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Identification of Differentially Expressed Genes between Immune Phenotypes in Breast Cancer

Abstract

Background: Breast cancer (BC) is the second leading cause of cancer-related deaths worldwide. Around 80% of all BC tumors are estrogen receptor positive (ER+); therefore, treatments for these cases target the estrogen receptor (SERMs, SERDs, etc.). Immunotherapy, or targeting the immune system to treat disease, is a novel approach of cancer therapy that has proven to have potential for triple negative breast cancer, but its efficacy in ER+ breast cancer has been understudied. Previously, Tamborero *et al.* curated a set of immune related gene signatures to investigate immune infiltration in TCGA tumors. Immune infiltration was quantified by the enrichment scores from gene set variation analysis (GSVA). They then used these GSVA scores to classify TCGA ER+ BC samples by their immune phenotype: as either low-immune phenotype (ImL) tumors or high-immune phenotype (ImH). In a study performed by Oesterreich *et al.*, it was found that PDL-1 signaling was upregulated in ImH tumors, thus indicating immune evasion. However, it is still not clearly known how ImL BC tumors might be preventing infiltration and what other properties distinguish them from ImH tumors. Studies have suggested that upregulation of certain extracellular matrix (ECM) factors in ImL tumors might be preventing immune infiltration. It is additionally not known what genes are aiding PD1/PDL-1 in immune evasion (i.e. genes that are also upregulated when PD1/PDL-1 is upregulated).
Methods: To identify if ECM factors exist in immune low breast cancer tumors, differential gene expression analysis (using DEseq2) was used on RNAseq data of tumor samples to identify differentially expressed genes (DEG) in ImH vs ImL tumors that might be driving the immune phenotypes. The DEG were filtered by function, and ECM genes were analyzed. To study other genes that could be working in combination with PD1/PDL-1, a spearman correlation of different genes with PD1/PDL-1 was conducted based on RNAseq expression data in ImH tumors.
Conclusions: I found that COL2A1, PCSK1, and SLIT1 were significantly upregulated ($logFC > 2$, $pval < 0.001$) in ImL tumors. Additionally, caspase genes and p53 were significantly correlated with high PD1/PDL-1 expression ($spearman > 0.1$, $pval < 0.001$). A future direction would be to validate the protein expression of these genes in-vitro through immuno-histochemistry or possibly use them as targets for treatment.

Introduction

Breast cancer (BC) is the most commonly diagnosed cancer in women. According to the American Cancer Society, about 2 out of every 3 cases of breast cancer are hormone receptor-positive[1]. The estrogen receptor positive (ER+) breast cancer phenotype composes the majority (80%) of all cases[2]. In ER+ tumors, cancer cells receive signals from estrogen that promote tumor growth by binding to ER-α. The estrogen receptor is then thought to function as a ligand-activated transcription factor to genes that control cell growth and proliferation. Currently, treatment for breast cancer has focused on the blockage of the estrogen receptor through endocrine therapy; however, resistance to treatment can be seen in some patients as tumor progresses[2]. Immunotherapy is another novel approach that has proven to have potential for triple negative BC, but its efficacy in ER+ breast cancer has so far been understudied[3]. Immunotherapy would focus on the activation of immune cells such as T-lymphocytes, monocytes, and natural killer cells found in some breast cancer tumors.

Previously, Tamborero *et al.* curated a set of immune related gene signatures to investigate immune infiltration in tumor samples (from The Cancer Genome Atlas) compared to normal tissue samples from GTEX[4] (Genotype-Tissue Expression project). For quantifying immune infiltration, they used gene set variation analysis (GSVA) to calculate enrichment scores of these signatures in each sample. GSVA is a method of quantifying upregulation of a certain gene set compared to all other genes, where a positive GSVA score signifies that the gene set in question is upregulated. The purpose of GSVA was to determine if the "immune gene set" was significantly deregulated compared to baseline genes. The samples were then given an immune phenotype score for classification based on GSVA score for each immune signature. These immune phenotype scores ranged from 1-6, with 1 representing the lowest immune infiltrate, and 6 representing the highest immune infiltrate. These immune phenotype scores were then extracted to classify tumor samples as either "immune-low" phenotype tumors (immune phenotype scores between 1-3) or "immune-high" phenotypes (scores between 4-6).

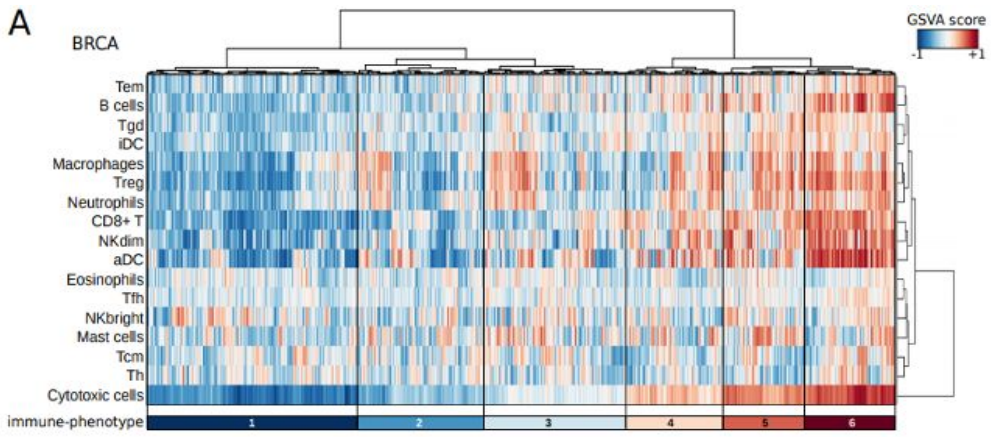


Figure A. Pan-cancer landscape of interactions between solid tumors and infiltrating immune cell populations. Based on GSVA score, an immune enrichment score is assigned (1-6, 1 = low immune infiltration, 6 = high immune infiltration)[4].

However, in a practical sense, both immune phenotype tumors have rapid growth, with no difference in patient outcome. This discovery subsequently raised the question of how "immune-high" tumors had the capability to proliferate if there truly was a greater amount of immune infiltrate in these tumors. In a following study performed by Oesterreich *et al.*, they showed that high immune phenotype tumors, despite their greater number of T-cells, were potentially able to evade immune responses by up-regulating the receptor PD1 and its ligand PD-L1 (programmed-death 1)[5]. This ligand-receptor checkpoint inhibitor, when activated, blocks the activation of T-cells in the tumor, and prevents the anti-cancer immune response in the body. These findings suggest that immunotherapy could be used along with other therapeutic methods to treat ER+ breast cancer, specifically by targeting the PD1/PD-L1 mechanism in tumors with high immune-infiltration[6].

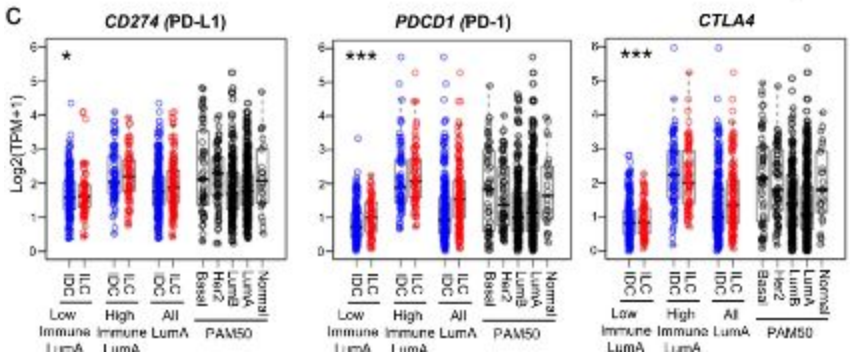


Figure B. Immune phenotypes categorized by histology (invasive ductal vs. lobular carcinoma). Tian Du *et al*. found higher activity levels of immune checkpoint pathways in immune high tumors in both histological subtypes[5].

Questions

Based on current literature, it is still not clearly known what properties distinguish immune low breast cancer tumors from immune high tumors, and how immune low tumors might be preventing immune infiltration. Studies have suggested that upregulation of certain extracellular matrix (ECM) factors in immune low tumors might be preventing immune infiltration. One of the main goals in this study is to identify any differentially expressed genes between the immune high and immune low phenotypes, or any gene upregulated in the immune low phenotype that may play a role in the prevention of immune cell (lymphocyte) infiltration.

Additionally, it is currently known which pathways or genes work in combination with PD1/PDL-1 in immune-high tumors. It was recently published by Xu *et al.* that p53 may have a possible role in the pathway of PD1/PDL-1 in cancer, as there was a strong positive association between the expression of the two genes in lung adenocarcinoma patients[6]. Furthermore, in the same paper, this relation was confirmed in-vitro in tumor samples through immuno-histochemistry staining. The additional goal of this study is to filter gene candidates that are positively correlated with high PD1/PDL-1 gene expression in immune-high breast cancer tumors.

Methods

Data Selection

All patient samples were obtained from The Cancer Genome Atlas project on immune signatures, originally started by Tambarero *et al*. 467 patient samples with immune low tumors and 277 immune high tumors were used in this study. RNA sequencing data for tumors was the primary method of analysis in this study. In addition, for verification purposes, corresponding biospecimen data (such as lymphocyte, monocyte, and tumor purity cell count) was obtained for each patient tumor sample.

Verifying Immune Classification

Because the immune classification for the ER+ tumors used in this study is based on gene signature, validation of the classification (based on immune cell counts) is necessary in order to make any conclusions about immune phenotypes. This verification was done by analyzing whether the immune high tumors had a higher percentage of T-lymphocytes and the immune-low tumors had a lower percentage of lymphocytes. Using a graphical representation, mean lymphocyte concentration was compared between the two phenotypes to see whether a significant difference was present.

Differential Gene Expression Analysis

In order to view what genes were significantly upregulated in the distinct phenotypic groups based on RNA sequencing data, differential gene expression analysis was conducted. The bioinformatics software DESeq2 was used to accomplish this. The DESeq2 pipeline involves a series of steps that first normalizes raw RNAseq counts, estimating gene dispersions, and fitting the data to a statistical model to compare mean gene expression between two groups.

DESeq2 Pipeline

The first step in the pipeline involves normalizing the raw RNAseq counts to account for differences in library depth, or the number of RNA reads per tumor samples. Next, the algorithm analyzes gene-wide dispersion, or the variability in the data. Dispersion is essentially an inverse measure of the mean gene expression, measuring variance in gene

expression for a given mean expression value. To generate more accurate estimates for variation, DESeq2 uses a method coined as “shrinkage”, which estimates variation based on the mean expression level of the gene. The dispersion estimates are then fitted to model a curve, where the expected dispersion value for genes of a given expression strength are generated from the plot. The algorithm then “shrinks” the gene-wide dispersion estimates to those that match the expected value of the curve. The curve allows for a more accurate identification of differentially expressed genes when sample sizes are small. Shrinkage of gene dispersions are dependent on the curve’s expected estimates, as well as sample size. This step proves very important, as it decreases the likelihood of false positives when reporting differentially expressed genes. Once the dispersions and means are calculated for each gene (among all tumor samples), the two groups are compared for any significant differences in gene expression.

The differentially expressed genes are depicted in a volcano plot, where genes upregulated in immune-low and immune-high phenotypes can be analyzed. Because this study aims to answer the question of upregulated genes in the immune-low phenotype, special attention was given to these genes. From this list of genes, the ones that were both significantly upregulated (based on log2 fold change and p-value) and ECM-related genes were filtered.

Spearman Correlation Analysis with PD1/PDL-1

In order to answer the second question of which genes were significantly correlated with high PD1/PDL-1 expression, a spearman correlation analysis was implemented. Using RNA sequencing data and the differential gene expression data, a volcano plot was made from expression of all immune-high tumors to correlate which other genes were significantly upregulated (log2 fold change > 2 and adjusted pal < 0.001) when PD1/PDL-1 expression was significantly upregulated. This graphical representation would give the possible genes that are working in conjunction with PD1/PDL-1, either within the same pathway in the same biological mechanism or as an effect of high PD1/PDL-1 expression.

Spearman Correlation

The theoretical basis of the spearman correlation is structured on the rank of variables being analyzed. In this case, we rank the correlation of different genes (for which differential expression differences were calculated) with PD1/PDL-1. This method essentially assesses how well two variables are related to each other using a correlation function. This correlation is similar to other measures, such as the Pearson correlation, that assess the linearity of the relationship between two variables, but differs in the fact that it calculates whether a relationship is linear in the first place. The correlation for each gene pair, a differentially expressed gene and PD1/PDL-1, is depicted in a graphical representation, to see which genes have the highest correlation score in terms of

expression values. A spearman correlation score of 1 or -1 indicates perfect linearity between variables being analyzed.

Fisher Exact Test on Extracellular Matrix Genes

Of all significantly differentially expressed genes, a Fisher's Exact test was conducted to see if there is any nonrandom association in these genes that belong to the ECM category compared to all other differentially expressed genes that are not ECM-related. A list of all ECM genes was taken from the Matrisome Project, a project led by Harvard University and Massachusetts Institute of Technology. The purpose of this project was to characterize and predict (using bioinformatics tools) the set of possible genes encoding the "matrisome", or the "ensemble of extracellular matrix and ECM-associated proteins". The results from this study include a comprehensive list of genes and gene families that play a vital role in the extracellular environment, whose mutated versions could potentially play a role in cancer pathogenesis[13].
In the Fisher's Exact test, the categorical variables tested were ECM-related genes versus non-ECM related genes, in the differentially expressed group of genes and not in the differentially expressed group.

Fisher's Exact Test

The Fisher's Exact test is used for categorical data that result from classifying objects in two different ways; specifically, it is used to examine the significance of the association (contingency) between the two kinds of classification. The structure of this test evaluates the probability of the null hypothesis - where there is no association between the categorical variables and the relationship is simply due to random chance. A p-value is given to test the statistical significance of the association of categories. This type of analysis is usually conducted on a small sample size and lower number of categorical variables, and a Chi Squared test is used for larger sample sizes.

Results

Immune high phenotype samples have greater lymphocyte infiltration

To verify the immune classification based on gene expression, available tumor sample data from TCGA was analyzed (ImH n = 277; ImL n = 467). Lymphocyte concentration for each tumor sample was plotted and segregated between the immune-low and immune-high tumor group. The results confirm the classification used, where immune-high tumors had a significantly higher lymphocyte concentration compared to immune-low tumors. The results were statistically significant to pvalue < 0.01.

Although there were a few outliers in the immune-low category that had a higher percentage of lymphocytes, this could be attributed to the fact that there were a greater number of immune-low samples to begin with, and a greater sample size inherently comes with greater

variation. Despite the outliers, the overwhelming majority of immune-low tumors had a lower concentration, as indicated by the median value in the boxplot. This result confirms that immune phenotypes have a difference both in immune signature and immune cell counts.

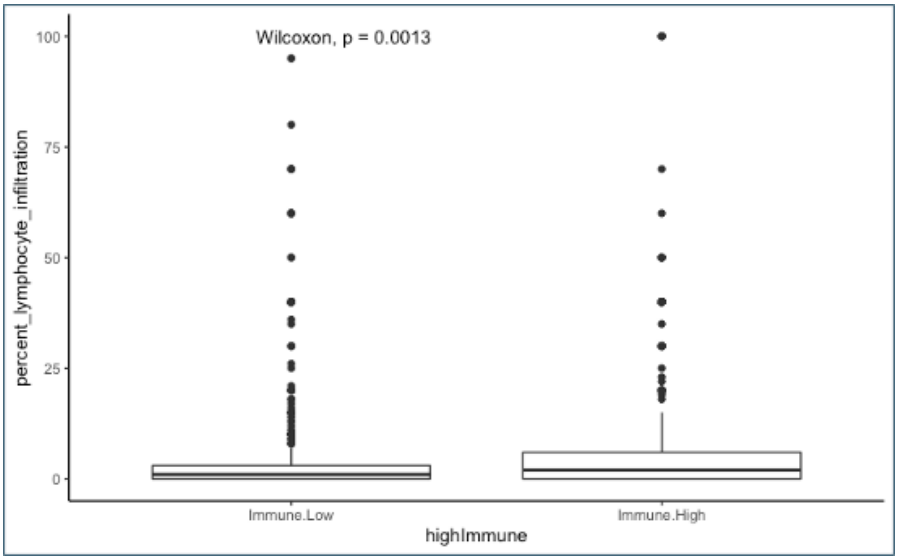


Figure 1. The boxplots depict the percentage of lymphocytes in immune-high and low tumor samples. Lymphocyte concentration was significantly higher in immune high tumors compared to immune low tumors, pval = 0.0013.

Volcano plot of significantly upregulated genes in immune-low phenotype

Depicted is the volcano plot that shows the results from DESeq2, where log2fold change and adjusted pvalue is measured for each gene between the immune phenotype groups. The genes labeled are the ones that cross the threshold of log2 fold change > 2, and adjusted pvalue < 10^{-5}. The genes with a significant negative log2 fold change are upregulated in the immune low phenotype, and genes with positive log2 fold change are upregulated in the immune high phenotype. Some notable genes are SLIT1, PCSK1, and COL2A1. These genes will be further analyzed for function to evaluate what role they could be playing in preventing immune infiltration in the tumors.

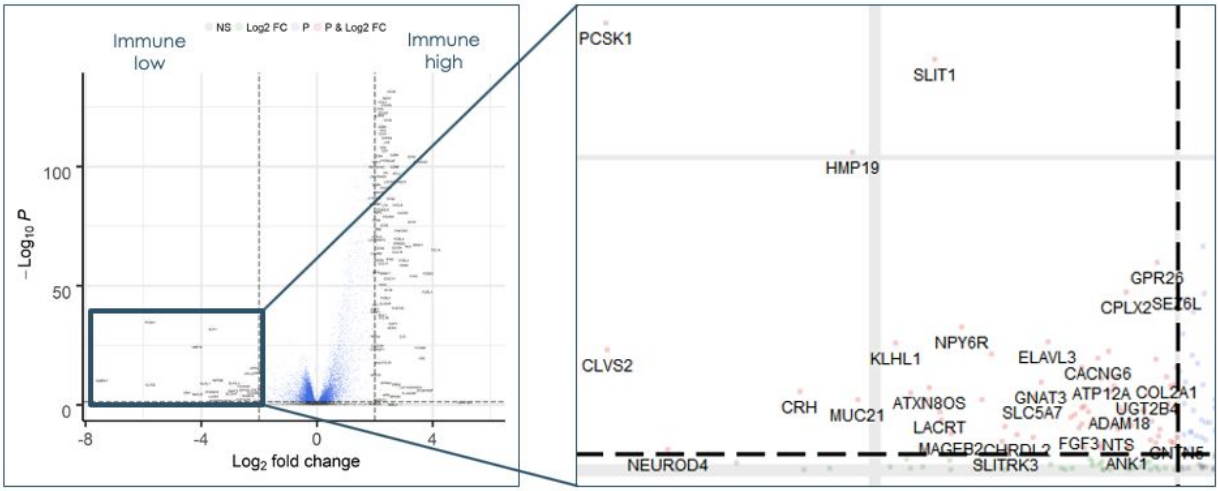


Figure 2. Volcano plot depicts the significantly upregulated genes with log2 fold change > 2 and –log(pvalue) < 50. The image on the right shows the genes upregulated in the immune-low phenotype. Among these genes are SLIT1, PCSK1, and COL2A1.

Extracellular matrix genes are significantly upregulated between immune phenotypes compared to other gene families

The table below shows the results to the Fisher Exact Test. This statistical test was conducted to see if extracellular matrix (ECM) genes as a group were significantly upregulated in immune phenotypes compared to all other differentially expressed genes. The pvalue exceeds the threshold of < 0.001, which suggests this group of genes is significantly upregulated. The ECM genes used for comparison (both DEG and not DEG) were from the ~1000 total genes compiled from the Matrisome Project. This result indicates that ECM genes play a role in differentiating the immune phenotypes, either by blocking entry of immune cells into immune-low tumors, or by promoting mobility of immune cells in the immune-high phenotype.

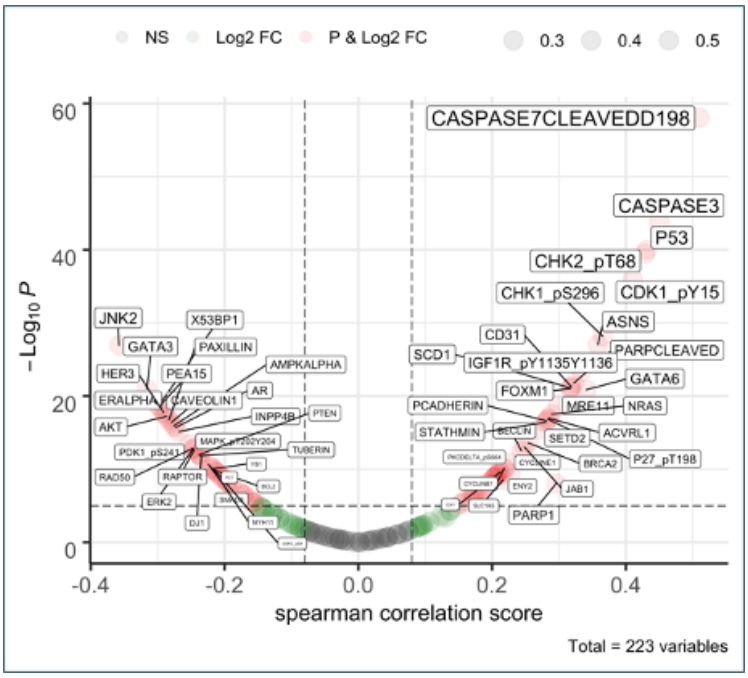
p-value: 5.523e-08	DEG ImH v ImL	Not DEG ImH v ImL
ECM related	4	990
Non-ECM related	218	22128

Figure 3. Of all the differentially expressed genes expressed between immune high and low phenotypes, ECM genes were significantly enriched (Fisher's Exact Test).

Volcano plot of genes correlated with high PD1/PDL1 expression

The volcano plot shows the spearman correlation of genes to PD1/PDL1. The genes that exceed a correlation score > 0.1 and a –log(pvalue) > 10 are labeled to be significantly correlated with PD1/PDL-1. The purpose of this plot is to show the genes that could be working in the same pathway or working in conjunction with PDL1. Caspase genes and P53 are notable correlated genes with increased PD1/PDL-1 expression, which suggests a possible biological link in function for the proteins encoded by these genes.

Figure 4. The volcano plot shows the spearman correlation scores of all genes with the PD1/PDL-1 receptor, where the correlations with a correlation score > 0.1 and –log(pvalue) > 10 are labeled. Highly correlated genes include Caspase7-Cleaved198, Caspase3, and P53.



Discussion

The results indicate that there are differentially expressed genes between immune-low and immune-high tumors that could potentially explain the differences in immune infiltration that are seen phenotypically. Figure 2 shows the potential gene candidates that have possible roles in immune modulation. There were three significantly upregulated genes in the immune-low phenotype: SLIT1, PCSK1, and COL2A1.

SLIT1 and Immune Modulation

SLIT1, or Slit guidance ligand 1, is a signal receptor binding protein, and acts as a molecular guidance cue in cellular migration. SLIT1 and SLIT2 both encode for large secreted proteins that function as ligands for Roundabout (Robo) receptors. These transmembrane receptors regulate axonal guidance and cell migration in the central nervous system. The SLIT-ROBO signaling pathway is implicated in numerous processes such as angiogenesis in endothelial cell migration and immune response through dendritic cell migration. In a paper by Katoh et al., which compares SLIT1, -2, -3 homologs through computational methods, it was additionally found that Mammalian SLIT1 orthologs were identified as part of the WNT/beta-catenin signaling pathway[9]. This signal-transduction pathway directs both cell-cell communication as well as same-cell communication. Although further research currently does not exist on this gene with immune infiltration, it could play a role in the blockage of immune cell migration into the tumor microenvironment.

PCSK1 and Immune Modulation

PCSK1, or Proprotein convertase 1, is thought to be expressed in the secretory pathways of neural and endocrine cells. Its main function is in the post-translational processing and activation of precursor proteins. In a study by Refaie *et al*., they found that disruption of Proprotein Convertase 1/3 (PC1/3) expression in a mouse model (in which PCSK1 was silenced) results in overexpression of the innate immune system, including uncontrolled cytokine secretion. Specifically, Plasma levels of proinflammatory cytokines (IL-6, IL-1β, and TNF-α), which are key components of the immune system, were shown to be significantly elevated in mice with the absence of PCSK1, which indicates the presence of uncontrolled inflammatory response[10]. Based on the results of their in-vivo study, they concluded that the normal function of PCSK1 has an important role in the regulation of the innate immune system, most likely through the regulation of cytokine secretion in macrophages. Therefore, its overexpression in the immune-low phenotype, as shown in this *in-silico* analysis, is synonymous with these findings of decreased immune response in the presence of PCSK1. This protein could play a role in the modulation of immune molecules and the prevention of immune penetration in the tumor, possibly by blocking lymphocytes, cytokines, and macrophages.

COL2A1 and Immune Modulation

Collagen 2A1 upregulation is the most interesting finding from this analysis, as it suggests the role of ECM genes involved in immune blockage. According to Figure 3, there are multiple ECM factors that are differentially expressed in immune phenotypes, although COL2A1 was the most significant out of these genes. As a gene family, collagens have been shown to play a role in dramatic remodeling of the surrounding extracellular matrix leading to the formation of a tumor-specific ECM that causes T-cell prevention. A high-density tumor-specific ECM could reduce the ability of T cells to kill cancer cells by making the environment more collagen fiber-rich and increasing stiffness of the extracellular matrix. A 3D ECM analysis in a cancer tumor was performed by Kuczek *et al*., where they found that overall, T cell (CD4+ and CD8+) proliferation was significantly reduced in a high-density matrix compared to a low-density matrix. In mammary tumors, there is a consistent reduction in the number of infiltrating T-cells with high collagen-density, which indicates that increased collagen has a role in regulating T cell abundance in human breast cancer[11]. This directly relates to the upregulation of COL2A1 in immune-low breast tumors, because of its evident role in preventing immune cells. As suggested by the study, remodeling of the ECM could be the factor that differentiates immune phenotypes, and the main reason why immune-low tumors continue to have little to no infiltration by the immune system.

The second part of this study looked into genes that were significantly correlated with PD1/PDL-1 expression. The caspase family of genes as well as p53 were notable genes that had significantly high spearman correlation scores.

Caspase Family and Immune Evasion

Both Caspase7Cleaved98 and Caspase3 are activated and play a role in induced apoptosis. The caspase family is categorized into two main functions, and Caspase 3 and caspase 7 are part of the apoptotic caspases, which function in the initiation and execution of programmed cell death. They are also classified as inflammatory caspases, which regulate the innate immune system. Two studies have shown that caspases-9, -3 and -7 involved in the intrinsic apoptotic pathway negatively regulate the induction of I-IFNs, a type of immune response, by controlling cGAS and STING signaling. STING signaling is a component of the innate immune system that triggers expression of inflammatory genes as a defense mechanism in response to pathogens. In addition, caspase 3 and 7 are often classified as the "executioner caspases", named for their role in cleaving cytokines in deactivation. This provides an explanation as to why immune-high tumors, which have immune evasion, consist of high expression of caspases — because of their deactivating function. In effort to negate any action of immune cells, similar to PD1/PDL-1, it has been proposed that these caspases have their own mechanism of killing cytokines, a major component of the immune system.

P53 and Immune Evasion

P53 is the most well-known gene implicated in cancer, where its main function lies in the DNA-damage checkpoints in the cell cycle. Additionally, its role is critical in regulating apoptosis, DNA damage and modulating immune response. Recent studies (Tojyo *et al*., Xu *et al.*) have shown the possible correlation between p53 and PD1/PDL-1 expression, and even suggested the possible mechanism by which these two proteins relate, where p53 controls PD1/PDL-1 expression[8,14]. In the in-vitro analysis performed by Tojyo *et al*., their immunohistochemical stainings of oral squamous cell carcinoma tissue revealed similar expressions of p53 and PD1/PDL-1 at the protein level. When quantified, they observed a statistically significant correlation between p53 and PD-L1 expression ($p = 0.0009$), with similar survival patterns in patients (from which the tumor samples belonged to)[14]. Although the true biological mechanism that links these two proteins is currently unknown, there is still evidence of similar expression patterns and similar immune response. This positively relates to Figure 4, which shows significant correlation between p53 and PD1/PDL-1 activity.

Sources of Error and Future Directions

Sources of Error

Some possible sources of error of this study could be due to the level of accuracy of the RNA reads or the biospecimen data used in the study. Any misreported RNA reads could lead to false positive genes upregulated in immune phenotypes. Furthermore, if the biospecimen data were inaccurate, then there is insufficient validation as to whether the immune cell counts in the tumor itself match the gene classification it received. Lastly, expression values across tumors within

each immune phenotype is heterogeneous, and requires further validation to see if expression differences are consistent.Additionally, the immune phenotype groups of immune-low and immune-high were classified with phenotype scores of 1-3 and 4-6 respectively. There is a possibility that using the middle scores of 3 and 4 could have skewed the data, leading to hidden genes that were not significant enough based on log2 fold change and pvalue, but were still differentially expressed between the two (false negatives).

Future Directions

There are multiple ways to expand on and improve the methods of this study. First, instead of using immune scores of 1-3 as immune-low and 4-6 as immune-high sample groups, taking the extreme groups, of scores 1-2 as immune-low and 5-6 as immune-high, may provide with more consistent results of differentially expressed genes between phenotypic groups. In addition, this study focused on immune classification based on gene signature. However, classifications can also be in transcriptome, epigenomic, and infiltration data. These additional categories could be used in conjunction with immune classification to view more concrete expression differences. Last, a wet lab confirmation is needed of this in-silico analysis to view protein expression of shortlisted upregulated genes in immune low phenotypes, as well as the genes hypothesized to work with PD1/PDL-1 in the biological mechanism. Protein expression can be viewed through immunohistochemistry staining, trichrome staining (for collagen proteins), or gel electrophoresis.

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