The Role of Skin-Specific TRAs in Skin Cancer

Data Analysis Project SS21

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ABSTRACT

Tissue restricted antigen genes have been researched on because of their ability to open ways to new personalized and specific cancer treatments which is not harmful for the rest of the organism, except for the concerned tissue. In this project, certain tissue restricted antigen genes in melanoma show upregulated expressions after being treated with MAPK pathway and MEK inhibitors. As they are analyzed further, genes that are expressed under the influence of the sun and genes related to melanosome biogenesis are found. The dependency of the upregulated genes on the genes representing the rest of the expressions with highest variance was questioned. For this analysis, methods such as principal component analysis, box plots, log fold change, and linear regression models have been used.

INTRODUCTION

Skin cancer makes up most of the malignancies all around the world, and consists of two main different types; melanoma and non-melanoma **(Linares *et al.*, 2015)**. Both are mainly caused by exposure to UV lights, but non-melanoma cancer develops in the outer layers of the skin **(Leiter & Garbe, 2008)**, whereas melanoma develops from the melanocytes **(Smith *et al.*, 2003).** Melanomas only make up a small amount of reported skin cancer diagnoses, however they still account for the majority of skin cancer deaths, making them extremely dangerous **(Smith *et al.*, 2003).** Melanomas have the highest rate of metastatic capacity of all tumors when they have delayed diagnoses **(Potrony *et al.*, 2015).**

More than half of all melanoma cases contain a mutation of the BRAF gene **(Alqathama, 2020)**. The B-Raf protein is a signaling molecule in the Mitogen-activated protein kinase (MAPK) pathway, activating MEK and leading to the phosphorylation of ERK **(Alqathama, 2020)**. This kinase cascade is known to direct important cell processes such as proliferation, differentiation, and survival **(Alqathama, 2020)**.

In this analysis, data from the melanoma cell line WM266-4 is used. This cell line has a BRAF-V600D mutation, meaning that the valine at codon 600 is changed into aspartic acid. This mutation leads to an overexpression of BRAF, therefore an uncontrolled increase in oncogenic signaling **(Alqathama, 2020)**. The cell line is treated with Trametinib, which is an inhibitor of MEK 1 and MEK2 **(Zeiser, 2014)**, ERK1/2-inhibitor, and doxycycline-shERK1 for different periods of time; 3 and 24 hours, 3 and 7 days. Later, the mRNA from these cells are extracted and processed into labeled cRNA fragments. These fragments are hybridized on microarrays and scanned, resulting in expression level quantifications of large amounts of genes. It is to mention that there are no units in this data, because it is simply light intensity scanned. The microarray data is further analysed in order to get a closer look at the gene expression of this melanoma cell line.

The focus in this microarray analysis is a mechanism cancer cells have been shown to use; the upregulation of tissue restricted antigen (TRA) genes. TRAs are highly expressed selectively in their tissues, and T-cells reacting to these self-antigens result in autoimmune diseases **(Kyewski & Derbinski, 2004)**. So that immature T-cells can gauge the self-reactivity of their antigen receptors, they go through a selection in the thymus, where T-cells reacting to TRAs undergo apoptosis **(Klein *et al.*, 2014)**. TRA genes are mostly upregulated in cancer cells for undiscovered reasons, making them good potential drug targets for therapy **(Rosenberg, 1999)**. Current treatments for melanomas include chemotherapy, radiation, immunotherapy, and surgical excision **(Gruber & Zito, 2020)**. Developing a new therapy, where the upregulated skin specific TRA genes are attacked, therefore other tissues don't get as damaged, would be a significant discovery in the medical field.

Therefore, the main aim of this project is to find upregulated skin specific genes in skin cancer. By looking into the gene expression data of TRA genes in a melanoma cell line, and comparing the variety of the expression levels between different treatments and treatment times, it is possible to gain insight into

how the genes affect the survival of the cancer cells. The genes which present significant changes in their expression would be potential drug targets to further investigate.

METHODS AND RESULTS

Quality Control and Normalization of the Data

The programming language R Studio Version 1.4.1106 **(R Core Team, 2021)** is used throughout this entire project. First, all of the data to be analyzed is downloaded; the numerical microarray data[1] with gene expression intensities of the melanoma cell line and the nominal TRA data **(Dinkelacker, 2007;2019)** with names of genes originating from different tissues. The TRA data has other information such as chromosome numbers of the given genes which will be put to use later. Before the analysis, the data must be investigated to see if there are any problems within, for instance a broken microarray chip, that would alter the results.

Single chips are controlled by visualizing raw microarray data, there aren't any abnormalities visible in all of the fifteen chips, it is concluded that they are of good quality. Gene expression profiling results are highly influenced by RNA quality and degradation **(Opitz *et al.*, 2010)**, therefore RNA degradation plots are made to investigate. All of the lines, each representing a chip, showed similar slope values, and there isn't any deviation visible. For this reason, all chips are to be included in the analysis as they are all qualitative.

The microarray data is normalized using the package vsn **(v3.58.0; Hüber, NA)** to remove any systematic variation that can affect the analysis. The pre-normalization and normalized data, which is glog2 transformed, are compared in the forms of boxplots and histograms, in order to see how much systematic variation existed. The boxplots didn't show a significant difference, even before normalization the median lines are almost in a linear line, indicating that the chips didn't have much variation to begin with. In both pre- and after normalization histograms, the chip lines follow the same path, and none of the chips stand out. After the normalization, a meanSdPlot is made, and from this plot, it is seen that the mean values of the chips are almost linear, once again proving that they are high quality chips.

Cleaning Data and Creating a Dataframe

TRA data is made up of genes from different tissues, different cell lines of humans and mice. All skin genes are extracted and combined in a vector, all NA values are erased. As the skin data available on mice is very little, only the expression of human genes is analyzed. One set of TRA data has the information of sun exposure, as this information might be valuable further into the analysis, these genes are separated into two vectors; exposed to the sun and not exposed to the sun. A pie chart is finalized to visualize how much of the TRA data comes from skin. It is found out that skin specific genes make up only 4.84% of all TRA data.

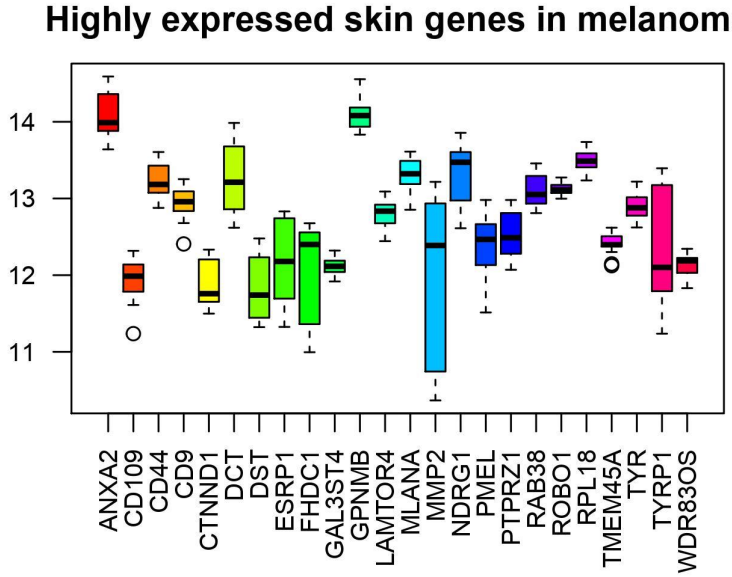
[1] GEO: https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE57721, [cited: 28.04.2021]

In the data from microarray chips genes are saved as numbers, to be able to view and analyse the data easier, these numbers are switched with their names using the ensembl IDs[2] that were loaded from Biomart. Later, all expression values are extracted from the microarray data, and put into a data frame with chip IDs as columns and gene symbols as rows. After empty spaces and NA values are removed from the data, skin specific antigens are extracted to create the main data frame of skin TRA expression levels in skin cancer to analyze. The column names are changed so that the treatment and time point of every chip is easily viewed.

Gene Expression Analysis

To begin the analysis a heat map is created, showing the expression levels of skin specific TRA genes in all chips. This heat map is useful in recognizing expression patterns to focus on, and it gives a starting point for the further analysis. It is observed that the expression levels of some genes increase as the treatment lasts longer. These are the genes that the cancer cells upregulate against the different treatments.

Another method used to visualize the gene expression in all chips is box plots. There are too many genes overall, therefore the boxes look like overlapping lines. This does not give much information about the expression levels. Instead, only the highly expressed genes are chosen to be visualized. It should be noted that a highly expressed gene doesn't have to be an upregulated gene, and an upregulated gene has the possibility of not being a highly expressed gene. A threshold of twelve is set according to the overall expression levels, to define the high expression margin. Aside from the two highest expressed genes GPNMB and ANXA2, the genes NDRG1, RPL18, DCT, CD44, and MLANA show varying expression levels between chips with levels above the threshold in some chips and below in others.



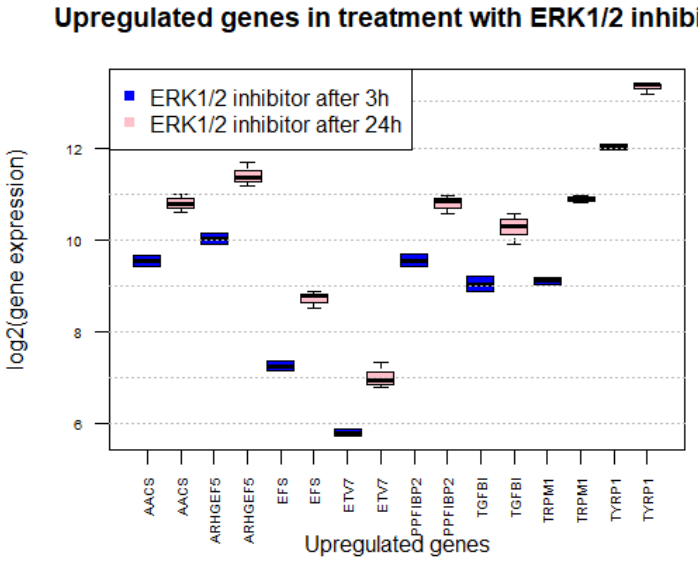
Img. 1: Boxplots of Highly Expressed Genes: ANXA2 and GPNMB are the highest expressed.

[2] https://www.ensembl.org/biomart/martview/74741c6cba3dea58c83e6520f12fa790, [cited: 01.05.2021]

The central purpose in this analysis was to find upregulated genes that could be used as drug targets in melanoma. A gene that is expressed more after treatment would be a gene that has been upregulated to help the cancer cell survive under the treatment, leading to drug resistance.

However, there isn't any data available from cells without any treatment, therefore for chips that have been treated with ERK1/2-inhibitor or Trametinib, the gene expression between the three hour and twenty four hour time points is compared to see which genes showed a higher expression after twenty one hours of treatment. The zero point for dox-shERK1 chips is taken as three days, as there isn't any earlier data available. The expression of the genes are therefore compared between the data from three and seven days after the beginning of treatment. Since a comparison between expression values under two different conditions are required, the log2 fold change is used as a method. The only changing condition is taken as the treatment time, fold change analysis is done within groups of chips with the same treatment types.

Not all genes encountered at the end of this analysis are significantly upregulated to become drug targets. Therefore, ten genes with the highest log-ratios from different treatments are chosen to be filtered through a t-test. Throughout the chips of dox-shERK1, only MPZL3 and BCL1A genes were detected as significantly upregulated genes. From ERK1/2-inhibitor data, the genes TRPM1, EFS, ARHGEF5, TYRP1, ETV7, AACS, PPFBIBP2, TGFBI and from the Trametinib data, the genes VANGL2, TRPM1, ETV7, DAPL1, EFS, DCT, TYRP1 are identified as significantly upregulated genes. Box plots were built to visualize the expressions of these genes.

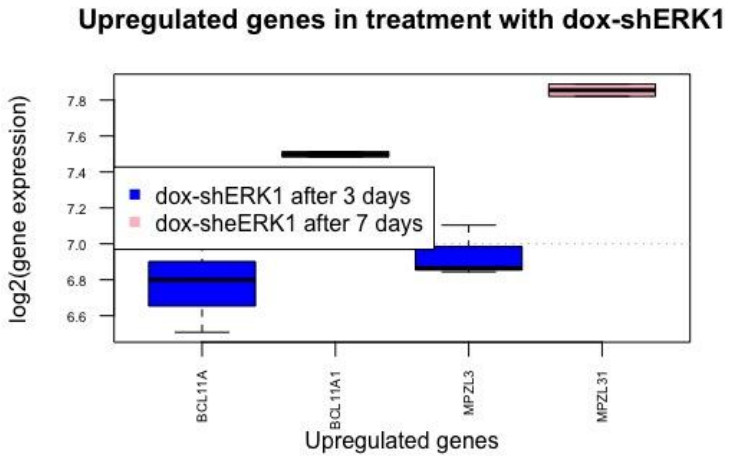


Img. 2: Upregulation Boxplots: The expression levels of the most significantly upregulated genes after three hours and twenty four hours of treatment with ERK1/2 are shown.

The package "Limma" is used as a model of comparison to the upregulated genes that were found manually using the log fold change. Limma takes the normalized data and performs statistical tests to discover any quantitative changes in the expression **(Ritchie *et al.*, 2015).** The results show mostly

identical genes to the already found upregulated genes. They are compared by looking into the intersection of the vectors with the upregulated genes and the ten highest positive differential expressions that were found in Limma.

Aside from upregulated genes, downregulated genes are of interest too. The existence of significantly downregulated genes indicates that the treatment has been successful, and that these were inducing tumors before the treatment. That is why, ten genes from different treatments with the lowest log-ratio are filtered out and evaluated under t-tests. The genes MMP2, TYRP1 from dox-shERK1 chips, CMSS1, KRT27, MPP4, CHTF18, POLA1, WEE1, REEP4, MFSD2A, CA12, C3of52 from ERK1/2 inhibitor chips, and RHNO1, MPP4, IRX3, MFSD2A, WEE1, CA12, C3orf52, POLA1 from Trametinib chips proved to be significantly downregulated. It should be noted that TYRP1 is significantly upregulated under Trametinib treatment, but significantly downregulated under dox-shERK1.



Img. 3: Downregulation Boxplots: of the expression levels of the most significantly downregulated genes after three days and seven days of dox-shERK1 treatment.

After this discovery of significantly upregulated and downregulated genes, a further category of gene expression is analyzed; expression under exposure to sun and no exposure to sun. Once again, box plots are used to visualize the expression levels of the two gene sets, there is not any significant difference seen in any of the chips. Yet, after a Wilcoxon signed rank test was run for each chip, a significant difference between the expression levels of the two gene groups was observed as all of the p-values were confirmed to be lower than 0.05. After removing genes that have expression data in both circumstances of exposure to sun, the genes that were only expressed under the influence of the sun are acquired. Four of these sun exposure related genes are also upregulated genes: ETV7, TGFBI, DAPL1, and ARHGEF5. The upregulation of these genes that are seen to be expressed under sun exposure is further proof that UV radiation has triggering effects on melanoma.

Since the expression difference of genes exposed to sun and genes that were not exposed to sun was not visible via boxplots, k-means is used as a method to try again. After running the elbow, silhouette, and one other method provided by the package NbClust **(v3.0; Charrad, 2015)**, it is decided that two

clusters are the optimal amount for this data. After clustering and plotting with the package factoextra **(v1.0.3; Kassambara, 2020)**, two clusters are defined with an average silhouette width of 0.65, indicating that there is indeed a clusterable difference between the expressions of these two gene groups.

Upregulated and downregulated genes were also tried to be clustered, thinking there might be signs of certain gene groups. However, after testing the up- and downregulated data using Hopkins statistics from the package clustertend **(v1.5; Wright, 2021)**; it was concluded that this data is not meaningfully clusterable.

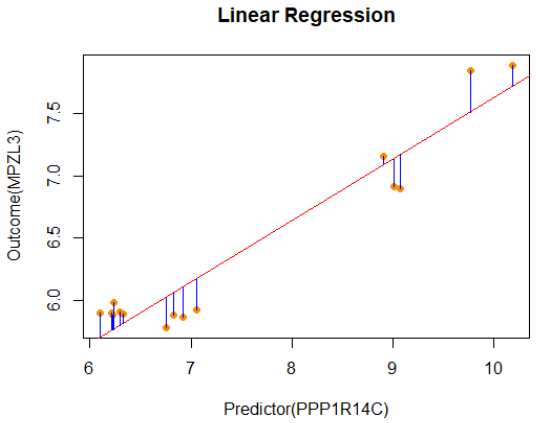
In order to develop therapies and case-specific combination treatments, one needs to be able to predict these upregulated genes, which make good drug targets. Nonetheless, there are too many genes as variables to be able to predict anything specific, hence a principal component (PC) analysis is carried out on the 24h and 7d chips to reduce the amount of dimensions. PC1 and PC2 represent 88% of all the variance of the melanoma dataset. Five genes with the top variance from each of these components are chosen to continue the analysis with: ANXA2, DCT, NDRG1, TYRP1, PTPRZ1 from PC1, and DSP, GJA1, PPP1R14C, TGFBI, UCN2 from PC2.

Linear regression models are used to see if there are any significant dependencies between the PC genes and the upregulated genes that would justify making a prediction of the expressions. Before the modeling, Pearson correlation is used to see if the upregulated genes and the PC genes have any correlations within their group. The package corrplot **(v0.90; Wei, Levy & Simko, 2021)** is used to visualize and calculate the correlations. All of the upregulated genes turn out to be highly correlated with each other except for MPZL3. Since the rest are highly correlated, a representative gene, AACS is chosen to do the modeling with. Even though not as much as the upregulated genes, PC genes are found to be somewhat correlated. In this case, ANXA2 will be representing DCT, PTPRZ1, TGFBI, TYRP1. NDRG1 is correlated with PPP1R14C, and the rest of the genes, DSP, GJA1, UCN2 are not correlated. To summarize: the upregulated genes MPZL3 and AACS are going to be predicted by ANXA2, NDRG1, DSP, GJA1, UCN2.

Next, the distribution of the residuals of the upregulated genes are investigated with a QQ-Plot, they are normally distributed. The residuals have to meet some conditions in order to be able to form a linear relationship with X, our PC genes; and Y, the upregulated genes. Their means have to be checked to see if they are close or equal to zero and they must not correlate with the PC genes. It is confirmed that the residuals have a mean of zero, and that they do not correlate with the PC genes.

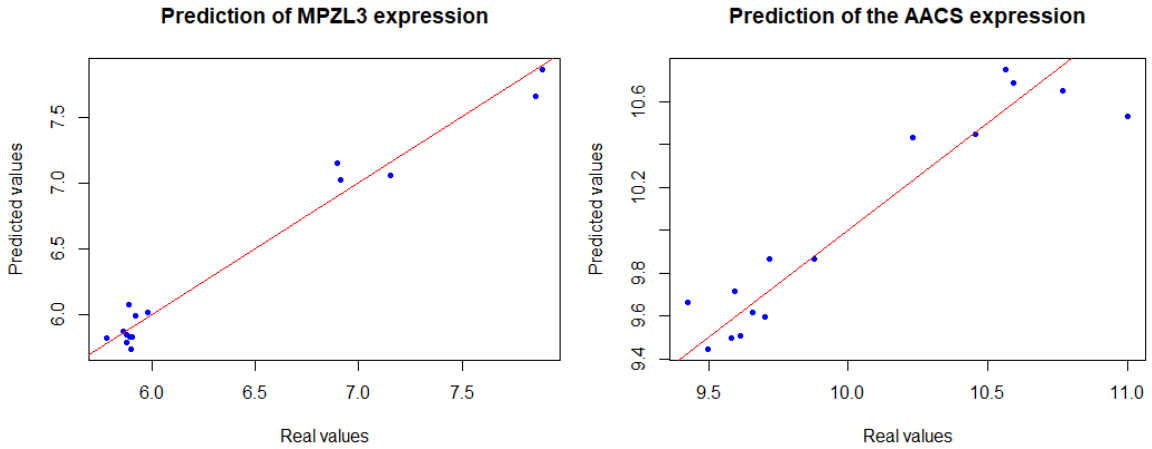
Multiple linear regression is carried out between MPZL3 and the aforementioned five PC genes, and a highly significant result is observed. With an adjusted R squared value of 0.9593 and a low p-value, 7*10^-7, it is right to say that MPZL3 expression can definitely be predicted by these five genes' expression levels. PPP1R14C appears to be able to predict the expression of MPZL3 the best out of all. This has been decided with help from the package QuantPsyc **(v1.5; Fletcher, 2012)**. The dependency between the AACS and the PC genes on the other hand, is not as significant. The linear relationship of the PC genes and AACS, with an adjusted R squared value of 0.8224, and a p-value of 0.0005, is weaker than

the relationship between MPZL3, yet still significant. The expression of AACS is represented best by DSP among all PC genes, however they show a negative linear relation.



Img. 4: Simple linear regression: Plot with the predictor that has the highest impact on the model. Residuals are also shown.

To confirm the accuracy of the predictions made by the PC genes on the expression of the two representative upregulated genes AACS and MPZL3, the predicted values are plotted against the real values of expression. They seem to be nearly identical. Since the gene AACS is highly correlated with the rest of the upregulated genes, it can be concluded that the expression of all of the upregulated genes can be predicted by the expression profiles of the PC genes.



Img. 5: Expression Prediction Plots: (left) Prediction of the MPZL3 expression according to the real expression values in the data set. (right) Prediction of the AACS expression representing expressions of all other upregulated genes according to the real expression values in the dataset.

For the last step of the analysis F-test is executed to make sure that the linear model refers to a significance. The p value is low enough to overthrow the H0 hypothesis. In both multiple regression models the p-value is lower than 0.01 indicating that the fit is good with five independent variables for both of the upregulated genes. The H0 hypothesis is overthrown, meaning that the model is significant, and the dependency between the genes is significant enough to predict upregulated genes.

DISCUSSION

Regardless of treatment type and time variation between different chips, GPNMB and ANXA2 proved to be highest expressed in all of them. ANXA2 is known as a biomarker in cancers, it is already seen as an ideal target for cancer therapy because of its crucial functions in cancer cell proliferation, migration, invasion, metastasis **(Wang *et al.,* 2019)**. GPNMB has been formerly identified as an aggressive pro-metastatic protein that is highly expressed in many tumor types, including melanoma **(Taya & Hammes, 2018)**. The fact that brings these two together is that they are both responsible for the genesis of melanosomes **(Setaluri & Jayanthy, 2013; Delevoye et al., 2015)**.

Melanoma develops from melanocytes, which normally assemble melanosomes; organelles in mammals responsible for melanin production **(Setaluri & Jayanthy, 2013)**. It makes sense that these two genes related to the assembly and maturation of melanosomes are found to be highly expressed in melanoma. The two other important genes TYRP1 and DCT found in this analysis, which are also involved in the development of melanosomes **(Setaluri & Jayanthy, 2013)**, are both upregulated and principal component genes at the same time. DCT also known as TYRP2 encodes proteins that play roles in the melanin synthesis, just as TYRP1 **(Setaluri & Jayanthy, 2013)**. The upregulation and high expression of these melanosome related genes, indicates that a therapy focusing to direct melanosome functions would be effective against melanoma.

Microphthalmia-associated transcription factor (MITF) is a key signal regulator with the subset target genes GPNMB and TYRP1 **(Rose *et al.,* 2016)**. MITF plays important roles in melanosomal function, pigmentation, but it is also required for sustaining the BRAF-mutant melanoma, and supporting the melanoma cell proliferation **(Rose *et al.,* 2016)**. It has been observed before that MITF related genes are upregulated under certain BRAF and MEK inhibitors, such as Trametinib **(Rose *et al.,* 2016)**. This would explain the expression results of TYRP1 in this project. It is upregulated as it is needed for the maintenance of the melanoma cell, and the Trametinib treatment, which is a MEK1/2 inhibitor further accelerates its expression. The other two treatments are inhibitors of ERK, which partially successfully seem to have worked, because one of the genes supporting the cancer cell is downregulated under them.

Other significant genes that have been found are the four upregulated genes that are only expressed under the sun. DAPL1 is a gene upregulated by the aforementioned MITF **(Ma *et al.,* 2018)**, and TGFBI regulates cyclin-dependent kinases, which are known to be in charge of the cell cycle, and allows a high metastatic potential to the melanoma **(Lauden *et al.,* 2014)**. Lastly, ARHGEF5 codes for a guanin exchange factor that is crucial for certain membrane formations that are related to the invasive potential of cancer cells **(Kuroiwa *et al.,* 2011)**. The knowledge that such genes, aiding the development of melanoma, have only been upregulated under sun exposure, confirms the already commonly known fact that the exposure to sun is a trigger for skin cancer.

The fourth upregulated gene under sun exposure, ETV7, codes for a transcription factor, which is usually seen to be downregulated in melanoma patients and correlates with a poor prognosis. **(Qu *et al.,* 2020)**. However, ETV7 is seen to be upregulated in our data under Trametinib, suggesting that it might have a role in attacking the tumor microenvironment, leading to a better prognosis. The fact that this gene is expressed under the sun suggests that it might be a natural response to the tumorigenic genes, upregulated under sun exposure. Trametinib helps upregulate it further to fight tumor proliferation. The relationship between ETV7 and the three genes mentioned above should be looked into with more detail, as a treatment upregulating its expression seems to be possibly effective against melanoma.

What's important to give attention to here is that almost all of the upregulated genes are either regulated by MITF or are from the data that was exposed to the sun. This indicates that the high expression of the genes that are related to MITF are as effective as exposure to sun when it comes to helping create or supporting the melanoma. Since all of the genes analysed on are human skin specific TRA genes, a new treatment where the expression of the genes mentioned above are targeted, would not harm healthy tissues. This opens countless opportunities for future research and treatment of melanomas, especially because melanoma is highly metastatic.

Through the opposite expression of TYRP1, and varying up- and downregulated genes between chips of different treatments, the different effects of MEK and ERK inhibitors on melanoma have been shown. Even though these treatments in this project have shown to downregulate some known tumor inducing genes such as WEE1, an important regulator in the cell cycle **(Tashnizi *et al.,* 2016)**, it should be further researched and discussed on if these inhibitors should be used on melanoma. If yes, a combination of other drugs are needed as treatment to inhibit the accelerating effects of MAPK inhibitors on melanosome related genes.

The strong linear dependency found between the expression of the gene DSP and most of the upregulated genes also raises new questions requiring additional research. DSP codes for a desmosomal protein, which helps human melanoma metastases to escape the immune system **(Salerno *et al.,* 2016)**. Some desmosomal proteins are used to identify melanomas, even when there are not any immune signatures seen **(Salerno *et al.,* 2016)**. This linear relationship between DSP and the upregulated genes could be used for better diagnosis of melanomas, however more information on the expression of DSP in melanomas is needed.

The findings that melanosome related genes, and genes highly expressed under sun exposure strongly influence the melanoma cells, and that there is a strong linear relationship between the upregulated and the high variance genes set base for further research in new treatments for melanoma. Therefore, it can be stated that this analysis has given successful results.

LITERATURE

Alboukadel, K. (2017). Practical Guide to Cluster Analysis in R: Unsupervised Machine Learning, STHDA

Alqathama A. (2020). BRAF in malignant melanoma progression and metastasis: potentials and challenges. American journal of cancer research, 10(4), 1103–1114.

Delevoye, C., Heiligenstein, X., Ripoll, L., Gilles-Marsens, F., Dennis, M. K., Linares, R. A., Derman, L., Gokhale, A., Morel, E., Faundez, V., Marks, M.S., Raposo, G. (2015). BLOC-1 Brings Together the Actin and Microtubule Cytoskeletons to Generate Recycling Endosomes, Current Biology, Vol 26(1), 1-13, https://doi.org/10.1016/j.cub.2015.11.020

Dinkelacker, 2007. A database of genes that are expressed in a tissue-restricted manner to analyse promiscuous gene expression in medullary thymic epithelial cells. Diplomarbeit, Albert-Ludwigs-Universitaet, Freiburg, Germany.

Dinkelacker, 2019. Chromosomal clustering of tissue restricted antigens, Dissertation, University Heidelberg, Germany.

Gruber, P., Zito, P. M. (2021). Jan, Skin Cancer. [Updated 2020 Nov 19]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; Available from: https://www.ncbi.nlm.nih.gov/books/NBK441949/

Klein, L., Kyewski, B., Allen, P. *et al.* **(2014)**. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat Rev Immunol 14, 377–391. https://doi.org/10.1038/nri3667

Kuroiwa, M., Oneyama, C., Nada, S., & Okada, M. (2011). The guanine nucleotide exchange factor Arhgef5 plays crucial roles in Src-induced podosome formation. Journal of cell science, 124(Pt 10), 1726–1738. https://doi.org/10.1242/jcs.080291

Kyewski, B., Derbinski, J. (2004) Self-representation in the thymus: an extended view. *Nat Rev Immunol* 4, 688–698. https://doi.org/10.1038/nri1436

Lauden, L., Siewiera, J., Boukouaci, W., Ramgolam, K., Mourah, S., Lebbe, C., Charron, D., Aoudjit, F., Jabrane-Ferrat, N., Al-Daccak, R. (2014). TGF-β-Induced (TGFBI) Protein in Melanoma: A Signature of High Metastatic Potential, Journal of Investigative Dermatology, Vol:134(6), 1675-1685, ISSN 0022-202X, https://doi.org/10.1038/jid.2014.20.

Leiter, U., & Garbe, C. (2008). Epidemiology of melanoma and nonmelanoma skin cancer--the role of sunlight. Advances in experimental medicine and biology, 624, 89–103. https://doi.org/10.1007/978-0-387-77574-6_8

Linares, M. A., Zakaria, A., & Nizran, P. (2015). Skin Cancer. Primary care, 42(4), 645–659. https://doi.org/10.1016/j.pop.2015.07.006

Ma, X., Hua, J., Zheng, G., Li, F., Rao, C., Li, H., Wang, J., Pan, L., & Hou, L. (2018). Regulation of cell proliferation in the retinal pigment epithelium: Differential regulation of the death-associated protein like-1 DAPL1 by alternative MITF splice forms. Pigment cell & melanoma research, 31(3), 411–422. https://doi.org/10.1111/pcmr.12676

Moloney, E. B., Moskites, A., Ferrari, E. J., Isacson, O., & Hallett, P. J. (2018). The glycoprotein GPNMB is selectively elevated in the substantia nigra of Parkinson's disease patients and increases after lysosomal stress. Neurobiology of disease, 120, 1–11. https://doi.org/10.1016/j.nbd.2018.08.013

Opitz, L., Salinas-Riester, G., Grade, M., Jung, K., Jo, P., Emons, G., Ghadimi, B. M., Beissbarth, T., & Gaedcke, J. (2010). Impact of RNA degradation on gene expression profiling. *BMC medical genomics, 3,* 36. https://doi.org/10.1186/1755-8794-3-36

Potrony, M., Badenas, C., Aguilera, P., Puig-Butille, J. A., Carrera, C., Malvehy, J., Puig, S. (2015). Update in genetic susceptibility in melanoma. Annals of translational medicine, 3(15), 210. https://doi.org/10.3978/j.issn.2305-5839.2015.08.11

Qu, H., Zhao, H., Zhang, X., Liu, Y., Li, F., Sun, L., & Song, Z. (2020). Integrated Analysis of the ETS Family in Melanoma Reveals a Regulatory Role of ETV7 in the Immune Microenvironment. Frontiers in immunology, 11, 612784. https://doi.org/10.3389/fimmu.2020.612784

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research, 43*(7), e47. https://doi.org/10.1093/nar/gkv007

Rose, A. A., Annis, M. G., Frederick, D. T., Biondini, M., Dong, Z., Kwong, L., Chin, L., Keler, T., Hawthorne, T., Watson, I. R., Flaherty, K. T., Siegel, P. M. (2016). MAPK Pathway Inhibitors Sensitize BRAF-Mutant Melanoma to an Antibody-Drug Conjugate Targeting GPNMB, DOI: 10.1158/1078-0432.CCR-16-1192

Rosenberg S. A. (1999). A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity, 10(3), 281–287. https://doi.org/10.1016/s1074-7613(00)80028-x

Salerno, E. P., Bedognetti, D., Mauldin, I. S., Deacon, D. H., Shea, S. M., Pinczewski, J., Obeid, J. M., Coukos, G., Wang, E., Gajewski, T. F., Marincola, F. M., & Slingluff, C. L., Jr (2016). Human melanomas and ovarian cancers overexpressing mechanical barrier molecule genes lack immune signatures and have increased patient mortality risk. Oncoimmunology, 5(12), e1240857. https://doi.org/10.1080/2162402X.2016.1240857

Setaluri, V., Jayanthy, A. (2013). Coat Color Mutations, Animals, Brenner's Encyclopedia of Genetics, Second Edition, Academic Press, 58-60, ISBN 9780080961569, https://doi.org/10.1016/B978-0-12-374984-0.00275-8.

Smith, R. A., Mettlin, C. J., Eyre, H. (2003). Melanoma and Nonmelanoma Skin Cancer, Holland-Frei Cancer Medicine. 6th edition. Hamilton (ON): BC Decker; https://www.ncbi.nlm.nih.gov/books/NBK13764/

Tashnizi, A. H., Jaberipour, M., Razmkhah, M., Rahnama, S., & Habibagahi, M. (2016). Tumour suppressive effects of WEE1 gene silencing in neuroblastomas. Journal of cancer research and therapeutics, 12(1), 221–227. https://doi.org/10.4103/0973-1482.165861

Taya, M., Hammes, S. R. (2018). Glycoprotein Non-Metastatic Melanoma Protein B (GPNMB) and Cancer: A Novel Potential Therapeutic Target. Steroids, 133, 102–107. https://doi.org/10.1016/j.steroids.2017.10.013

Volz, A., Korge, B. P., Compton, J. G., Ziegler, A., Steinert, P. M., & Mischke, D. (1993). Physical mapping of a functional cluster of epidermal differentiation genes on chromosome 1q21. Genomics, 18(1), 92–99. https://doi.org/10.1006/geno.1993.1430

Wang, T., Wang, Z., Niu, R., & Wang, L. (2019). Crucial role of Anxa2 in cancer progression: highlights on its novel regulatory mechanism. Cancer biology & medicine, 16(4), 671–687. https://doi.org/10.20892/j.issn.2095-3941.2019.0228

Zeiser R. (2014). Trametinib. Recent results in cancer research. Fortschritte der Krebsforschung. Progrès dans les recherches sur le cancer, 201, 241–248. https://doi.org/10.1007/978-3-642-54490-3_15