Impact of PTP4A3 Expression on Ovarian Cancer Cell Migration

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Abstract

Overexpression of protein tyrosine phosphatase 4A3 is common in many cancers and often indicates poor prognosis and survival. Knockdown of PTP4A3 has hindered ovarian cancer migration, and overexpression has shown increased migration capabilities. PTP4A3 knockdown was also associated with the overexpression of several extracellular matrix proteins in murine colorectal tumor cells. We targeted PTP4A3 to better develop targeted therapies for cancers with overexpression of this protein. This study chose to focus on an ovarian cancer cell line because ovarian tumor cells in particular frequently migrate within the peritoneal cavity, and a majority of diagnoses present themselves at advanced stages. The link between PTP4A3 overexpression and enhanced migration has not been established since the substrate and functional pathway of PTP4A3 in tumor progression and migration are not established. To identify correlations between PTP4A3 and extracellular matrix proteins, we investigated cancer genomics data from both patient samples and cell lines using publicly available databases cBioPortal and CellMinerCDB. We found some evidence to indicate in patient samples that PTP4A3 was mutually exclusive to FBLN1. Increasing copy number of PTP4A3 also corresponded with increased mRNA expression levels. Finally, the highest mRNA Pearson correlation to PTP4A3 occurred in CNNM3, indicating a potential interaction and influence on migration. Our plans for this semester were to establish several OVCAR4 cell lines with varying expression of PTP4A3 and phenotypically measure their migration characteristics while also visualizing the quantity and localization of PTP4A3 using fluorescently labeled protein tags. While these experiments could not be carried out, the primary database collection can hopefully be validated in the future with *in vitro* mRNA and protein quantification as well as fluorescence images to determine localization.

Introduction

Cancer is the unregulated proliferation of cells in a part of the body. These cells, more so than non-neoplastic cells, migrate to and invade other tissues where metastatic growth can occur[1]. Metastasis normally occurs by tumorigenic cells entering the bloodstream or lymphatic system, traveling to a distant part of the body, and attaching themselves to other tissues. Metastasis accounts for 90% of deaths from solid tumors[2]. Ovarian carcinoma, in addition to migrating through pelvic lymph nodes, can also metastasize through direct extension from the tumor to the bladder or colon or by cell clusters breaking away[3]. In 2020, there will be an estimated 22,000 cases of ovarian cancer and 14,000 deaths[4]. This high death rate highlights the severity of ovarian cancer and how important it is to determine new drug targets.

One example of a widely researched drug target are protein tyrosine kinases, enzymes that catalyze the addition of a phosphate group to molecules. Phosphatases, which have the opposite function, are now emerging as potential oncogenic drug targets. Phosphatases were originally thought to be undruggable because researchers were focusing on its active sites. Active site inhibitors for phosphatases are generally difficult to create because they must have a negative charge to mimic the substrate and have a high affinity to the catalytic site of phosphatases. However, negatively charged molecules are difficult to transport through the plasma membrane of cells[5]. Studies have identified Protein Tyrosine Phosphatase 4A3 (PTP4A3) as a key protein of interest. First, PTP4A3 is overexpressed in many cancers including ovarian[6]. Second, genetic suppression or deletion of PTP4A3 hinders cancer cell migration. This may be because PTP4A3 is localized to the cell membrane via C-terminal prenylation. If localization to the membrane is important to this protein's function, it is reasonable to hypothesize that PTP4A3 may be involved with the extracellular matrix (ECM) protein production and secretion which are

thought to be important for cancer cell metastasis[7]. The degree of influence PTP4A3 has on cell migration is currently not fully established.

Originally, this study focused on how varying levels of PTP4A3 protein expression would affect overall ovarian cancer cell migration, but due to unfortunate circumstances, a bioinformatics approach was adopted to supplement the original research question. Data mining was done to understand the genomic and mRNA expression of various ECM and secretome proteins would be influenced by PTP4A3 mRNA expression to indirectly analyze protein expression on cell migration.

Results

cBioPortal

While cBioportal contains a significant amount of data, we chose to focus primarily on genomics and mRNA expression to predict protein expression to the best of our abilities. Upon querying our desired genes, there was more information on DNA mutations and expression compared to mRNA expression. Both mutations and changes in copy number were reported for all queried genes for a majority of patients. For PTP4A1, 1.5-2% of patients had alterations, a majority of those being amplifications and a minority being deep deletions (Fig. 1). For PTP4A2, 3-6% of patients had alterations, again, a majority being amplifications. In the PanCancer Atlas study, only two patients had a

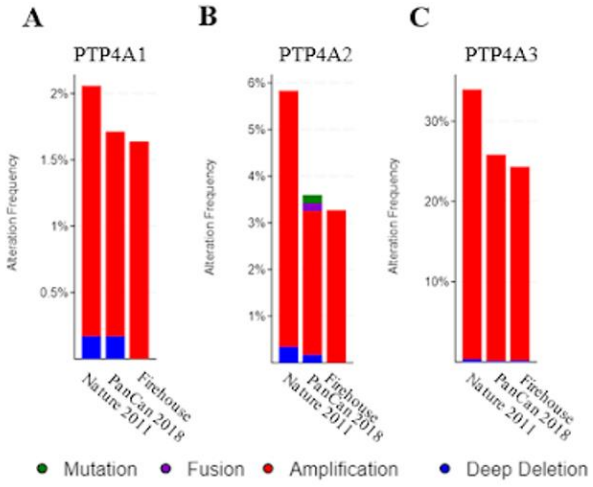

Fig. 1. Alteration frequency of PTP4A Genes by Study. A) Alteration frequency of PTP4A1, B) PTP4A2, and C) PTP4A3. PTP4A3 is more frequently altered than its homologous proteins. Very few mutations occur, most often there is amplification.

mutated form of PTP4A2, one truncating mutation and one fusion. Most significantly, PTP4A3 was amplified in about 25-34% of patients. There was also a lack of mutations in PTP4A3.

Five extracellular matrix genes were investigated alongside the PTP4A proteins for notable alterations in mutation and copy number as well. Those selected were EMILIN1, FBLN1, FBLN5, IGFBP2, and MMP2. Previous correlations between EMILIN1 and PTP4A3 have been published in a murine colorectal model[8]. The other genes were identified from a published ovarian secretome[9]. If PTP4A3 was involved, there could be a correlation between its overexpression and the underexpression of these ECM proteins. However, since MMP2 encodes for type IV collagenase, that would be overexpressed. We found deep deletions with FLBN1; the other genes were amplified, which was the opposite of what was expected. EMILIN1, FBLN5, and IGFBP2 were primarily amplified in ovarian carcinoma patients ranging from 1-3% (Fig. 2).

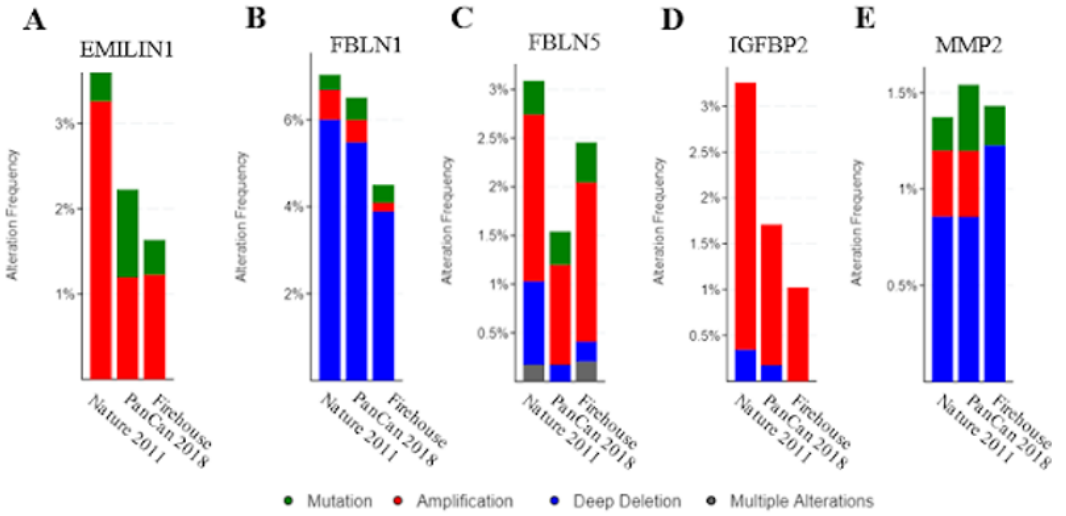

Fig. 2. Alteration Frequency of Ovarian Carcinoma Secretome Extracellular Matrix Proteins by Study. Alteration frequencies of A) EMILIN1, B) FBLN1, C) FBLN5, D) IGFBP2, and E) MMP2. These data account for genetic mutations and copy number alterations.

MMP2 was deeply deleted in less than 1% of cases in all three studies. FBLN not only followed the trend we expected, but was the most frequently altered in 4-6% of cases. More mutations were observed in the ECM genes, but they constitute a very small fraction of alterations.

Next, we investigated the mRNA expression of PTP4A3 to determine if the copy number alteration (CNA) is representative of protein expression. Two of the studies provided mRNA expression levels for PTP4A3, Nature and Firehouse Legacy. When normalized to nearby healthy tissue of the ovary, the z-score plotted on a logarithmic scale is indicative of gains or losses of mRNA. When plotted against increasing CNA, there is an increasing trend of mRNA as well (Fig. 3). This suggests CNA and mRNA do have a direct relationship, but since cBioPortal does not report any protein expression data, protein expression cannot be approximated. Ideally, mRNA expression would reinforce the role of PTP4A3 in increased metastasis, but that cannot be assumed here.

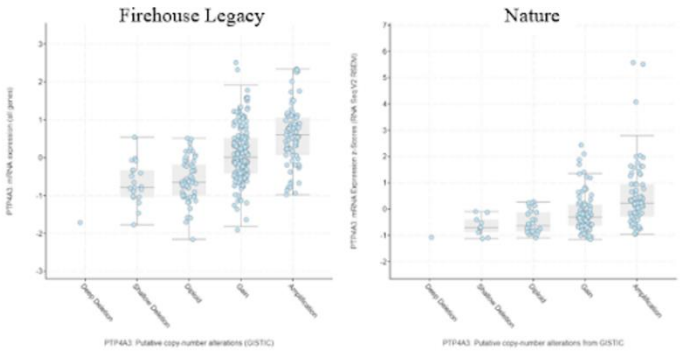

Fig. 3. mRNA Expression of PTP4A3 in Ovarian Carcinoma. Fire house Legacy and Nature studies measured mRNA z-score values against copy number alterations. As copy number increases, mRNA expression increases.

Mutual exclusivity is a tool used to predict the relationship between genes. If two are mutually exclusive, it means that one gene is altered without the other, and changes in that gene's expression are due to the change in the first gene rather than random mutations; this might indicate two proteins being involved in the same functional pathway. On the other hand, genes that exhibit co-occurrence often mutate simultaneously. These calculations were done using Equation 1 and the incidences of alterations in the genes of PTP4A3 and the selected ECM genes

A	B	Neither	A Not B	B Not A	Both	Log_2 Odds Ratio	p-Value	q-Value	Tendency
PTP4A3	EMILIN1	807	306	16	9	0.569	0.233	0.698	Co-occurrence
PTP4A3	MMP2	813	313	10	2	-0.945	0.311	0.698	Mutual exclusivity
PTP4A3	IGFBP2	803	306	20	9	0.240	0.410	0.698	Co-occurrence
PTP4A3	FBLN1	769	296	54	19	-0.130	0.431	0.698	Mutual exclusivity
PTP4A3	FBLN5	806	308	17	7	0.108	0.513	0.699	Co-occurrence

Table 1. Mutual Exclusivity of PTP4A3 with Ovarian Carcinoma Secretome Extracellular Matrix Proteins. Two proteins, MMP2 and FBLN1, are predicted to be mutually exclusive, but the data is not statistically significant with an $\alpha = 0.05$.

(Table 1). Two of the five pairings suggested mutual exclusivity, yet with a statistical significance of p = 0.05, these data did not reach our defined level of statistical significance. That is not to say they are incorrect; this merely suggests that further experiments in addition to genomics studies are needed to solidify or disprove these relationships.

CellMinerCDB

Unlike cBioPortal, CellMinerCDB does not give as much information because the database contains small scale datasets and they are solely cell data instead of data from patient studies. Only mRNA expression data was collected from each of the four cell lines sets for 14 different extracellular matrix and secretome proteins, three of which were from the PTP4A family.

Emilin1 was one of the investigated proteins and is known to have a correlation with PTP4A3. As PTP4A3 protein expression increases, Emilin1 protein expression also seems to increase. However, when analyzing the mRNA expression data from CellMinerCDB, the Pearson correlation coefficient for the two proteins was 0.16, which is an extremely low correlation, when research has shown the opposite (Fig. 4). A statistical Pearson Correlation test yielded a p-value of 0.08075, which is greater than $\alpha = 0.05$, showing insignificance. This was the case with a majority of the proteins when compared with PTP4A3.

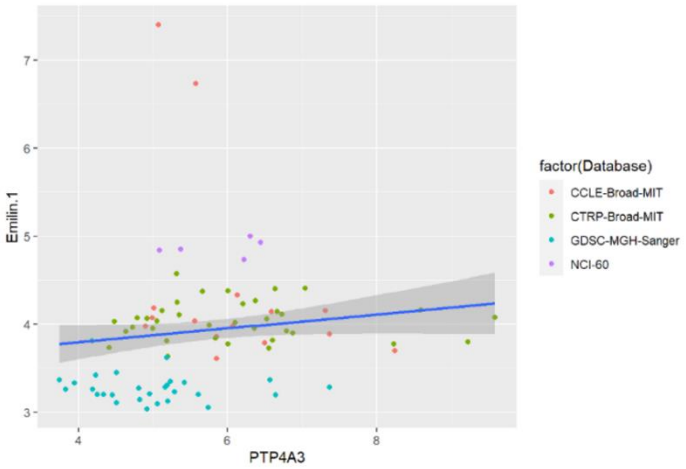

Fig. 5. mRNA Expression of Emilin1 and PTP4A3. Scatter plot of the Emilin1 and PTP4A3 mRNA expression of ovarian epithelial tumor samples from four datasets. A linear regression line shows the correlation between the two proteins.

	PTP4A3	
	Pearson Correlation	P-value
Emilin1	0.1600	0.0807
ECM1	0.0834	0.3648
Fibulin1	0.2605	0.0040
LTBP2	0.1472	0.1085
EZR	0.0252	0.7843
COL6A3	0.0845	0.3584
COL6A2	0.2867	0.0015
CNNM1	0.2279	0.0123
CNNM2	0.3108	0.0005
CNNM3	0.4160	2.294E-6
CSF1	0.2366	0.0092

Table 2. Pearson Correlation and p-Values of ECM Proteins Compared to PTP4A3. The CNNM family has the highest correlation and is statistically significant compared to the other ECM and secretome proteins.

However, of the proteins analyzed, the CNNM protein family gave a surprising result. All three of the proteins had some of the highest Pearson correlation values with PTP4A3 and all had significant p-values that were less than an alpha value of 0.05. CNNM3, specifically, had the highest correlation value of 0.416 (table 2).

CNNM3 is a transmembrane protein that is a magnesium ion transporter. Magnesium is extremely important in cell metabolism and migration. It is also key in influencing key cell adhesion proteins. CNNM3 and PTP4A2 create a complex that when inhibit ed decreases breast cancer proliferation and tumor growth[10]. Although this study looked specifically at PTP4A2, the data shows that PTP4A3 might also be involved with CNNM3 and is an interaction that should be further investigated.

Discussion

Using cBioPortal and CellMinerCDB as sources of genomic and mRNA data, we investigated the connection between PTP4A3 and various ECM proteins to determine if any were in the same functional pathway. The expectation was to see deletions of the selected ECM genes, but rather there were amplifications. FBLN1 was the one gene that exhibited deep deletions. Also, while not statistically significant, mutually exclusive tendencies exist between the two genes. Further research into the relationship between PTP4A3 and FBLN1 must be done.

The mRNA expressions of multiple ECM and secretome proteins were analyzed in the hopes of finding a correlation between them and PTP4A3. However, most of the correlations were low and were not statistically significantly different indicating that mRNA expression of these proteins had little influence on PTP4A3. The CNNM family had the highest correlations and were statistically significant. The data showed that in terms of mRNA expression there was a relationship between CNNM3 and PTP4A3, but further research must be done to understand the protein expression relationship between the two proteins.

The results of our bioinformatics results demonstrate that PTP4A3 is often amplified and its mRNA expression is increased in human ovarian cancer. We know, however, that mRNA expression does not always accurately reflect protein levels[11]. The cause of PTP4A3 amplification is unknown, but we believe likely contributes to increased migration of ovarian tumor cells. PTP4A3, also known as PRL-3, has been implicated in metastasis as early as 2003 in colorectal models[12,13]. The history of research done on this protein suggests that it is a valuable predictive marker indicating prognosis of disease and likelihood of metastasis[14].

Several groups, including the Fiske Drug Discovery Laboratory, are focusing efforts on small molecule allosteric inhibitors of PTP4A3. One that has been of note has been JMS-053, a novel iminothienopyridone[15]. It is potent, selective, and reversible, but its exact mechanism of inhibition is currently under investigation. By better understanding how PTP4A3 interacts with its substrates, more knowledge can be gained to better develop not only inhibitors for PTP4A3, but all phosphatase inhibitors.

Materials and Methods

Bioinformatics

Two database systems were utilized to mine cancer cell data from, cBioPortal and CellMineerCDB. Both of these databases have multiple types of expression information for various types of cancers. cBioPortal is a cancer genomics database containing over 200 large-scale patient studies. It allows the user to visualize gene alterations in single and multiple cancer patient studies. In order to determine expression of extracellular matrix and secretome proteins, three cancer studies were used totaling 1680 samples. CellMinerCDB is a genomic and pharmacological analysis of six cancer cell sets. It was used to visualize mRNA expression of extracellular matrix and secretome proteins as an indirect measure of their protein expression. Four cell line sets were used, totalling to 120 ovarian epithelial tumor cell samples. Table 3 lists the studies and cell line sets used from cBioPortal and CellMinerCDB, respectively. It also lists the number of samples taken from each study or set.

cBioPortal		CellMinerCDB	
Cancer Genomics Study	Number of Samples	Cell Line Set	Number of Samples
TCGA, Firehouse Legacy	606	NCI-60	5
TCGA, Nature 2011	489	CCLE-Broad-MIT	51
TCGA, PanCancer Atlas	585	GDSC-MGH-Sanger	28
		CTRP-Broad-MIT	36

Table 3. Summary of Studies and Samples Analyzed from Both Databases. The cBioPortal analysis used three large-scale patient studies with a total of 1680 samples. The CellMinerCDB analysis used four small-scale cell line sets with 120 ovarian epithelial tumor samples.

cBioPortal Analysis

Data from three serous ovarian cancer studies were used to generate data, those being Firehouse Legacy, Nature 2011, and PanCancer Atlas. Queried genes included *Ptp4a3* and five

extracellular matrix proteins that were reported to be a part of the ovarian serous carcinoma: *Emilin1, Fbln1, Fbln5, Igfbp2, and Mmp2.* Genetic mutations, copy number alterations, and mRNA expression levels were used. Genetic mutation and copy number alteration data from all three studies was combined and plotted on a percentage scale for each gene. mRNA expression levels were plotted based on CNA and box-and-whisker plots were generated. All mRNA data measured using RNASeq V2, normalized to healthy ovarian tissue for each patient, and plotted on a $\log_2$ scale. Mutual exclusivity comparisons between two genes were calculated using the following equation:

$$OR = (A * D)/(B * C)$$

where OR is the odds ratio, A is the number of cases altered in both genes, B is the number of cases altered in G1 but not G2, C is the number of cases altered in G2 but not G1, and D is the number of cases altered in neither gene. Statistical significance for these values is set at $\alpha = 0.05$[16].

CellMinerCDB Analysis

The mRNA expression using a log2 scale for each protein listed in Table 2 was found using the univariate analysis option for each cell line set. These proteins were chosen based on a paper that analyzed the secretome of ovarian serous carcinoma which highlighted these specific proteins[9]. The data set only included samples that were from ovarian epithelial tumors which totaled to 120 samples. The expression data was downloaded into excel and then used in Rstudio to compare the mRNA expression levels of the different ECM proteins with all three proteins from the PTP4A family. Correlation was calculated using the Pearson Correlation coefficient to determine if the mRNA expression levels had any relationship to each other.

In vitro techniques

The following techniques would have been used to understand how varying PTP4A3 expression levels influences overall ovarian cancer cell migration. It would be a more direct approach than focusing on PTP4A3 influence on individual proteins. These techniques were started but could not be completed due to unforeseen circumstances. These techniques can be used to confirm the data mining results in the future.

Cell Culture Techniques

OVCAR4 cells were obtained from Charles River Laboratories (New York, NY) and were cultured in RPMI Medium 1640 with 10% fetal bovine serum (FBS). Cells were passed fewer than 20 times to maintain desired PTP4A3 expression levels[17].

Bacterial Plasmid Amplification and Purification

The GFP-PTP4A3 plasmid used contained GFP attached to PTP4A3 in order to visualize the presence of PTP4A3 under a fluorescence microscope. The plasmid also contains an ampicillin resistance gene which will give ampicillin resistance to bacteria that take up the plasmid and kill the ones that do not. The plasmid was mixed with the DH5a bacteria, streaked onto an agar plate with ampicillin, and incubated overnight. The next day, 5 mL of agar and ampicillin were put into a snap cap tube and bacterial cells from the plate were placed into the tube for 8 hours. Then 200 uL of the agar-bacteria solution in the snap cap tube was placed into 200 mL of more agar-ampicillin solution in a flask, which was incubated overnight. The Qiagen Plasmid Maxi Kit was used to purify the plasmid DNA and purify it for nucleofection. The manufacturer's instructions were strictly adhered to for purification.

Nucleofection

The nucleofection was done using the 4D-Nucleofector System. However, the system did not already have a protocol in place for OVCAR4 cells, so the protocol had to be optimized for those specific cells. Each sample had one million cells and four Nucleofector system protocols were tested, SKOV-3, HEK-293, SH-SY5Y, and MDA-MB-453. The amount of plasmid was also varied between 2.5ug and 5 ug and a control vector was used for each system protocol. After the nucleofection, the cells were transferred to fresh media and allowed to incubate in order for the cells to heal and grow. Although a large majority of the cells died, the SKOV-3 protocol was determined to be the best for nucleofecting the OVCAR4 cells.

Transwell Migration Protocol

OVCAR4 cells were cultured in FBS-containing RPMI media until 60% confluency in T75 flasks. Cells were removed from the plate with 0.25% trypsin, counted using celltiter blue in a hemocytometer, and diluted in FBS-containing media. They were centrifuged in 1 mL quantities for five minutes at 10,000 g, the supernatant was removed, and the cells were resuspended in FBS-free media. In a 24-well plate, 100 µL of the cell solution was seeded on top of each 8 µm Transwell insert porous membrane and incubated for 10 minutes. Following incubation, 600 µL of FBS-supplemented media was added into the bottom chamber of each well. The cells were left to incubate at 37℃ with 5% CO_2 for 48 hours[18]. Since OVCAR4 are adherent, the migrated cells remained on the bottom of the membrane. Each membrane was fixed with 70% ethanol for 10 minutes and allowed to fry for 10-15 minutes.

Cells were stained with 600 µL of 0.2% crystal violet in methanol for 5-10 minutes and left to dry[19]. The membranes were to be carefully removed, placed on slides, and imaged under the light microscope. Cells were to be counted in three representative areas of the membrane,

averaged, and multiplied by the area of the membrane to obtain representative cell counts for each cell line. Cells were stained with 600 µL of 0.2% crystal violet in methanol for 5-10 minutes and left to dry. The membranes were to be carefully removed, placed on slides, and imaged under a light microscope. Cells were to be counted in three representative regions of the membrane, averaged, and multiplied by the area to estimate the total cell count.

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