Effects of Thymoquinone and Cisplatin on C-MYC, KRAS, p53 and EGFR Gene Expression in Lung Cancer Cell Lines

Bayrakçi Onur [a,b*#], Karaman Taşdemir Demet [b], Ilhan Sedat [b] and Işik Ahmet Ferudun [b,c]

[a] *Thoracic Surgery Department, Ersin Arslan Education and Research Hospital, Gaziantep, Turkey.*
[b] *Gaziantep University School of Medicine, Cell Culture Laboratory, Gaziantep, Turkey.*
[c] *Thoracic Surgery Department, Gaziantep University School of Medicine, Gaziantep, Turkey.*

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Lung cancer is one of the most common causes of death.It is known that genetic reasons in its etiology.Lung cancer has been shown to be associated with the EGFR,P53,KRAS and c-MYC genes.Thymoquinone is an antitumoral and antineoplastic bioactive substance procured from Nigella sativa plant.Cisplatin is a frequently used chemotherapeutic agent in the treatment of lung cancer.Our study has been conducted to examine the effects of Tq and Cis on gene expressions on lung cancer cell lines.Potential effects of Tq and Cis on A549,HTB54, CRL5820 and BEAS2B cell lines and cell viability using MTT has been evaluated.Cell culture has been effectuated with RPMI supplemented with 10% FBS,1% antibiotic and DMEM(37°C, %5 CO_2).Cells were cultured for 24 h in 96 well plates(2500/ml cells) 10% FBS RPMI appropriate medium.The cells have been exposed 100 μM Tq and 200 μM Cis for 4h under incubation conditions.DMSO has been used for negative control.RT PCR has been conducted using SYBR Green qPCR Master Mix(reference gene GAPDH).As a result, p53 gene suppression has been shown in lung adenocarcinoma with Tq and Cis and epidermoid carcinoma with Cis only.EGFR gene suppression has been shown in lung adenocarsinoma with Tq only and epidermoid carcinoma with Cis only.C-MYC gene suppression

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[#]*ORCID: 0000-0001-8681-3931*
Corresponding author: Email: dronurbayrakci@gmail.com;

has been shown in lung adenocarsinoma with both substances(more at Tq).It has been shown that KRAS gene suppression does not occur in any cell line.In addition, it has been shown that no gene expression is suppressed after Tq and cis exposure in the mesothelioma cell line.

Keywords: Thymoquinone; lung cancer; cisplatin; gene expression; cell lines.

1. INTRODUCTION

Lung cancer is one of the most common cancers that cause death in men and women. It has been shown that one of its etiologies genetic causes. It has been reported that p53 mutation, EGFR hyperexpression, c-MYC and KRAS oncogenes are associated with lung cancer [1]. The p53 gene is the tumor suppressor gene, the most frequently mutated and the cause of lung cancer [2]. c-MYC is an oncoprotein that promotes cancer cell growth and survival. This protein is anti-apoptotic and plays a role in the cycle of cancer cell [3]. Mutant KRAS activation is common in lung and epithelial cancers. Cancers driven by this activation are among the resistant of treatments [4]. The EGFR gene is the driver gene frequently mutated in lung cancer. Such that mutation of this gene is has a worse prognosis in squamous cell carcinoma than adenocarcinoma in lung cancer [5]. The presence or mutation of these genes in oncological treatments is important in the form of treatment, in the clinical course of the disease in the lung cancers. Thymoquinone is an antitumoral and antineoplastic bioactive substance procured from Nigella sativa plant [6]. Our study had been carried out on cell lines (adenocarcinoma, epidermoid carcinoma, lung mesothelioma and bronchus epithelial). Them had been evaluated at the effective concentrations of cisplatin and thymoquinone in cell culture. According to the effect of this bioactive component on genes in cell cultures under in vitro medium, it was thought and aimed that it could be used in oncological treatments as a result of additional scientific studies.

2. MATERIALS AND METHODS

2.1 Biological Activity Assay

Human alveolar adenocarcinoma (A459), human lung mesothelioma (CRL-5820), human lung epidermoid carcinoma (HTB-54) and bronchus epithelial (virus transformed 12-SV40, BEAS-2B) cell lines was used for evaluated potential effect of thymoquinone. In our previous works cell viability was assessed using MTT (3-(4,5-dimetiltiazol-2-il) 2,5-difenil tetrazolyum bromid) assay. According to results we selected 200 µM cisplatin and 100 µM thymoquinone for working concentrations.

2.2 Cell Culture

A549, CRL-5820 and HTB-54 human lung cancer cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and %1 antibiotic (Gibco, USA) BEAS-2B human normal cell line cultured DMEM with same supplemented at 37°C, %5 CO_2 [7].

2.3 Chemical Exposure

Cells were cultured for 24 h in 96 well plates (2500/ml cells) in 10% FBS RPMI appropriate medium. Before chemical exposure, the media was replaced with serum free medium for 16h. The cells were treated with 100 µM thymoquinone and 200 µM cisplatine for 4h under incubation conditions. We used DMSO (dimetil sülfoksit) for negative control (Thymoquinon and cisplatin solved in DMSO).

2.4 Measured of Genes Expression by RT-PCR

QIAamp RNA isolation Mini Kit (Qiagen, Germany) was used to extract total RNA from cultured cell lines, which was then applied to reverse transcription using a Reverse Transcription Kit (Qiagen, Germany). RT PCR was conducted using SYBR Green qPCR Master Mix (Qiagen, Germany). Expression data were standardized to the reference gene GAPDH in order to control the variability in expression levels and calculated as CT of candidate genes versus CT of GAPDH, where CT represents the threshold cycle for each transcript. The average for each gene and sample was calculated and the experiments were independently repeated.

The primer sequences for the RT-PCR of KRAS, MYC, EGFR, TP53 and GAPDH were as follows:

KRASF:5–
GGTGGAGTATTTGATAGTGTATTAACC–3

KRAS R:5-GAATGGTCCTGCACCAGTAA-3,
MYC F:5-CCTGGTGCTCCATGAGGAGAC-3
MYC R:5- CAGACTCTGACCTTTTGCCAGG-3,
EGFR F:5 AACACCCTGGTCTGGAAGTACG-3
EGFR R: 5- TCGTTGGACAGCCTTCAAGACC-3,
TP53 F:5- CCTCAGCATCTTATCCGAGTGG-3
TP53 R: 5- TGGATGGTGGTACAGTCAGAGC-3,
GAPDH F:5- GTCTCCTCTGACTTCAACAGCG
GAPDH R:5- ACCACCCTGTTGCTGTAGCCAA-3 [8].

2.5 Statistical Analysis

Data were tested for normality using Shapiro-wilk test. Multiple comparisons were performed using one-way variance analysis, ANOVA or the Kruskal-Wallis test. Mann-Whitney U test were used for non-normally distributed data in comparison of treatment groups. Results are expressed as mean ± SEM or median ± interquartile ranges (Q1 and Q3). p values < 0.05 were considered to be significant. Prism 6 program used for statistical analysis (GraphPad Software, Inc, San Diego, USA).

3. RESULTS

3.1 HIF Inhibitors Suppressed the Expression of HIF Targeted Genes

We treated BEAS-2B, A549, CRL5820 and HTB-54 cells with 200 µM concentrations of cisplatin and 100 µM thymoquinone incubated the cells for 4 hours. Then, extracted the RNA and evaluated the mRNA levels of lung cancer targeted genes KRAS, EGFR, MYC and TP53 via quantitative PCR assay.

The results proved that the cisplatin decreased expression on EGFR (median = 0.96, Q1= 0.94 and Q3= 1.11 %, p = 0.010 vs median = 1.15, Q1= 1.13 and Q3= 1.18), myc (median = 0.93, Q1= 0.90 and Q3= 1.04 %, p = 0.010 vs median = 1.15, Q1= 1.12 and Q3= 1.16) and tp53 (median = 1.06, Q1= 0.99 and Q3= 1.11 %, p = 0.030 vs median = 1.13, Q1= 1.12 and Q3= 1.15) genes in A549 cells compared to negative control DMSO (cisplatin).Similarly thymoquinone decreased expression on tp53 (median = 1.11, Q1= 0.91 and Q3= 1.11 %, p = 0.026 vs median = 1.26, Q1= 1.11 and Q3= 1.35) genes for same cell line. Therefore, thymoquinone showed the same effect at a lower dose 100 µM than the cisplatin 200 µM concentration for tp53 gene in A549. When thymoquinone compared to cisplatin with Mann –Whitney t test. The thymoquinone decreased to EGFR (median = 1.07, Q1= 1.00

and Q3= 1.14 %, p = 0.004 vs median = 1.29, Q1= 1.22 and Q3= 1.37) and tp53 (median = 1.10, Q1= 1.02 and Q3= 1.16 %, p = 0.026 vs median = 1.19, Q1= 1.12 and Q3= 1.25) genes compared to cisplatin in HTB-54 cell line. For A549 cell line, thymoquinone decreased only myc and tp53 (median = 1.11, Q1= 1.05 and Q3= 1.17 %, p = 0.008 vs median = 1.25, Q1= 1.03 and Q3= 1.26) gene.

4. DISCUSSION

The p53 gene function inside the cell; gene transcription, DNA synthesis and repair, preservation of genetic stability, cell cycle arrest and programmed cell death. It is the most common mutant gene in cancer and located at the 17p13 locus. It has been shown in small cell lung cancer 90%, epidermoid carcinoma 65%, large cell cancer 60%, adenocarcinoma 33% and all cancers 50% [9]. If repair of DNA damaged gene is not possible p53 gene trigger apoptosis in a normal cell cycle. However, a dysfunctional p53 causes cancer. For these reasons, control of p53 gene expression is required in the treatment of lung cancer. Cisplatin is a chemotherapeutic agent, often preferred in combinations in lung cancers. In our study, the effects of timoquinone and cisplatin on cell lines at in vitro effective doses ($EC50_{Tq}$: 100 µM, $EC50_{Cis}$: 200 µM) had been compared. Both substances were compared to DMSO. It was observed that Tq and Cis in the A549 cell line decreased p53 gene expression (p<0.05). Tq has been shown to have a similar effect at lower concentration. It was observed that Cis in the HTB54 cell line decreased p53 gene expression (p<0.05) but Tq had no effect. It was shown that both substances had no effect on p53 gene expression in CRL5820 and BEAS2B cell lines (Fig. 1).

The EGFR is a transmembrane glycoprotein with tyrosine kinase activity. Its abnormal stimulation and dysregulation are associated with tumor grow. EGFR's has been shown to act a part in inhibition of apoptosis, adhesion, invasion, differentiation, angiogenesis and metastasis [10]. Hyperexpression of EGFR has been reported with a rate of 58% in non-small cell lung cancer and 64.9% in lung epidermoid carcinoma [11]. Mutation of this gene is also being investigated in chemotherapy protocol. Detection of EGFR mutation is important in the decision to give a tyrosine kinase inhibitor in oncological treatment [12]. In our study, it was observed that significantly decreased Tq EGFR gene expression (p<0.05) in A549 cell lines but Cis

had no effect. It was observed that significantly decreased Cis EGFR gene expression (p<0.01) in HTB54 cell lines but Tq had no effect. Both substance have been shown to no effect on EGFR gene expression in BEAS2B and CRL5820 cell lines (Fig. 2).

The c-MYC oncogene is frequently amplified from cells derived from lung tumors and is associated with malignancy. Analysis of lung cancer tumors showed that dysregulated expression of c-MYC may occur more frequently in metastases than in the primary tumor [13]. In addition, c-MYC mutation is a poor prognostic factor in lung adenocarcinoma [14]. In our study,

it was observed that Tq and Cis decreased c-MYC gene expression in the A549 cell line (p<0.01). Tq has been shown to decrease gene expression more than cis (Fig. 3).

KRAS activating mutations are found in 25% to 30% of non-squamous cell NSCLCs [15]. KRAS mutations common in NSCLC are associated with poor prognosis, possibly due to poor responses to most systemic therapies and lack of targeted drugs [16]. Therefore KRAS inhibitor is an important option among oncological treatments. In our study, it has been observed that the rates of KRAS gene expression did not decrease significantly in all cell lines (Fig. 4).
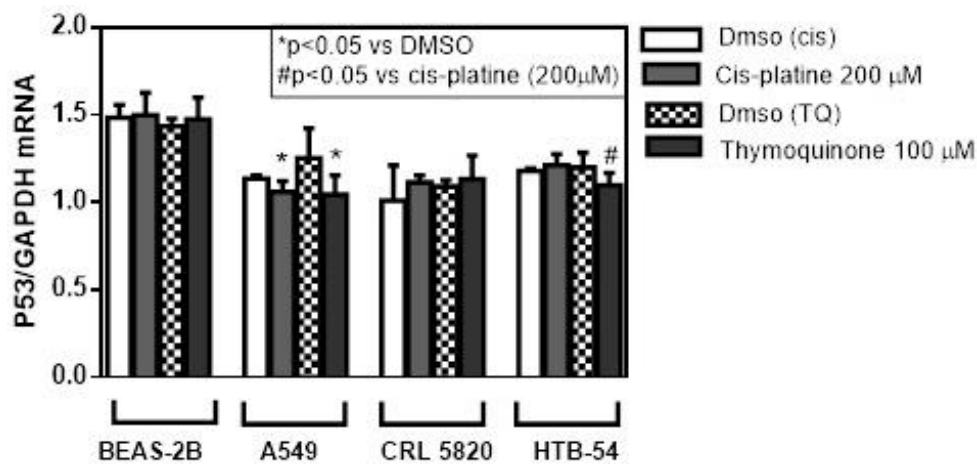

Fig. 1. Expression of mRNA for TP53/GAPDH
Under following incubation with compounds tymoquinone (100 µM) and cisplatine (200 µM) for 4 hours. Results are shown expressed as median and interquartile ranges
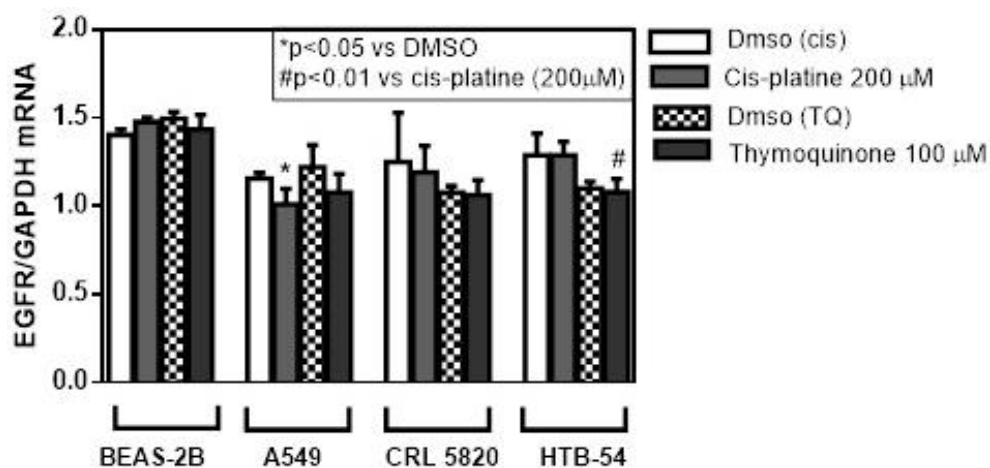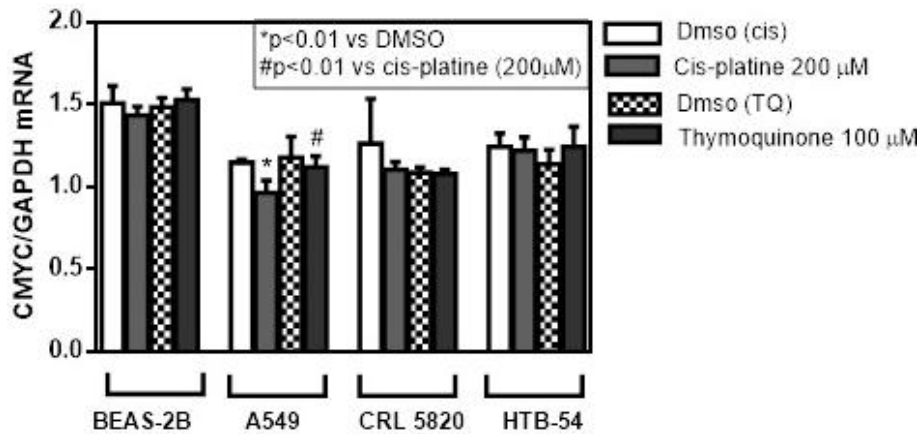

Fig. 2. Expression of mRNA for EGFR/GAPDH
Under following incubation with compounds tymoquinone (100 µM) and cisplatine (200 µM) for 4 hours. Results are shown expressed as median and interquartile ranges

Fig. 3. Expression of mRNA for CMYC/GAPDH
Under following incubation with compounds tymoquinone (100 µM) and cisplatine (200 µM) for 4 hours. Results are shown expressed as median and interquartile ranges
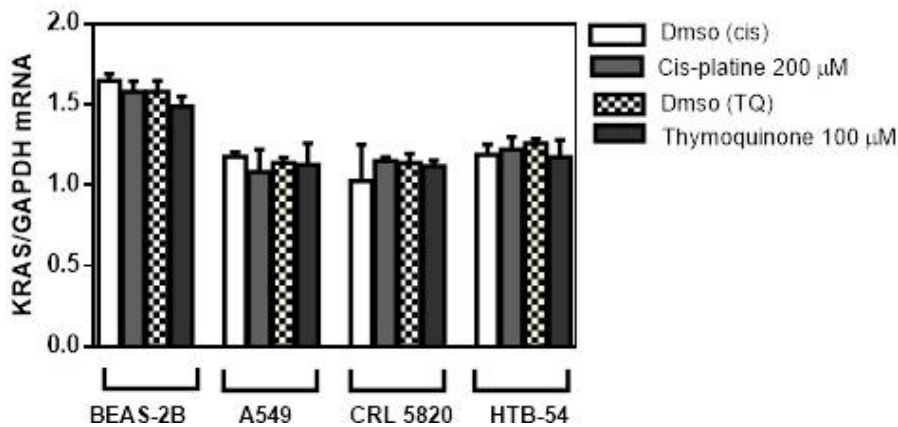

Fig. 4. Expression of mRNA for KRAS/GAPDH
Under following incubation with compounds tymoquinone (100 µM) and cisplatine (200 µM) for 4 hours. Results are shown expressed as median and interquartile ranges.

5. CONCLUSIONS

p53 gene suppression has been shown in lung adenocarcinoma with Tq and Cis and epidermoid carcinoma with Cis only. EGFR gene suppression has been shown in lung adenocarsinoma with Tq only and epidermoid carcinoma with Cis only. C-MYC gene suppression has been shown in lung adenocarsinoma with both substances (more at Tq). It has been shown that KRAS gene suppression does not occur in any cell line. In addition, it has been shown that no gene expression is suppressed after Tq and cis exposure in the mesothelioma cell line.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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