Research Article

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Investigation into drug resistance to cisplatin in cancer stem cell-enriched population in non-small cell lung cancer

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Abstract

Objectives: understanding drug resistance in cancer is of importance in treatment. Cancer stem cells are main factor for drug resistance. Therefore, the possible gene/gene interactions/proteins were explored in our study using a cancer stem cell-enriched population (H1299/S) derived from a parental non-small cell lung cancer cell line (H1299/P).
Methods: response to cisplatin, which is the main drug for the treatment of lung cancer, was evaluated with the Adenosine triphosphate (ATP) viability test. As a result of the gene expression analysis, while 14 genes were not evaluated, expression profiles were obtained for 37 genes out of 51 genes. By the drug-protein interaction analyses, Topoisomerase I (TOPI), Topoisomerase 2 alpha (TOP2A), Topoisomerase 2 beta (TOP2B), Cyclin-dependent kinases 4 (CDK4), Cyclin-dependent kinases 6 (CDK6), ATP binding cassette subfamily B member 1 (ABCB1), ATP binding cassette subfamily C member 1 (ABCC1), ATP binding cassette subfamily C member 3 (ABCC3), B-cell leukemia/lymphoma 2 (BCL2), Poly (ADP-ribose) polymerase 1 (PARP1), Breast cancer gene 1 (BRCA1) and Cyclin dependent kinase inhibitor 1A (CDKN1A) genes and protein products were statistically significantly found to be in association with drug resistance.
Results and discussion: in bioinformatics analyses, it was observed that 13 pathways were affected due to expression changes and 12 genes related to these pathways were determined to activate multidrug resistance mechanisms.
Conclusions: platinum-based drugs, as well as a broad range of other agents including topoisomerase and PARP1 inhibitors, and anthracyclines, have been shown to potentially possess multiple drug resistance.

Keywords: cisplatin; drug resistance; gene; lung cancer; stem cell

Introduction

Non-small cell lung cancer (NSCLC) accounts for approximately 80 % of lung cancer cases. Cancer 5-year survival is 24 %, with a median survival of 18 months on average from diagnosis [1]. The most important problem in oncology is failure to respond well to chemotherapy or recurrence of cancer. This condition is usually fatal and patients die shortly after metastasis as they no longer respond to the drugs administered. The most important reason for this resistance mechanism and metastasis is cancer stem cells (CSC) with tumour forming capacity. These cells are also the cause of the tumour heterogeneity seen in the clinic. The more these cells, the lower the success of the treatment. Despite developing treatment strategies, chemotherapy failure due to cellular drug resistance is an important problem in many cancer cases. Various drug resistances that produce changes in target proteins have been characterized using cell lines resistant to anticancer agents. Multi-drug resistance (MDR) in cancer cells continues to pose a major challenge for clinicians and pharmacologists [2]. MDR is

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defined as the insensitivity of cancer cells to the cytotoxic and cytostatic effects of a number of structurally and functionally unrelated drugs. Cancer cells are intrinsically resistant to anticancer agents due to genetic and epigenetic heterogeneity. These cells also acquire resistance to a wide variety of chemotherapeutic drugs through alterations in the absorption, metabolism, and excretion of a drug [3]. Although many mechanisms are associated with drug resistance in lung cancer, there is a long way to go as to exactly how to overcome drug resistance [4]. In the light of the mechanisms described so far, studies aimed at predicting existing or emerging resistance mechanisms during treatment rather than preventing drug resistance gain more importance. In our study, genes for the pre-detection of NSCLC cancer stem cell-derived drug resistance were investigated. For this purpose, potential gene clusters that may be responsible for drug resistance and chemotherapy drugs targeting them were determined by *in vitro* experiments using a CSC-enriched population.

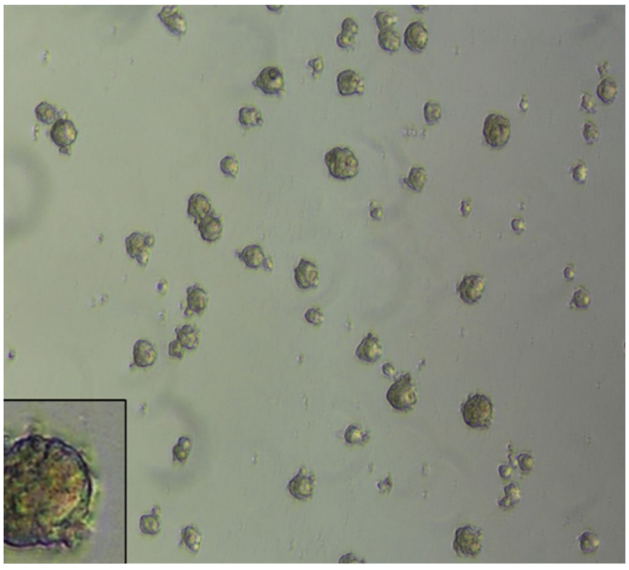

Figure 1: Microscopic images of spheroid structures obtained from H1299/P cell line (day 7, 10×). Inner is higher magnification of one of the spheres.

Materials and methods

Cell culturing and formation of spheres (CSC-enriched population)

The parental H1299 (H1299/P) human NSCLC cell line was used to obtain CSC-enriched population (H1299/S) following the propagation. The cell line was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 [DMEM/F-12 (Lonza)] medium containing 10 % fetal bovine serum (FBS) and 0.5 % primocin (Invivogen) at 37 °C in 5 % CO_2 medium. 3×10^5 H1299/P cells were grown in 75 cm^2 "ultra-low attachment" cell culture dishes in DMEM:F12 [1:1] growth medium with 0.5 % primocin, B27 supplement (Gibco) without vitamin A, 2 µg/mL heparin (Santa Cruz), 20 ng/mL Epidermal growth factor (EGF) (Stem cell Technologies) and 10 ng/mL Fibroblast growth factor (FGF) (Stem cell Technologies) for 7 days to form CSC-rich spheroid structures (H1299/S, 1st Generation). The grown cells were centrifuged at 1,200 rpm for 10 min. The resulting pellet was treated with 1 ml of trypsin (Gibco) for 10–13 min at room temperature. By pipetting, the spheroid structures were separated as single cells. Then, trypsin was removed by centrifugation at 1,200 rpm for another 10 min. The pellet was resuspended with the medium and the cells were counted, followed by culturing again in T75 "ultra-low attachment" cell culture dishes for 7 days, with the cell number of 3×10^5 to form spheroid structures (H1299/S, 2nd generation) [5]. Thus, human lung CSCs were enriched under non-adherent, serum-free culture conditions where only cancer stem/progenitor cells were able to survive and proliferate as tumorspheres. Subsequent experiments were performed on 2nd generation H1299/S cells (Figure 1).

ATP viability assay

In H1299/P and H1299/S cells, cisplatin response was evaluated with ATP viability test to evaluate the resistance of lung to therapy. For cisplatin response, cells were treated with Cisplatin in the 25–200 % test drug concentrations (TDC) for 48 and 72 h 100 % TDC is similar to plasma peak concentration. H1299/P cells were seeded at 2×10^3 cells/well. First day after seeding, cells were treated with 25, 50, 100 and 200 TDC of cisplatin. H1299/S cells were seeded into cell culture dishes at 2×10^3 cells/well and spheroid formation was observed for 3 days (2nd Generation). spheres were treated with respective doses of cisplatin for 48 and 72 h. ATP content was measured in a luminometer (LUMIStar omega) by a luciferin-luciferase bioluminescence reaction. Results were taken as relative light unit (RLU). Viability was calculated with the following formula. Viability (%)=[100 × (Sample RLU)/(Control RLU)].

Gene expression analyses

Gene expression levels expected to change in relation to aggressive character/drug resistance in H1299/S cells compared to H1299/P were determined by simultaneous PCR

Reverse transcription-polymerase chain reaction (RT-PCR). After the spheroids were obtained, total RNA isolation from both cell groups (PureLink RNA mini kit, Life Sciences) was performed in accordance with the kit content. Quantification and quality of RNAs were performed spectrophotometrically on a microplate reader (LUMIStar Omega). cDNA translation was performed with High capacity cDNA reverse transcription kit, Applied biosystems kit. Target cDNAs were amplified with sequence-specific primers with DNA polymerase in power SYBR green PCR master mix (Applied biosystems), with a total PCR reaction volume of 20 µL for the relevant genes. Relative expression levels of genes were normalized with the expression level of housekeeping genes (GAPDH and β-Actin) and using the delta delta Ct (ΔΔCt) method, with the help of RT-PCR Stepone plus (Applied biosystems) device. The relevant primer sequences are given in Supplementary Table 1 A, B, C.

Gene enrichment analysis

Gene enrichment and pathway analyses were performed using R/bioconductor 'clusterProfiler' package and 'GO, KEGG, Reactome' databases [6]. Genes with FDR<0.05 (false discovery rate) were considered significant and relevant pathways were visualized using the 'Cytoscape v3.9.1' software [7].

Gene-drug interaction analysis

Drug-protein interaction analyses were performed using KEGG, DrugBank [7, 8] and DGIdb databases [9]. For this purpose, analyses were carried out in three different categories depending on the expression: "Clinically approved drugs with FDA approval", "Drugs in Clinical Trials" and "Drugs with proven *in vitro* or *in vivo* efficacy". All analyses were performed in R/bioconductor using the "Rcpi" [10] and "rDGIdb" packages. The data were visualized in the 'Cytoscape v3.9.1' software.

Statistical analysis

Statistical analyses were evaluated in GraphPad Prism 6.0 program. Significance between groups was calculated using one-way analysis of variance (one-way ANOVA) and Tukey's multiple comparison (*post-hoc*) test. $p<0.01$ and $p<0.05$ data were considered significant. Results are given as the mean of three replicates with standard deviation.

Results

Obtaining Cancer Stem Cell Rich Population (H1299/S)/Formation of Spheres.

For CSC propagation, H1299/P cells were successfully grown and the formation of CSC-rich spheres was photographed (Figure 1). Spheres were grown in proper shape with around 200–400 µm in diameter.

Response to cisplatin

No significant change in H1299/S cell viability was observed compared to H1299/P cells (Figure 2 (A)) 48 h after the treatment with cisplatin. However, after 72 h, a clear difference was observed in the viabilities of H1299/P and H1299/S cells. CSC-enriched population's viability was not affected by the treatment while the parental cells viability was greatly reduced in a statistically significant fashion ($p<0.01$; $p<0.05$), depending on the dose (Figure 2). IC_{50} value of H1299/P cells was found to be lower than H1299/S cells, proving the drug resistance in CSC-enriched population, compared to the parental cells (Table 1).

Evaluation of gene expression levels associated with drug resistance.

While performing the expression analysis of drug resistance-related genes, 14 genes (*CDK1; ZEB1; ABCC2; CDKN2A; CDKN2D; FN1; CDKN2B; G6PH; LDHA; XIAP; TWIST; ABCC1; ABCC5; ATM*) were excluded because of "multiple Tm peak". The expression profile for 37 genes in H1299/S compared to H1299/P is given in Table 2.

Gene enrichment and pathway analysis

In the analyses made based on the expression data obtained as a result of RT-PCR, it was observed that they had a statistically significant effect on a total of 13 pathways (Table 3). In addition, detailed analysis of the affected pathways related to gene expression was performed using the Cytoscape program (Figure 3).

Gene-drug interaction analysis

As a result of the analysis, it was observed that statistically significant *TOP1, TOP2A, TOP2B, CDK4, CDK6, ABCB1, ABCC1, ABCC3, BCL2, PARP1, BRCA1* and *CDKN1A* gene or protein

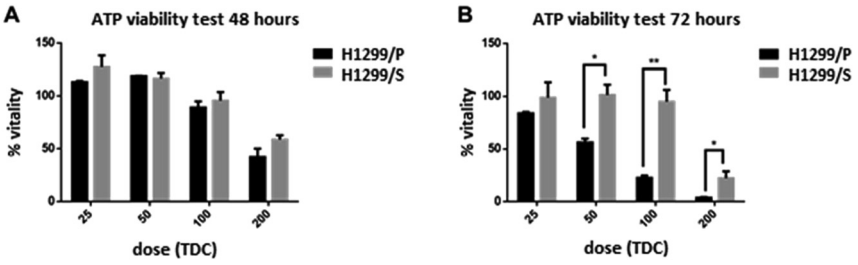

Figure 2: Evaluation of cisplatin response in H1299/P and H1299/S cells. Viability was measured by ATP viability assay as explained in the materials and methods. * statistically significant compared to H1299/P, p<0.05) ** statistically significant compared to H1299/P, p<0.01).

Table 1: IC_{50} values after Cisplatin administration in H1299/P and H1299/S cells.

Duration of treatment		IC_{50}	
		48 h	72 h
Cell	H1299/P	183.1	59.2
	H1299/S	>200	161.8

Table 2: H1299/S gene expression profile.

Gene	Change rate			Average	STD
CDK6	−3,70	−3,57	−2,78	−3,35	0,50
CDKN1C	−1,79	−1,43	−1,47	−1,56	0,20
PKM	−2,78	−3,45	−3,03	−3,09	0,34
PDK1	−2,70	−2,78	−2,44	−2,64	0,18
ENO1	1,33	1,11	1,20	1,21	0,11
BCL2	−1,85	−2,08	−1,32	−1,75	0,39
BCL2L1	−1,18	−1,96	−1,43	−1,52	0,40
MCL1	−4,00	−4,00	−4,00	−4,00	0,00
ZEB2	−1,82	−1,85	−1,59	−1,75	0,14
CLDN1	−2,56	−2,50	−2,63	−2,57	0,07
ABCB1	2,04	−1,56	1,08	0,52	1,87
CDKN1A	−4,76	−5,26	−4,55	−4,86	0,37
XPA	−50,00	−9,09	−11,11	−23,40	23,06
XPC	−2,38	−2,56	−2,56	−2,50	0,11
PARP1	−1,43	−1,79	−1,45	−1,55	0,20
TOP1	−1,64	−2,00	−1,96	−1,87	0,20
ATR	−2,08	−2,63	−2,38	−2,37	0,27
SNAI1	−2,33	−3,13	−3,13	−2,86	0,46
ABCG2	−4,17	−4,35	−4,35	−4,29	0,10
CCNE1	−2,56	−2,56	−2,70	−2,61	0,08
CCNA2	−2,00	−1,85	−2,86	−2,24	0,54
CDK4	−1,30	−1,45	−1,69	−1,48	0,20
CCNB1	−1,64	−1,75	−1,79	−1,73	0,08
CCND1	−1,61	−1,89	−1,82	−1,77	0,14
BRCA2	−1,69	−2,13	−2,56	−2,13	0,43
TOP2A	−1,92	−1,79	−1,89	−1,87	0,07
TOP2B	−2,08	−2,44	−2,33	−2,28	0,18
CDH2	−1,43	−1,64	−1,41	−1,49	0,13
SNAI2	1,12	1,55	−1,09	0,53	1,41
ABCC6	−1,33	−1,72	−2,70	−1,92	0,71
ABCC1	1,90	1,90	1,95	1,92	0,03
CDK2	−1,54	−1,45	−1,37	−1,45	0,08
BRCA1	−1,45	−1,37	−1,35	−1,39	0,05
CCNA1	−11,11	−10,00	−9,09	−10,07	1,01
KRT18	1,21	1,26	1,30	1,26	0,05
ABCC3	13,82	12,98	16,52	14,44	1,85
VIM	−3,13	−10,00	−6,25	−6,46	3,44

products can form resistance to various drugs. A resistance mechanism was observed against platinum-based drugs frequently used in lung cancer treatment. The increased resistance of H1299/S cells after cisplatin administration confirms the results of gene-drug interaction analyses. It is also seen that it can create an effective resistance against topoisomerase inhibitors, which are a targeted therapy option against *TOP1, TOP2A* and *TOP2B*. Protein drug interaction analysis is given in Figure 4.

Discussion

After starting chemotherapy treatment, either pre-existing (intrinsic) or acquired drug resistance is one of the main causes of failure in lung cancer treatment [11, 12]. Thirty seven genes that may be responsible for CSC-induced drug resistance in NSCLC were screened. In the bioinformatic analyses, it was seen that 13 pathways were affected due to expression changes and 12 genes related to these pathways activate multidrug resistance mechanisms. The most striking one among the statistically significant genes is the *TOP* gene family. Understanding the cellular effects of the *TOP* gene family's function and inhibition has been a springboard for the development of effective topoisomerase inhibitors derivatives in cancer therapy. Since *TOP1* functions in normal cells and cancer cells, the use of low doses of *TOP1* inhibitors is actively sought to treat cancers of highly malignant and proliferative nature based on the function of *TOP1*. Topoisomerase inhibitor resistance may develop due to mutations in the *TOP* gene family or methylation status [13, 14]. In the gene/protein drug interaction analysis; It has been observed that topoisomerase inhibitors may be quite ineffective due to decreased gene expression in the TOP gene family (Figure 4). Another point is the platinum-based drug

Table 3: Pathways found statistically significant as a result of gene enrichment and pathway analysis.

Metabolic pathway	p-Value, FDR
p53 signalling pathway	1.7×10^{-10}
Cell cycle	7.79×10^{-10}
Cellular senescence	1.83×10^{-7}
Lung cancer	5.86×10^{-7}
Platinum drug resistance	1.77×10^{-6}
ABC transporters	5.05×10^{-5}
PI3K-Akt signalling pathway	7.58×10^{-5}
Pathways in cancer	0.00175
Non-small cell lung cancer	0.00368
Jak-STAT signalling pathway	0.0111
HIF-1 signalling pathway	0.0128
FoxO signalling pathway	0.0331
Apoptosis	0.0357

resistance observed in NSCLC-derived CSCs. When H1299/P and its CHDs were compared, it was observed that the drug response to cisplatin was statistically different (Figure 2). Drug resistance against platinum-based drugs may develop due to expression differences in the ABC transporter gene family [15, 16]. In the gene expression results, we observed that the ABC transport gene family generally followed an increased expression pattern in CSCs. Observation of resistance to platinum-based drugs, including cisplatin, in parallel with the increase in expression in bioinformatics analyses also confirms the ATP cell viability result. Increased expression of the BRCA1 gene causes cispatin resistance due to increased DNA repair due to *BRCA*1 [17]. Considering the statistically significant platinum drug resistance observed as a result of drug-gene/protein interaction analysis and the ATP cell viability test results as a result of cisplatin administration, it can be thought that the low *BRCA1* expression in CSCs is not sufficient to break the resistance against platinum-based drugs. In addition to topoisomerase inhibitors and platinum-based drugs, *PARP1* inhibitors, a new approach in lung cancer, have also become the focus of attention [18]. Olaparib, which is currently approved for use in ovarian cancer, has also been shown to be effective in NSCLC in clinical phase studies (Clinical Trails: NCT01562210,

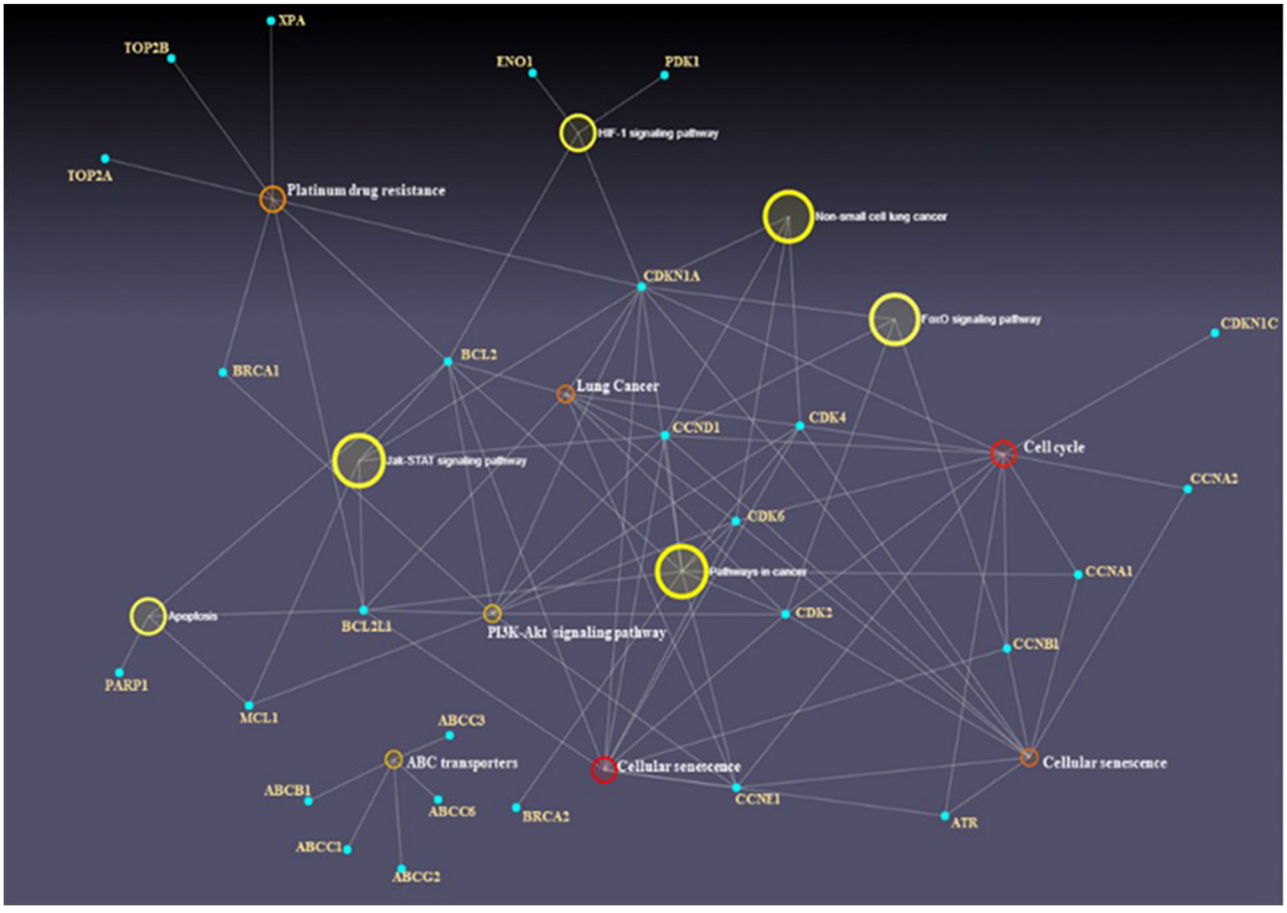

Figure 3: Representation of effective molecular pathways as a result of gene network analysis.

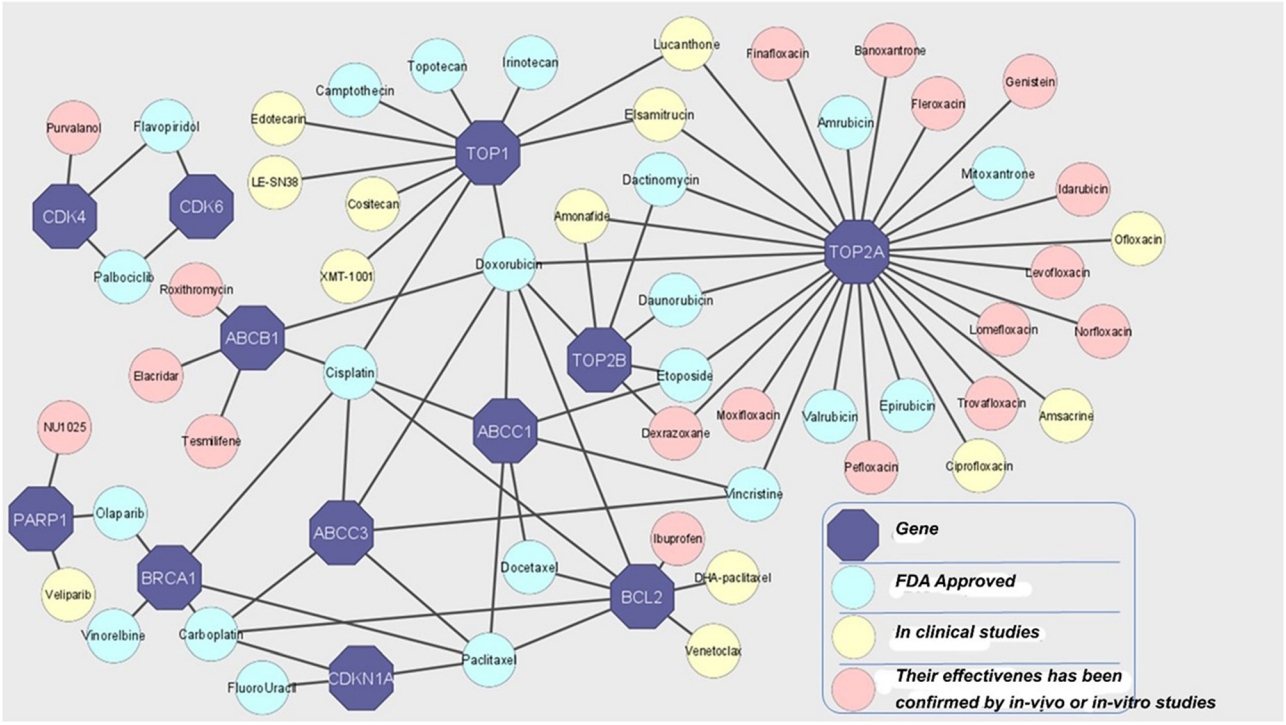

Figure 4: Gene drug interaction analysis result.

NCT03775486) and its application for FDA approval continues. Although studies have shown the presence of high PARP1 expression in lung cancer [19, 20]. low PARP1 expression level was observed in cancer stem cells in our study. For this reason, it has been observed that PARP1 inhibitor therapy may be ineffective in drug interaction analyses.

As a result, 37 genes and drug resistance mechanisms related to these genes were analysed in the study conducted with NSCLC cancer stem cells. It has been shown that they can have multi-drug resistance on a wide range of 13 genes, including platinum-based drugs, topoisomerase and parp1 inhibitors, and anthracyclines.

This study was conducted with a population enriched with H1299 cancer stem cells. For the development of cancer treatments and drug testing, analyses should be carried out using lung cancer cell lines from different subtypes.

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Research ethics: The local Institutional Review Board deemed the study exempt from review. No humans or animals were used in the study. Experiments were conducted in cell culture.

Informed consent: Informed consent was obtained from all individuals included in this study.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Use of Large Language Models, AI and Machine Learning Tools: None declared.

Conflict of interest: There is no conflict of interest between the authors.

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