The Search for Secreted Proteins in Prostate Cancer by the *Escherichia coli* Ampicillin Secretion Trap: Expression of NBL1 Is Highly Restricted to the Prostate and Is Related to Cancer Progression

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**Key Words**

CAST · NBL1 · Prostate cancer

**Abstract**

**Aims:** Genes expressed only in cancer tissue or specific organs will be useful molecular markers. To identify genes that encode secreted proteins present in prostate cancer (PCa), we generated *Escherichia coli* ampicillin secretion trap (CAST) libraries from PCa and normal prostate (NP).

**Methods and Results:** We identified 15 candidate genes that encode secreted proteins present in PCa and NP. Quantitative RT-PCR analysis revealed that *MSMB*, *NBL1* and *AZGP1* were expressed with much higher specificity in PCa and NP than in 14 other kinds of normal tissue. We focused on *NBL1*, which was originally identified as a putative tumor suppressor gene. Western blot analysis revealed that NBL1 protein was highly expressed in both cell lysate and culture media of the DU145 PCa cell line. Immunohistochemical analysis showed that NBL1 expression was highly detected in and restricted to NP and PCa and was significantly down-regulated in PCa. NBL1 expression was significantly reduced according to the tumor stage, Gleason grade and preoperative prostate-specific antigen (PSA) value.

**Conclusion:** NBL1 is a secreted protein that is highly restricted to the prostate. Underexpression of NBL1 correlated with PCa progression. NBL1 might be a candidate tumor marker for PCa in addition to PSA.

**Introduction**

Prostate cancer (PCa) is one of the most common human male cancers. Cancer develops as a result of multiple genetic and epigenetic alterations [1]. Better knowledge of changes in gene expression that occur during prostatic carcinogenesis may lead to improvements in its diagnosis, treatment and prevention [2]. Genes encoding secretory proteins expressed specifically in cancers or specific organs may be ideal biomarkers for cancer diagnosis. To identify novel genes that encode secreted protein present in PCa, we used the *Escherichia coli* ampicillin secretion trap (CAST) method. We have previously identified several PCa-specific genes encoding transmembrane proteins with the CAST method and reported their high potential as therapeutic targets [3, 4]. To our knowledge, however, CAST analysis of secreted protein in PCa has not been reported.
Prostate-specific antigen (PSA), a serine protease found in semen, is the most widely used serum marker for detecting and monitoring PCAs [5, 6]. The rapid incorporation of aggressive PSA testing has resulted in dramatically earlier identification of PCAs and is attributed with the decrease in mortality from PCAs [7]. However, there are limitations to the use of PSA. PSA levels are also increased in benign prostatic hyperplasia (BPH) and general inflammatory responses. PSA testing has the potential disadvantage of low specificity and has led to a tremendous increase in the number of unnecessary prostate biopsies [8]. Furthermore, blood PSA level is not significantly increased in patients with poorly differentiated PCAs and patients receiving androgen deprivation therapy [9, 10]. Moreover, the prognosis of castration-resistant PCAs remains unsatisfactory [11]. Therefore, increasing emphasis has been placed on the need to determine new protein biomarkers for use in the diagnosis of PCAs.

In the present study, to identify genes that encode secreted proteins, we generated CAST libraries from 2 PCA cell lines, LNCaP and DU145, and normal prostate (NP). CAST is a signal sequence trap method developed by Ferguson et al. [12]. Signal peptides target secreted and transmembrane proteins to their appropriate subcellular localization [13]. A consensus sequence for the signal peptide has not been identified, and, thus, standard molecular techniques are not well suited to identify such proteins. CAST exploits the ability of mammalian signal sequences to confer ampicillin resistance to a mutant β-lactamase lacking the endogenous signal sequence [14]. We report here the identification of several genes that encode secreted proteins expressed in PCAs and NPs. Among these, we focused on the NBL1 gene because this gene is highly restricted in PCAs and NPs. NBL1 (neuroblastoma suppression of tumorigenicity 1) was originally identified as a putative tumor suppressor gene in a transformed fibroblast rat model [15]. We confirmed the expression of NBL1 in cell lysate and culture media of PCAs. We examined the expression and distribution of NBL1 in human PCAs and NPs by immunohistochemistry and compared them with clinicopathological characteristics. We also studied the expression of NBL1 in normal systemic organs because NBL1 has the possibility of being a serum marker for PCAs.

**Materials and Methods**

**CAST Library Construction**

Plasmid CAST (pCAST) was designed to contain the kanamycin resistance gene and the β-lactamase gene lacking the first 69 nucleotides encoding the endogenous signal peptide. EcoRI and BamHI sites were placed upstream of the mutant β-lactamase gene for directional cloning. CAST library construction was performed as described previously [3, 12]. In brief, CAST cDNA libraries were generated from 2 μg of mRNA with a random primer containing a BamHI restriction site for reverse transcription (SuperScript Choice System; Invitrogen, Carlsbad, Calif., USA). The EcoRI-adapted cDNA was digested with BamHI, size fractionated, ligated into pCAST and plated onto Luria-Bertani/ampicillin medium. Individual colonies were picked and grown in 1.0 ml of this medium with kanamycin in a 96-well format. Plasmid DNA was sequenced in a 96-well format using a primer located within the β-lactamase gene.

**Tissue Samples**

In total, 219 primary tumor samples were collected from patients diagnosed with PCAs and those with NP. Patients were treated at the Hiroshima University Hospital or an affiliated hospital. Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

For quantitative RT-PCR, 16 PCAs samples and 9 non-neoplastic samples were used. Samples were frozen immediately in liquid nitrogen and stored at –80 °C until use. We confirmed microscopically that the tumor specimens consisted mainly (>50%) of cancer cells. Noncancerous samples of heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain and spinal cord were Purchased from Clontech (Palo Alto, Calif., USA).

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 127 PCAs patients treated by radical prostatectomy, 54 PCAs patients who underwent prostate biopsy, and 13 patients treated by suprapubic prostatectomy for BPH. Tumor staging was in accordance with the TNM classification system [16], and histological classification of PCAs was made in accordance with the World Health Organization classification [17]. The clinical characteristics of the PCAs patients are shown in table 1. In addition, we used archival formalin-fixed, paraffin-embedded tissues from normal systemic organs including 5 speci-
menses each of the brain, spinal cord, heart, lung, esophagus, stomach, small intestine, colon, liver, spleen, pancreas, kidney, adrenal, ureter, bladder, testis, skin, skeletal muscle and blood vessels.

**Table 2. List of genes encoding secreted protein from the CAST libraries**

<table>
<thead>
<tr>
<th>Sample name</th>
<th>LNCaP</th>
<th>DU145</th>
<th>Normal Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes encoding secreted protein</td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Gene name</td>
<td>TFPI</td>
<td>TFPI</td>
<td>MSMB</td>
</tr>
<tr>
<td></td>
<td>SPP1</td>
<td>CDDC126</td>
<td>AZGPI</td>
</tr>
<tr>
<td></td>
<td>FN1</td>
<td>CLU</td>
<td>SPP1</td>
</tr>
<tr>
<td></td>
<td>COL4A5</td>
<td>DMKN</td>
<td>TFPI</td>
</tr>
<tr>
<td></td>
<td>CALU</td>
<td>NBL1</td>
<td>C1RL</td>
</tr>
<tr>
<td></td>
<td>SFTPA1B</td>
<td>ARMETL</td>
<td>FGB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SRGN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NTN4</td>
</tr>
</tbody>
</table>

**Quantitative RT-PCR and Western Blot Analysis**

Quantitative RT-PCR was performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, Calif., USA) as described previously [18]. Quantitation of NBL1 mRNA levels was done by real-time fluorescence detection as reported in a previous study [19]. The NBL1 primer sequences were 5'-TCAACAAGCTGGCAGTTC-3' and 5'-GCAGGAGTCA-CAGTGAACCA-3' (for more information, see online suppl. material at www.karger.com/doi/10.1159/000341396).

For Western blot analysis, tissue samples or cells were lysed as described previously [20]. The primary antibody against NBL1 (R&D Systems, Inc., Minneapolis, Minn., USA) was used (see online suppl. material).

**Evaluation of Specificity of Gene Expression**

To evaluate the specificity of expression in each gene, a specificity index was calculated as follows: first, we identified the 14 normal tissues, which are indispensable for survival, in which the target gene expression was highest among tissues analyzed by quantitative RT-PCR. We then identified the PCa among 16 tissues in which the target gene expression was highest by quantitative RT-PCR to identify genes expressed specifically in PCa, we generated CAST libraries from 2 PCa cell lines (LNCaP and DU145) and NP, as previously described [3, 4]. We identified 6, 7 and 8 genes encoding secreted proteins from the respective cell lines and NP. The names of these 21 genes are shown in table 2. We performed quantitative RT-PCR to identify genes expressed specifically in PCa and NP. Representative results are shown in figure 1. We then identified the PCa among 16 tissues in which the target gene expression was highest (mRNA expression levels are shown as A; table 3) and NP among 9 tissues in which the target gene expression was highest (mRNA expression levels are shown as B; table 3). We performed quantitative RT-PCR to identify genes expressed specifically in PCa and NP. Representative results are shown in figure 1. We then identified the PCa among 16 tissues in which the target gene expression was highest (mRNA expression levels are shown as A; table 3) and NP among 9 tissues in which the target gene expression was highest (mRNA expression levels are shown as B; table 3). Next, the PCa specificity index (A/B ratio) for each gene was calculated. We could not find a gene specific only to PCa, but we could detect genes specific to both PCa and NP. Because
PSA is also specific for prostate and not specific for PCa, we examined these prostate-specific genes. Of the 15 candidates, 3 genes, MSMB, NBL1 and AZGP1, were found to show high specificity for the prostate, and 3 genes, C1RL, NTN4 and DMKN, were found to show low specificity for the prostate. Of the 3 genes showing high specificity, MSMB (microseminoprotein-β) and AZGP1 (zinc-α2-glycoprotein) have already been studied in PCa, and their utility as serum tumor marker and therapeutic target have been reported [24–27]. Therefore, we focused on NBL1. NBL1 has been reported as one of the genes whose expression is reduced in PCa compared with NP by profiling of expressed sequence tags and quantitative RT-PCR analysis [28, 29]. However, there is no report, to our knowledge, in which NBL1 expression in human systemic organs is compared and NBL1 expression in PCa and NP determined by immunohistochemical analysis has not been reported.

We compared NBL1 mRNA expression between prostate and systemic normal organs. The average NBL1 mRNA expression levels in NP and PCa were 19.9- and 8.1-fold greater, respectively, than in brain, in which NBL1 is most highly expressed of the systemic normal organs. In comparison with that in the other systemic organs, the expression of NBL1 mRNA is highly restricted to the prostate.

**NBL1 Protein Expression in Cell Lysate and Culture Medium**

To study whether NBL1 is a secreted protein, we performed Western blot analysis in 3 PCa cell lines. In cell lysate, moderate NBL1 expression was detected in DU145 cells as a band of approximately 27 kDa. LNCaP cells showed low NBL1 expression, and NBL1 expression was absent in PC3 cells (fig. 2a). In culture medium, very high NBL1 expression was noted in DU145 cells, low NBL1 ex-

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**Fig. 1.** Quantitative RT-PCR analysis of candidate genes in 16 PCa samples, 9 NP samples, and 14 kinds of normal tissues. MSMB, NBL1 and AZGP1 were found to show high specificity for the prostate. C1RL, NTN4 and DMKN were found to show low specificity for the prostate.
pression was seen in LNCaP cells, and no expression of NBL1 was noted in PC3 cells. In the CAST analysis, colonies containing the NBL1 gene were detected in the DU145 CAST library, indicating that Western blot analysis of NBL1 protein was consistent with CAST analysis. Next, we examined transition of NBL1 expression by Western blot analysis of cell extracts of DU145 transfected with NBL1-specific siRNAs. Two types of siRNAs (siRNA1 and siRNA2) and negative control siRNA were transfected into DU145 cell extracts. Expression of NBL1 in DU145 was suppressed by treatment with siRNA1 and siRNA2 in both cell lysate and culture medium (fig. 2b).

**Immunohistochemical Analysis of NBL1 Expression in Normal Systemic Organs**

We performed immunohistochemical analysis of NBL1 in 19 kinds of non-cancerous systemic tissues in 5 samples of each tissue. NBL1 expression was detected only in epithelium of the small intestine and colon, islets of the pancreas, and nerve cells in brain and spinal cord (fig. 3a–d). We did not detect the expression of NBL1 in heart, lung, esophagus, stomach, liver, spleen, pancreas, kidney, adrenal, ureter, bladder, testis, skin, skeletal muscle and vessel. When the level of NBL1 expression is compared in small intestine and colon, islets of the pancreas, and nerve cells in brain and spinal cord, expression in the brain nerve cells is higher than in the other tissues. These results are consistent with our quantitative RT-PCR results.

**Immunohistochemical Analysis of NBL1 Expression in PCa and NP**

We also performed immunohistochemical analysis of NBL1 in a total of 194 prostate samples, which included BPH in 13 patients and PCa in 181 patients. The clinical characteristics of the PCa patients are summarized in table 1. NBL1 staining was observed in the cytoplasm of both NP epithelium and cancer cells. In some acini, NBL1 expression was stronger at the apical side of luminal cells.

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**Table 3. Summary of quantitative RT-PCR analysis of candidate genes specifically expressed in PCa and NP samples**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Name of normal organ with highest expression</th>
<th>PCa with highest mRNA expression level (A)</th>
<th>NP with highest mRNA expression level (B)</th>
<th>PCa specificity index (A/B)</th>
<th>PCa cases with mRNA expression level ≥10-fold normal organ with highest expression</th>
<th>NP cases with mRNA expression level ≥10-fold normal organ with highest expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>High specificity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSMB</td>
<td>Stomach</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>0.13</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>NBL1</td>
<td>Brain</td>
<td>29.6</td>
<td>71.2</td>
<td>0.42</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>AZGP1</td>
<td>Pancreas</td>
<td>21.5</td>
<td>22.4</td>
<td>0.96</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Low specificity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1RL</td>
<td>Liver</td>
<td>7.7</td>
<td>8.2</td>
<td>0.94</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NTN4</td>
<td>Pancreas</td>
<td>5.2</td>
<td>3.7</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMKN</td>
<td>Pancreas</td>
<td>2.1</td>
<td>7.0</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No specificity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FNI</td>
<td>Liver</td>
<td>0.78</td>
<td>0.37</td>
<td>2.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>COL4A5</td>
<td>Stomach</td>
<td>0.72</td>
<td>0.69</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCDC126</td>
<td>Skeletal muscle</td>
<td>0.58</td>
<td>0.07</td>
<td>8.3</td>
<td>0</td>
<td>0</td>
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<tr>
<td>SPP1</td>
<td>Kidney</td>
<td>0.56</td>
<td>0.34</td>
<td>1.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CALU</td>
<td>Heart</td>
<td>0.32</td>
<td>0.26</td>
<td>1.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CLU</td>
<td>Liver</td>
<td>0.3</td>
<td>1.0</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SRGN</td>
<td>Bone marrow</td>
<td>0.27</td>
<td>0.67</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FGB</td>
<td>Liver</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TFPI</td>
<td>Kidney</td>
<td>&lt;0.01</td>
<td>0.022</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SFTPA1B</td>
<td>Lung</td>
<td>0.057</td>
<td>&lt;0.01</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ARMETL</td>
<td>Skeletal muscle</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Target mRNA expression levels were standardized to 1.0 μg total RNA from the normal organ with the highest expression set as 1.0 (16 PCa and 9 NP samples).
and was detected in the prostatic ducts. Expression of NBL1 was not detected in stromal cells. Although NBL1 expression in PCa cells has some heterogeneity, NBL1 expression was detected in 1–95% of all samples (fig. 3 e–h). All prostate samples were considered NBL1 positive if any cell stained positive. These immunohistochemical results suggest that NBL1 was highly expressed in and restricted to the prostate.

Next, we compared clinicopathological parameters with NBL1 expression scores in the prostate samples (fig. 4a–d). Mean NBL1 expression score was significantly higher in BPH samples and NP adjacent to PCa than in PCa itself (p < 0.0001). The mean NBL1 expression score was significantly higher in PCa classified as stage B than in PCa classified as stages C and D (p = 0.0014) and was significantly higher in PCa with Gleason score 6 than in PCa with Gleason score 7–10 (p = 0.0024). The mean NBL1 expression score was also significantly higher in PCa with PSA level ≤20 than in PCa with PSA level >20 (p < 0.0001).

Effect of NBL1 Inhibition on Cell Growth and Invasive Activity of PCa Cells

We studied the biological role of NBL1 using the DU145 cell line because of the high expression of NBL1 in this cell line. To investigate the possible proliferative effects of NBL1 knockdown, we performed an MTT assay 4 days after siRNA transfection (fig. 4e). Cell viability was not significantly different between NBL1 siRNA-transfected DU145 and negative control siRNA-transfected DU145. Next, to determine the possible role of NBL1 in invasiveness, a transwell invasion assay was performed (fig. 4f). Invasion ability 1 day after siRNA transfection was not significantly different between NBL1 siRNA-transfected DU145 and negative control siRNA-transfected DU145.

Discussion

In the present study, we identified several genes that encode secreted proteins present in PCa and NP from CAST libraries. Quantitative RT-PCR revealed that MSMB, NBL1 and AZGP1 were expressed to a much higher extent in PCa and NP than in 14 types of normal tissues. MSMB, prostatic acid phosphatase and PSA are the three most abundant proteins found in semen. It was reported that MSMB also has high specificity for the prostate and has utility as a serum biomarker for PCa [24]. In addition, a single-nucleotide polymorphism of MSMB has been reported to increase the risk of developing PCa [25]. It was also reported that immunohistochemical staining of AZGP1 was a predictor of tumor recurrence and could be used as a specific serum biomarker for PCa [26, 27]. Although we could not detect PSA in our CAST library, we think that PSA might be one of the genes difficult to ligate into the pCAST vector. In contrast, little is known about NBL1 expression in PCa. NBL1 can stimulate differentiation of neuroblastoma cells in culture in the presence of retinoic acid [30], and its growth-suppressive activity has been noted in sarcoma cells [31]. NBL1 may play an important role in preventing cells from entering into the S phase [32]. NBL1 mRNA was reported to be down-regulated in PCa compared with corresponding NP by quantitative RT-PCR analysis [28, 29].

Because the genes in the present study were identified by CAST analysis of PCa cell lines and quantitative RT-PCR analysis of bulk PCa and NP tissues, immunohistochemical analysis was required to determine which cells expressed these genes. With this analysis, we confirmed that NBL1 was highly expressed in the epithelium of PCa.
and NP. In prostate samples, NBL1 expression was high in NP and lower in PCa. Furthermore, average NBL1 expression was significantly reduced according to the progression of stage, Gleason grade and preoperative PSA value. Because NBL1 functions as a tumor suppressor gene, these results were consistent with those of previous reports. NBL1 expression was detected in epithelium of the intestine, pancreatic islets and nerve cells, but was absent in other non-cancerous systemic tissues and stromal cells in adult humans. Ozaki et al. [33] examined NBL1 expression in rat tissue by Northern blot analysis and showed that NBL1 was detected in brain, intestine, kidney and lung. They did not test NBL1 expression in rat prostate, but their results were similar to those of our present study in humans.

We also confirmed with Western blot analysis that high NBL1 expression was detected in DU145 cells in culture medium. Nakamura et al. [15] also reported that NBL1 was observed in the culture medium, and the amount of NBL1 secreted from the cells was calculated to be 80% of the total NBL1 protein. Furthermore, NBL1
expression was detected in prostatic ducts in PCa and NP. Therefore, NBL1 may be secreted into semen as well as MSMB, prostatic acid phosphatase and PSA. PSA is produced by secretory epithelial cells in the acini and ducts, and it is secreted directly into the lumen. A characteristic feature of PCa is disruption of the basal cell layer and basement membrane, and this loss of the normal glandular architecture appears to allow PSA increased direct access to the peripheral circulation. PSA is normally found at lower levels in paraurethral and perianal glands, apocrine sweat glands, breast, thyroid and placenta, but these sites do not normally contribute measurable levels of PSA to the circulation [34]. Therefore, in spite of the fact that PSA expression is also higher in NP than in PCa in immunohistochemical analysis, serum PSA is increased in patients with PCa. Although establishment of an enzyme-linked immunosorbent assay system for serum samples is needed to clarify whether NBL1 can serve as a serum marker for detecting and monitoring PCa, we believe that NBL1 might be useful as a serum biomarker for PCa. NBL1 might be beneficial in addition to PSA in situations where PSA is less useful, such as in patients with low PSA level or castration-refractory disease.

The regulation of NBL1 is poorly understood, especially in PCa. Because NBL1 is thought to be a tumor suppressor gene, it is thought that NBL1 in PCa has mutations, deletions and methylation. Ozaki et al. [35] reported that two transcription sites were present in the rat NBL1 gene, suggesting the possibility of transcriptional regulation of NBL1. Further studies will clarify how NBL1 is regulated and whether androgen and the androgen receptor axis regulate NBL1. The biological function of the NBL1 protein is also poorly understood in PCa. NBL1 expression correlated with PCa progression, but NBL1 knockdown did not reduce viability and invasiveness relative to the negative control. Because NBL1 was also reported to act as a bone morphogenetic protein (BMP) antagonist by binding to BMPs [36] and BMPs are known to participate in the progression of PCa [37, 38], we speculate that interaction between NBL1 and BMP may play a more important role during growth and development in PCa than in cell cycle inhibition.

In summary, the present study yielded a list of genes that encode secreted proteins present in PCa and NP from CAST analysis. NBL1 expression is narrowly restricted to the prostate and is higher in NP than in PCa. Underexpression of NBL1 is associated with tumor progression. NBL1 has high potential as a biomarker of PCa and its progression.

Acknowledgments

We thank Mr. Shinichi Norimura for his excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. We thank the Analysis Center of Life Science, Hiroshima University, for the provision of their facilities. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan; in part by a Grant-in-Aid for the Third Comprehensive 10-Year Strategy for Cancer Control and for Cancer Research from the Ministry of Health, Labor and Welfare of Japan, and in part by a grant (07-23911) from the Princess Takamatsu Cancer Research Fund.

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