Activation of the Osteopontin/Matrix Metalloproteinase-9 Pathway Correlates with Prostate Cancer Progression
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Abstract Purpose: Prostate cancer remains the second most frequent cause of tumor-related deaths in the Western world. Additional markers for the identification of prostate cancer development and progression are needed. Osteopontin (OPN), which activates matrix metalloproteinases (MMP), is considered a prognostic biomarker in several cancers. “In silico” and experimental approaches were used to determine whether OPN-mediated MMP activation may be a signal of prostate cancer progression.

Experimental Design: Pearson correlation coefficients were computed for each OPN/MMP pair across seven publicly available prostate cancer gene expression data sets. Using Gene Set Enrichment Analysis, 101 cancer-related gene sets were analyzed for association with OPN and MMP-9 expression. OPN, MMP-9, MMP-2 tissue inhibitor of metalloproteinase-1 plasma levels, and MMP gelatinase activity were measured by ELISA and zymography in 96 and 92 patients with prostate cancer and benign prostatic hyperplasia, respectively, and 125 age-matched healthy men.

Results: Computational analyses identified a significant correlation only between MMP-9 and OPN, and showed significant enrichment scores in “cell proliferation”, “genes constituting the phosphoinositide-3-kinase predictor”, “proliferation signature”, and “tumor metastasis” gene sets in association with both OPN and MMP-9. Plasma analyses revealed a significant increase in OPN and MMP-9 levels and activity in patients with prostate cancer in association with clinical variables (prostate-specific antigen >4 ng/mL and Gleason score >7). Significant correlation between OPN and MMP-9 levels were also observed. Mean plasma levels of OPN and MMP-9 decreased in patients with prostate cancer within 6 months after prostatectomy.

Conclusions: The concordant computational and experimental data indicate that the extent of OPN pathway activation correlates with prostate cancer progression.

Prostate cancer is the most common malignant neoplasm in men in Western countries. Up to 40% of men diagnosed with advanced disease develop metastasis at 5 years (1). Since its discovery in 1970, the prostate-specific antigen (PSA) test remains the main diagnostic tool associated with prostate cancer (2). Several markers have been proposed in recent years, but none are currently considered adequate to diagnose and predict the outcome of prostate cancer (3, 4). New markers that correlate with prostate cancer progression are needed.

In recent years, the overexpression of osteopontin (OPN) in tissue samples and in body fluids has been proposed as a progression and metastasis prognostic biomarker for several tumor types (5, 6). In prostate cancer, previous studies suggest a role for increased OPN tissue expression in the malignant transformation of prostate epithelial cells and as an important determinant of tumor progression (7) and patient survival (8). Moreover, elevated plasma OPN levels in patients with hormone-refractory prostate cancer as compared with those in healthy male volunteers have been reported, as well as the value of such elevation in predicting the survival of patients with prostate cancer (9, 10).

OPN (also named bone sialoprotein I, secreted phosphohistone I, 2ar, uropoietin, early T-lymphocyte activation-1) is a secreted glycosylated phosphoprotein involved in cell migration, cell survival, and immunity (11). OPN is constitutively expressed in several cell types in which it is involved in many physiologic processes, whereas induced expression has been detected in remodeling processes such as inflammation,
ischemia-reperfusion, bone resorption, and tumor progression. OPN is a substrate for several extracellular proteases and is cleaved in vitro and in vivo by thrombin coagulation factor and matrix metalloproteinases (MMP). OPN contributes to malignancies through both inhibition of apoptosis and activation of various matrix-degrading proteases such as activation of MMP-2 and MMP-9 and urokinase-type plasminogen activator, leading to cancer cell motility, tumor growth, and metastasis (reviewed in ref. 5). Moreover, Das et al. have reported OPN-induced urokinase-type plasminogen activator expression in a breast cancer model (12).

MMPs are extracellular matrix–degrading enzymes that play a crucial role in embryogenesis, tissue remodeling, inflammation, and angiogenesis. Each MMP is classified by substrate activity and contains a catalytic domain, a propeptide regulatory domain, and carbonyl-terminal structural domains. MMP expression is regulated at the level of gene transcription, enzymatic activation, and inhibition of proteolytic activity by tissue inhibitors of metalloproteinases (TIMP). MMP-2 and MMP-9 (also called gelatinase A and B, respectively) have been regarded as critical molecules in promoting tumor cell metastasis (13, 14).

Increased MMP-9 secretion has been observed in short-term cultures of prostate tumor cells (15); furthermore, in vitro and in vivo xenograft studies showed that OPN-regulated signaling cascades lead to cyclooxygenase-2 (COX-2) up-regulation, COX-2–mediated prostaglandin E2 (PGE2) production and MMP-2 activation (16). recent data, obtained using in vitro–modified prostate cancer cell lines, suggested the involvement of OPN in the activation of MMP-9 during migration of prostate cancer cells (17), confirming previous evidences determined in melanoma (18).

The present study was designed to (a) identify molecular correlates between OPN and all known MMPs using a published computational procedure (19); (b) examine in silico the association of OPN and MMP-9 with specific cancer-related gene sets using Gene Set Enrichment Analysis (GSEA; ref. 20); and (c) investigate the plasma levels of OPN, MMP-9, MMP-2, TIMP-1, and PGE2 in a series of patients with prostate cancer, patients with benign prostatic hyperplasia (BPH), and age-matched healthy donors (HD). Our findings suggest that OPN and active MMP-9 function in concert to promote tumor progression and that OPN plasma levels could have diagnostic/prognostic significance.

Materials and Methods

Gene expression data sets and MMP list. Nine publicly available gene expression data sets of prostate cancer samples were analyzed, one of which was excluded due to the absence of the OPN clone on the platform (21), and one was excluded due to the high number of cases null for OPN gene expression (22). Table 1 lists the characteristics of the seven data sets explored. Data sets I and II were generated by hybridization on cDNA, for which no additional data manipulation was done to the downloaded processed gene expression matrices. Data sets III to VII were generated by oligonucleotide DNA chips, for which the raw data for all genes across the samples were normalized using the robust multivariate average, including probe-level quantile normalization and background correction, as implemented in the Bioconductor software suite. All probe sets and cDNA clones from each platform were assigned National Center for Biotechnology Information gene identifiers. When more than one cDNA clone or probe set matched a given gene, all possible pairs were considered and that with the highest variance were selected.

GSEA. GSEA was done using software v2.01 (20) to assess OPN and MMP-9 associations with specific cancer-related gene sets in the five prostate cancer data sets (III–VII in Table 1) generated using similar platforms. For this purpose, OPN and MMP-9 expressions were considered as continuous variables. Genes represented by more than one probe were collapsed using the XCollapseProbes utility to the probe with the maximum value. The gene set database was obtained by extracting gene lists from the Cancer GEArray DNA Microarrays (63 gene sets), the Kyoto Encyclopedia of Genes and Genomes for cancer-related pathways (29 gene sets), and known signatures from published transcriptional profiles (9 gene sets). The final database was composed of 101 cancer-related gene sets. The complete list of gene sets and genes is reported in the Supplementary Table S1.

Patients and samples. Blood samples from 313 subjects, including 92 patients with BPH, 96 patients with prostate cancer, and 125 HD were collected by the Department of Biomedical Sciences, University of Catania, Catania, Italy. The local scientific ethics committee approved all procedures. All participants gave written consent for blood collection. Venous blood obtained from all subjects was placed in tubes with or without heparin. Samples were centrifuged and the separated plasma or serum was placed in aliquots and stored at -80°C until analysis. None of the subjects had received any medication known to interfere with bone metabolism or had signs of infection or immunologic disease. The control group consisted of 125 age-matched men with no history of prostate disease or metabolic bone disease. The BPH clinical diagnosis was confirmed by histologic examination of material obtained by transurethral resection of the prostate or by transrectal ultrasound-guided prostate biopsy. Prostate cancer was histopathologically diagnosed by prostate biopsy and confirmed by tissue material obtained after radical prostatectomy (77 cases). Prostate cancers were graded according to the Gleason system (23). Multiple blocks were identified based on the presence of adequate tumor and the representative nature of the overall grade; for statistical evaluations, tumors with Gleason scores of 6 or lower were considered low-grade (35 cases), whereas tumors scoring 7 or higher were considered high-grade (61 cases). Table 2 lists the essential clinical data and serum PSA levels of BPH, prostate cancer, and HD subjects. PSA serum levels were <4 ng/mL in all HD, 53 BPH, and 27 prostate cancer subjects. In 16 prostate cancer cases, whose presurgery PSA levels were >7 ng/mL, blood was also collected during follow-up at 6 months after surgery.
Immunohistochemistry. All clinical prostate cancer specimens used in this study were obtained with Institutional Review Board approval and informed consent to use excess biological material for investigative purposes from all participating patients. The OPN, MMP-9, and COX-2 expression were detected by immunohistochemistry on serial sections obtained from 10 tumor specimens using specific antibody: mouse monoclonal anti-OPN antibody (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-MMP-9 antibody (Chemicon), and mouse monoclonal anti-COX-2 (Cayman Chemical). The processing of samples and immunohistochemical procedures were done according to the instructions of the manufacturer.

Serum/plasma assays of PSA, OPN, MMP-2, MMP-9, PGE2, and TIMP-1. PSA serum levels were measured by the Hybritech Tandem method (Hybritech). OPN, MMP-2, MMP-9, PGE2, and TIMP-1 plasma concentrations were measured using ELISA kits (R&D Systems Europe). The MMP-2 and MMP-9 assays recognized both pro and active forms. Plasma samples were diluted and the immunoassay was done according to the instructions of the manufacturer. Marker concentrations for each sample were calculated from the standard curve. All assays were in duplicate. A subset of samples was assayed five times in every ELISA plate for quality control. No significant cross-reactivity to or interference with various proteins was observed.

Gelatin zymography. Gelatinolytic activity of MMPs was analyzed using gelatin zymography as described (24). Gel images were acquired with a Duoscan T1200 scanner (AGFA), and MMP levels (pro and activated forms) were quantified by densitometric analysis of the bands obtained from 10 tumor specimens using specific antibody: mouse monoclonal anti–COX-2 (Cayman Chemical). The processing of samples and immunohistochemical procedures were done according to the instructions of the manufacturer.

Statistical analysis. For computational analysis of gene expression correlation, the Pearson correlation coefficients were computed for each pair across each data set and the P value was computed using the function cor.test of the software package R9 version 2.5.0 (accessed March 23, 2007). All samples in each data set were considered except for normal adjacent prostate to prostate cancer derived from the same patient (data sets I, III, and VII) to avoid overfitting of the results (see Table 1).

In the GSEA, the ranking of genes according to OPN and MMP-9 correlation was based on a Pearson metric and the weighted enrichment statistic was selected. P values were calculated by permuting the phenotype labels 1,000 times and the false discovery rate (FDR) was considered. We considered as statistically significant enrichments with both normalized P < 0.01 and FDR < 0.25 or one of the two variables below the fixed threshold.

Analysis of experimental data started with descriptive statistics, including mean and SD for continuous variables, and frequency for categorical variables. The Kruskal-Wallis and the Dunn multiple comparison tests were used to compare biomarker levels and MMP activity between patients and control subjects. An α level of 0.05 was used for all statistical tests.

Results

Computational correlation between OPN and MMP gene expression profiles. The MMPs paired with OPN and extractable from the seven selected prostate cancer microarray data sets ranged from 16 (in two data sets) to 23 (in three data sets). Computation of a Pearson correlation coefficient for each OPN/MMP pair in each data set revealed no significant correlation for 10 pairs in any data set and a significant correlation (P < 0.05) for 11 pairs in at least one data set, subsequent analysis of these 11 pairs across all data sets to search for concordant significant correlations identified a robust correlation between OPN and MMP-9 only, which were significantly correlated in five of seven data sets (Fig. 1A; Supplementary Table S2a, for the complete list of extracted correlations for each data set). To check the possibility that the observed correlations were due to chance alone, data set I, containing the highest number of analyzed samples (see Table 1), was used for selecting genes not previously associated to OPN and matched for mean expression level to MMP genes. Among these genes, a set of 23 genes were randomly picked and their expression was correlated with that of OPN in all the data sets. None of them showed a significant correlation in more than two data sets (see Supplementary Table S2b). Furthermore, we evaluated in the same data sets COX-2/OPN gene expression correlation. A significant correlation (r = 0.58; P < 0.0001) was observed between OPN and COX-2 gene expression profiles only in data set VII.

In addition to the correlation analysis, the expression data of OPN and MMP-9 were evaluated separately for their possible correlation with transformation and progression. On the basis of the number and tissue origin of samples that were informative for OPN and/or MMP-9 genes, these evaluations were carried out: in data set VII, for intraindividual comparison between paired samples of prostate cancer and normal adjacent prostate; in data sets I and V, for interindividual comparisons of

Table 1. Characteristics of the explored prostate cancer data sets

<table>
<thead>
<tr>
<th>Code</th>
<th>Author (ref.)</th>
<th>Year</th>
<th>Platform</th>
<th>PCA primary</th>
<th>Met-PCA</th>
<th>Normal prostate*</th>
<th>BPH</th>
<th>Others †</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Tomlins et al. (41)</td>
<td>2007</td>
<td>20K Hs6</td>
<td>32</td>
<td>20</td>
<td>23</td>
<td>11</td>
<td>18</td>
<td>104</td>
</tr>
<tr>
<td>II</td>
<td>True et al. (42)</td>
<td>2006</td>
<td>cDNA v4</td>
<td>15</td>
<td>15</td>
<td>16</td>
<td></td>
<td></td>
<td>31</td>
</tr>
<tr>
<td>III</td>
<td>Nanni et al. (43)</td>
<td>2006</td>
<td>U133A</td>
<td>22</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>IV</td>
<td>Best et al. (44)</td>
<td>2005</td>
<td>U133A</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>V</td>
<td>Varambally et al. (45)</td>
<td>2005</td>
<td>U133A</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td></td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>VI</td>
<td>Xia et al. (GSE1431)</td>
<td>2004</td>
<td>HG-U95A</td>
<td>88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>88</td>
</tr>
<tr>
<td>VII</td>
<td>Singh et al. (46)</td>
<td>2002</td>
<td>U95Av2</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>102</td>
</tr>
</tbody>
</table>

NOTE: Data sets I and II, samples were microdissected by laser capture microdissection; data set III, epithelial culture from explanted tissues. Abbreviations: PCA, prostate cancer; Met-Pca, metastatic prostate cancer.

*Data set I, 17 normal adjacent prostate (NAdjP) to cancer; the expression values for MMP-9 are missing in these samples; 6 NAdjP to cancer derived from different patients. Data set II, 16 NAdjP to cancer derived from different patients. Data set VII, 50 NAdjP to cancer.

† Data set I, 5 atrophic epithelium, 13 prostatic intraepithelial neoplasia; data set III, 3 basaloid tissues.

http://www.r-project.org/
prostate samples with different status of disease progression. Intraindividual comparison by paired $t$ test between 33 paired samples of prostate cancer and normal adjacent prostate from data set VII indicated significantly higher expression levels of both OPN and MMP-9 (Fig. 1B) in the tumor specimens. Interindividual comparisons of prostate samples from data set I and V showed increased expression with progression of the disease for OPN but not for MMP-9 (Fig. 1C and D).

**GSEA.** GSEA to identify OPN and MMP-9 relationships to specific cancer-related gene sets indicated the enrichment of 22 gene sets in association with OPN expression (Supplementary Table S3a) and 28 gene sets enriched in association with MMP-9 (Supplementary Table S3b) among a total of 101 gene sets analyzed. The FDR in GSEA highlighted that, probably due to the limited number of samples and informative genes, respectively, all the correlations observed in data sets IV and VII had a FDR value near or equal to 1; thus, we decided to restrict our analysis to the three remaining data sets and to consider of potential significance only the gene sets with correlations above our statistical threshold in at least two of the three remaining valuable data sets. According to this analysis, 7 and 10 gene sets were associated with OPN and MMP-9, respectively, 4 of them being associated with both. The plots in Fig. 2A and B show the enrichment results of OPN and MMP-9, respectively, analyzed in data set VI. Supplementary Table S4 lists the genes significantly enriched in the four gene sets.

**High OPN and MMP-9, and low TIMP-1 plasma levels in patients with prostate cancer.** To validate the computational results, median OPN, MMP-9, MMP-2, TIMP-1, and PGE$_2$ plasma levels were compared in the three groups of subjects (Table 2). OPN and MMP-9 levels were significantly higher in patients with prostate cancer compared with those from BPH patients and HD, and significantly higher in BPH patients compared with HD; no significant differences in MMP-2 plasma levels were observed among any of the groups. TIMP-1 and plasma levels in patients with prostate cancer were significantly lower than those in BPH and HD individuals, whereas these levels did not differ significantly between BPH and HD subjects. PGE$_2$ levels were significantly increased only in plasma from patients with prostate cancer.

**Expression of OPN, MMP-9, and COX-2 proteins in prostate cancer specimens.** Immunohistochemical analysis was carried out to evaluate the expression and distribution of OPN, MMP-9, and COX-2 in the tumor specimens. Two examples of reactivity are reported in Fig. 3. Very intense COX-2 and OPN immunostaining was observed in all tumor cells for all cases. MMP-9 immunostaining was observed in all tumor cells and in parts of the extracellular matrix for all cases. These results clearly indicated that OPN and MMP-9 proteins, which are present at high plasma levels in patients with prostate cancer, may arise from prostate cancer cells. Increased plasma levels of PGE$_2$, a metabolite of the COX-2 enzyme, might be linked to the overexpression of COX-2 in prostate cancer cells.

### Table 2. Characteristic of study groups and OPN, MMP-9, MMP-2, TIMP-1, and PGE$_2$ plasma levels

<table>
<thead>
<tr>
<th>(A) Clinical characteristics</th>
<th>HD ($n = 125$)</th>
<th>BPH ($n = 92$)</th>
<th>PCa ($n = 96$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y median (range)</td>
<td>71 (46-79)</td>
<td>70 (56-80)</td>
<td>72 (47-80)</td>
<td>—</td>
</tr>
<tr>
<td>&lt;60</td>
<td>8</td>
<td>2</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>60-70</td>
<td>47</td>
<td>43</td>
<td>31</td>
<td>—</td>
</tr>
<tr>
<td>&gt;70</td>
<td>70</td>
<td>47</td>
<td>58</td>
<td>—</td>
</tr>
<tr>
<td>PSA (ng/mL) mean ± SE</td>
<td>1.47 ± 0.42</td>
<td>3.67 ± 0.14</td>
<td>8.62 ± 0.67</td>
<td>0.2026*</td>
</tr>
<tr>
<td>&lt;4 ng/mL</td>
<td>125</td>
<td>53</td>
<td>27</td>
<td>—</td>
</tr>
<tr>
<td>≥4 ng/mL</td>
<td>—</td>
<td>39</td>
<td>69</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Type of surgery</td>
<td></td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>TURP</td>
<td>NA</td>
<td>92</td>
<td>19</td>
<td>—</td>
</tr>
<tr>
<td>Prostatectomy</td>
<td>NA</td>
<td>—</td>
<td>77</td>
<td>—</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Low</td>
<td>NA</td>
<td>NA</td>
<td>35</td>
<td>—</td>
</tr>
<tr>
<td>High</td>
<td>NA</td>
<td>NA</td>
<td>61</td>
<td>—</td>
</tr>
<tr>
<td>(B) Plasma levels</td>
<td></td>
<td>Median, upper, and lower quartiles (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPN</td>
<td>46.7 (28.9-79.9)</td>
<td>116.5 (91.4-139.5)</td>
<td>170.6 (142.2-234.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MMP-9</td>
<td>17.2 (12.9-23.7)</td>
<td>55.2 (35.5-75.3)</td>
<td>97.3 (77.6-127.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MMP-2</td>
<td>832 (598-1,103)</td>
<td>971 (622-1,165)</td>
<td>937 (598-1,297)</td>
<td>NS</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>139.8 (95.3-175.8)</td>
<td>120.1 (91.4-138.9)</td>
<td>89.7 (65.1-119.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>0.16 (0.14-0.20)</td>
<td>0.17 (0.14-0.21)</td>
<td>0.70 (0.59-0.86)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Abbreviations: PCa, prostate cancer; TURP, transurethral resection of the prostate; NA, not applicable; NS, not statistically significant.

*Age: by $\chi^2$ test among all subgroups; prostate cancer vs. BPH, $P = 0.0552$; prostate cancer vs. HD, $P = 0.7118$; BPH vs. HD, $P = 0.1865$. PSA level: by $\chi^2$ test prostate cancer vs. BPH.

<sup>1</sup> By one-way ANOVA with Dunnett’s multiple comparison posttest; prostate cancer vs. BPH, $P < 0.0001$, by unpaired $t$ test.

<sup>2</sup> By Kruskal-Wallis test; Dunn’s posttest of BPH vs. HD and prostate cancer vs. BPH revealed a significant difference ($P < 0.001$).

<sup>3</sup> By Kruskal-Wallis test; Dunn’s posttest of prostate cancer vs. BPH or HD revealed a significant difference ($P < 0.001$) but no significant difference between BPH and HD.
High MMP-9 activity in patients with prostate cancer. Zymography and densitometric quantitation of band intensity for MMP-9 revealed significantly higher activity in patients with prostate cancer than in BPH (P < 0.0001) and HD (P < 0.0001), whereas no significant differences in MMP-9 activity were observed between BPH and HD subjects (see Supplementary Fig. S1 for representative examples of MMP-9 gelatinolytic activity and quantified activities in all tested plasma). No difference in MMP-2 activity was detected among the three groups of subjects (data not shown).

Fig. 1. Correlation analysis of OPN and MMP-9 and intraindividual and interindividual comparison of OPN and MMP-9 expression levels in prostate samples. A. Heat map of Pearson correlations for each OPN/MMP pair in each data set. Red, positive correlation; yellow, negative correlation; black, no correlation. For the actual values of Pearson coefficient correlations and P value, see Supplementary Table IA. B. Scatter plots of OPN and MMP-9 expression, respectively, in the 33 paired samples of prostate tumors (PCa) and adjacent prostate tissue not containing tumor (NAdjP) that were informative for both genes (Data set VII); differences were assessed by two-tailed paired t test. C and D, scatter plots of OPN and MMP-9 expression, respectively, in the samples from BPH, prostatic intraepithelial neoplasia (PIN), primary prostatic tumor (PCa), and metastatic tumor (Met-PCa) from datasets I and V; differences were assessed by Kruskal-Wallis test. Mean expression levels are reported.

A significantly positive Spearman correlation between plasma MMP-9 levels and activity was calculated in HD (r = 0.43, P < 0.0001), BPH (r = 0.55, P < 0.0001), and prostate cancer patients (r = 0.61, P < 0.0001).

OPN and MMP-9 plasma levels and their relationship with clinical variables. A significant Spearman correlation between OPN and MMP-9 plasma levels in HD (r = 0.75, P < 0.0001), in BPH (r = 0.88, P < 0.0001), and in patients with prostate cancer (r = 0.89, P < 0.0001) was observed. Figure 4A shows the correlation of OPN and MMP-9 for all plasma samples tested.
As expected, OPN levels also correlated with MMP-9 activity (HD, \( r = 0.58, P < 0.001 \); BPH, \( r = 0.64, P < 0.0001 \); prostate cancer, \( r = 0.77, P < 0.0001 \)). Although PGE\(_2\) was significantly increased in prostate cancer, a marginal correlation between plasma levels of OPN, MMP-9, and MMP-2 with PGE\(_2\) was found: OPN/PGE\(_2\) \((r = 0.22; r^2 = 0.03 \ P = 0.0271)\), MMP-9/PGE\(_2\) \((r = 0.13; r^2 = 0.01; P = 0.2153)\), MMP-2/PGE\(_2\) \((r = -0.01; r^2 = 0.00; P = 0.8991)\).

The potential association of OPN and MMP-9 plasma levels with two main prostate cancer prognostic factors was examined in patients stratified according to PSA levels (<4 and ≥4 ng/mL) and Gleason score (low and high). Median OPN (Fig. 4B) and MMP-9 (Fig. 4C) plasma levels were significantly higher in patients with PSA >4 ng/mL or high Gleason score than in those with PSA <4 ng/mL or low Gleason score. Note that among the prostate cancer patients with PSA <4 ng/mL and low Gleason score, only 1 of 27 and 2 of 35 cases, respectively, had OPN levels below the upper 95th percentiles of HD.

Analysis of blood samples collected 6 months after surgery from a selected group of 16 patients with prostate cancer with a presurgery level of PSA >7 ng/mL (17.89 ± 1.40, mean ± SE) indicated a decrease in both OPN and MMP-9 levels below the upper 95th percentile of HD, concomitant with normalization of PSA levels, in the 15 cases judged as tumor-free based on clinical and laboratory test criteria; in the single prostate cancer patient in whom metastases was identified, the levels of all three markers increased (PSA from 28 to 55 ng/mL; OPN from 266.3 to 538.0 ng/mL; and MMP-9 from 176.5 to 278.0 ng/mL).

### Discussion

Recent studies strongly support the crucial role of OPN in tumor progression through the regulation of highly integrated signaling events, including those critically dependent on MMP bioavailability (5). Our computational analysis of more than 300 prostate samples from seven different gene expression data sets, together with our serologic analysis of well-defined clinical material including more than 300 blood samples, show a significant correlation between OPN and both levels and activity of MMP-9 but not of MMP-2.

### Table 3. List of enriched gene sets correlated with OPN

<table>
<thead>
<tr>
<th>Name</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>1.68</td>
<td>0.550</td>
<td>0.2288</td>
<td>1.79</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>1.89</td>
<td>0.0076</td>
<td>0.0672</td>
<td>1.43</td>
</tr>
<tr>
<td>Genes constituting the PI3K predictor</td>
<td>1.40</td>
<td>0.2090</td>
<td>0.5758</td>
<td>1.78</td>
</tr>
<tr>
<td>Proliferation signature</td>
<td>1.48</td>
<td>0.1637</td>
<td>0.4510</td>
<td>1.71</td>
</tr>
<tr>
<td>Recurrence score</td>
<td>1.05</td>
<td>0.4041</td>
<td>0.7800</td>
<td>1.57</td>
</tr>
<tr>
<td>Tumor metastasis</td>
<td>1.66</td>
<td>0.0303</td>
<td>0.2160</td>
<td>1.27</td>
</tr>
<tr>
<td>Underhill proliferation</td>
<td>1.55</td>
<td>0.0726</td>
<td>0.4131</td>
<td>1.58</td>
</tr>
</tbody>
</table>

NOTE: A significant \( P \) value or an FDR of <0.25 in at least two of the three prostate cancer data sets analyzed was found. The gene sets cell proliferation, genes constituting the PI3K predictor, proliferation signature, and tumor metastasis concomitantly associated with OPN are in italics. Abbreviations: NES, normalized enrichment score; PI3K, phosphoinositide-3-kinase.

### Table 4. Lists of enriched gene sets correlated with MMP-9

<table>
<thead>
<tr>
<th>Name</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>1.56</td>
<td>0.0229</td>
<td>0.4117</td>
<td>1.03</td>
</tr>
<tr>
<td>Cell adhesion molecules (KEGG)</td>
<td>1.52</td>
<td>0.0214</td>
<td>0.4111</td>
<td>0.75</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>1.49</td>
<td>0.1680</td>
<td>0.3076</td>
<td>1.45</td>
</tr>
<tr>
<td>ECM cell-cell adhesion</td>
<td>1.54</td>
<td>0.0122</td>
<td>0.4131</td>
<td>1.28</td>
</tr>
<tr>
<td>ECM cell-matrix adhesion</td>
<td>1.63</td>
<td>0.0236</td>
<td>0.3986</td>
<td>1.03</td>
</tr>
<tr>
<td>Genes constituting the PI3K predictor</td>
<td>0.52</td>
<td>0.8686</td>
<td>0.9734</td>
<td>1.61</td>
</tr>
<tr>
<td>Proliferation signature</td>
<td>1.51</td>
<td>0.0204</td>
<td>0.4818</td>
<td>1.82</td>
</tr>
<tr>
<td>TNF L and R</td>
<td>1.79</td>
<td>0.0316</td>
<td>0.3379</td>
<td>1.10</td>
</tr>
<tr>
<td>Tumor metastasis</td>
<td>1.70</td>
<td>0.0390</td>
<td>0.3097</td>
<td>0.87</td>
</tr>
<tr>
<td>Underhill proliferation</td>
<td>1.17</td>
<td>0.3231</td>
<td>0.5186</td>
<td>1.74</td>
</tr>
</tbody>
</table>

NOTE: A significant \( P \) value or an FDR of <0.25 in at least two of the three prostate cancer data sets analyzed was found. The gene sets cell proliferation, genes constituting the PI3K predictor, proliferation signature, and tumor metastasis concomitantly associated with MMP-9 are in italics. Abbreviations: NES, normalized enrichment score; KEGG, Kyoto Encyclopedia of Genes and Genomes; ECM, extracellular matrix; PI3K, phosphoinositide-3-kinase; TNF, tumor necrosis factor.
cDNA microarray analyses have identified OPN as one of the most abundantly expressed genes in colon carcinomas and metastatic melanoma (25), and the overexpression was confirmed in numerous studies both at the computational and immunohistochemical levels. Our computational intra-individual and interindividual comparisons of prostate samples indicated an increased expression of OPN in association with the transformation and progression of disease, suggesting its potential usefulness as a marker of prostate cancer progression. Several reports have implicated MMP-9 in the regulation of tumor cell migration and tumor-induced angiogenesis that result in the development and metastasis of several adenocarcinomas, including breast and prostate, and the protein was detected immunohistochemically in tumor cells as well as in the stromal compartment (15, 26). Our computational analysis indicated a modest increase in MMP-9 expression associated with transformation but not with progression. Although we cannot rule out the possibility that the association of MMP-9 with prostate cancer progression was less stringent than that of OPN, it should be taken into account that expression analysis could not discriminate between the pro and active forms of MMP-9, such that its final involvement in progression might be underestimated. Notwithstanding the less evident increase in MMP-9 gene expression, we found a significant positive correlation between OPN and MMP-9 expression in five of the seven data sets analyzed. Support for the validity of computational analysis to identify an association between OPN and MMPs comes from a similar approach applied to a published microarray data set of hepatocellular carcinoma, which reveal concordant elevated expression of OPN and MMP-9 in primary tumors and their secondary metastatic lesions (27). It has been reported that OPN induces COX-2 expression in prostate cancer (16). The authors found that COX-2 plays a role in OPN-induced PGE2 production and MMP-2 activation that ultimately regulates prostate tumor progression. Moreover, it has been shown that increased MMP-2 expression is linked with decreased disease-free survival in patients with prostate cancer (28). In contrast, in the present study, computational analysis reveals no correlation between OPN and MMP-2 or COX-2 expression levels.

Immunohistochemistry analysis was applied to discover the origin/source of the OPN, MMP-9, and COX-2 expression in a fraction of prostate cancer samples. Our results are in agreement with previous data showing that PGE2, OPN, and

![Fig. 2. Relationship of OPN and MMP-9 with cancer-related gene sets by GSEA. A and B, Illustration of the running enrichment score of the proliferation signature and tumor metastasis gene sets with OPN and MMP-9, respectively, in the context of data set VI. The enrichment score is plotted as a function of the position within the ranked gene lists (green). Black bars, position of all genes belonging to the two indicated gene sets. The ranked list metrics (gray) illustrate the correlation between the signal-to-noise values of all individually ranked genes according to OPN and MMP-9 expression.](image-url)
MMP-9 are mostly released from tumor cells. These data are also supported by previous studies demonstrating the secretion of active MMP-9 in prostate cancer cell cultures (15) and OPN-induced activation of pro-MMP-9 (17), raising the possibility that both markers are coregulated in tumor cells.

Increasing evidence suggests that OPN blood levels can serve as a prognostic or diagnostic marker in several malignancies. Clinical studies have revealed a clear correlation between OPN plasma levels and tumor burden and prognosis in patients with breast cancer metastasis (29), and between OPN levels and the presence of metastasis to bone and survival prediction in patients with prostate cancer (9, 10, 30). Fewer studies have correlated circulating gelatinases or their inhibitors with clinical course or tumor burden in cancer, and their role in prostate cancer progression is, at present, not yet defined. In particular, MMP-2 serum levels have been reported to correlate with the extent of disease in patients with prostate cancer (31), whereas more recent reports, including ours, suggest good prognostic accuracy for MMP-9 but not for MMP-2 (32, 33). It is well established that increased levels of PGE2 production and MMP-2 activation are associated with enhanced expression of COX-2 in many cancers. Our ELISA data confirm that PGE2, as a metabolite of COX-2, is significantly and specifically increased in prostate cancer. However, the marginal correlation found between OPN and PGE2 plasma levels may be referred to 3% of the observed data. On the contrary, ELISA and zymographic analyses of plasma from patients with prostate cancer and BPH and age-matched HD identified a significant increase in OPN levels and MMP-9 levels and activity associated with disease progression, but no significant variation in MMP-2 levels and activity, and only a marginal, highly variable change in TIMP-1. Furthermore, increased levels of both OPN and MMP-9 were significantly associated with the clinical variables PSA ≥4 ng/mL and high Gleason score. Although we did not specifically analyze the samples in relation to metastatic status at the time of surgery, the high levels of OPN in patients with prostate cancer with a PSA of <4 ng/mL suggests that OPN can be elevated in patients with no skeletal metastasis. These data are in line with numerous studies in other malignancies reporting increased OPN concentrations in patients without distant metastases. On the other hand, no differences in OPN plasma concentrations between patients with prostate cancer without metastases and controls have been recently found by Ramankulov et al. (30). It is well known that OPN is highly

Fig. 3. Immunohistochemical analysis for COX-2 (A and B), MMP-9 (C and D), and OPN (E and F) on serial sections from two prostate cancer specimens. Immunostaining for COX-2 and OPN is present in most tumor cells; extracellular matrix is negative; weak immunostaining for OPN in fibromuscular stroma is observed. MMP-9 immunostaining is visible in all tumor cells and in part of the extracellular matrix.
heterogeneous due to differential RNA splicing, glycosylation, sulfation, and susceptibility to proteases. Thus, the discrepancy of the results regarding the measurement of OPN levels may be partially attributed to the different ELISA systems used. Conclusions about the immediate clinical applicability of OPN assay await further investigations.

In agreement with our computational identification of a direct correlation between OPN and MMP-9 at the gene expression level, our serologic analysis provided clinical evidence that the protein plasma concentrations of the two markers were significantly and concomitantly increased in prostate cancer as compared with HD or BPH samples. A correlated expression between the two markers, albeit at lower levels in non–cancer plasma, was observed in all three plasma groups analyzed either separately or as a single series ($r^2 = 0.91$, $P < 0.0001$), suggesting that the same physiologic process underlies the association between OPN and MMP-9 expression. Furthermore, the evident increase in MMP-9 protein concentration was accompanied by activation of the proenzyme that resulted in the highest Spearman correlation between OPN and MMP-9 activity ($r = 0.77$, $P < 0.0001$) in patients with prostate cancer.

![Fig. 4. Positive correlation between OPN and MMP-9 plasma levels and their relationship with clinical variables. A, the relative amounts of OPN (X-axis) and MMP-9 (Y-axis) plasma concentrations measured using an ELISA test in HD, BPH, and prostate cancer plasma samples. The $r^2$ is indicated; diagonal line, the least-squares linear regression fit to the data. Correlated expressions are evident in each of the three groups. B and C, median plasma of OPN and MMP-9, respectively, in prostate cancer patients stratified into groups according to PSA levels (<4 and ≥4) and Gleason scores (low and high). Levels of both proteins were significantly higher in patients with PSA levels ≥4 or high Gleason score than in patients with PSA <4 and low Gleason score. Dotted lines, upper 95th percentiles of HD, OPN: 96.1 ng/mL; MMP-9: 60.0 ng/mL.](https://www.aacrjournals.org/clin-cancer-research)
The mechanism by which OPN mediates MMP-9 activation in tumors and consequently increases cancer aggressiveness remains poorly understood; however, previous studies have indicated that OPN stimulates the secretion of urokinase-type plasminogen activator, which regulates pro–MMP-9 activation, cell motility, invasion, and tumor growth (18). Furthermore, OPN was implicated in the regulation of phosphoinositide-3-kinase–dependent Akt phosphorylation, cell adhesion and migration in prostate cancer cells (34), and activation of protein kinase Ca/nuclear factor–inducing kinase/nuclear factor κB–dependent signaling cascades (16). Because correlated expression of two genes at the mRNA level, as found in our computational analysis, suggests that the genes are regulated by a common mechanism (35), we applied GSEA to the available gene expression data sets to gain further insight into this aspect. Only 4 of 101 cancer-associated gene sets explored were positively enriched in association with both OPN and MMP-9 in at least two data sets. The significant enrichment of the tumor metastasis gene set was clearly compatible with the reported association of both OPN and MMP-9 to the metastatic process. Although the function of OPN (6) and MMP-9 (36) in cell proliferation is still in debate, the enrichment of the “cell proliferation” gene set (37) and the “proliferation signature” gene set, originally identified in highly proliferative breast tumor cells (38), is consistent with reports of the indirect contribution of OPN and MMPs to tumor growth through processing of several growth factors. Our GSEA data suggests a more stringent association of OPN with cell proliferation than that of MMP-9 (see Fig. 2), consistent with experimental evidence that OPN can stimulate LNCaP cell proliferation by enhancing the association of its receptor integrin-β1 with the epidermal growth factor receptor, and in turn, stimulating the sustained phosphorylation/activation of epidermal growth factor receptor (6). Furthermore, in keeping with the implication of the phosphoinositide-3-kinase/Akt pathway in the downstream OPN signals that regulate tumor progression and invasive behavior (18, 39), we observed concomitant enrichment of the ‘genes constituting the phosphoinositide-3-kinase predictor’ gene set, originally described in ref. (40), with both OPN and MMP-9. Although the associations per se are not sufficient to identify the mechanism underlying OPN overexpression and OPN-mediated MMP-9 activation, due to the preliminary nature of our analysis, the GSEA findings may help to generate new hypothesis and to focus on further research of specific signaling pathways.

Overall, the concordant computational and serologic identification of a correlated expression between OPN and MMP-9 suggests the promise of the analysis of activation of the OPN/MMP-9 pathway as a marker for prostate cancer progression, and together with the preliminary indications by GSEA, provide an avenue to therapeutically interfering with the signaling pathway(s) regulating the OPN-mediated MMP-9 activation in prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References


Activation of the Osteopontin/Matrix Metalloproteinase-9 Pathway Correlates with Prostate Cancer Progression

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