

**Effects of thymoquinone and cisplatin on c-MYC, KRAS, p53 and EGFR gene expression in lung cancer cell lines**

**Keywords:** *thymoquinone, lung cancer, cisplatin, gene expression, cell lines*

**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<sub>2</sub>). 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 adenocarcinoma with Tq only and epidermoid carcinoma with Cis only. c-MYC gene suppression has been shown in lung adenocarcinoma 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*

**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 [1,2]. Thymoquinone is an antitumoral and antineoplastic bioactive substance procured from *Nigella sativa* plant [3]. Our study had been carried out on cell lines (adenocarcinoma, epidermoid carcinoma, lung mesothelioma and bronchus epithelial). They had been evaluated at the effective concentrations of cisplatin and thymoquinone in cell culture.

## Materials and Method

**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 were used for evaluating the potential effect of thymoquinone. In our previous works cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. According to results we selected 200  $\mu$ M cisplatin and 100  $\mu$ M thymoquinone for working concentrations.

**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 in DMEM with same supplement at 37°C, 5% CO<sub>2</sub> [4].

**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  $\mu$ M thymoquinone and 200  $\mu$ M cisplatin for 4h under incubation conditions. We used DMSO (dimethyl sulfoxide) for negative control (Thymoquinone and cisplatin dissolved in DMSO).

**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:

KRAS F: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 [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  $\pm$  SEM or median  $\pm$  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).

## Results

### *HIF inhibitors suppressed the expression of HIF targeted genes*

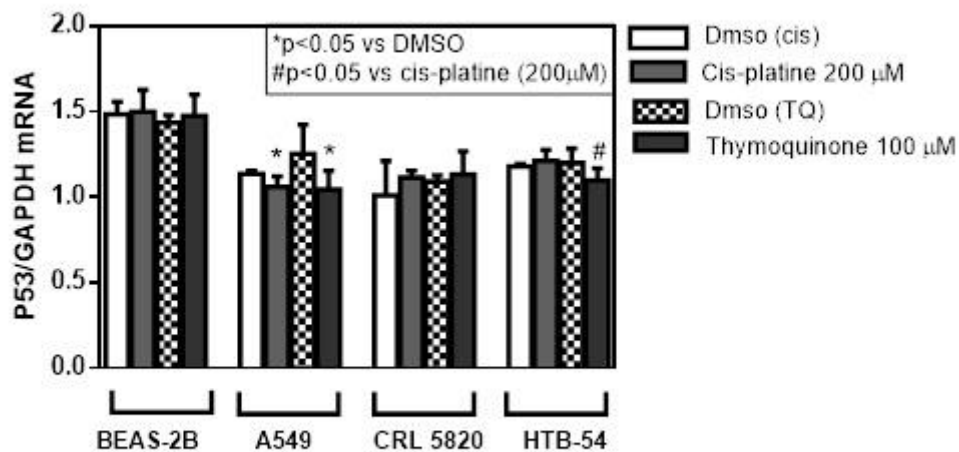
We treated BEAS-2B, A549, CRL5820 and HTB-54 cells with 200  $\mu$ M concentrations of cisplatin and 100  $\mu$ M thymoquinone incubated the cells for 4 hours. Then, extracted the RNA and evaluated the mRNA levels of lung cancer targeted genes KRAS, EGFR, MYC and TP53 via quantitative PCR assay.

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  $\mu$ M than the cisplatin 200  $\mu$ M 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.

## Discussion

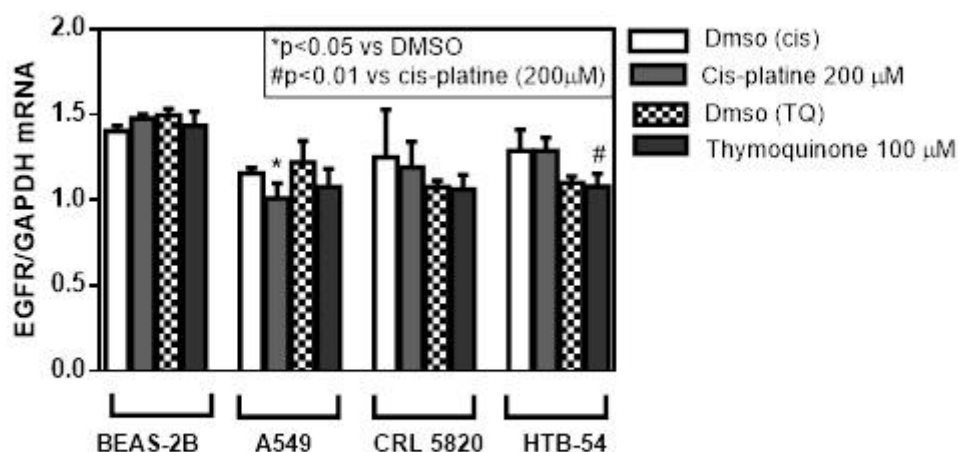
The p53 gene function inside the cell; gene transcription, DNA synthesis and repair, preservation of genetic stability, cell cycle arrest and programmed cell death. It is the most common mutant gene in cancer and located at the 17p13 locus. It has been shown in small cell lung cancer 90%, epidermoid carcinoma 65%, large cell cancer 60%, adenocarcinoma 33% and all cancers 50% [6]. When DNA damage occurs in a normal cell, p53 is obtain genomic stability and inhibits cell cycle in G1. If repair is not possible, cell apoptosis occurs. In case of loss of P53 function, preneoplastic and neoplastic cells increase. Cell proliferation continues uncontrolled without DNA repair. Mutations in the 157,248 and 278th codons of the p53 gene are important for lung cancer [7]. For these reasons, control of p53 gene expression is required in the treatment of lung cancer. Cisplatin has been an important agent for the treatment of lung cancer and provided statistically and clinically significant improvement in survival [8]. In our study, the effects of timoquinone and cisplatin on cell lines at in vitro effective doses ( $EC_{50-Tq}$ : 100  $\mu$ M,  $EC_{50-Cis}$ : 200  $\mu$ M) 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 (figure 1).



**Figure 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.*

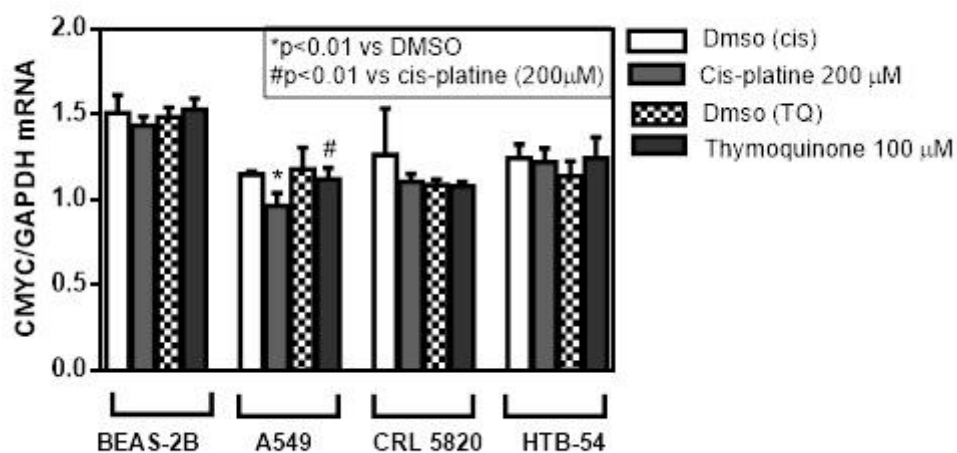
The EGFR is a transmembrane glycoprotein with tyrosine kinase activity. Its abnormal stimulation and dysregulation are associated with tumor grow. EGFR's has been shown to act a part in inhibition of apoptosis, adhesion, invasion, differentiation, angiogenesis and metastasis [9]. Hyperexpression of EGFR has been reported with a rate of 58% in non-small cell lung cancer and 64.9% in lung epidermoid carcinoma [10]. 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 (figure 2).



**Figure 2:** Expression of mRNA for EGFR/GAPDH

*\*Under following incubation with compounds tymoquinone (100  $\mu$ M) and cisplatine (200  $\mu$ M) for 4 hours. Results are shown expressed as median and interquartile ranges.*

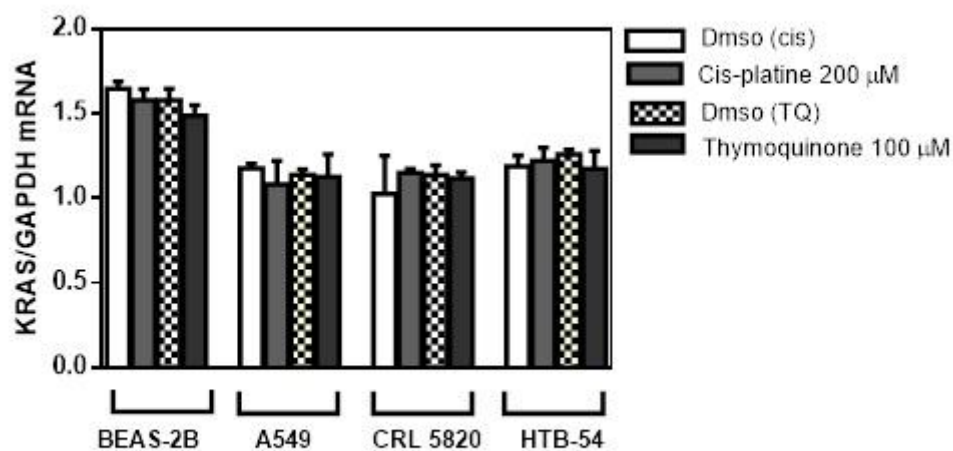
It is a member of the myc gene family known as the c-MYC oncogene. Myc genes, encode phosphoprotein binding to DNA, which is effective in cell proliferation and differentiation. Takes part in starting DNA synthesis. This gene transform into oncogene by amplification and dysregulation. It has been reported myc activation rate in small cell lung cancer 18-31% and non-small cell lung cancer 8-20% [11]. 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 (figure 3).



**Figure 3:** Expression of mRNA for CMYC/GAPDH

*\*Under following incubation with compounds tymoquinone (100  $\mu$ M) and cisplatin (200  $\mu$ M) for 4 hours. Results are shown expressed as median and interquartile ranges.*

The KRAS, a member of the Ras gene family, is an important oncogene. Because it inhibits the mutant ras protein GTPase, its gene products increase uncontrolled. KRAS mutation is the most common and usually on the 12th codon. It has been reported KRAS activation rate in non-small cell lung cancer 15-50%, adenocarcinoma and large cell lung cancer 30-60%, lower in epidermoid carcinoma [12]. In our study, it has been observed that the rates of KRAS gene expression did not decrease significantly in all cell lines (figure 4).



**Figure 4:** Expression of mRNA for KRAS/GAPDH

*\*Under following incubation with compounds tymoquinone (100  $\mu$ M) and cisplatin (200  $\mu$ M) for 4 hours. Results are shown expressed as median and interquartile ranges.*

## Conclusions

In lung adenocarcinoma cell lines; it has been shown to decrease p53 and c-MYC gene expressions both substances (Tq and Cis). However, with Tq exposure, p53 gene expression has been suppressed at a lower concentration and c-MYC more at effective doses. Tq has been shown to significantly suppress EGFR gene expression and Cis no effect. Both substance has any effect on the expression of KRAS gene.

In lung epidermoid carcinoma cell lines; Cis has been shown to decrease EGFR and P53 gene expression and Tq no effect. Both substances have no effect on KRAS and c-MYC gene expressions.

In lung mesothelioma and broncus epithelial cell lines; it has been shown that Tq and Cis have no effect on all gene (c-MYC, KRAS, EGFR, P53) expressions.

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 adenocarcinoma with Tq only and epidermoid carcinoma with Cis only. C-MYC gene suppression has been shown in lung adenocarcinoma 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.

### **Availability of data and materials**

All data obtained in our study are record and available.

### **Ethics approval and consent to participate**

The study has been carried out on cancer cell lines in the cell culture laboratory and accordance with international standards.

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