Proteomic Profiling of a Mouse Model for Ovarian Granulosa Cell Tumor Identifies VCP as a Highly Sensitive Serum Tumor Marker in Several Human Cancers

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Abstract

The initial aim of this study was to identify novel serum diagnostic markers for the human ovarian granulosa cell tumor (GCT), a tumor that represents up to 5% of all ovarian cancers. To circumvent the paucity of human tissues available for analyses, we used the Ctnnb1tm/+-Pten±/±mouse model, which features the constitutive activation of CTNNBI signaling combined with the loss of Pten in granulosa cells and develops GCTs that mimic aggressive forms of the human disease. Proteomic profiling by mass spectrometry showed that vinculin, enolase 1, several heat shock proteins, and valosin containing protein (VCP) were more abundantly secreted by cultured mouse GCT cells compared to primary cultured GC. Among these proteins, only VCP was present in significantly increased levels in the preoperative serum of GCT cancer patients compared to normal subjects. To determine the specificity of VCP, serum levels were also measured in ovarian carcinoma, non-Hodgkin’s lymphoma and breast, colon, pancreatic, lung, and prostate cancer patients. Increased serum VCP levels were observed in the majority of cancer cases, with the exception of patients with lung or prostate cancer. Moreover, serum VCP levels were increased in some GCT, ovarian carcinoma, breast cancer, and colon cancer patients who did not otherwise display increased levels of widely used serum tumor markers for their cancer type (e.g. inhibit A, inhibit B, CA125, CEA, or CA15.3). These results demonstrate the potential use of VCP as highly sensitive serum marker for GCT as well as several other human cancers.

Introduction

Serum markers are of considerable value for the clinical screening, diagnosis, and follow-up of cancers. An ideal marker should have high sensitivity and/or high specificity, in order to discriminate cancer patients from healthy subjects as well as from patients with benign tumors or unrelated conditions. Most currently used serum markers are hormones, glycoproteins, or other proteins overexpressed by cancer cells. These markers are usually not specific to a unique cancer type and sometimes lack sensitivity [1]. Several strategies have been recently proposed to identify new serum tumor markers [2]. Differential proteomic analyses of serum specimens from cancer patients and healthy subjects are approaches of choice, but are hampered by the paucity of material in rare cancers as well as by the difficulty in detecting proteins that are expressed at low levels compared to abundant normal serum proteins. An alternative two-step approach involves the initial identification of proteins that are differentially expressed and/or secreted between normal and tumor cells, followed by the identification of these proteins in the serum of cancer patients. This approach ideally requires the isolation of primary tumor cells and corresponding normal cells. In this context, the use of relevant animal models of tumor development can provide essential starting materials for proteomic or genomic analysis, and lead to the identification of tumor-specific candidate proteins or genes whose expression can be then be investigated in human samples, as demonstrated in ovarian cancer [3,4,5].

The ovarian granulosa cell tumor (GCT) is the most prevalent of the sex cord/stromal subgroup of ovarian tumors in women, and is thought to represent up to 5% of all ovarian cancers. Over the past few years, our group has focused on the elucidation of the molecular etiology of human GCT, as well as on the creation of
relevant animal GCT models. We found evidence that misregulation of both the WNT/CTNNB1 (β-catenin) and PI3K/AKT signaling pathways occur in human GCT [6,7]. Transgenic mice with constitutive activation of CTNNB1 in granulosa cells (GC, \textit{Ctnnb1}^\text{tm1Mmt/+};\textit{Amhr2}^\text{tm1Hov/+};\textit{Pten}^\text{tm5(cre)Bhr/+}) develop precancerous ovarian lesions that often progress into GCT later in life [6]. The loss of the PI3K/AKT signaling antagonist gene \textit{Pten} in GC rarely causes GCT, but concomitant activation of CTNNB1 and loss of \textit{Pten} in the \textit{Ctnnb1}^\text{tm1Mmt/+};\textit{Amhr2}^\text{tm1Hov/+};\textit{Pten}^\text{tm5(cre)Bhr/+} (CPA) model results in the development of aggressive, metastatic GCT with 100% penetrance [7]. We have proposed that the CPA mouse is the best model currently available for the analysis of GCT biology as well as for preclinical animal studies aimed at developing novel therapeutic interventions and/or diagnostic tools.

We hypothesized that the CPA mouse model could be used for the identification of differentially expressed, GCT-associated proteins, and that these would translate to clinically useful novel serum diagnostic markers of the human disease. Here we present a differential secretome analysis comparing primary cultured GC from normal mice to GCT cells from CPA mice. This approach led to the identification of vasolin containing protein (VCP) as a potentially clinically relevant serum marker for human GCT, as well as for other forms of cancer.

Results

Identification of proteins secreted selectively in mouse GCT

Proteomic profiling was used with the objective of identifying secreted proteins that could represent new serum diagnostic markers specific for GCT. Spent culture media were obtained from cultured GCT cells from CPA mice or from normal GC from eCG-stimulated ovaries. Proteins from the media were separated by SDS-PAGE and fourteen protein bands observed in GCT but not in GC medium were subjected to mass spectrometry analysis (Figure 1). This process identified a number of proteins that were more abundantly secreted or shed by GCT cells relative to normal GC (Table 1). VCL, ENO1, several heat shock proteins (HSPA8/HSC70, the constitutive and inducible HSP90 alpha isoforms HSP90ab1 and HSP90aa1, and HSPA4), and VCP were the most consistently identified proteins from the GCT secretome.

VCP is a serum marker for GCT and other cancer types

We next investigated whether the proteins secreted by GCT cells from CPA mice could serve as serum markers in human GCT patients. Human homologs of nine proteins identified in the secretome analyses (B2M, CTSB, HSPA4/HSP70, HSP90, SPARC, TIMP-2, VCP, VCL, and WIF-1) were selected for further study based on known gene functions and antibody availability. Serum samples were obtained from healthy volunteers and from patients with GCT prior to treatment. No significant differences in the levels of B2M, CTSB, HSP90, SPARC, TIMP-2, VCP or WIF-1 were observed by immunoblot analyses in the serum of GCT patients compared to healthy subjects (data not shown). Marginally increased levels of HSPA4 were observed in the serum of some GCT patients compared to normal subjects, however the differences were not statistically significant, and deemed unlikely to be clinically useful (Figure 2A). VCP levels were low in immunoblot analyses of serum samples from healthy subjects, but were significantly increased in the majority of serum samples from GCT patients \((P<0.05)\). With a reference value for VCP levels set at the mean of immunoblot band intensity values of the healthy controls plus twice the standard deviation, we observed that 8 out of 9 women with GCT displayed increased serum VCP levels (Figure 2B, Table 2). To determine the specificity of VCP as a tumor marker for GCT, serum VCP levels were assessed in patients with ovarian carcinomas, as well as in small cohorts of patients with major non-ovarian cancers of known histology and grade (Table 3). Surprisingly, elevated serum VCP levels were detected in clinically significant proportions of patients with ovarian carcinoma (8 of 8), breast cancer (5 of 12), colon cancer (7 of 12), pancreatic cancer (8 of 12) or non-Hodgkin’s lymphoma (5 of 12) (Figure 2B and Table 3). However, serum VCP levels were not meaningfully increased in patients with lung or prostate cancer.

Specificity and sensitivity of VCP relative to widely-used serum diagnostic markers

We next compared the relevancy of VCP to that of other commonly-used serum diagnostic markers for various cancer types. Presently, the most useful serum markers for GCT in postmenopausal women are inhibin A and inhibin B [8]. In our cohort, inhibin A and inhibin B serum levels were increased in 2 out 9 and 7 out of 9 of patients with GCT (Table 2). There was no clear correlation between preoperative levels of inhibin A, inhibin B and VCP in GCT patients, nevertheless one patient had undetectable serum levels of inhibin A and inhibin B but highly increased levels of VCP (Table 2). The sensitivity of VCP therefore appears to compare favorably to that of the inhibins, and VCP could serve to identify rare inhibin-negative GCT patients. Serum CA125 is increased in approximately 50% of women with early ovarian carcinoma and in over 80% of women with advanced disease and is useful for monitoring therapy [9]. In our small ovarian carcinoma cohort, 4 of 8 patients tested positive for CA125, whereas VCP measurement detected cancer
in all patients, including those negative for CA125 (Figure 3). CEA is the most widely used serum tumor marker for colon cancer. Serum CEA is elevated in less than 25% of early stage colon cancer and 75% of late-stage cancer and is useful for determining prognosis, monitoring therapy and surveillance [10]. In the 12 colon cancer patients that we tested, 5 were positive for CEA. VCP measurement detected cancer in all CEA-positive patients, in addition to two that were CEA-negative (Figure 3). CEA and CA15.3 are the most commonly used serum markers for breast cancer. Assessment of these markers is not recommended for prognosis but rather for postoperative follow-up as well as monitoring in advanced diseases [10]. In the 12-case cohort that we examined, 3 patients tested positive for either CEA or CA15.3, with the remainder negative for both. VCP levels were elevated in all three patients that were CEA- or CA15.3-positive, and also in three patients that were negative for both markers (Figure 3). Thus, VCP serum levels were significantly increased in the majority of the tested cancer patients, including in some otherwise negative for established serum tumor markers.

**Discussion**

The VCP protein is a AAA+ ATPase associated with diverse cellular activities. VCP is necessary for the maintenance of cellular protein homeostasis and regulates the expression of proteins involved in many functions such as DNA replication, mitosis, protein degradation, endocytosis, membrane fusion, and organelle biogenesis. Specifically, VCP acts on ubiquitinated substrates

| Table 1. Mass spectrometry analysis of the secretome of mouse GCT from CPA transgenic mice. |
|----------------------------------|--------|---|
| Protein Identification | Protein Description | Protein Score | Peptides |
| IPI00405227 | Vcl Vinculin | 746 | 21 |
| IPI00323357 | Hsp8 Heat shock protein 8/Hsc70 | 488 | 27 |
| IPI00554929 | Hsp90ab1 Heat shock protein 90 alpha (cytosolic), class B member 1 | 468 | 13 |
| IPI00622235 | Vcp Valosin containing protein/p97 | 372 | 12 |
| IPI00462072 | Eno1 Enolase 1 alpha non-neuron | 349 | 9 |
| IPI00330804 | Hsp90a1 Heat shock protein 90, alpha (cytosolic), class A member 1 | 346 | 10 |
| IPI00110827 | Acta1 Actin alpha 1 skeletal muscle | 228 | 9 |
| IPI00124707 | Fst1 Follistatin-like 1 | 228 | 5 |
| IPI00117312 | Got2 Glutamate oxaloacetate transaminase 2, mitochondrial | 224 | 4 |
| IPI00331556 | Hspa4 Heat shock protein 4 | 201 | 5 |
| IPI00466069 | Eef2 Eukaryotic translation elongation factor 2 | 178 | 5 |
| IPI00135231 | Idh1 Isocitrate dehydrogenase 1 (NADP+), soluble | 171 | 4 |
| IPI00223231 | Qsox1 quiescin Q6 sulfhydryl oxidase 1 | 157 | 4 |
| IPI00126343 | Sparc Secreted acidic cysteine rich glycoprotein | 132 | 4 |
| IPI00313900 | Lum Lumican | 119 | 3 |
| IPI00113517 | Ctsb Cathepsin B | 113 | 3 |
| IPI00133208 | Hspa1 Heat shock protein 1A/Hsp70-3 | 106 | 3 |
| IPI00221402 | Aldoa Aldolase A, fructose-bisphosphate | 105 | 7 |
| IPI00228548 | Eno3 Enolase 3 beta muscle | 101 | 3 |
| IPI00124441 | Wif1 Wnt inhibitory factor 1 | 82 | 2 |
| IPI00331286 | B2m beta-2-microglobulin | 79 | 3 |
| IPI00113863 | Timp2 Tissue inhibitor of metalloproteinase 2 | 79 | 2 |
| IPI00313296 | Rnh1 Ribonuclease/angiogenin inhibitor 1 | 78 | 2 |
| IPI00762198 | Hbb-b1 Hemoglobin, beta adult major chain | 66 | 2 |
| IPI00109061 | Tubb2b Tubulin beta-2B | 63 | 2 |
| IPI00109073 | Tubb4 Tubulin beta-4 | 63 | 2 |
| IPI00131547 | Serpine1 Serine (or cysteine) peptidase inhibitor, clade E, member 1 | 55 | 2 |
| IPI00130391 | Psp1 Protease, serine, 1 | 54 | 2 |
| IPI00407502 | C1r Complement C1r-A subcomponent precursor | 50 | 2 |
| IPI00114209 | Glud1 Glutamate dehydrogenase 1 | 49 | 3 |
| IPI00127407 | Pld1 Phospholipase D1, 2-oxoglutatate 5-dioxygenase 1 | 43 | 2 |
| IPI00119809 | Lgl13b3p Lectin, galactoside-binding, soluble, 3 binding protein | 39 | 2 |
| IPI00112252 | Tgfb1 Transforming growth factor-beta-induced | 31 | 2 |

Protein identification (International Protein Index identifier) and protein description are given along with the overall score and the number of peptides identified by mass spectrometry as described in Materials & Methods. Proteins in bold were studied further.

doi:10.1371/journal.pone.0042470.t001
molecules and regulates protein turnover by balancing the proteasomal degradation of soluble proteins or misfolded protein aggregates localized in the ER lumen or the cytosol. Depending on conformational recoverability of complexes with VCP and target protein substrates, VCP either promotes their degradation, segregates them from large protein complexes, or modulates their ubiquitination by competing ubiquitin conjugation and deconjugation machineries (reviewed in [11]). VCP associates with numerous ubiquinated substrates, and the functions of VCP in different cell types relates in part to the specific tissue expression of substrates and co-factors. In neurons and muscle cells, VCP interaction with NF-1, UNC45b, and caveolin-3 regulates...

Figure 2. Serum levels of HSPA4 and VCP in healthy volunteers and in cancer patients. (A) HSPA4 levels in the serum of women with GCT or ovarian carcinoma. Equal amounts of serum protein were separated by SDS-PAGE and were subjected to immunoblot analysis for HSPA4 levels (representative blots are shown in the top panel, each lane represents a single donor). Densitometry analyses of signals obtained with the HSPA4 immunoblot analyses are reported in a graph in which each dot represents a single donor (bottom, horizontal bar = mean). No significant difference in HSPA4 levels was detected among groups by one-way ANOVA. (B) VCP levels are increased in the serum of cancer patients. Sera were analyzed as in (A) for the presence of VCP in patients with breast, colon, lung, pancreatic or prostate cancer, ovarian carcinoma, GCT, or non-Hodgkin’s (NH) lymphoma. Statistically significant differences (P<0.05) were detected between the control (normal) and GCT and colon cancer groups.

doi:10.1371/journal.pone.0042470.g002
VCP as a Serum Cancer Marker

Table 2. Serum levels of GCT markers in healthy women or in women with GCT.

| Group          | Diagnosis    | Menopausal status | INHA (ng/L) | INHB (ng/L) | CA-125 (U/ml) | VCP (arbitrary units) |
|----------------|--------------|-------------------|-------------|-------------|---------------|----------------------|
| control        | Post         | NE                | <20.0       | 6           | 110           |                      |
| control        | Pre          | NE                | <20.0       | 6           | 46            |                      |
| control        | Post         | NE                | <20.0       | 6           | 121           |                      |
| control        | Post         | NE                | 24.7        | 9           | 0             |                      |
| control        | Pre          | NE                | <20.0       | 20          | 241           |                      |
| control        | Post         | NE                | <20.0       | 7           | 95            |                      |
| control        | Post         | NE                | <20.0       | 14          | 117           |                      |
| GCT            | Adult GCT    | Pre               | <13         | 26.7        | NE            | 1010                 |
| GCT            | Adult GCT    | 92                | >1136.0     | NE          | 921           |                      |
| GCT            | Adult GCT    | Post              | <13         | 63.8        | NE            | 1110                 |
| GCT            | Adult GCT    | Pre               | <13         | <20.0       | NE            | 1478                 |
| GCT            | Adult GCT    | Pre               | 28.1        | 59.7        | 57            | 2212                 |
| GCT            | Adult GCT    | Post              | 213.5       | >1136.0     | 231           | 1357                 |
| GCT            | Adult GCT    | Pre               | 145.1       | >1136.0     | 120           | 964                  |
| GCT            | Adult GCT    | Pre               | 21.1        | <20.0       | 31            | 308                  |
| GCT            | Adult GCT    | Post              | <13         | 391         | NE            | 83                   |

Measurements that exceeded the normal reference range are indicated in bold. For inhibin A and B (INHA and INHB), values beneath the detection thresholds by ELISA were defined as normal. For VCP, the reference value was set as the mean of healthy control band intensity in immunoblot analyses+2SD (253 arbitrary units). Note that serum inhibin usually becomes undetectable after menopause in healthy women. Interpretation of premenopausal inhibin values can be difficult due to their secretion both by growing ovarian follicles and by GCTs. NE: not evaluated.

doi:10.1371/journal.pone.0042470.t002

Serum of cancer patients. The present report is the first to identify increased VCP levels in the context of disease progression [18,19,20,21,22,23,24,25]. However, the overexpression of VCP can be due to increased expression levels of VCP by tumor cells often correlate with tumorigenesis in humans. We report that VCP is overexpressed in the serum of cancer patients, and that it may represent a new clinically useful marker for cancer detection.

Materials and Methods

Mice

Ctnnb1tm1Mmt/+;Pten+tm1Hwu/tm1Hwu;Amhr2tm3(cre)Bhr/+ (CPA) transgenic mice were generated as previously described [7] and maintained on a C57BL/6 genetic background. In these mice, constitutive activation of CTNNB1 signaling is due to the deletion of the third exon of Ctnnb1, resulting in the production of a dominant-stable CTNNB1 mutant protein that lacks the phosphorlation sites required for its progresional degradation [6]. CPA mice develop GCT perinatally and die before 8 weeks of age [7]. All animal procedures were approved by the Institutional Animal Care and Use Committee and were conform to the USPHS Policy on Humane Care and Use of Laboratory Animals.

Cell culture

Normal GC were isolated using the method described by Zeleznik et al. [26]. Briefly, immature (23–26 day-old) C57BL/6 mice were injected IP. with 5 IU equine chorionic gonadotropin (eCG, Folligon, Intervet, Schering-Plough) to induce follicular growth. Forty-eight hours later, animals were sacrificed and the ovaries punctured with a 25-gauge needle to free GCs into the culture media.
### Table 3. Tumor clinical features and VCP serum level in tested cancer patients.

| Sample Number | Histology          | Grade/Differentiation | VCP levels |
|---------------|--------------------|-----------------------|------------|
| **Healthy donor** |                    |                       |            |
| 1             |                    |                       | 110.0      |
| 2             |                    |                       | 46.0       |
| 3             |                    |                       | 121.0      |
| 4             |                    |                       | 0.0        |
| 5             |                    |                       | 240.9      |
| 6             |                    |                       | 94.8       |
| 7             |                    |                       | 116.6      |
| **GCT**       |                    |                       |            |
| AM3           | Adult GCT          | IA                    | 1010.4     |
| AM4           | Adult GCT          | IA                    | 921.1      |
| AM5           | Adult GCT: Relapse | X                     | 1109.6     |
| AM6           | Adult GCT          | IA                    | 1478.0     |
| AM7           | Adult GCT          | IIIC                  | 2212.0     |
| AM8           | Adult GCT          | IA                    | 1357.0     |
| AM9           | Adult GCT          | IA                    | 964.0      |
| AM10          | Adult GCT          | IC                    | 308.0      |
| AM11          | Adult GCT          | IIIC                  | 83.0       |
| **ovarian cancer** |                |                       |            |
| AM12          | CL adenocarcinoma  | IC                    | 362.0      |
| AM13          | CL adenocarcinoma  | IIIB                  | 266.0      |
| AM14          | EM adenocarcinoma  | I                     | 2069.0     |
| AM15          | EM adenocarcinoma  | IIIC                  | 1048.0     |
| AM16          | mucinous cystadenocarcinoma | IA | 261.6 |
| AM17          | mucinous cystadenocarcinoma | IIIC | 618.0 |
| AM18          | serous cystadenocarcinoma | IIIC | 1046.4 |
| AM19          | serous cystadenocarcinoma | III | 1836.7 |
| **breast cancer** |                |                       |            |
| B00404105     | invasive mammary NOS | III                | 121.8      |
| B00405105     | invasive mammary NOS | I                  | 708.8      |
| B00515113     | invasive mammary NOS | III                | 394.8      |
| B00516111     | invasive mammary NOS | II                 | 343.9      |
| B00596105     | invasive mammary NOS | II                 | 126.7      |
| B00649114     | invasive mammary NOS | II                 | 237.6      |
| F00020105     | IDC                | I                    | 65.2       |
| F00048105     | IDC                | II                   | 72.3       |
| F00049105     | IDC                | III                  | 1060.0     |
| F00116105     | IDC                | II                   | 30.9       |
| F00121405     | IDC                | III                  | 351.1      |
| F00372103     | IDC                | II                   | 25.4       |
| **colon cancer** |                |                       |            |
| B00266111     | Adenocarcinoma     | II                   | 2452.8     |
| B00279112     | Adenocarcinoma     | II                   | 2510.2     |
| B00443114     | Adenocarcinoma     | II                   | 8.1        |
| B00457113     | Adenocarcinoma     | II                   | 2.6        |
| B00502102     | Adenocarcinoma     | IV                   | 11.3       |
| B00530114     | Adenocarcinoma     | II                   | 31.8       |
| B00674115     | Adenocarcinoma     | II                   | 1157.1     |
| B00703104     | Adenocarcinoma     | IV                   | 1513.5     |
| B00728115     | Adenocarcinoma     | IV                   | 44.6       |
| Sample Number | Histology | Grade/Differentiation | VCP levels |
|---------------|-----------|-----------------------|------------|
| B01057112     | Adenocarcinoma | IV                    | 1595.2     |
| B01157113     | Adenocarcinoma | IV                    | 2120.2     |
| B01595113     | Adenocarcinoma | IV                    | 498.3      |
| B00537112     | Adenocarcinoma | III                   | 4.23       |
| B00627112     | Adenocarcinoma | X                     | 367.56     |
| B02973106     | Ductal adenocarcinoma, NOS | II | 277.7 |
| D00086102     | ductal adenocarcinoma | II | 756.32 |
| D00205104     | adenocarcinoma | III                   | 1445.88    |
| D00408101     | adenocarcinoma | X                     | 1268.59    |
| D00544102     | adenocarcinoma | II                    | 372.54     |
| D01000101     | ductal carcinoma | II | 779.26 |
| D01013101     | ductal carcinoma | II | 761.58 |
| E01584101     | invasive ductal adenocarcinoma | II | 72.89 |
| F00302101     | ductal adenocarcinoma | II | 77.15 |
| A00195101     | adenocarcinoma | III                   | 41.58      |
| A00242102     | LC undifferentiated carcinoma | X | 158.4 |
| A00327102     | adenocarcinoma | I                     | 195.6      |
| A00392101     | adenocarcinoma | I                     | 148.76     |
| A00404102     | squamous carcinoma | II | 86.63 |
| A00699101     | adenocarcinoma | X                     | 272.46     |
| A00710101     | adenocarcinoma | III                   | 168.24     |
| A00728102     | adenocarcinoma | I                     | 208.09     |
| A01015104     | LC undifferentiated carcinoma | X | 14.4 |
| A01341101     | squamous carcinoma | III | 82.37 |
| A01429101     | squamous carcinoma | II | 351.27 |
| A02117112     | squamous carcinoma | II | 119.04 |
| A01598101     | adenocarcinoma | T3b                   | 89.97      |
| A01738101     | adenocarcinoma | T3b                   | 44.24      |
| B02131103     | adenocarcinoma | T3b                   | 42.92      |
| B02408102     | adenocarcinoma | T2c                   | 298        |
| B02409102     | adenocarcinoma | T2c                   | 114.31     |
| B02469101     | adenocarcinoma | T3, NOS               | 11.07      |
| B02682102     | adenocarcinoma | T2c                   | 147.48     |
| B02683103     | adenocarcinoma | T2c                   | 124.46     |
| B02704103     | adenocarcinoma | T2c                   | 135.62     |
| B02725101     | adenocarcinoma | T2c                   | 3.72       |
| B03083102     | adenocarcinoma | T3b                   | 37.73      |
| F00339102     | adenocarcinoma | T3a                   | 34.9       |
| A00730101     | mixed; follicular and diffuse large B cell lymphoma | IE | 0 |
| A01749102     | diffuse large B cell lymphoma | X | 0 |
| B01423113     | small bowel lymphoma, NOS | IVE | 2753.45 |
| B01563111     | testicular lymphoma, NOS | IV | 181.93 |
| B01824113     | follicular lymphoma | IV | 0 |
| B02332112     | mixed; follicular and diffuse large B cell lymphoma | III | 252.9 |
| B02337111     | mixed; follicular and diffuse large B cell lymphoma | IV | 0 |
medium (0.9% NaCl). The cell suspension was centrifuged at 1,000 g for 5 min and resuspended GC were plated into 24 well plates at 70% confluence in DMEM/F12 medium (Sigma Aldrich) supplemented with 5% FBS (Invitrogen), penicillin and streptomycin (P/S). GCT from 21 to 25 day-old CPA mice were excised, minced with a size 10 scalpel blade and digested for 2 h with 0.1% collagenase from *Clostridium histolyticum* (Sigma) in serum-free DMEM with P/S. Cells were then centrifuged at 1,000 g for 10 min and resuspended in DMEM supplemented with 10% FBS and P/S prior to plating into 100 mm cell culture dishes (25–6 × 10^6 cells/dish).

Differential secretome analyses

Twenty-four hours after plating, cultured GC and GCT cells were washed with HBSS (Invitrogen) and incubated for 24 h in serum-free DMEM. Supernatants were collected and concentrated 80-fold using Amicon Ultra-4 centrifugal filter units (Millipore) with a 3 kDa molecular weight cut-off. Protein concentrations were quantified using the method of Bradford (Bio-Rad protein assay) and 4–6 mg protein samples were separated by SDS-PAGE. Following silver staining, proteins bands found in GCT but not in GC samples (n = 14) were excised from the gel. The gel slices were destained with 50% methanol then reduced in 10 mM DTT for 1 hour at 56 °C and alkylated in 55 mM chloroacetamide for one hour at room temperature. After washing in 50 mM ammonium bicarbonate, the gel pieces were shrunk in 100% acetonitrile (ACN). Digestion was performed using trypsin in 50 mM ammonium bicarbonate for 8 hours at 37 °C. The peptides were finally extracted in 90% ACN/0.5 M urea and dried in a speed vacuum. Samples were resolubilized in 5% ACN with 2% formic acid (FA) and separated on a homemade C18 column (150 mm × 610 cm) using an Eksigent nanoLC-2D system. A 56-min gradient from 5–60% ACN (0.2% FA) was used to elute peptides from a homemade reversed-phase column (150 mm i.d. ×100 mm) with a flow rate set at 600 nanoliter/min. The column was directly connected to a nanoprobe interfaced with an LTQ-Orbitrap Velos mass spectrometer (Thermo-Fisher). Each full MS spectrum was followed by twelve MS/MS spectra (thirteen scan events), where the twelve most abundant multiply-charged ions were selected for MS/MS sequencing. Tandem MS experiments were performed using collision-induced dissociation in the linear ion trap. The data were processed using the 2.1 Mascot (Matrix Science) search engine with tolerance parameters set to 15 ppm and 0.5 Da for the precursor and the fragment ions respectively. The selected variable modifications were carbamidomethyl (C), deamidation (NQ), oxidation (M) and phosphorylation (STY). The selected database was human IPI database v.3.54 with 150858 sequences.

**Table 3.**

| Sample Number | Histology                                   | Grade/Differentiation | VCP levels |
|---------------|--------------------------------------------|-----------------------|------------|
| D0076101      | mixed; follicular and diffuse large B cell lymphoma | I                     | 1203.38    |
| D02063102     | follicular lymphoma                         | X                     | 2911.73    |
| D02342103     | marginal zone lymphoma                      | X                     | 447.32     |
| D02482102     | NA                                         | X                     | 1136.11    |
| E00273103     | follicular lymphoma                         | IV                    | 0          |

For VCP, the reference value was set as the mean of healthy control band intensity in immunoblot analyses ± 2SD (253 arbitrary units). Positive VCP values are indicated in bold. X: unknown grade/differentiation; CL: clear cell, EM: endometroid, NOS: not otherwise specified, IDC: invasive ductal carcinoma, LC: large cell, NHDG: non-Hodgkin’s lymphoma, NA: not available.

doi:10.1371/journal.pone.0042470.t003

**Figure 3.** Assessment of serum levels of VCP compared to serum tumor markers currently used for ovarian carcinoma, colon cancer, and breast cancer. Sera from patients were tested for VCP levels by immunoblot analyses and the dotted line shows VCP cutoff values established in healthy donors. In addition, sera were tested by ELISA for the presence (+) or absence (−) of increased levels of CA125 in ovarian carcinoma, CEA in colon cancer, or CEA and CA15.3 in breast cancer. The normal ranges of CA125, CEA, and CA15.3 are below 35 U/ml, 7 μg/l, and 29 kU/l, respectively.

doi:10.1371/journal.pone.0042470.g003
Serum samples

Serum samples from patients with breast, colon, lung, pancreatic or prostate cancer, or non-Hodgkin’s lymphoma (n = 12 for each cancer type) were obtained from the Ontario Tumour Bank. Serum samples from healthy volunteers (n = 7) and patients with GCT (n = 9) or ovarian carcinoma (n = 8; 2 clear cell, 2 serous, 2 mucinous and 2 endometrioid ovarian cancers) were obtained from the Réseau de recherche du cancer du Fonds de recherche Québec - Santé. Procedures were approved by the FROs-RR Cancer Research Committee and the Ontario Cancer Research Ethics Board, as well as the Comité d’éthique de la recherche sur les sujets humains de the Centre hospitalier de l’Université de Montréal. Inhibin A and inhibin B levels were determined by ELISA at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. Values for inhibin A and B beneath the detection threshold (13 ng/l and 20 ng/l, respectively) were defined as normal. CA15.3 and CEA levels were determined by les Laboratoires du Centre Hospitalier de l’Universite´ de Montre´al. Inhibin A and B levels were determined by ELISA at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. Values below 7 µg/l and 29 kU/l for CEA and CA 15.3, respectively, were considered normal. CA125 levels were determined using a commercially available ELISA assay kit and levels were considered as normal when below 35 U/ml (Abnova).

Immunoblot analyses

Serum samples (4 µl i.e. approximately 200 µg) were separated by SDS-PAGE on 10% acrylamide gels. The gels were transferred to polyvinylidene fluoride membranes (GE, Amersham/VWR). Immunodetection was performed with HSPA4- or VCP-specific antibodies (clones ab75977 or ab11433 respectively, Abcam) and horseradish peroxidase-conjugated secondary antibodies (GE, Amersham/VWR) and revealed using ECL Detection Reagents (GE, Amersham/VWR). Quantification of the protein bands was performed by densitometry analyses using a Kodak Image Station 440CF and Kodak 1D v.3.6.5 software (Eastman Kodak, Rochester, NY). In order to normalize VCP or HSPA4 protein levels between immunoblots, each gel contained two or three common serum samples as references. The reference value for VCP was set as the mean of healthy control band intensities in immunoblot analyses+2SD.

Statistical analyses

One-way ANOVA with Dunnett’s post-test was used to compare VCP levels in healthy women and cancer patients.

Acknowledgments

The authors thank Meggie Girard, Kathleen Théroix, Jennifer Kendall-Dupont, and Manon de Ladurantaye for their technical help and the Ontario Tumour Bank (Jeanette Joanes) and the Réseau de recherche du cancer du Fonds de la Recherche en Santé du Québec for providing sera from cancer patients and normal subjects.

Author Contributions

Performed the experiments: M-NL EB AB NP PT. Analyzed the data: RR-M M-EN DB. Contributed reagents/materials/analysis tools: A-MM-M. Wrote the paper: RR-M M-EN DB. Conceived the experiments: M-NL EB AB NP PT. Designed the experiments: M-EN DB.

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