1 **Down-regulation of protein phosphatases PPM1K and PPP1R3C in high-grade serous**
2 **ovarian cancers.**

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6 Ovarian cancer is the most lethal gynecologic cancer¹. We sought to identify genes associated
7 with high-grade serous ovarian cancer (HGSC) by comparing global gene expression profiles of
8 normal ovary with that of primary tumors from women diagnosed with HGSC using published
9 microarray data^{2,3}. We found significant differential expression of the genes encoding protein
10 phosphatases PPM1K and PPP1R3C in high-grade serous ovarian tumors.

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24 **Keywords:** ovarian cancer, high-grade serous ovarian cancer, HGSC, targeted therapeutics in
25 ovarian cancer, systems biology of ovarian cancer, PPM1K, PPP1R3C.
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1 The five-year survival rate for women diagnosed with high-grade serous ovarian cancer
2 is between 30-40% and has not changed significantly in decades^{6,7}. The development of novel,
3 targeted therapeutics to treat HGSC can be facilitated by an enhanced understanding of the
4 transcriptional behavior of ovarian tumors relative to that of the normal ovary. We mined
5 published microarray data^{2,3} to compare global gene expression profiles between HGSC ovarian
6 tumors and that of normal ovarian tissue. We identified the genes protein phosphatases
7 PPM1K and PPP1R3C as among the most differentially expressed in HGSC tumors of the
8 ovary. PPM1K and PPP1R3C may be genes of interest when prioritizing the study of target
9 genes and pathways for the development of novel therapeutic interventions in high-grade
10 serous ovarian cancers.
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12 **Methods**

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14 We used microarray data from datasets GSE124766² and GSE146556³ for this
15 differential gene expression analysis of high-grade serous ovarian carcinomas. The Benjamini
16 and Hochberg method of p-value adjustment was used for ranking of differential expression but
17 raw p-values were used for assessment of statistical significance of global differential
18 expression. Log-transformation of data was auto-detected, and the NCBI generated category of
19 platform annotation was used. GSE124766² was generated using Agilent-014850 Whole
20 Human Genome Microarray 4x44K G4112F with n=3 of for normal ovarian tissue and n=8 for
21 tumors from patients with high-grade serous ovarian cancer. GSE146556³ was generated using
22 Affymetrix Human Gene 1.0 ST Array with n=3 for normal ovarian tissue and n=40 for tumors
23 from patients with high-grade serous ovarian cancer. GEO2R provides mRNA expression levels
24 only for the top 250 most differentially expressed genes.
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26 A statistical test was performed to evaluate whether PPM1K or PPP1R3C expression
27 was significantly different when comparing normal ovarian tissue from control subjects and
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primary tumors from women diagnosed with HGSC using a two-tailed, unpaired t-test with Welch's correction. Only p-values less than 0.05 were considered statistically significant. We used PRISM for all statistical analyses (Version 8.4.0)(455).

Results

We mined published microarray data^{2,3} to identify differentially expressed genes in high-grade serous ovarian cancer (HGSC), the type of ovarian cancer responsible for 70-80% of deaths resulting from the most lethal gynecologic malignancy.

PPM1K is differentially expressed in ovarian tumors from women diagnosed with HGSC.

We identified PPM1K as among the genes whose expression was most different when comparing primary HGSC tumors to normal ovarian tissue² (Table 1). When sorting all of the transcripts measured by microarray based on change in expression between HGSC and the normal ovary, PPM1K ranked 105 out of 41093 total transcripts (Table 1). Differential expression of PPM1K in HGSC tumors was statistically significant (Table 1; $p=2.32E-06$).

We analyzed a second microarray dataset³ generated using normal ovarian tissues and tumors from women diagnosed with HGSC to determine whether differential expression of PPM1K could be observed in tumors from a separate group of patients. We again found that PPM1K was differentially expressed in primary HGSC tumors (Table 1). When sorting all of the transcripts measured by microarray based on change in expression between HGSC and the normal ovary, in this data set, PPM1K ranked 343 out of 29088 total transcripts (Table 1). Differential expression of PPM1K in HGSC tumors in this second microarray dataset was statistically significant (Table 1; $p=1.8E-06$).

PPM1K is expressed at significantly lower levels in HGSC when compared to the normal ovary.

We obtained exact mRNA expression levels for the differentially expressed PPM1K transcript from both normal ovarian tissue and from high-grade serous ovarian tumors. PPM1K

was expressed at significantly lower levels in high-grade serous ovarian cancers when compared to the normal ovary (Figure 1; $p=0.0023$). We calculated a mean fold change of 0.7136 ± 0.0526 in PPM1K expression when comparing HGSC tumors to normal ovarian tissue (Table 1).

PPP1R3C is differentially expressed in ovarian tumors from women diagnosed with HGSC.

Our analyses revealed that a second protein phosphatase, PPP1R3C, was among the genes whose expression was most different when comparing primary HGSC tumors to normal ovarian tissue² (Table 2). When sorting all of the transcripts measured by microarray based on change in expression between HGSC and the normal ovary, PPP1R3C ranked 53 out of 41093 total transcripts (Table 2). Differential expression of PPP1R3C in HGSC tumors was statistically significant (Table 2; $p=4.84E-07$).

We analyzed a second microarray dataset³ generated using normal ovarian tissues and tumors from women diagnosed with HGSC to determine whether differential expression of PPP1R3C could be observed in tumors from a separate group of patients. We again found that PPP1R3C was differentially expressed in primary HGSC tumors (Table 2). When sorting all of the transcripts measured by microarray based on change in expression between HGSC and the normal ovary, in this data set, PPP1R3C ranked 1702 out of 29088 total transcripts (Table 2). Differential expression of PPP1R3C in HGSC tumors in this second microarray dataset was statistically significant (Table 2; $p=5.51E-04$).

PPP1R3C is expressed at significantly lower levels in HGSC when compared to the normal ovary.

We obtained exact mRNA expression levels for the differentially expressed PPP1R3C transcript from both normal ovarian tissue and from high-grade serous ovarian tumors. PPP1R3C was expressed at significantly lower levels in high-grade serous ovarian cancers

when compared to the normal ovary (Figure 2; $p=0.0016$). We calculated a mean fold change of 0.6173 ± 0.0612 in PPP1R3C expression when comparing HGSC tumors to normal ovarian tissue (Table 2).

Thus, we found using published microarray data that PPM1K and PPP1R3C were among the genes most differentially expressed in tumors from women with high-grade serous carcinomas and that the expression of both PPM1K and PPP1R3C was significantly lower in HGSC tumors when compared to the normal ovary.

Discussion

Transcriptional profiling of HGSC in a Cancer Genome Atlas integrated genomic analysis classified HGSC into four subtypes based on gene clustering: immunoreactive, differentiated, proliferative, and mesenchymal⁶. We sought to continue to describe the transcriptional landscape of high-grade serous ovarian cancers⁶ and identify genes whose differential expression was associated with HGSC by using published microarray data from primary tumors of women diagnosed with HGSC compared to transcriptome data from normal ovarian tissue^{2,3}. In both datasets analyzed, PPM1K and PPP1R3C were among the genes whose expression changed most significantly when comparing the normal ovary to primary HGSC tumors.

BCAA (branched chain amino acids) are critical for metabolic function. BCAA levels can be regulated by the BCKDH (branched-chain α -keto acid dehydrogenase complex). Experiments showed PPM1K/PP2Cm is the endogenous phosphatase for the BCKDH; PP2Cm could interact with the BCKDH and in the presence of BCKDH substrates, dephosphorylate Ser293⁷. An alternative substrate of the BCKDH is ATP-citrate lyase (ACL). Over-expression of the kinase that antagonizes PPM1K function, BDK, results in increased ACL phosphorylation and *de novo* lipogenesis. Over-expression of PPM1K in the livers of Zucker fatty rats by tail vein administration of recombinant adenovirus resulted in decreases in phosphorylation at

Ser293 of BCKDH, as well as significant decreases in hepatic TG content and decreased glucose excursion during an i.p. glucose tolerance test⁸.

Genome sequencing of a patient with a mild variant of maple syrup urine disease, a disorder resulting from defects in the branched-chain α -keto acid dehydrogenase complex (BCKDH), revealed a mutation in PPM1K. The mutation c.417_418delTA, was predicted to result in translation of a truncated protein due to generate of a premature stop codon; while mRNA was detectable by quantitative real-time PCR, there was no protein detectable by Western blot, leading authors to conclude the mutation resulted in the production of a truncated, non-functional and unstable protein. Rescue experiments expressing wild-type PPM1K restored 35% of wild-type BCKDH activity⁹.

In INS1 β -cell culture, increasing the concentration of glucose cells resulted in a decrease in PPM1K gene expression, and elevated ChREBP (carbohydrate response element binding protein) activity, an important molecule in controlling gene expression changes in response to carbohydrate availability¹⁰. Importantly, over-expression of PPM1K in INS-1 cells with recombinant adenovirus significantly increased β -cell proliferation¹⁰.

A study identifying genetic variants associated with Type II diabetes (T2D) by measuring insulin response to glucose and glycemic control (HbA1c) and performing gene expression microarray and genome-wide association studies (GWAS) in the same individuals found that PPM1K was among the top 20 genes associated with T2D. They used correlation of gene expression in islets with secretion of insulin, correlation of gene expression with HbA1c, SNPs within a 50kb region of the gene associated with T2D, differentiation expression in pancreatic islets from diabetic and non diabetic donors, and computational analysis of differentiation expression in pancreatic beta cells from diabetics and non-diabetics to make these associations¹¹.

PPM1K could promote metabolism of branched-chain amino acids (BCAA) in hematopoietic stem cells (HSC), maintaining p21 and MEIS levels through control of CDC20.

1 Treatment of HEK293T with BCAA resulted in a significant increase in CDC20 levels. In normal
2 HSC, loss of PPM1K impaired glycolysis, resulted in significant increases in BCAA in long-term
3 HSC, and the repopulation capacity of PPM1K deficient HSC was severely impaired upon serial
4 transplantation¹². In the MLL-AF9-induced mouse model of acute myeloid leukemia, deletion of
5 PPM1K resulted in a significant decrease in Lin⁻ IL7-R⁻ cKit⁺ CD34⁺ FcγR⁺ L-GMP cells,
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7 decreases in protein levels of Meis1 and p21, and prolonged survival¹². ATP content,
8 extracellular acidification (ECAR) and oxygen consumption (OCAR) were are reduced in
9 PPM1K ^{-/-} Mac-1⁺ c-Kit⁺ leukemia initiating cells, demonstrating that glycolytic flux as a system
10 was impaired in PPM1K null cells¹².

11 Interestingly, PPP1R3C is another phosphatase important for regulation of glucose and
12 glycogen levels. PPP1R3C depletion corrects hyperglycemic defects found in the db/db mouse
13 model of Type 2 diabetes¹³. Over-expression of PPP1R3C in the mouse liver supported hepatic
14 glucose production and expression of gluconeogenic genes, and silencing PPP1R3C in the
15 livers of wild-type and db/db mice resulted in significant decreases in blood glucose and hepatic
16 glucose production¹³.

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18 PPP1R3C deficient mice manifest general enhanced whole body sensitivity to insulin,
19 characterized by increased whole body glucose turnover and glycolysis. Male but not female
20 PPP1R3C null mice are more sensitive to insulin tolerance test¹⁴.

21 Another context in which PPP1R3C has been identified as important is in hypoxic
22 conditions. In MCF7 cells, hypoxia leads to induction of PPP1R3C gene expression;
23 investigators identified a hypoxia response element 229bp upstream of the PPP1R3C gene.
24 Concomitant with PPP1R3C induction was significant accumulation of glycogen¹⁵. Depletion of
25 HIF1α or PPP1R3C lessened accumulation of glycogen induced by hypoxia¹⁵.

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27 Literature describing a role for PPP1R3C in human cancers is limited. Changes in
28 methylation of the promoter of PPP1R3C are found in colorectal cancer and in melanomas.
Promoter sequences at PPP1R3C were methylated in 81% (97/120) of plasma samples from

1 colorectal cancer patients but in 19% (18/96) of plasma from patients without cancer¹⁶. A
2 PPP1R3C promoter CpG island is hypermethylated in 47% of primary melanomas, but in 0% of
3 common nevus and dysplastic nevus¹⁷. Thus, while DNA methylation at regulatory sequences
4 in the PPP1R3C gene are altered in colorectal cancer and in melanomas, and while PPM1K is
5 important for progression and survival in MLL-AF9 induced murine acute myeloid leukemia, we
6 could not identify any literature describing a role for PPM1K or PPP1R3C in ovarian cancers.
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8 Differential expression of PPM1K and PPP1R3C in HGSC should be validated in larger
9 and separate cohorts of women with HGSC. If validated, mouse models of high-grade serous
10 ovarian cancers¹⁸⁻²⁰ can be utilized to assess the effect of PPM1K conditional expression on
11 HGSC tumor growth post-development and on overall survival *in vivo*.
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Rank	ID	p-value	t	B	FC	Gene	Gene name
105	A_24_P586264	2.32E-06	-8.4990363	5.26829	0.7136 ± 0.0526	PPM1K	protein phosphatase, Mg2+/Mn2+ dependent 1K
343	8101701	1.8E-06	-5.4969435	4.9307332		PPM1K/// PPM1K	protein phosphatase, Mg2+/Mn2+ dependent 1K

Table 1: PPM1K is differentially expressed in high-grade serous ovarian carcinomas.

The rank of differential expression, the probe/transcript ID, the p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, the fold change of PPM1K in HGSC tumors when compared to the normal ovary, the gene and gene name are listed in this chart. Data in this table is from GSE124766 (probe ID A_24_P586264) and GSE146553 (probe/transcript ID 8101701).

Rank	ID	p-value	t	B	FC	Gene	Gene name
53	A_23_P35414	4.84E-07	-9.877917	6.72913	0.6173 ± 0.0612	PPP1R3C	protein phosphatase 1 regulatory subunit 3C
1702	7934997	5.51E-04	-3.7235229	-0.5126153		PPP1R3C	protein phosphatase 1 regulatory subunit 3C

Table 2: PPP1R3C is differentially expressed in high-grade serous ovarian carcinomas.

The rank of differential expression, the probe/transcript ID, the p-value with respect to global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups compared, the fold change of PPM1K in HGSC tumors when compared to the normal ovary, the gene and gene name are listed in this chart. Data in this table is from GSE124766 (probe ID A_23_P35414) and GSE146553 (probe/transcript ID 7934997).

Figure 1: PPM1K is expressed at significantly lower levels in HGSC tumors when compared to the normal ovary.

The mRNA expression of PPM1K in normal ovarian tissues from control subjects (left) and in the primary tumors of patients with HGSC (right) is represented with mean mRNA expression level marked and the result of a statistical test evaluating the significance of difference in PPM1K expression between the normal ovary and primary tumors from patients with HGSC, a p-value, listed above.

Figure 2: PPP1R3C is expressed at significantly lower levels in HGSC tumors when compared to the normal ovary.

The mRNA expression of PPP1R3C in normal ovarian tissues from control subjects (left) and in the primary tumors of patients with HGSC (right) is represented with mean mRNA expression level marked and the result of a statistical test evaluating the significance of difference in PPP1R3C expression between the normal ovary and primary tumors from patients with HGSC, a p-value, listed above.