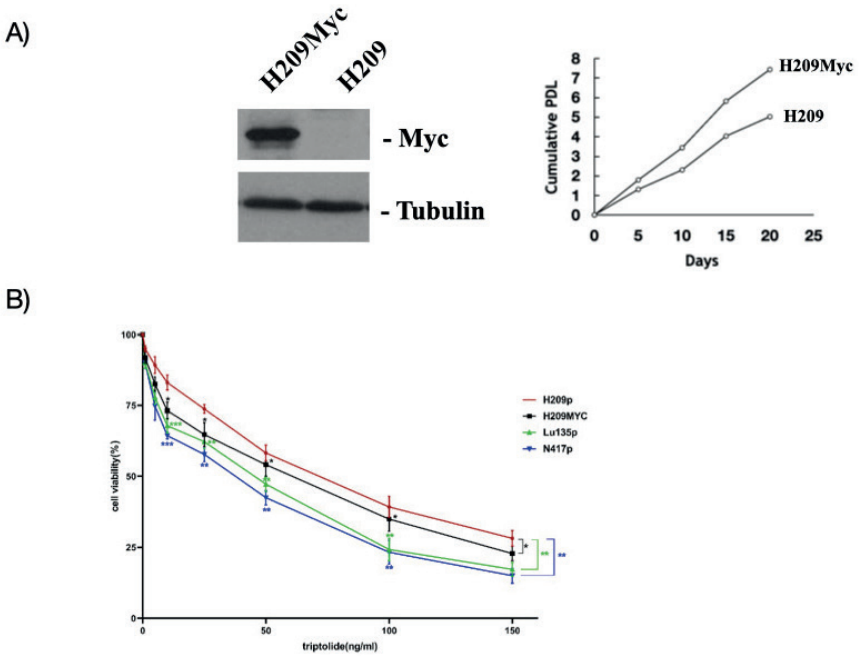

Figure 1. A) Overexpression of Myc induce cellular proliferation of H209 cells after lentiviral vector infection. B) Triptolide inhibit cellular proliferation of SCLC cells

in H209myc cells promotes cellular proliferation compared to H209 cells (Fig. 1a).

Triptolide inhibited the proliferation of SCLC cells through repressing c-Myc expression

For the detection of possible cytotoxic activity of triptolide, H209, H209Myc, Lu135 and N417 cell lines treated with different concentrations of triptolide (0,1,5,10,25,50,100 and 150 ng/ml) for 72hrs. Dose and time-dependent response to Triptolide was evaluated. Figure 1b indicates that Triptolide had cytotoxic effects on the cellular proliferation of SCLC cells.

We also calculated IC50 values for Triptolide (72hrs) and represented them in Table 1. Depending on IC values, we observed that H209myc cells were more sensitive to Triptolide than H209 cells.

Table 1. IC50 values were estimated after 72h treatment with Triptolide

	IC50 Value (ng/ml)
H209	67,59
H209myc	47,86
Lu135	31,69
N417	25,83

Depending on IC values, we observed that H209myc cells were more sensitive to Triptolide than H209 cells. Furthermore, considering the Myc expression status of SCLC cells, we concluded that Triptolide induced the inhibition of cell proliferation of SCLC cells in a Myc-dependent manner. To investigate whether this decrease in cell proliferation was caused by Myc reduction, western blot analysis was performed 48h after triptolide treatment. Western blot analysis results showed that Myc expression was significantly decreased after triptolide treatment on H209Myc, Lu135, and N417 cell lines (Figure 2a). Gel band intensities were quantified by the Image J program (Figure 2b).

Triptolide represses cellular proliferation by regulating cell cycle regulatory molecules

Since growth suppression was observed upon triptolide treatment in SCLC cells, we evaluated the expressions of cell cycle regulator molecules after triptolide treatment by western blotting (p21, cyclin D1) (Figure 3a) and qRT-PCR (Myc, p21, Cyclin D1, CDK6, PTEN) (Figure 3b).

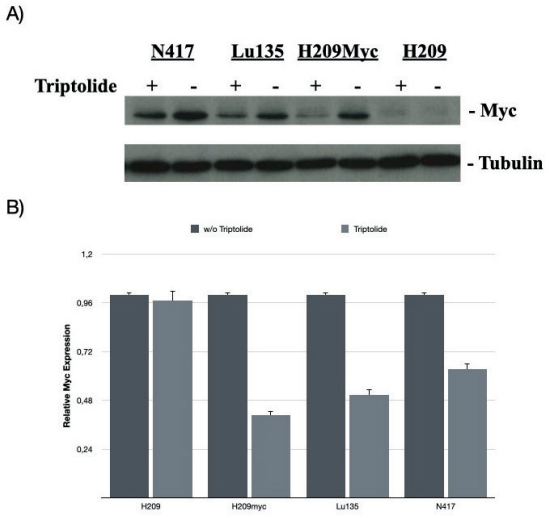

Figure 2. A) Effects of triptolide treatment on Myc protein levels. B) Band intensity of Myc protein

Because N417 cells are PTEN deficient, we did not check PTEN expression. Depending on qRT-PCR and western blot analysis results, c-Myc expression was significantly decreased after triptolide treatment. Additionally, we obtained that the triptolide treatment suppressed CDK6 and Cyclin D1 and overexpressed p21 and PTEN. So, reduced expressions of cell cycle activators and increased cell cycle inhibitors'

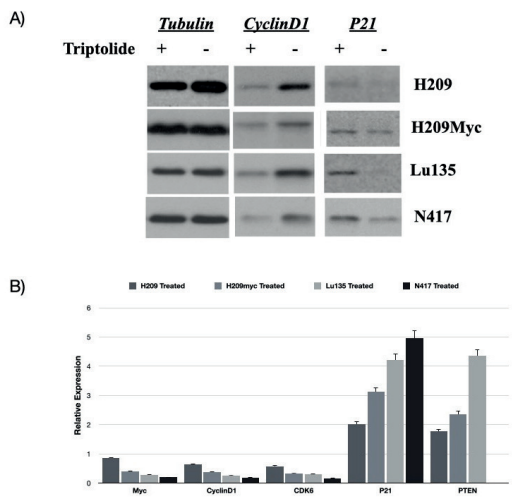

Figure 3. A) Triptolide treatment regulates p21 and cyclinD1 protein levels. B) Triptolide repressed cellular proliferation via regulating cell cycle regulatory genes

expression probably contribute to Triptolide's antiproliferative effects on SCLC cells.

Triptolide suppresses glucose metabolism and glucose uptake

Triptolide's mechanism-induced decrease of glucose consumption in SCLC cells was investigated using a Glucose Uptake-Glo™ Assay kit. Glucose uptake assay results indicated that glucose consumption was reduced after triptolide treatment (Figure 4a). Next, we evaluated glucose metabolism expressions related to genes in Triptolide-treated SCLC cells by using qRT-PCR analysis. Depending on PCR results, we obtained the decreased expressions of Glut1, Glut4, HK2, LDHA, and Eno1 (Figure 4b).

These results pointed out that Triptolide regulates glucose metabolism by repressing c-MYC expression in SCLC.

Triptolide treatment changes the expressions of cellular proliferation and metabolism-related lncRNAs

To check expression changes of lncRNAs after triptolide treatment, we performed qRT-PCR analysis for ANRIL, PVT1, PTENP1, H19, and lincRNA-p21. Our results demonstrated that Triptolide affected the expression levels of lncRNAs that have roles in proliferation (PTENP1 and LincRNA-p21) and cellular metabolism (ANRIL, PVT1, PTENP1, H19), as shown in Figure 5. Because N417 cells are PTEN deficient, we did not check PTENP1 expression.

Our previous manuscript explored the expression status of ANRIL, PVT1, PTENP1, H19, and lincRNA-p21changes depending on Myc expression status. Similarly, in this study, we obtained that triptolide treatment can suppress Myc expression, and this suppression regulates the expressions of ANRIL,

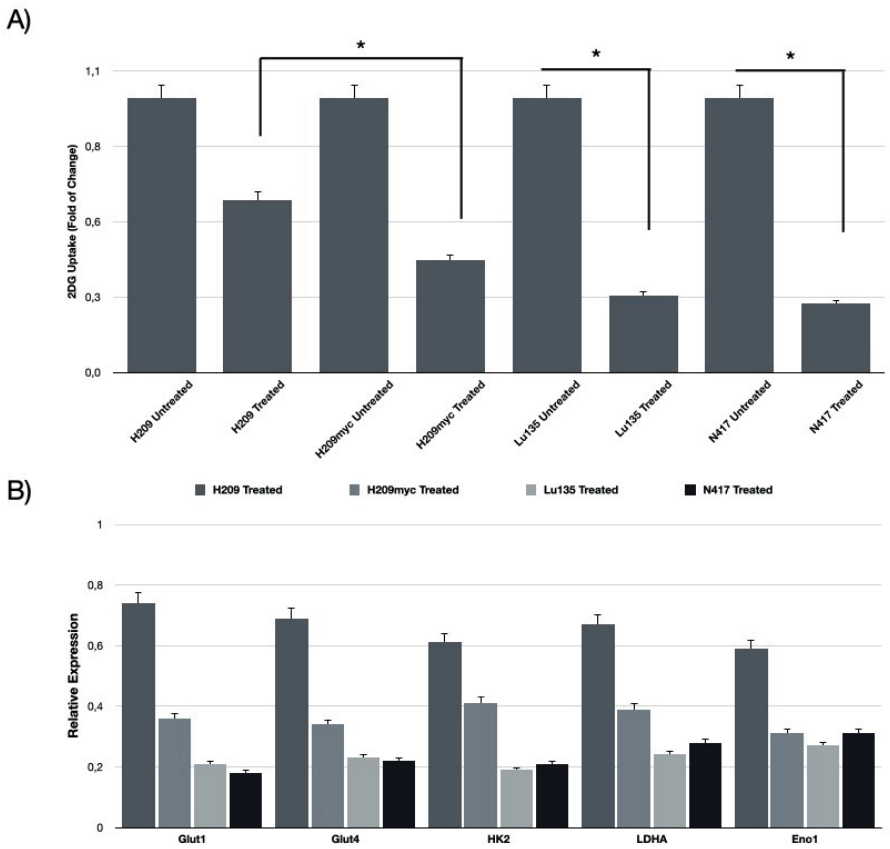

Figure 4. A) 2-DG uptake in triptolide-treated SCLC cells. B) Triptolide treatment regulates glucose metabolism-related gene expression levels.

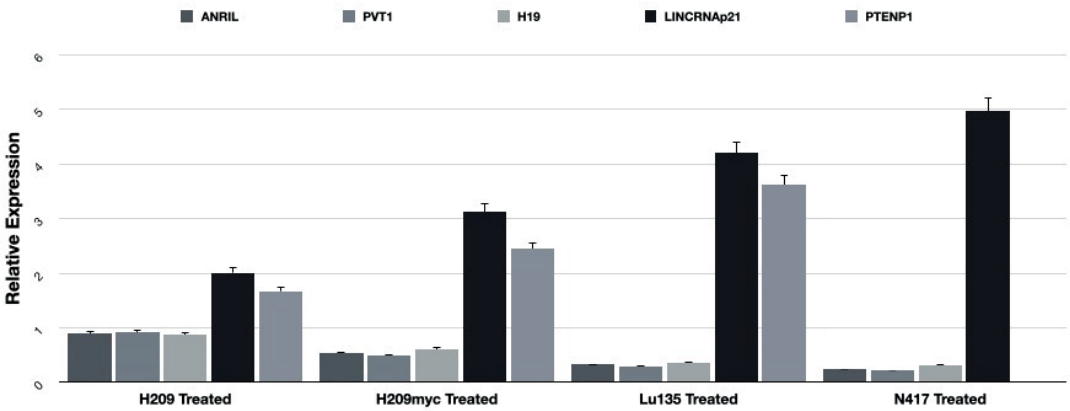

Figure 5. LncRNA expression changes depending on triptolide treatment

PVT1, PTENP1, H19, and lincRNA-p21. As a result of lncRNA expression experiments, triptolide treatment inhibited cellular proliferation by regulating Myc-dependent lncRNAs that play cellular proliferation and glucose metabolism roles.

DISCUSSION

Small cell lung cancer is a subtype of lung cancer that accounts for approximately 15% of lung cancer and has unique genomic features (2). Many clinical studies indicated that SCLC displays distinct characteristics, including extreme genomic instability, genetically amplified and/overexpressed different transcription factors, and poor prognosis (3,4). Moreover, current diagnostic strategies for early detection and standard treatment protocols are insufficient to increase patients' overall survival. Consequently, the 5-year survival of patients is much less than 10% (13,14). Therefore, there is a need for new effective molecules that also have low cytotoxicity for patients. In the literature, studies have shown that Myc is the oncogenic driver in SCLC, and genetic aberrations in Myc have been identified to change different cellular mechanisms as particularly metabolism, in SCLC (17). Therefore, we believe that targeting Myc might be a significant therapeutic strategy for SCLC.

Triptolide, a natural product, has been investigated as a new agent for treating a broad range of cancers. Triptolide has been demonstrated to exert anti-tumor effects by reducing proliferation and inducing apoptosis in different cancer types, including cholangiocarcinoma, hepatocellular carcinoma, pancreatic cancer, and non-small cell lung cancer. Many studies to date have shown that Triptolide can inhibit various human solid tumors in vivo and in vitro. It is also known that this inhibitory effect is achieved by affecting multiple mechanisms, especially inhibitory heat shock factor-1 (HSF-1), suppression of DNA damage response, and regulating mRNA stability. Although Triptolide's anticancer property has been shown in the literature with some studies in lung cancers, there is no study showing its effect on small cell lung cancer. Studies are showing the effect of Triptolide on MYC expression in various cancer types. Therefore, we have foreseen that Triptolide might be an effective anticancer molecule in small cell lung cancers where Myc overexpression or amplification is observed at a rate of 15%. Hence, in this study, we examined Triptolide's effect on SCLC cell lines with or without Myc overexpression. Previous studies have shown that Triptolide can downregulate the expression of c-Myc in non-small cell lung carcinoma (7). Correlatively, our study revealed that Triptolide decreased the expression of Myc in SCLC cell lines.

Furthermore, we observed that when we treated the Triptolide cells, H209myc cells were more sensitive than H209 parental cells. These results indicated that SCLC cells are more addicted to MYC for their growth and survival than the cells without MYC amplification and/or overexpression. Therefore, they are susceptible to MYC inhibition by triptolide treatment. Moreover, we have shown that Triptolide repressed cellular proliferation via regulating cell cycle regulatory genes,

which Myc regulates. The literature indicates that cell cycle regulatory genes like cyclin D1 and CDK6 are a transcriptional target of Myc (14). In this study, we also found that expressions of cell cycle regulatory genes like cyclin D1 and CDK6 were significantly decreased with Myc in SCLC cells. Conversely, triptolide treatment increased the expressions of PTEN and p21. These data strengthen the evidence that triptolide treatment represents a common inhibitor for the MYC protein in vitro and further supports Triptolide's utility as a therapeutic strategy for SCLC.

Cellular metabolism and proliferation are closely linked to cellular processes. MYC oncogene has a critical role in the regulation of aerobic glycolysis. MYC transcriptionally regulates almost all glycolytic genes by binding the E-box sequence (12). We analyzed the expression changes of glucose metabolism-related genes after triptolide treatment in light of this information. Our results indicated that triptolide treatment decreased the expression of glucose metabolism-related genes. These results support the inhibition of glucose metabolism and repression of cellular proliferation in SCLC cells by triptolide treatment.

Many experimental studies have revealed that long-noncoding RNAs (lncRNAs) have a role in regulating cancer cell metabolism. Different lncRNAs have pivotal roles in metabolic reprogramming of various cancer types such as non-small cell lung cancer (lncRNA-NEF), hepatocellular carcinoma (lncRNA Ftx, HOTAIR, TUG1, LINC01554), osteosarcoma (TUG1, PVT1), ovarian cancer (SNHG3), gastric cancer(MACC1-AS1)(26). Increasing pieces of evidence have shown that lncRNAs regulate essential genes in glucose metabolism. Our previous studies indicated that Myc regulates various lncRNAs in SCLC and breast cancer (14,16). The present results demonstrated that triptolide treatment could regulate cellular proliferation and glucose metabolism via Myc-regulated lncRNAs (ANRIL, PVT1, PTENP1, H19, and lincRNA-p21) in SCLC.

CONCLUSION

In summary, we demonstrated the promising use of Myc as a target for SCLC therapy by using Triptolide of its expression. The results have strongly indicated that triptolide treatment reduces cellular proliferation and glucose metabolism by regulating lncRNA expressions. Accordingly, it was concluded from this study that triptolide treatment would be a promising therapeutic strategy for SCLC.

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Author Contributions: Conception/Design of Study- O.T.; Data Acquisition- O.T., K.İ.; Data Analysis/Interpretation- O.T., K.İ.; Drafting Manuscript- O.T., K.İ.; Critical Revision of Manuscript- O.T.; Final Approval and Accountability- O.T., K.İ.

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