Neuropilin-2 is an independent prognostic factor for shorter cancer-specific survival in patients with acinar adenocarcinoma of the prostate

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Neuropilin-2 (NRP2) is a member of the neuropilin receptor family and known to regulate autophagy and mTORC2 signaling in prostate cancer (PCa). Our study investigated the association of immunohistochemical NRP2 expression with clinicopathological data in PCa patients. For this purpose, we generated a tissue microarray with prostate tissue specimens from 400 PCa patients treated by radical prostatectomy. We focused on patients with high-risk factors such as extraprostatic extension (pT ≥ 3), Gleason score ≥ 8 and/or the presence of regional lymph node metastases (pN). Protein levels of NRP2, the vascular endothelial growth factor C (VEGFC) and oncogenic v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) gene as an indicator for TMPRSS2-ERG fusion was assessed in relation to the patients’ outcome. NRP2 emerged as an independent prognostic factor for cancer-specific survival (CSS) (hazard ratio 2.360, 95% confidence interval = 1.2–4.8; p = 0.016). Moreover, the association between NRP2 expression and shorter CSS was also especially pronounced in patients at high risk for progression (log-rank test: p = 0.010). We evaluated the association between NRP2 and the TMPRSS2-ERG gene fusion status assessed by immunohistochemical nuclear ERG staining. However, ERG staining alone did not show any prognostic significance. NRP2 immunostaining is significantly associated with shorter CSS in ERG-negative tumors (log-rank test: p = 0.012). No prognostic impact of NRP2 expression on CSS was observed in ERG-positive tumors (log-rank test: p = 0.153). Our study identifies NRP2 as an important prognostic marker for a worse clinical outcome especially in patients with a high-risk PCa and in patients with ERG-negative PCa.

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Additional Supporting Information may be found in the online version of this article.

Key words: ERG, neuropilin-2, prognosis, prostate cancer, VEGFC

Abbreviations: CSS: cancer-specific survival; EGFR: epithelial growth factor receptor; ERG: v-ets avian erythroblastosis virus E26 oncogene homolog gene; ETS: E26 transformation-specific; NRP: neuropilin; OS: overall survival; PCa: prostate cancer; RP: radical prostatectomy; TMA: tissue microarray; TMPRSS: transmembrane protease serine; VEGF: vascular endothelial growth factor

Conflict of interest: M.R. declares a potential financial conflict of interest due to honoraria for lectures by Amgen. All other authors declare no conflict of interest.

Grant sponsor: Deutsche Forschungsgemeinschaft/German Research Foundation; Grant sponsor: Ferdinand-Eisenberger-Fellowship of the German Society of Urology

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DOI: 10.1002/ijc.32679

History: Received 7 Apr 2019; Accepted 7 Aug 2019; Online 11 Sep 2019

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Introduction

Prostate cancer (PCa) is the most common malignant disease in men in the western world. In most patients with PCa, the disease is characterized by a long survival time and clinicopathological parameters are very important predictors for treatment response or survival. However, in a subgroup of patients, the disease progresses after local treatment. Therefore, it is of utmost importance to reliably identify men with aggressive disease, but prognostic biomarkers are still lacking for this subgroup.

Neuropilin-2 (NRP2) is a member of the neuropilin family of receptor proteins; the other member is Neuropilin-1 (NRP1). NRP2 is a non-tyrosine kinase receptor and functions as a co-receptor of vascular endothelial growth factor (VEGF)-receptors or plexins by modulating various cellular pathways including angiogenesis, cellular communication and migration. NRP2 plays a substantial role in the development of vascular capillaries and lymphatic vessels. NRP2 is expressed in numerous human cancers like PCa. During carcinogenesis, it is involved in proliferation, survival, migration and therapy resistance. One of the ligands for NRP2 is the VEGF. VEGFC promotes autophagy upon binding to NRP2 to escape chemotherapeutic stress in PCa cells. Together with NRP2, VEGFC promotes mTORC2 activation under oxidative stress. Moreover, NRP2, VEGFC and their co-expression emerged as predictive markers for radio-chemotherapy in bladder cancer patients. NRP2 also cross-talks with the insulin-like growth factor 1 receptor (IGF-1R) axis and promotes PCa progression and therapy resistance by regulating BMI-1 signaling.

Recently, the two most abundant isoforms of NRP2—NRP2a and NRP2b—have proven to elicit different functions in cancer cells. In this respect, the hepatocyte growth factor (HGF) plays an important role for cancer progression by inducing NRP2b-mediated Akt phosphorylation. NRP2b is also an important mediator for the signaling events initiated by transforming growth factor β (TGF-β). For PCa, it has been demonstrated that NRP1 was associated with increased tumor stage and Gleason grading as well as with nodal status. Although the expression of NRP2 in several tumor tissues and its association with tumor progression was described previously, its prognostic impact in primary PCa has not been investigated so far.

The PCa-specific gene fusion of the promoter and 5′-untranslated region of transmembrane protease serine 2 (TMPRSS2) with the oncogenic v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) gene, which belongs to the E26 transformation-specific (ETS) family of transcription factors, is an early event in PCa onset. However, the role of TMPRSS2-ERG gene fusion as prognostic marker for PCa progression remains contradictory. This fusion event results in an increased protein expression of ERG. In our study, we evaluated the staining of NRP2 and VEGFC in primary PCa tissue in a large cohort of 400 patients who underwent radical prostatectomy (RP) due to PCa. We investigated the role of NRP2 as potential predictor of unfavorable PCa outcome. Furthermore, we analyzed the role of ERG alone and in combination with NRP2 staining as prognostic marker in this PCa cohort.

Methods

Patient cohort for tissue microarray (TMA) analyses

For the generation of a TMA, paired malignant and non-malignant prostate tissue specimens from 400 patients with PCa were used. Patients were treated between 1996 and 2005 by RP at the Department of Urology of the Technische Universität Dresden, Germany. The study was approved by the Institutional Review Board of the Medical Faculty of the Technische Universität Dresden (EK194092004 and EK195092004), and written informed consent was obtained from each patient. Follow-up data regarding cancer-specific survival (CSS) and overall survival (OS) were obtained from medical records and by contacting the treating urologists, oncologists and general practitioners. CSS as the primary endpoint was defined as time between RP and the date of cancer-specific death. Data of patients not succumbing to PCa were censored at the date of last follow-up or date of death from any other cause. OS was defined as the time between RP and the date of death. Patient data without reported date of death were censored at the day of last follow-up.

For the calculation of potential associations with clinicopathological and survival parameters, patient groups differing in pathological tumor stages (pT), Gleason scores (GS) and pathological lymph node stages (pN) were compared. Patients were stratified according to prognostic risk factors as follows: low-risk (pT2 / GS ≤6 / pN0), intermediate-risk (pT2 / GS 7 / pN0) and high-risk (pT ≥3, GS ≥8 and/or pN1). As reported previously, the cohort was enriched for patients representing high-risk PCa. None of the patients showed distant metastases.
Staining scores for NRP2 and ERG were dichotomized into total of 14 paraffin blocks. Diagnostic hematoxylin and eosin stained tissue sections from the RP specimens were reviewed by an experienced board-certified uropathologist (M.H.M.) and representative tumor areas were assigned. Four tumor cores were selected per patient within these areas. Cores were mounted into a tumor areas were assigned. Four tumor cores were selected into a HRP Kit (Vector Laboratories; Burlingame, CA) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Steinheim, Germany) as substrate or the BrightVision plus poly-HRP Kit (Immunologic, Duiven, the Netherlands) and the DAB-3S Kit BULK (Nicheirei Biosciences Inc., Chuo, Tokyo, Japan). Immunohistochemical staining of ERG served as surrogate marker for ERG gene fusion events that otherwise be detected by fluorescence in situ hybridization.25–27 The percentage of stained cells (0–100%) (cytoplasmic staining) and the intensity of staining (1 = low, 2 = medium, 3 = high) were assessed in case of VEGFC staining. For the scoring, we multiplied the percentage with the intensity. Because NRP2 and ERG staining allow a clear distinction between a positive and a negative staining, we scored only “positive” or “negative” in case of NRP2 and ERG. In case of NRP2, a distinction between membranous and cytoplasmic staining was not performed. For ERG, only the nuclear staining was counted. Evaluation was performed by two board-certified uropathologists (M.T. and M.H.M.).

Immunohistochemistry
The protein levels of NRP2, VEGFC and ERG were analyzed by immunohistochemistry using commercially available antibodies against total NRP2 (monoclonal mouse anti-human antibody C-9 / sc13117; Santa Cruz Biotechnology, Dallas, TX), VEGFC (polyclonal rabbit anti-human antibody PAD Z-CVC7, Thermo Fisher, Schwerte, Germany) and ERG (monoclonal rabbit anti-human antibody EP111; Zeta; Arcadia, CA). Staining was performed on 2 μm TMA sections using the immunoperoxidase-based universal VECTASTAIN Elite ABC HRP Kit (Vector Laboratories; Burlingame, CA) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Steinheim, Germany) as substrate or the BrightVision plus poly-HRP Kit (Immunologic, Duiven, the Netherlands) and the DAB-3S Kit BULK (Nicheirei Biosciences Inc., Chuo, Tokyo, Japan). Immunohistochemical staining of ERG served as surrogate marker for ERG gene fusion events that otherwise be detected by fluorescence in situ hybridization.25–27

Statistical analyses
Data were analyzed using SPSS v24.0 (IBM Corp, Armonk, NY). Categorical data are presented as absolute and relative frequencies. Continuous variables are described as means with standard deviation, complemented by median and interquartile range. The Student’s t-test was used to compare means of independent groups. The Chi-square test was used to compare absolute frequencies. The prognostic impact of NRP2 and ERG (positive vs. negative) as well as of VEGFC expression (low expression vs. high expression) were assessed by Kaplan–Meier analyses. Differences in CSS and OS were evaluated by the log-rank test, respectively. These analyses were performed in the whole cohort and in patient subgroups stratified according to the risk (low- and intermediate- vs. high-risk, tumor stage (pT2 vs. pT ≥3), Gleason score (GS ≤7 vs. GS ≥8) and lymph node stage (pN0 vs. pN1)). Uni- and multivariate Cox’s proportional hazard regression analyses were performed to assess the prognostic impact of NRP2, ERG and VEGFC protein levels on CSS adjusted to pT stage (pT ≥3 vs. pT2), GS (GS ≥8 vs. GS ≤7) and pN (pN1 vs. pN0). Statistically significant parameters in univariate Cox’s proportional hazard regression analysis were included in multivariate analysis. A p-value of <0.05 was considered as statistically significant.

Data availability
Data available on request due to privacy/ethical restrictions.

Results
Demographic and clinicopathological characteristics of PCa patients
Patients’ and histopathological characteristics are depicted in Table 1. In total, 81% (n = 325) of the analyzed tumors were staged as high-risk PCa with ≥pT3, GS ≥8 or pN1. Only 51 and 24 patients presented low- and intermediate-risk PCa, respectively.

| Table 1. Patients and histopathological characteristics | All (n = 400) |
|--------------------------------------------------------|--------------|
| Age (years) (mean ± SD)                                 | 65 ± 5.64    |
| PSA (ng/ml) (mean ± SD)                                 | 13.0 ± 12.9  |
| pT2 (n)                                                 | 178          |
| pT3 (n)                                                 | 154          |
| pT4 (n)                                                 | 68           |
| pN0 (n)                                                 | 294          |
| pN1 (n)                                                 | 106          |
| Low-risk PCa (n)                                        | 51           |
| Intermediate-risk PCa (n)                              | 24           |
| High-risk PCa (n)                                       | 325          |
| Gleason score ≤ 6 (n)                                   | 78           |
| Gleason score 7 (n)                                     | 75           |
| Gleason score ≥ 8 (n)                                   | 247          |
| Follow-up (years) (mean ± SD)                           | 9.5 ± 2.9    |
| NRP2 expression (n (%))                                 | 128 (32%)    |
| High VEGFC expression (n (%))                           | 137 (34%)    |
| ERG expression (n (%))                                  | 182 (46%)    |
Accordingly, the cohort was enriched for patients with high-risk PCa and does not represent the distribution of a natural RP cohort. High-risk PCa was significantly associated with shorter CSS. CSS was 15.9 years in patients with low- and intermediate-risk PCa versus 11.2 years in patients with high-risk PCa (95% confidence interval [CI] = 15.33–16.57; \( p = 0.003 \)). While the 10-year CSS was 100% in patients with low- or intermediate-risk PCa, it was only 89% in patients with high-risk PCa (data not shown).

**Immunohistochemical staining of NRP2, ERG and VEGFC and association with clinical parameters**

Membranous and cytoplasmic staining of NRP2 could be assessed in 396 patients (Fig. 1a). Two hundred sixty-eight patients (68%) showed positive staining for NRP2 (Fig. 1a), whereas in 128 patients (32%) NRP2 staining was negative. NRP2 was detected in 33% (107/325) of high-risk PCa, in 33% (74/222) with a pathological stage ≥pT3, in 34% (85/247) with a GS ≥8 and in 31% (33/106) with the evidence of locoregional lymph node metastasis (pN1). In 28% of patients (n = 21) with low- and intermediate-risk PCa (n = 75), NRP2 staining was detected.

Nuclear staining for ERG (Fig. 1b) and cytoplasmic staining for VEGFC (Fig. 1c) could be assessed in all patients (n = 400). In 42% of the patients (168/400), nuclear ERG staining (Fig. 1b) was detectable, whereas in 58% (232/400) ERG staining was negative. Furthermore, ERG was detected in 45% (146/325) of high-risk PCa, in 48% (107/222) with a pathological stage ≥pT3, in 44% (109/247) with a GS ≥8 and in 49% (52/106) with the evidence of lymph node metastasis. High VEGFC protein levels were detected in 34% (137/400) of all cases, whereas 66% (263/400) of the cases showed low VEGFC levels. High VEGFC levels were also observed in 33% (107/325) of high-risk PCa, in 33% (74/222) with a pathological tumor stage ≥pT3, in 34% (83/247) with a GS ≥8 and in 41% (33/106) with the evidence of lymph node metastasis.

Patients with detectable NRP2 staining, high VEGFC levels and ERG staining did not show significantly different preoperative PSA serum levels or age compared to patients without NRP2 expression, low VEGFC levels or negative ERG staining. Concerning the histopathological parameters, there was no significant association of the NRP2 status, VEGFC level or the ERG status, with tumor stage, lymph node status and the GS (data not shown).

**Association of outcome and NRP2 staining**

NRP2 staining in the whole patient cohort indicated a significantly shorter CSS with a mean survival time of 15.5 years.
Figure 2. Association of NRP2 staining with CSS of the whole cohort (a), in patients with high-risk PCa (b), with pT ≥ 3 (c), with GS ≥ 8 (d) and with pN1 (e). The number of patients in each group is indicated, in which the values in brackets represent the number of events.

(95% CI = 14.5–16.5) compared to 16.1 years (95% CI = 15.6–16.6; \( p = 0.007 \)) for patients without NRP2 staining (Fig. 2a). Accordingly, the 5- and 10-year survival rates were lower for patients with positive NRP2 staining in comparison to patients without NRP2 protein expression (5-year CSS 92% vs. 97% and 10-year CSS 86% vs. 94%; Fig. 2a). In contrast, immunohistochemical staining of NRP2 was not associated with OS in the whole patient cohort (Supplemental Fig. S1).

In the subgroup of patients with high-risk PCa, the NRP2 expression was significantly associated with CSS (log-rank test: \( p = 0.010 \); Fig. 2b). Furthermore, patients with non-organ confined tumors (≥pT3), high-grade tumors (GS≥8) or with lymph node metastasis (pN1) were characterized by a significantly shorter CSS when NRP2 was expressed (Figs. 2c–2e).

In the univariate Cox’s proportional hazard regression analysis, patients with NRP2-positive staining presented a 2.5-fold higher probability of cancer-specific death (hazard ratio [HR] = 2.5, 95% CI = 1.27–5.10; \( p = 0.009 \)). More importantly, expression of NRP2 appeared as an independent prognostic indicator of cancer-specific death (HR = 2.4, 95% CI = 1.17–4.75, \( p = 0.016 \)) as revealed by multivariate Cox’s regression analyses and adjustment to stage (pT), Gleason Score and regional lymph node metastases (pN) (Table 2).

**Association of outcome and VEGFC staining**

We detected no prognostic impact of VEGFC levels with regard to OS and CSS in the whole cohort (Supplemental Figs. S2a and S2b) and to CSS in patients with high-risk PCa (Supplemental Fig. S2c). Moreover, VEGFC protein levels showed no significant association with CSS in PCa patients with non-organ confined tumor stage, high GS or the evidence of lymph node metastasis (Supplemental Figs. S2d–S2f). Cox’s regression analysis also revealed no prognostic influence of the VEGFC staining on CSS (univariate analysis: HR = 1.1, 95% CI = 0.51–2.21; \( p = 0.867 \); Table 2).
Table 2. Uni- and multivariate Cox regression analysis: parameters for the prediction of CSS in patients with PCa

| Parameter | Comparison | Univariate Cox analysis HR (95% CI) | Univariate Cox analysis p-value | Multivariate Cox analysis HR (95% CI) | Multivariate Cox analysis p-value |
|-----------|------------|------------------------------------|---------------------------------|--------------------------------------|-----------------------------------|
| Age (median 65 years) | ≤ vs. > median | 0.693 (0.338–1.422) | 0.317 | - | - |
| Gleason score (GS) | GS ≥ 8 vs. GS ≤ 7 | 5.165 (1.803–14.791) | 0.002 | 3.871 (1.333–11.240) | 0.013 |
| pT | pT ≥ 3 vs. pT2 | 26.764 (3.65–196.12) | 0.001 | 16.168 (2.18–120.12) | 0.007 |
| pN | pN1 vs. pN0 | 3.841 (1.896–7.781) | <0.001 | 3.085 (1.509–6.306) | 0.002 |
| VEGFC intensity | High vs. low | 1.065 (0.513–2.209) | 0.867 | - | - |
| ERG staining | Positive vs. negative | 0.942 (0.468–1.895) | 0.867 | 2.360 (1.174–4.747) | 0.016 |
| NRP2 staining | Positive vs. negative | 2.542 (1.267–5.102) | 0.009 | - | - |

Association of outcome and NRP2 and VEGFC co-expression

A 49% of patients with high levels of VEGFC (67/136) and 23% of patients (61/260) with low levels of VEGFC showed a positive NRP2 staining. Focusing on patients for high levels of VEGFC, there was no significant difference in CSS between patients with or without positive NRP2 staining (p = 0.109; Fig. 3a). However, in patients with low VEGFC levels, patients with NRP2-positive tumors showed a significantly shorter CSS than those with NRP2-negative tumors (13.1 years, 95% CI = 12.0–14.3 vs. 16.0 years, 95% CI = 15.3–16.6; p = 0.022; Fig. 3b).

Association of outcome and ERG staining as a surrogate marker for fusion positive PCa

In the whole patient cohort, there was no significant association between ERG status and OS (Supplemental Fig. S3a). Moreover, ERG positivity was not associated with CSS (p = 0.867; Supplemental Fig. S3b). The same was true for patients with high-risk PCa (p = 0.892; Supplemental Fig. S3c). In accordance with these observations, immunohistochemical ERG staining was not a prognostic marker as assessed by univariate Cox’s regression analysis (HR = 0.94, 95% CI = 0.47–1.89; p = 0.867; Table 2).

Figure 3. Association of NRP2 staining with CSS in patients stratified according to high levels of VEGFC (a) and low levels of VEGFC (b). The number of patients in each group is indicated, in which the values in brackets represent the number of events.
Association of outcome and NRP2 and VEGFC expression in fusion positive and negative PCa

Out of the patients with positive ERG staining, 18% of the patients (71/182) showed an NRP2 expression. When considering the outcome of patients with positive ERG staining, no significant differences in CSS were detected between NRP2-positive and NRP2-negative patients (Fig. 4a). In contrast, in the subgroup of ERG-negative patients those with NRP2 staining (57/214) showed a significantly shorter CSS than those without NRP2 expression (log-rank test: \( p = 0.012 \); Fig. 4b).

When the patients were stratified into subgroups according to their VEGFC levels, no differences in CSS were detected between patients with ERG-positive and ERG-negative tumors for both VEGFC subgroups (data not shown).

Discussion

For men with localized PCa undergoing surgical treatment with curative intent, it is important to define prognostic markers for those at the highest risk to develop metastases and to die from the disease. The identification of useful clinicopathological factors and suitable biomarkers might lead to an individualized follow-up and tumor treatment. For this purpose, the prognostic potential of NRP2 and its ligand VEGFC as well as ERG expression as surrogate marker for TMPRSS2-ERG were evaluated in a large RP cohort enriched with high-risk PCa patients.

We previously reported an important role of the NRP2 axis in inducing antiapoptotic signaling during oxidative stress in PCa.\(^1\) Subsequent studies from our group showed that this axis also regulates autophagy and trafficking of the epithelial growth factor receptor (EGFR) in PCa cells.\(^9,28\) Both molecular pathways are important for cancer progression. The finding that NRP2 regulates Bmi-1 and modulates IGF-1R signaling in PCa underscores its importance in PCa and as possible marker for treatment response for drugs targeting the IGF-1R pathway.\(^13\) Moreover, calpain-mediated promoter methylation of the antimetastatic collapsing response mediator protein-4 (CRMP4) also promotes PCa metastases through the VEGFC/NRP2 axis.\(^29\) Furthermore, it has already been shown that immunohistochemical staining of NRPI, the other member of the neuropilin family, in PCa tissues was associated with increased tumor stage and Gleason grading as well as with nodal status.\(^14\)

Accordingly, we evaluated the role of immunohistochemical staining of NRP2 and VEGFC as ligand of NRP2 on a TMA as potential prognostic markers for PCa patients treated by RP. Cancer-specific death was considered as the primary end point in these analyses. NRP2 protein staining emerged as an independent predictor for shorter CSS in patients with PCa. Stratifying by risk factors, the prognostic value of NRP2 staining became more pronounced in patients with high-risk PCa (pT \( \geq 3 \), GS \( \geq 8 \) and/or pN1) compared to those with low- and intermediate-risk PCa. In high-risk PCa, especially in
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NRP2 expression. These whereby lymph node and bone metastases showed robust invasive potential of PCa and lymph node or bone metastases especially in patients with unfavorable PCa. However, the authors did not report any association of NRP2 expression with CSS. 

We observed predominantly cytoplasmic staining of NRP2 in PCa cells, which was similar to our previous study in bladder cancer. Most of the evaluated cases were characterized by cytoplasmic staining. Preliminary data from our work group clearly show that the localization of NRP2 inside the cells plays an important role for tumor progression and metastases. In addition, we have previously shown that the VEGFC/NRP2 axis influences the endocytosis of EGFR and thus, cell survival and proliferation.

We could not detect any prognostic value for the VEGFC tissue staining in PCa, although VEGFC is an important ligand for NRP2. In earlier studies, we demonstrated that VEGFC protects PCa cells from oxidative stress and regulates the maturation of autophagosomes as well as EGFR trafficking in PCa cells. Other groups have also shown an important role of VEGFC in PCa progression by evaluating the presence of VEGFC on protein level in primary PCa tissues and in experimental models. In bladder cancer, we reported an association of VEGFC and NRP2 expression with resistance to radiochemotherapy. Other ligands for NRP2 might also be important for cancer progression like the HGF receptor derived NRP2 suggesting a possible regulatory function of ERG for PCa innervation. Interestingly, in our cohort, NRP2 expression was significantly associated with shorter time to cancer-specific death in ERG-negative patients. This suggests that the prosurvival and prometastatic function of ERG could be substituted by molecules like NRP2. Taken together, this implies a prognostic impact of NRP2 expression especially in ERG-negative patients.

The commercially available antibody against NRP2 used in our study detects total NRP2 including the two most abundant isoforms—NRP2a and NRP2b. Recently, it has been demonstrated that the isoform NRP2b plays an important role for disease progression in a subcutaneous xenograft model of lung cancer. Further studies with specific antibodies detecting individual isoforms are needed.

In summary, we have shown an association between NRP2 protein levels in PCa tissues and CSS in patients with localized PCa treated by RP. NRP2 was associated with a worse outcome especially in high-risk PCa patients and patients with negative ERG status. NRP2 emerged as independent prognostic marker for cancer specific death. Therefore, it could be considered as a novel biomarker that can indicate aggressive PCa.

Acknowledgements

A.B. was supported by the Ferdinand-Eisenberger-Fellowship of the German Society of Urology (DGU). This work was supported by the Deutsche Forschungsgemeinschaft/German Research Foundation (DFG MU2687/5-1 to M.H.M. and L.C.H.) and the SKELMET Consortium funded by the DFG. The tumor and normal tissue bank of the University Cancer Center Dresden, Technische Universität Dresden provided the primary PCa tissues for the creation of the investigated tissue microarray.
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