

Ankaferd Hemostat Affects Etoposide Resistance of the Malignant Melanoma Cells

Mehdi GHASEMI¹, Mufide OKAY², Umit Yavuz MALKAN³, Seyhan TURK⁴, Javaid JABBAR⁵,
Helin HOCAOGLU⁶, Ibrahim Celalettin HAZNEDAROGLU²

¹ Lokman Hekim University, Faculty of Medicine, Department of Medical Microbiology, Ankara, TURKEY

² Hacettepe University, School of Medicine, Department of Hematology, Ankara, TURKEY

³ University of Health Sciences, Diskapi Yildirim Beyazit Training and Research Hospital,
Department of Hematology, Ankara, TURKEY

⁴ Hacettepe University, Faculty of Pharmacy, Department of Biochemistry, Ankara, TURKEY

⁵ Bilkent University, Department of Molecular Biology and Genetics, Ankara, TURKEY

⁶ UTSouthwestern University, Department of Physiology, Texas, USA

ABSTRACT

The development of resistance towards chemotherapeutic drugs has become an obstacle in treatment of cancer. Ankaferd Hemostat [ABS] has shown to suppress the proliferation of melanoma cells, but little is known about its' mechanism. In this study, we demonstrate that ABS can make some melanoma cell lines such as A2058 more sensitive towards etoposide by altering the genes involved in oxidative phosphorylation [OXPHOS] pathway. ABS treatment has shown to increase the sensitivity of A2058 towards etoposide and showed no effect for SK-MEL-5. Previously known to be more resistant to etoposide, SK-MEL-30 showed least amount of sensitivity to ABS. We found mitochondrion cluster to be the most relevant to genes altered by ABS. To validate our claim, we compared two sets of melanoma cell lines; A375 with A2058 and A375 with SK-MEL-2. The clusters that we obtained from A375 and A2058 comparison did contain mitochondrial related clusters, their corresponding p value was not significant. Whereas, the clusters from A375 and SK-MEL-2 comparison contain 72 genes in 'oxidoreductase' cluster with enrichment score of 2.52. To get insight of the oxidoreductase cluster, we put the genes in that cluster to Enrichr. We found that majority of the genes among oxidoreductase cluster participate in oxidative phosphorylation and electron transport chain. Our study suggests that the use of ABS prior to etoposide treatment can increase the response of melanoma cell lines because of the alteration of OXPHOS genes.

Keywords: Ankaferd hemostat, Etoposide, Oxidative phosphorylation, Melanoma, Drug sensitivity

ÖZET

Ankaferd Hemostat'ın Malign Melanom Hücrelerinde Etoposid Direncine Etkisi

Kemoterapötik ilaçlara karşı direnç gelişimi, kanser tedavisinde bir engel haline gelmiştir. Ankaferd Hemostat'ın (ABS), melanom hücrelerinin proliferasyonunu baskıladığı gösterilmiştir; ancak mekanizması hakkında çok az şey bilinmektedir. Bu çalışmada, ABS'nin oksidatif fosforilasyon (OXPHOS) yolağında yer alan genleri değiştirerek, A2058 gibi bazı melanom hücre dizilerini etoposide karşı daha duyarlı hale getirilebileceğini gösterdik. Bu çalışmada, ABS tedavisinin A2058'in etoposide duyarlılığını artırdığı gösterildi, ancak SK-MEL-5 için herhangi bir etki gösterilemedi. Daha önce etoposide daha dirençli olduğu bilinen SK-MEL-30, bizim çalışmamızda ABS'ye karşı en az hassasiyet gösterdi. Analizimiz sonucunda, mitokondri kümelerinin ABS tarafından değiştirilen genlerle ilişkili olduğunu gördük. İddiamızı doğrulamak için iki set melanom hücre çizgisini (A375'i A2058 ile ve A375'i SK-MEL-2 ile) karşılaştırdık. A375 ve A2058 karşılaştırmasından elde ettiğimiz kümeler mitokondriyal ilişkili kümeler içermektedir, ancak p değerleri anlamlı değildi. Öte yandan, A375 ve SK-MEL-2 karşılaştırmasından elde edilen kümeler, 2.52 zenginleştirme skoruna sahip 'oksidoredüktaz' kümesinde 72 gen içermektedir. Oksidoredüktaz kümesini analiz etmek için, bu kümedeki genleri Enrichr'e koyduk. Oksidoredüktaz kümesi içindeki genlerin çoğunun oksidatif fosforilasyona ve elektron taşıma zincirine katıldığını bulduk. Sonuç olarak bu çalışma, etoposid tedavisinden önce ABS kullanımının, OXPHOS genlerinin değişmesi nedeniyle melanom hücre çizgilerinin tepkisini artırabileceğini öne sürmektedir.

Anahtar Kelimeler: Ankaferd hemostat, Etoposid, Oksidatif fosforilasyon, Melanom, İlaç duyarlılığı

INTRODUCTION

Ankaferd Hemostat (ABS) is a drug composed of five different plant extracts.^{1,2} It is commonly used as a blood stopper.¹⁻³ There are several studies suggesting that ABS is an effective antibacterial agent.^{4,5} Moreover, studies have shown that ABS stops the proliferation of cancer cells and cell lines, specifically malignant melanoma.^{6,7} Melanoma is caused by over proliferation of melanocytes,⁸ which are known to produce melanin.⁹ Malignant melanoma is known as the most aggressive skin cancer type with the highest amount of deaths among the types of melanomas.¹⁰ The primary reason for melanoma being such a deadly disease is the resistance towards known chemotherapy drugs.¹¹

Compared to normal cells, cancer cells have up regulated glycolysis that results in high consumption of glucose and high lactate production.¹² Most of the times, cancer cells exhibit Warburg effect that states the dependence of cancer cells on glycolysis and lactic acid fermentation as the primary source of ATP production.¹³ With regard to Warburg effect, it was conceived that Oxidative phosphorylation [OXPHOS] is down regulated in cancer cells.¹⁴ Although, it is true for many types of cancers, new studies indicate that OXPHOS genes can be up regulated in some cancers such as lymphomas, leukemias, endometrial carcinoma and pancreatic ductal adenocarcinoma.¹⁵ During carcinogenesis, a significant level of OXPHOS genes is maintained in the cancer cells that allow the cells to switch from glycolysis to OXPHOS.¹⁶ Recently, Vellinga and colleagues have reported an increase in the OXPHOS level of the patients treated with chemotherapy.¹⁷ The demand for ATP is significantly increased when chemotherapy is administered as the enzymes involved in DNA repair, drug detoxification and drug efflux need ATP to function.^{18,19} Since, the main feature of the chemotherapeutic drug is to create lesions in the DNA that results in apoptosis,^{20,21} DNA repair plays a critical role in drug resistance of cancer cells administered with DNA damaging drugs.²²

DNA topoisomerase II is the target enzyme for etoposide.²³ DNA topoisomerase II unwinds the DNA during DNA replication.²⁴ DNA topoisomerase II expression is known to be a predictive mark-

er for cancer which makes it a suitable target for chemotherapeutic drugs.²⁵ Previously, it has been shown that the expression level of topoisomerase II is essential for cancer cells to acquire resistance against topoisomerase II inhibitors such as etoposide.²⁶ In this study, we hypothesize that ABS alters the drug resistance of melanoma cell lines and makes them more sensitive towards etoposide. Our study demonstrates that alterations in basal expression level of OXPHOS can lead to drug resistance in melanoma, which could be overcome by ABS treatment.

MATERIALS AND METHODS

Determination of the Association Between Melanoma and Etoposide Resistance

We used © 2018 Tableau Software for the selection of cancer drug to be used in this study. Tableau software is a tool that uses the data from Genomics of Drug Sensitivity in Cancer to show the association between cancer drugs and gene expression for any particular type of cancer. The p-value we used for this study is 0.05. Spearman's rank correlation coefficients are calculated by the tool. Red color indicates positive correlation; green color indicates negative correlation between the expression of the respective gene and the IC50 value of the selected drug. A negative correlation suggests that the drug is more effective when the gene expression is high whereas, a positive correlation indicates that the drug is less effective when that gene is expressed.²⁷

Cell Culture

A2058 [ATCC® CRL-11147™], SKMEL9 [CVCL_U934], SKMEL-5 [ATCC® HTB-70™] and SKMEL-30 [CVCL_0039] cell lines were grown in 75 cm² flask until confluency in Dulbecco's Modified Eagle's Medium [Corning, catalogue # 10-017-CV] supplemented with 10% Fetal Bovine Serum [Corning, catalogue # 35-015-CV] Cultures were incubated at 37°C and 5% carbon dioxide. When cells were at confluency, they were seeded to 4 different 75 cm² flask. Flasks were treated with media containing 0, 0.1 and 0.05% ABS, respectively. Cells were incubated with etoposide for 72 hours prior to other experiments.

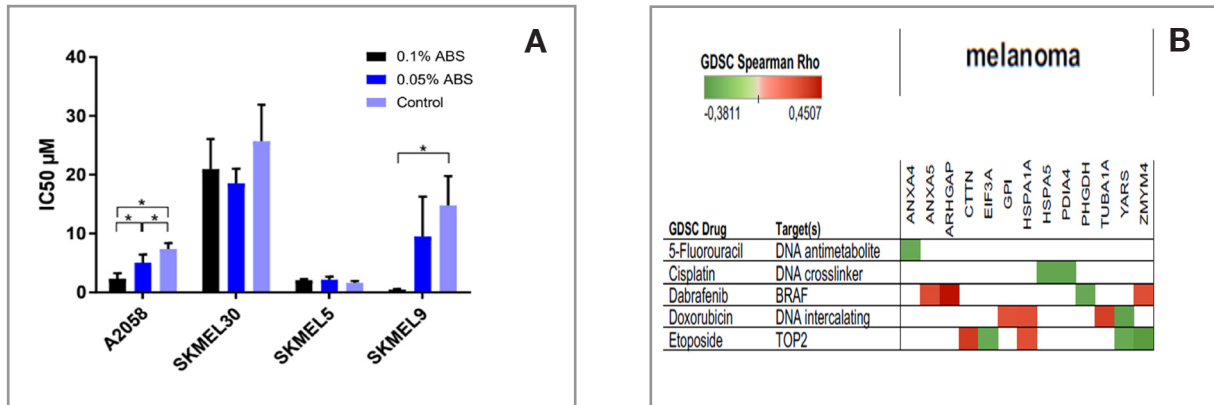


Figure 1. A. The graph represents the IC₅₀ values of the cell lines for Etoposide upon ABS treatment $p < 0.05$. **B.** Etoposide is the drug that is The differentially expressed genes after ABS treatment as are put into the Tableau Software and 5 different cancer drug types are compared according to their GDSC Spearman Rho score. Only the data with $p < 0.05$ were used. Red and green colors show positive and negative correlation respectively between the gene expressions and drug resistance.

Cell Viability Assay

After 72 hours of incubation with ABS, the cells were washed, detached by Trypsin [Corning, catalogue # 25-052-CI] and seeded to 96 well plates [Corning® 96 Well TC-Treated Microplates catalogue # CLS3997]. For each concentration of ABS, 24 wells were seeded. Cells were treated with 20 μM , 10 μM , 5 μM , 2.5 μM , 1.25 μM , 0.625 μM , 1.3125 μM and 0 μM Etoposide diluted with DMEM. Each concentration was performed as 3 replicates. The cells were incubated with different Etoposide concentration for 72 hours. CellTiterGlo® [Promega, catalogue # G7570] was performed to assess the effect of Etoposide upon ABS treatment according to the manufacturer's manual.²⁸

Calculation of Half Maximal Inhibitory Concentration

The percent viability for each well was calculated on Microsoft Excel 2016 using the data collected by the plate reader after CellTiterGlo® was performed. Log [IC₅₀] values for each trial and each concentration were calculated using Six Model Analysis algorithm developed by our lab on R 3.1.1. In this method, 6 different IC₅₀ values were generated and IC₅₀ value which is generated with the lowest error was selected to find the most reliable IC₅₀ value for the respective trial. Two Tailed Student's T-test was performed to calculate the p-

values to assess the significance of the difference in IC₅₀ values. The p-values were calculated and Figure 1A was generated on GraphPad Prism 7.0.

Acquisition and Analysis of Microarray Datasets

We selected five studies in total [accession number: GSE8332, GSE7153, GSE57083, GSE51115 and GSE32474] as listed in Table 1 to find the differentially expressed genes between five different melanoma cell lines: A2058, A375, SK-MEL-2, SK-MEL-5 and SK-MEL-30. The datasets we chose to work with were untreated samples and we compared samples from multiple studies that had common microarray platforms. In case when the number of samples for a cell line was less than 2, we grouped different studies that utilized the same microarray platform and performed the analysis. We grouped GSE8332, GSE7153 and GSE57083 together and compared it with GSE32474 to determine the differentially expressed genes between A2058 and SK-MEL-5. We used the study GSE51115 to find differentially expressed genes between A2058 and SK-MEL-30, A375 and SK-MEL-2, A375 and A2058. For each comparison, the samples were normalized using Robust Multi-Array [RMA] function on R 3.5.1. To find the most significant differentially expressed genes, a Two Tailed Student's T-Test was performed and genes

Table 1. Gene set enrichment analysis results for A2058, SKMEL5 and SKMEL30

Cluster	Number of Genes	%	p-Value	Enrichment Score
Metal-binding	365	20.4025	3.90E-05	2.43
Mitochondrion	127	7.09894	2.53E-04	2.15
Basement membrane	11	0.61487	0.00128	2.39
Cell junction	90	5.03074	8.75E-06	2.24

The table represents the clusters of genes that are selected upon GSEA using DAVID. The analysis is done with the genes that are differentially expressed in A2058 compared to both SKMEL5 and SKMEL30. Enrichment score, p-value and number of genes in the cluster are taken into account for the selection.

with p value less than 0.01 were chosen for further analysis.

Functional Analysis of the Differentially Expressed Genes

We used DAVID 6.8 for Gene Set Enrichment Analysis. Clusters utilized were selected based upon p-values and enrichment scores calculated by DAVID.²⁹ We used STRING 10.5 to see the associations between the proteins of interest. The edges represent co-expression and co-occurrence of the proteins.³⁰ We used Enrichr to determine the molecular functions of the genes which we were interested in. We obtained two different visual representations of the molecular functions by this enrichment analysis tool. We used the clusters determined by Enrichr based on the data provided by Gene Ontology Consortium.³¹

RESULTS

Upon ABS treatment, 2 out of 4 cell lines acquired etoposide sensitivity compared to control samples [Fig. 1A]. Even though both A2058 and SK-MEL-9 seem to be affected by ABS, due to unavailability of data on SK-MEL-9, we proceeded to our analysis with A2058 and compared it to the other two cell lines that are not affected by ABS. ABS treatment has shown to increase the sensitivity of A2058 towards etoposide and showed no effect for SK-MEL-5 (Figure 1A). Previously known to be more resistant to etoposide, SK-MEL-30 showed least amount of sensitivity to ABS (Figure 1A). We performed head to head comparison of melanoma

cell lines to each other i.e A2058 with SK-MEL-5 and A2058 with SK-MEL-30; to elucidate if the differentially expressed genes could be the cause of difference in response to ABS in these cell lines. The GEO datasets being used in our study contain data from different platforms hence; we decided to compare the datasets with common platform to each other to prevent any loss of information.

We put all the genes that were differentially expressed between A2058 and SK-MEL-5 together with A2058 and SK-MEL-30 to DAVID and performed Gene Set Enrichment Analysis (GSEA). As a result, we found clusters of genes with different enrichment score and their corresponding p values (Table 2). To see the association between the genes, we put all the genes from each cluster in Table 2 and the gene list from the previous study to STRING. We found mitochondrion cluster to be the most relevant to genes altered by ABS (Figure 2). We think that the network between the mitochondrion cluster and the gene list might be the indication of ABS having a significant role in acquiring sensitivity for etoposide. From the mitochondrion cluster, we took the genes which were co-expressed and co-occurred with the genes in the list from the previous study and found their common biological function using Enrichr (Figure 3). The result indicates that these genes are mostly related to oxidative phosphorylation, ATP synthesis and electron transport chain.

To validate our claim on mitochondrial cluster being responsible for variable sensitivities in melanoma cell lines, we decided to do a comparison between two sets of melanoma cell lines; A375 with A2058 and A375 with SK-MEL-2. We expect

Table 2. Gene set enrichment analysis results for A375 and A2058. GSEA was performed using DAVID database to obtain the clusters from differentially expressed genes between A375 and A2058

Cluster	Count	%	p-Value	Enrichment Score
Glycoprotein	507	28.40336	3.49E-12	9.99
Transmembrane region	478	26.77871	0.018175	2.51
Cell junction	84	4.705882	4.57E-04	1.91
Immunity	75	4.201681	2.07E-06	3.18
Extracellular matrix	39	2.184874	6.50E-04	3.94
MHC II	11	0.616246	2.22E-07	3.54
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	8	0.448179	0.287637	0.59
Positive regulation of release of cytochrome c from mitochondria	7	0.392157	0.04476	0.75
Release of cytochrome c from mitochondria	6	0.336134	0.061363	0.93
Electron transport	6	0.336134	0.958857	0.01
Respiratory chain	3	0.168067	0.975188	0.01

to see mitochondrial genes being differentially expressed between A2058 and SK-MEL-2 as their IC50 values are different. Whereas, we do not expect to see any differentially expressed mitochondria related genes between A2058 and A375. We used the GEO dataset to find the differentially expressed genes between the two sets of melanoma cell lines mentioned above. The most significant differentially expressed genes with the p value less than 0.01 from both sets were put into DAVID for GSEA, separately. Although, the clusters that we obtained from A375 and A2058 comparison did contain mitochondrial related clusters, their corresponding p value was not significant (Table 3). Whereas, the clusters from A375 and SK-MEL-2 comparison contain 72 genes in 'oxidoreductase' cluster with enrichment score of 2.52 (Table 4). To get insight of the oxidoreductase cluster, we put the genes in that cluster to Enrichr. We found that majority of the genes among oxidoreductase cluster participate in oxidative phosphorylation and electron transport chain (Figure 4). Since, there were no significant mitochondrial related clusters when the differentially expressed genes between A375 and A2058 were put onto DAVID, we believe that one reason could be the difference in etoposide

sensitivity of these respective cell lines. We think that oxidative phosphorylation genes are differentially expressed when the etoposide resistance is different between two cell lines.

DISCUSSION

There are many studies showing the anti-cancer effect of Ankaferd Hemostat.^{1,6,7,32} Even though many cancer types are known to be affected by ABS, we decided to use melanoma cell lines in our study. Since ABS is a blood stopper,¹⁻³ we think that it is more important to find an association between ABS and melanoma. If such a relation can be deduced, its future applications would be more conveniently designed compared to any association between ABS and any other cancer type. To elucidate the effect of ABS treatment, we obtained the gene list from a previous study by Haznedaroglu et al.³³ which contains all the genes that are altered upon ABS treatment for two colon cancer cell lines. We used an online tool, Tableau Software, to determine which drug would be more suitable to test the efficacy of ABS treatment of melanoma cell lines. We uploaded the gene list from Haznedaroglu et

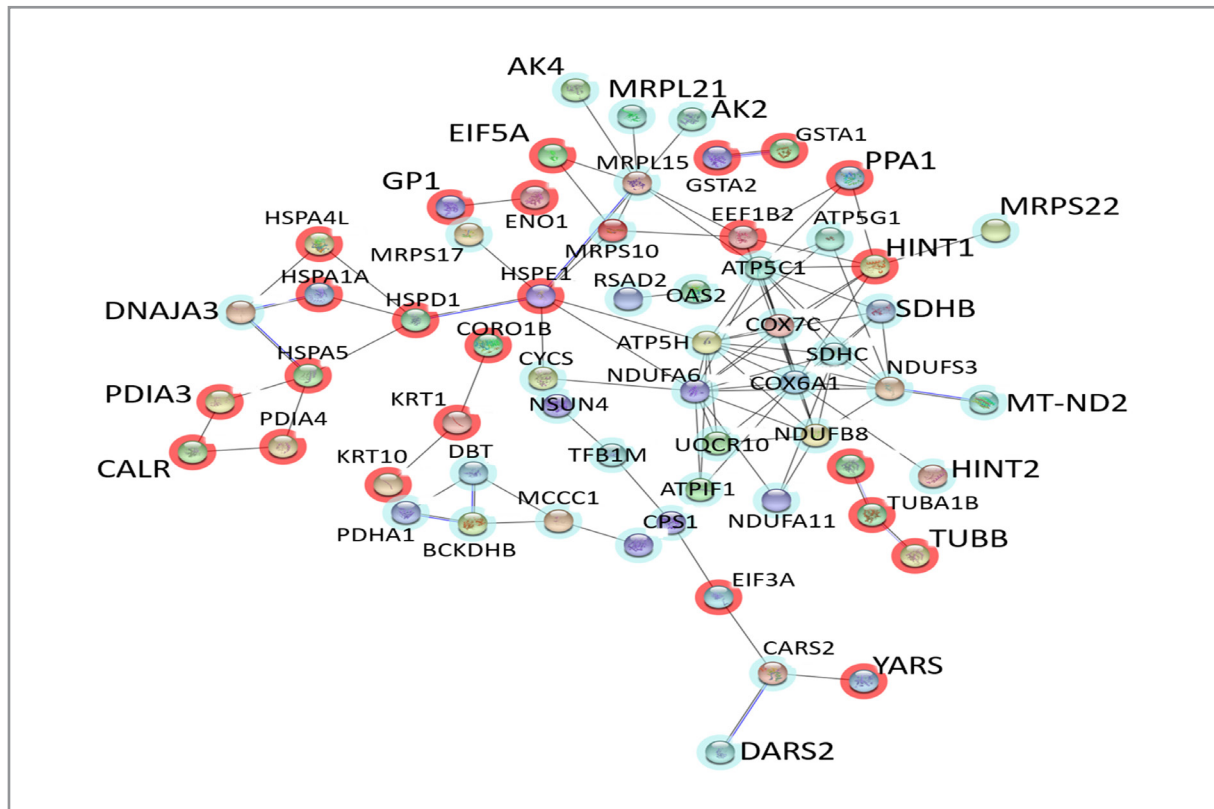


Figure 2. Interactions between the genes in the mitochondrion cluster which was obtained after GSEA and the genes that are differentially expressed upon ABS treatment. The interactions are depicted based on co-expression and co-occurrence of the genes through STRING database. The nodes that are not connected to other nodes are not shown. The red halos represent the ABS related genes and blue halos represent the genes in the mitochondrion cluster.

al.³³ and chose five commonly used cancer drugs to see the relationship between the proteins that have altered their level upon ABS treatment and the cancer drugs. Although, etoposide is not a melanoma drug, it has an influence on more genes than other common cancer drugs (Figure 1B). Hence, we decided to use etoposide and hypothesized that ABS

might have an effect on etoposide resistance of melanoma cell lines.

Before setting up our ABS doses in our cell lines, we have examined the study published by Turk et al. which showed no significant decrease in the viability of melanoma cells when treated with concen-

Table 3. Gene set enrichment analysis results for A375 and SKMEL2. The genes that are differentially expressed between A375 and SKMEL2 were analyzed through DAVID. The clusters with highest enrichment score, gene number; and the lowest p-value are shown.

Cluster	Count	%	p-Value	Enrichment Score
Signal peptide	429	23.85984	9.46E-19	8.5
Oxidation-reduction process	79	4.393771	0.002141	2.52
Oxidoreductase	72	4.004449	0.00164	2.52
Immunity	74	4.115684	4.97E-06	2.84
Cell-cell adherens junction	48	2.669633	0.001267	1.92
Antiviral defense	26	1.446051	1.39E-05	3.91

Table 4. The GEO datasets used for Gene Set Enrichment Analysis. The microarray datasets that are processed and used for GSEA are shown for each cell line.

GEO accession no	Cell line Sample	Number of Samples	Platform	Reference
GSE8332	A2058	1	Affymetrix Human Genome U133 Plus 2.0 Array	58
GSE7153	A2058	1	Affymetrix Human Genome U133 Plus 2.0 Array	59
GSE57083	A2058	1	Affymetrix Human Genome U133 Plus 2.0 Array	60
GSE51115	A375, A2058, SKMEL2, SKMEL30	2, 2, 2, 2	Microarray	36
GSE32474	SKMEL5	3	Affymetrix Human Genome U133 Plus 2.0 Array	57

trations lower than 0.39% of ABS.⁷ For our study, we chose to work with 0.1%, 0.05% and 0% ABS to ensure that the viability of melanoma cells is not affected by ABS treatment. In our study the viability of melanoma cells maintained successfully with these doses.

In the literature it was shown that SK-MEL-30 cell line is shown to be resistant to etoposide.^{34,35} Interestingly, in our study SK-MEL-30 showed least amount of sensitivity to ABS. As stated in previously published studies, the IC₅₀ values for A375, A2058 and SK-MEL-2 are 0.664 μM, 0.484 μM and 22.6 μM, respectively.^{34,35} Therefore, the mitochondrial gene expressions of these cell lines were expected to be different. In our study, the dif-

ference between mitochondrial gene expressions of A375 and A2058 was not significant whereas A375 and SK-MEL-2 mitochondrial gene expression comparison was significant. We have used the GEO dataset from a study conducted by Litvin et al. during the comparison of A375, A2058 and SK-MEL-2 cell lines.³⁶

After our literature search, we saw a correlation between the expression of OXPHOS genes and drug sensitivity in many different types of cancer.^{15,37,38} Cancer cells reduce their OXPHOS gene expression which reduces the amount of reactive oxygen species (ROS) being produced. Thus, it lowers the amount of damage to these cells.^{39,40} However, the opposite effect can be observed in other types of

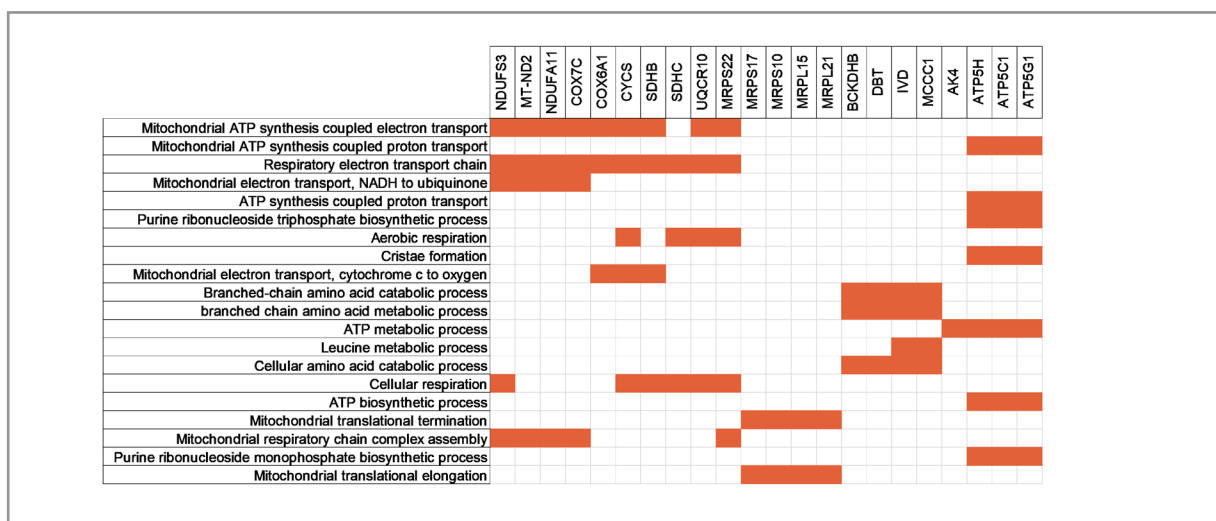


Figure 3. Biological functions of the genes that are in the mitochondrion cluster. The genes that are in the mitochondrion cluster after GSEA are analyzed through Enrichr, and 20 most common biological functions of these genes are shown. Only the data with p-value less than 0.05 are shown.

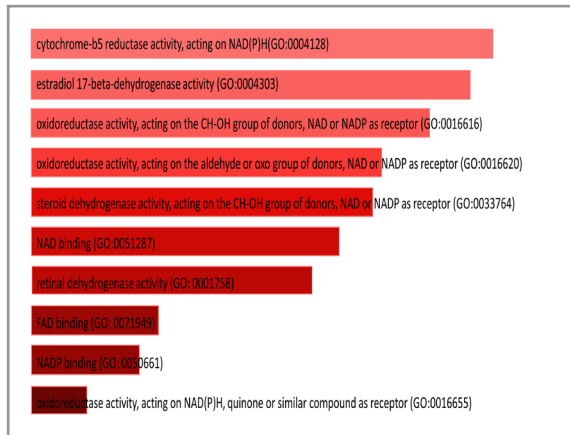


Figure 4. Molecular functions of the genes in the oxidoreductase cluster. Molecular function analysis was done by Enrichr for the genes that are in the oxidoreductase cluster after performing GSEA for differentially expressed genes between A375 and SKMEL2. The data taken from Enrichr are based on Gene Ontology Consortium 2018 and have a p-value less than 0.05.

cancer. For example, studies on acute myeloid leukemia (AML) show that AML stem cells rely on OXPHOS more when they are chemotherapy resistant. When the genes are altered to reduce OXPHOS, AML stem cells are more sensitive towards chemotherapy drugs.^{15,41} Another study on AML suggests that chemo resistance can be induced by changes in OXPHOS and mitochondrial metabolism.⁴² In prostate and colon cancer, increase in OXPHOS promotes cancer cell survival by making them more drug resistant.^{17,43} Furthermore, OXPHOS has been suggested to be associated with etoposide-induced cell death, specifically in colon and prostate cancer.⁴⁴

The consensus is that the change in OXPHOS levels can affect the sensitivity of cancer cells towards chemotherapy drugs. The same phenomenon can be observed for melanoma cells as well. Many studies suggest the presence of a relationship between mitochondria function and drug sensitivity in Melanoma.^{45,46} Melanoma cells have been studied as two different groups and this classification is done due to the differences between OXPHOS level as well as its correlation with drug resistance and clinical outcomes.^{47,48} The increase in mitochondrial mass and capacity is known to be associated with developing resistance in melanoma

cells towards BRAF inhibitors.⁴⁸⁻⁵¹ Moreover, it has been shown that in melanoma, especially the OXPHOS genes have crucial functions in regulating the drug resistance.⁵² Based on all these studies and our results, we think that alterations in OXPHOS genes in melanoma cells can be related to etoposide resistance.

From the list of drugs on Genomics of Drug Sensitivities in Cancers (GDSC), etoposide was chosen as it affects the most number of genes from the gene list provided in Haznedaroglu et al.³³ Although, the genes provided in Haznedaroglu et al.³³ are the differentially expressed upon ABS treatment in colon cancer. Our data shows that these genes interact directly or indirectly with genes in OXPHOS pathway and electron transport chain. Hence, it is possible that ABS treatment alters the expression of genes published in Haznedaroglu et al.³³ which affects OXPHOS pathway gene expression. As a result, the sensitivity of particular melanoma cell lines towards etoposide increases.

Experimental tumor models of preclinical setting for the anticancer drug target discovery and validation shall follow distinct strategies.⁵³ Experimental methods include assessing in vivo effects on tumor growth kinetics in transplanted tumors, engineered through gain-of-function by overexpressing transgene or knock-in or loss-of-function by gene silencing using knockdown or knockout, mutation via mutagenesis procedures, using genetically engineered mouse models and/or patient-derived xenografts resembling patient genetics and histopathology in order to describe specific pharmacology protocols in numerous cancer modeling stages.⁵³ RNAi-mediated knockdown of YY1 in cancer cells significantly decreased ATP6V1A mRNA and protein expression, while YY1 overexpression increased ATP6V1A expression level.⁵⁴ YY1 is a transcription factor enhanced by Ankaferd hemostat (ABS).⁵⁵ ABS has the potential to affect iron-regulated genomics as well.⁵⁶ Therefore, performing knock-down and over-expression experiments to test connections with etoposide/ABS treatment and further in vivo validation of the present research findings could lead to future potential mechanistic and clinical implications of ABS in human tumors. It is hoped that our present study represents a seminal catalytic spark for those kinds

of experimental and clinical future scientific work.

To conclude, our study utilizes microarray data from several other studies for the extraction of differentially expressed genes between different melanoma cell lines. Some studies contained only one sample for the respective melanoma cell line therefore; many studies had to be combined that utilized the same microarray platform to perform the analysis. For future studies, RNAseq could be used to identify specific mitochondrial genes and genes in the OXPHOS pathway to circumvent the limitations of microarray chips. Furthermore, other types of cancers should be tested for efficacy of ABS in making the cancer cells less resistant to chemotherapy. All in all, ABS makes melanoma cells especially A2058 more sensitive towards etoposide by altering the basal expression level of genes involved in OXPHOS pathway and electron transport chain, which can be used as a therapeutic approach in the future.

REFERENCES

- Mumcuoglu M, Akin D, Ezer U, Akar N. Ankaferd Blood Stopper induces apoptosis and regulates PAR1 and EPCR expression in human leukemia cells. *Egypt J Med Hum Genet* 16: 19-27, 2015.
- Kurt M, Onal I, Akdogan M, et al. Ankaferd Blood Stopper for controlling gastrointestinal bleeding due to distinct benign lesions refractory to conventional antihemorrhagic measures. *Can J Gastroenterol* 24: 380-384, 2010.
- Haznedaroglu B, Beyazit Y, Walker S, Haznedaroglu I. Pleiotropic cellular, hemostatic, and biological actions of Ankaferd hemostat. *Crit Rev Oncol Hematol* 83: 21-34, 2012.
- Saribas Z, Sener B, Haznedaroglu I, et al. Antimicrobial activity of Ankaferd Blood Stopper® against nosocomial bacterial pathogens. *Open Med* 5: 198-202, 2010.
- Koluman A, Akar N, Haznedaroglu I. Antibacterial activities of ankaferd hemostat [ABS] on shiga toxin-producing *Escherichia coli* and other pathogens significant in foodborne diseases. *Turk J Haematol* 34: 93-98, 2017.
- Kocyigit A, Guler E, Haznedaroglu I, Malkan U. Ankaferd hemostat induces DNA damage, apoptosis and cytotoxic activity by generating reactive oxygen species in melanoma and normal cell lines. *Int J Clin Exp Med* 10: 2116-2126, 2017.
- Turk S, Malkan UY, Ghasemi M, et al. Growth inhibitory activity of Ankaferd hemostat on primary melanoma cells and cell lines. *SAGE Open Med* 5: 205031211668951, 2017. doi: 10.1177/2050312116689519.
- Schadendorf D, Fisher DE, Garbe C, et al. Melanoma. *Nat Rev Dis Primers* 1: 15003, 2015.
- Cichorek M, Wachulska M, Stasiewicz A, Tyminska A. Skin melanocytes: biology and development. *Postepy Dermatol Alergol* 1: 30-41, 2013.
- Bandarchi B, Ma L, Navab R, et al. From melanocyte to metastatic malignant melanoma. *Dermatol Res Pract pii: 583748*, 2010.
- Kalal B, Upadhyay D, Pai V. Chemotherapy resistance mechanisms in advanced skin cancer. *Oncol Rev* 11: 326, 2017.
- Jiang B. Aerobic glycolysis and high level of lactate in cancer metabolism and microenvironment. *Genes Dis* 4: 25-27, 2017.
- Xie J, Wu H, Dai C, et al. Beyond Warburg effect – dual metabolic nature of cancer cells. *Sci Rep* 4: 4927, 2014.
- Moreno-Sánchez R, Rodríguez-Enríquez S, Marín-Hernández A, Saavedra E. Energy metabolism in tumor cells. *FEBS J* 274: 1393-1418, 2007.
- Ashton T, McKenna W, Kunz-Schughart L, Higgins G. Oxidative phosphorylation as an emerging target in cancer therapy. *Clin Cancer Res* 24: 2482-2490, 2018.
- Jose C, Bellance N, Rossignol R. Choosing between glycolysis and oxidative phosphorylation: A tumor's dilemma?. *Biochim Biophys Acta* 1807: 552-561, 2011.
- Vellinga TT, Borovski T, de Boer VC, et al. SIRT1/PGC1-dependent increase in oxidative phosphorylation supports chemotherapy resistance of colon cancer. *Clin. Cancer Res* 21: 2870-2879, 2015.
- Gottesman M, Fojo T, Bates S. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2: 48-58, 2002.
- Osley M, Tsukuda T, Nickoloff J. ATP-dependent chromatin remodeling factors and DNA damage repair. *Mutat Res* 618: 65-80, 2007.
- Huschtscha L, Bartier W, Ross C, Tattersall M. Characteristics of cancer cell death after exposure to cytotoxic drugs in vitro. *Br J Cancer* 73: 54-60, 1996.
- Walker PR, Smith C, Youdale T, et al. Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res* 51: 1078-1085, 1991.
- Salehan M, Morse H. DNA damage repair and tolerance: a role in chemotherapeutic drug resistance. *Br J Biomed Sci* 70: 31-40, 2013.
- Hande K. Etoposide: four decades of development of a topoisomerase II inhibitor. *Eur J Cancer* 34: 1514-1521, 1998.
- McClendon A, Osheroff N. DNA topoisomerase II, genotoxicity, and cancer. *Mutat Res* 623: 83-97, 2007.
- An X, Xu F, Luo R, et al. The prognostic significance of topoisomerase II alpha protein in early stage luminal breast cancer. *BMC Cancer* 18: 331, 2018.

26. Ganapathi R, Ganapathi M. Mechanisms regulating resistance to inhibitors of topoisomerase II. *Front Pharmacol* 4: 89, 2013.
27. Qin Y, Conley A, Grimm E, Roszik J. A tool for discovering drug sensitivity and gene expression associations in cancer cells. *PLOS ONE* 12: e0176763, 2017.
28. CellTiter-Glo® Luminescent Cell Viability Assay Protocol [2018]. Worldwide.promega.com. Available at: <https://worldwide.promega.com/resources/protocols/technical-bulletins/0/celltiter-glo-luminescent-cell-viability-assay-protocol/> [Accessed August 17, 2018].
29. Huang DW, Sherman BT, Tan Q, et al. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. *Nucleic Acids Res* 35: 169-175, 2007.
30. Mering C. STRING: a database of predicted functional associations between proteins. *Nucleic Acids Res* 31: 258-261, 2003.
31. Kuleshov MV, Jones MR, Rouillard AD, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res* 44: 90-97, 2016.
32. Haznedaroglu I. Acute in Vitro effects of ABS [Ankaferd Hemostat] on the lymphoid neoplastic cells [B-CLL and RAJL Tumor Cell Lines]. *UHOD* 4: 253-259, 2014.
33. Türk C, Okay M, Türk S, et al. The impact of JAK/STAT inhibitor ruxolitinib on the genesis of lymphoproliferative diseases. *Turk J Med Sci* 49: 661-674, 2019.
34. Yang W, Soares J, Greninger P, et al. Genomics of Drug Sensitivity in Cancer [GDSC]: a resource for therapeutic biomarker discovery in cancer cells. *Nucleic Acids Res* 41: 955-961, 2012.
35. Drug: Etoposide - Cancerrxgene - Genomics of Drug Sensitivity in Cancer [2018]. Cancerrxgene.org. Available at: <https://www.cancerrxgene.org/translation/Drug/134>. Accessed January, 2020.
36. Litvin O, Schwartz S, Wan Z, et al. Interferon α/β Enhances the Cytotoxic Response of MEK Inhibition in Melanoma. *Mol Cell* 57: 784-796, 2015.
37. Solaini G, Sgarbi G, Baracca A. Oxidative phosphorylation in cancer cells. *Biochim Biophys Acta* 1807: 534-542, 2011.
38. Molina JR, Sun Y, Protopopova M, et al. An inhibitor of oxidative phosphorylation exploits cancer vulnerability. *Nat Med* 24: 1036-1046, 2018.
39. Denko N. Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nat Rev Cancer* 8: 705-713, 2008.
40. Bhattacharya B, Mohd Omar M, Soong R. The Warburg effect and drug resistance. *Br J Pharmacol* 173: 970-979, 2016.
41. Kuntz EM, Baquero P, Michie AM, et al. Targeting mitochondrial oxidative phosphorylation eradicates therapy-resistant chronic myeloid leukemia stem cells. *Nat Med* 23: 1234-1240, 2017.
42. Bosc C, Selak M, Sarry J. Resistance Is Futile: Targeting mitochondrial energetics and metabolism to overcome drug resistance in cancer treatment. *Cell Metab* 26: 705-707, 2017.
43. Ippolito L, Marini A, Cavallini L, et al. Metabolic shift toward oxidative phosphorylation in docetaxel resistant prostate cancer cells. *Oncotarget* 7: 61890-61904, 2016.
44. Yadav N, Kumar S, Marlowe T, et al. Oxidative phosphorylation-dependent regulation of cancer cell apoptosis in response to anticancer agents. *Cell Death Dis* 6: 1969, 2015.
45. Luo C, Puigserver P, Widlund H. Breaking BRAF[V600E]-drug resistance by stressing mitochondria. *Pigment Cell Melanoma Res* 29: 401-403, 2016.
46. De Moura MB, Vincent G, Fayewicz SL, et al. Mitochondrial Respiration - An Important Therapeutic Target in Melanoma. *PLoS ONE* 7: 40690, 2012.
47. Feichtinger RG, Lang R, Geilberger R, et al. Melanoma tumors exhibit a variable but distinct metabolic signature. *Exp Dermatol* 27: 204-207, 2018.
48. Pollak M. Targeting Oxidative Phosphorylation: Why, When, and How. *Cancer Cell* 23: 263-264, 2013.
49. Lakhter AJ, Hamilton J, Dagher PC, et al. Ferroptosis: A cell death from modulation of oxidative phosphorylation and PKM2-dependent glycolysis in melanoma. *Oncotarget* 5: 12694-12703, 2014.
50. Roesch A, Vultur A, Bogeski I, et al. Overcoming Intrinsic multidrug resistance in melanoma by blocking the mitochondrial respiratory chain of slow-cycling JARID1B^{high} cells. *Cancer Cell* 23: 811-825, 2013.
51. Haq R, Shoag J, Andreu-Perez P, et al. Oncogenic BRAF Regulates Oxidative Metabolism via PGC1 α and MITF. *Cancer Cell* 23: 302-315, 2013.
52. Fischer GM, Vashisht Gopal YN, McQuade JL, et al. Metabolic strategies of melanoma cells: Mechanisms, interactions with the tumor microenvironment, and therapeutic implications. *Pigment Cell Melanoma Res* 31: 11-30, 2017.
53. Kopec KK, Bozyczko-Coyne D, Williams M. Target identification and validation in drug discovery: the role of proteomics. *Biochem Pharmacol* 69: 1133-1139, 2005.
54. Wang P, Wang L, Sha J, et al. Expression and transcriptional regulation of human ATP6V1A gene in gastric cancers. *Sci Rep* 7: 3015, 2017.
55. Yilmaz E, Gülec S, Torun D, et al. The effects of Ankaferd [R] Blood Stopper on transcription factors in HUVEC and the erythrocyte protein profile. *Turk J Haematol* 28: 276-285, 2011.
56. Gulec A, Gulec S. Ankaferd Influences mRNA expression of iron-regulated genes during iron-deficiency anemia. *Clin Appl Thromb Hemost* 24: 960-964, 2018.

57. Pfister TD, Reinhold WC, Agama K, et al. Topoisomerase I levels in the NCI-60 cancer cell line panel determined by validated ELISA and microarray analysis and correlation with indenoisoquinoline sensitivity. *Mol Cancer Ther* 8: 1878-1884, 2009.
58. Wagner KW, Punnoose EA, Januario T, et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. *Nat Med* 13: 1070-1077, 2007.
59. Packer LM, Pavey SJ, Boyle GM, et al. Gene expression profiling in melanoma identifies novel downstream effectors of p14ARF. *Int J Cancer* 121: 784-790, 2007.
60. Edgar R, Domrachev M, Lash A. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30: 207-210, 2002.

Correspondence:

Dr. Umit Yavuz MALKAN
Saglik Bilimleri Üniversitesi
Diskapi Yildirim Beyazit Eğitim ve
Arastırma Hastanesi
Hematoloji Klinigi, Diskapi, Altindag
ANKARA / TURKEY

Tel: (+90-532) 778 00 87

e-mail: umitmalkan@hotmail.com

ORCID:

<i>Mehdi GHASEMI:</i>	0000-0002-4697-7605
<i>Mufide OKAY:</i>	0000-0001-5317-0597
<i>Umit Yavuz MALKAN:</i>	0000-0001-5444-4895
<i>Seyhan TURK:</i>	0000-0003-3843-4173
<i>Javaid JABBAR:</i>	0000-0001-8217-3412
<i>Helin HOCAOGLU:</i>	0000-0003-1894-9962
<i>Ibrahim Celalettin HAZNEDAROGLU:</i>	0000-0001-8028-9462