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Abstract

Background. Perineural invasion (PNI) is generally accepted as a major route of cancer dissemination in malignancies associated with highly enervated organs. However, the effect of cancer cells on vasa nervorum remains unknown. We studied this effect in locally advanced prostate cancer, a high-risk feature associated with approximately 20 % of prostate cancer specific mortality.

Methods. We used immunohistochemistry for CD34, fibroblast growth factor-2 (FGF-2), FSHR, podoplanin, vascular endothelial growth factor (VEGF), and VEGFR-2 as well as histochemical methods to examine the vasa nervorum of nerves invaded by cancer cells in tissue samples from 85 patients.

Results. The percentage of the nerve area occupied by CD34-positive vasa nervorum endothelial cells in nerves with PNI was much higher than in nerves without PNI (7.3 ± 1.2 versus 1.9 ± 0.4; $p<0.001$ and 5.8 ± 0.6 versus 1.23 ± 0.8; $p<0.001$ in pT3a and pT3b prostate cancer specimens, respectively). In 19/85 of the patients the CD34-positive vasa nervorum microvessels have a thick basement membrane, similar to the vessels in diabetic microangiopathy. This subendothelial layer contains collagen fibers. Vasa nervorum endothelia and Schwann cells express FGF-2 (nuclear localization) and FSHR (plasma membrane and cytoplasmic staining). Prostate cancer cells invading nerves express VEGF, a critical cytokine in tumor angiogenesis. The vasa nervorum of prostatic nerves with PNI did not express detectable levels of VEGFR-2. No podoplanin-positive lymphatic vessels were seen in nerves.

Conclusion. In locally advanced prostate cancer, perineural invasion of cancer cells is associated with formation of new endoneurial capillaries and changes of vasa nervorum morphology.

INTRODUCTION

Tumor metastasis is a complex and highly selective process whereby cancer cells leave the primary tumor site and disseminate to other locations[1] via distinct routes involving blood vessels, lymphatic vessels, caelomatic cavities, and migration of cancer cells along nerves[2]. The latter route is encountered frequently in prostate and other cancers (pancreas, colon, rectum, biliary tract, stomach, skin, salivary gland, head and neck)[3]. Migration of cancer cells in close proximity of the nerve and/or within any of the three layers of the nerve (epineurium, perineurium, and endoneurium) constitutes the perineural invasion process[4]. Secreted neurotrophic factors that form a concentration gradient along nerves[5,6] may play a pivotal role in perineural invasion[7]. This is the case of the glial-derived growth factor that activates RET-receptor mediated cancer cell chemotaxis, which in turn guides the directional cancer cell migration towards and along the nerves[8]. Nerves not only harbor cancer cells, but they appear to actively promote cancer cell penetration of the nerves[9] and survival, and decreased apoptosis of the invading cells[10].

Recent studies have demonstrated that perineural invasion results in i) cancer-related axogenesis/neurogenesis[11] and ii) cancer cell proliferation and migration[11,12]. These biological processes are highly dependent on a local supply of oxygen and nutrients provided by blood vessels that accompany the nerves and/or penetrate into the nerves (vasa nervorum). However, basic questions about blood supply for perineural invasion zones remain unanswered. For example, it is not known if the invading cancer cells in human tumors utilize the same strategies as those well documented in animal models of prostate cancer (ex., neovascularization by sprouting angiogenesis from pre-existing endothelial cells in established vessels)[13]. A potential alternative is that perineural invasion develops the ability to progress independently of neovascularization. The answers to these questions should critically impact the potential of new therapeutic

approaches to prevent cancer cells from leaving the primary tumor site and disseminate to distant organs.

Antibodies for endothelial cell markers (Table 2), were used to study the effect of perineural invasion on vasa nervorum in patients diagnosed with locally advanced prostate cancer. In this type of cancer the tumor cells penetrated through the fibromuscular pseudocapsule covering the prostate gland, but did not spread to lymph nodes or to distant areas (T3-N0-M0, in the TNM staging system[14]. We analyzed locally advanced prostate cancer specimens because this type of cancer comprises about 5-10 % of all newly diagnosed prostate cancers and it is associated with approximately 20 % of prostate cancer specific mortality[15]. Modifications in the morphology of vasa nervorum have been also analyzed by histochemical methods.

MATERIALS AND METHODS

Tissue specimens

Paraffin sections of locally advanced prostate cancer tissue fixed in 10% neutral formalin were obtained from the biorepositories of Paris Hospitals Lariboisière (57 patients) and Tenon (28 patients) (Table1). The prostate cancer tissues were included in paraffin in the period 2009-2015. Two pathologists (C.C. and J-F.C.) reviewed all cases for the present study. The pathologic stage T3 (pT3) was assigned according to the World Health Organization guidelines[16]. Extraprostatic extension (pT3a) was diagnosed if tumor cells were present in the periprostatic soft tissue or penetrated through a fibromuscular pseudocapsule and came out on the other side. The seminal vesicle invasion (pT3b) was defined as tumor tissue present within the fibromuscular wall of the seminal vesicles. All pelvic lymph nodes were evaluated for the presence of metastatic disease. All cases were assigned a Gleason score.

The protocol was approved by the institutional ethics committee at each study site. Written informed consent was obtained at the time of surgery from all living donors from whom samples were obtained.

Immunohistochemistry

Paraffin sections (5 µm) of both pT3a and pT3b tumors were immunolabeled with antibodies directed against several endothelial markers (Table 2). Immunohistochemistry was carried out using an automated immunohistochemical stainer according to the manufacturer's guidelines (Leica Bond RX, Leica Biosystems). Antigen retrieval was conducted by treatment with high temperature at pH 6 or pH 9 (Table 2).

Image and statistical analysis

In our study we have analyzed all the nerves (invaded or not invaded by cancer cells) present on the tissue paraffin sections (the range was 3 – 20 nerve profiles per section). The normal nerve profiles were located at least 500 µm from the tumor border, outside the prostatic stroma. The nerves with PNI were analyzed inside and outside the prostatic stroma. We have quantitatively determined the density of FSHR-, CD34-, D2-40-positive blood vessels. This was done by counting the number of marker-positive vessels on digital images from whole images of serial sections obtained by using the Philips Digital Ultra-Fast Scanner 1.6 RA and Philips Image Management System 2.2RA, available in Curie Institute. The area occupied by CD34-positive endoneurial endothelial cells was calculated using the formula: % of endoneurial endothelial cells area = Σ pixels of endoneurial endothelial cells area × 100 / Σ pixels of the nerve area, as previously described[17]. The pixel density of the image was measured with the "color deconvolution function" of FIJI software in the range of 0 – 255, where 0 means presence of endothelial cells and 255 means absence. The computations were performed separately for nerve areas in which tumoral invasion was present or absent. The values presented are means

± standard deviations (SD). Relationships between the vascular expression of CD34 and FSHR, and clinicopathological data were examined by Pearson's correlation coefficient. The statistical significance was evaluated using the 2-tailed t-test.

RESULTS

Effect of cancer invasion of nerves on vasa nervorum

In order to obtain reliable results, we had to analyze the effect of PNI on vasa nervorum in nerve-reach prostate areas. Published data indicate that the innervation of prostate is richest in the “capsular” regions, with density of innervation increasing towards the seminal vesicles[18]. These areas correspond to the pathological T3a and T3b prostate tumors[16], which we have analyzed in the present study. We noticed the presence of prostate cancer cells along these nerves (Fig. 1A). In the majority of the cases PNI was detected before metastasis, which is the result of dissemination through lymphatic or blood vessels.

The presence of cancer cells within any of the three layers of the nerve (i.e., epineurium, perineurium, and endoneurium) may cause compression and destabilization of vasa nervorum, which should lead to reduced perfusion and hypoxia. Hypoxia, in turn, is known to induce secretion of angiogenic factors involved in prostate cancer progression and metastasis[19]. Because the formation of new endoneurial capillaries *in vitro* depends on the presence of angiogenic factors[20], we have analyzed by immunohistochemistry the expression of two potent angiogenic factors: VEGF and FGF-2. VEGF was present in the cytoplasm of most cancer cells, including those surrounding the nerves (Fig. 1B). FGF-2 was observed in the nuclei of vasa nervorum and Schwann cells, both in nerves that have or do not have perineural invasion (Fig. 1C). A strong FGF-2 staining was also noticed in both tumor and peritumoral endothelial cells (not illustrated). These results suggest that VEGF and FGF-2, via their endothelial receptors,

may activate pathways leading to endothelial cell proliferation and new vasa nervorum formation.

Angiogenesis of vasa nervorum induced by PNI is expected to lead to increased number of endothelial cells per mm^2 of nerves. To find out whether this is the case, we determined the percentage of nerve area occupied by endothelial cells, as detected by the endothelial marker CD34[21]. The pictures in Figs. 2A and 2B are representative for the distribution of CD34-positive vasa nervorum of nerves without or without PNI, respectively. A similar pattern was noticed for two other neurotropic cancers, pancreatic cancer and colon cancer (not illustrated).

In the pT3a prostate cancer specimens, the percentage of the area occupied by CD34-positive endothelial cells associated to the nerves with PNI was much higher than that in nerves without PNI (7.3 ± 1.2% *versus* 1.9 ± 0.4%; $p < 0.001$) (Fig. 2C). In the pT3b prostate cancer specimens, the percentage of the area occupied by CD34-positive endothelial cells associated to the nerves with PNI was also much higher than that in nerves without PNI (5.8 ± 0.6 *versus* 1.23 ± 0.8; $p < 0.001$). No significant difference was found between pT3a and pT3b cases ($p = 0.59$). The percentage of the nerve area occupied by CD34-positive endothelial cells in the nerves with PNI correlated with the clinical stage ($r = 0.62$; $p = 0.008$) and with the tumor size ($r = 0.62$; $p = 0.01$).

Although it is generally thought that cancer is associated with increased microvessel density in a variety of human cancers, this view is controversial for prostate cancer[22]. We have determined the CD34-positive vessel density in the prostate in regions of cancer and in the benign peritumoral area. Our results showed that tissue sections without cancer were significantly more vascularized than were tumors (3.28 ± 0.5 % *versus* 2.01 ± 0.32%; $p = 0.03$).

All these results indicate that an angiogenic process occurred for vasa nervorum of cancer invaded nerves.

In 19/85 (22%) of patients with pT3 tumors, the vasa nervorum in nerves with perineural invasion had a thick subendothelial layer containing collagen fibers (Fig. 3A), in comparison with non-invaded vasa nervorum) (Fig. 3B). Standard histochemical staining (hematoxylin-eosin-saffron staining, periodic acid-Schiff reaction and Masson's trichrome technique) indicated that in 19/85 (22%) of patients with pT3 tumors, the vasa nervorum in nerves with perineural invasion had a thick subendothelial layer containing collagen fibers, characterized by the absence of pericytes (Fig. 3).

The vasa nervorum of prostatic nerves with PNI did not express detectable levels of VEGFR-2. No podoplanin-positive lymphatic vessels were seen in nerves.

Work from our laboratory showed that FSHR is expressed by the tumor endothelial cells, most frequently at the periphery of prostate cancer tissue[23]. However, no information was available for the vascular FSHR expression in zones of perineural invasion in locally advanced prostate cancer. The density of FSHR-positive vasa nervorum (number of vessels/mm^2 of nerve tissue) of nerves with perineural invasion was not significantly different from that of nerves without perineural invasion (47.8 ± 12.5 *versus* 46.8 ± 10.4; p = 0.9). The density of FSHR-positive vasa nervorum correlated with both the final Gleason score (r = 0.48; p = 0.02) and tumor size (r = 0.69; p = 0.003). We noticed also intense FSHR staining of Schwann cells in all nerve ganglia and nerve fibers in peripheral areas affected by perineural invasion (Fig. 3D). No immunohistochemical signal was obtained by using an irrelevant mouse antibody of the same IgG2a subtype as FSHRA02 (not illustrated). In benign peritumoral tissues of cancer patients the nerves show also intense FSHR staining of Schwann cells and vasa nervorum at all analyzed Gleason scores (5-9). On a radical prostatectomy specimen obtained for a non tumor related disease, the majority of nerves and their associated vessels in the peripheral areas of the prostate do not express FSHR (not illustrated). The absence of staining in nerves and blood vessels was also noticed in a nerve ganglion in the peripheral zone.

The above results suggest that perineural invasion by prostate cancer cells could have important effects on the nerve microvessels by inducing angiogenesis and vascular remodeling.

DISCUSSION

Our study indicates that an angiogenic process occurs at the level of vasa nervorum of cancer invaded nerves, in comparison with the nerves that are not invaded. The increased blood supply is expected to facilitate further growth and invasion of tumor cells along the nerves, and progression of the disease.

The density of blood vessels in the cancer invaded nerves is higher than in the non-invaded nerves. This indicates that the angiogenic process in the cancer invaded nerves is caused by the proximity of both nerve and tumor cells. Angiogenesis could be induced by angiogenic factors released by the tumor cells, and the release can be induced by the adjacent nerves, or vice-versa. Angiogenesis was shown to occur in nerve cell grafts and at spinal cord lesions during peripheral nerve regeneration, and can be triggered by physiological tasks that increase neural function and synaptic activity[24,25]. It is possible that such increased neural function is induced by the tumor cells that invade the nerves.

In mouse models of prostate cancer, noradrenaline generated by nerves induces angiogenesis by activating adrenergic receptors on the endothelial cells, which in turn alter the endothelial cell metabolism[26]. Therapies attempting to starve the tumor by inhibiting angiogenesis had limited long term therapeutic benefit in various cancers, including that of the prostate[27], most likely due to resistance mechanisms[28]. Co-targeting angiogenesis, neural signals and/or endothelial cell metabolism may provide a multipronged therapeutic approach to inhibit cancer invasion via the nerves[26].

Analysis of the morphology of blood vessels in nerves with perineural invasion indicated that in 22% of patients with locally advanced prostate cancer the vasa

nervorum had a thick collagenous subendothelial layer in comparison with normal vasa nervorum. Another characteristic of these microvessels is the absence of pericytes associated with the capillaries and postcapillary venules. Pericytes are distinctive regulators of angiogenesis and are thought to provide vessel stability and control of endothelial proliferation[29]. The modified walls of these vessels could constitute an adaptation to ensure constant blood flow. The walls of normal vessels constantly adjust the vessel diameter to adapt to the metabolic needs of the tissue. The thick walls and the absence of contractile cells indicate that this process does not occur in the modified vessels, ensuring a constant, possibly maximal blood flow.

Thickening of the capillary basement membrane is a hallmark of diabetic microangiopathy, and may lead to occlusive angiopathy and to tissue hypoxia and damage[30].

The vasa nervorum of prostatic nerves with perineural invasion do not express detectable levels of VEGFR-2. This observation indicates that the angiogenic process induced by perineural invasion could not involve VEGF/VEGFR-2 /signaling.

The data were obtained from locally advanced prostate cancer, which is associated with approximately 20% of prostate cancer specific mortality. Most likely the data have a more general significance, based on the fact that migration of cancer cells along the nerves is encountered in other cancers in highly enervated organs (pancreas, colon, rectum, biliary tract, stomach, skin, salivary gland, head and neck). This study should stimulate investigations in these other cancer types.

CONCLUSION

Perineural invasion of cancer cells is associated with angiogenesis and vascular remodeling of vasa nervorum in locally advanced prostate cancer.

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Preliminary portions of this work have been presented elsewhere[31].

CONFLICT OF INTEREST

None

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Acquisition of data: Robin, Leclere, Nicolas

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Drafting of the manuscript: Ghinea, Radu

Critical revision of the manuscript for important intellectual content: Robin, Leclere, Nicolas, Pichon, Chnecker, Côté, Guillonneau, Radu, Ghinea

Statistical analysis: Robin, Ghinea

Supervision: Ghinea, Guillonneau

Other: None

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FIGURE LEGENDS

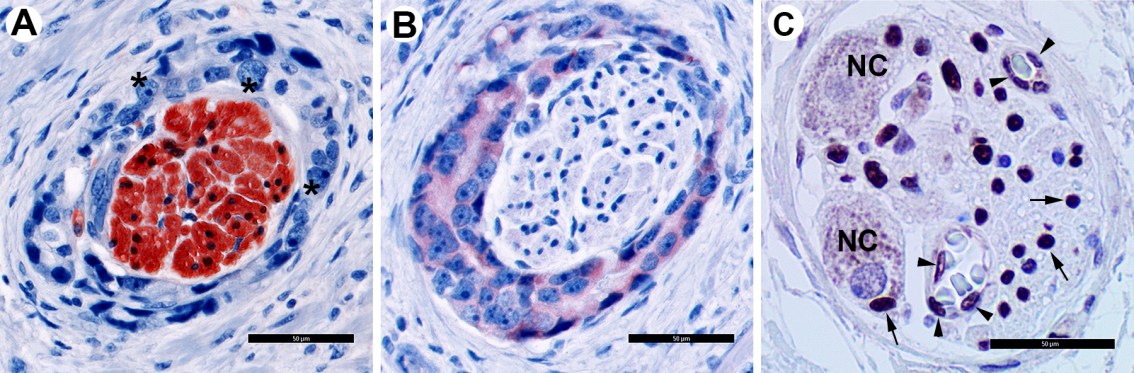
FIGURE 1. Prostate cancer cells that left the primary tumor site are visible along nerves. Immunohistochemical analysis was performed on paraffin-embedded sections of

human prostate cancer tissues using as primary antibodies a rabbit polyclonal antibody against S100 (a nerve marker), a rabbit polyclonal antibody against VEGF, and a mouse monoclonal antibody against FGF-2. Panel A: Nerve fibers (red color) surrounded by cancerous prostatic glands (asterisks). Panel B: Epithelial cancer cells (red-brown color) expressing VEGF. Panel C: Vasa nervorum endothelial cells (arrowheads) and Schwann cells (arrows) expressing FGF-2 (brown color). NC, nerve cells. Bars: 50 µm.

FIGURE 2. Expression of CD34 by vasa nervorum endothelial cells in locally advanced prostate cancer. Immunohistochemical analysis was performed on paraffin-embedded sections of prostate tumors using the monoclonal antibody CD34 followed by a secondary peroxidase-coupled goat anti-mouse immunoglobulin visualized by the red-brown peroxidase-reaction product of aminoethyl-carbazole. Sections were also stained with hematoxylin. Vasa nervorum (arrowheads) of prostatic nerves without perineural invasion (panel A) showed faint CD34 staining, whereas a strong presence of CD34 was noticed on vasa nervorum of prostatic nerves with perineural invasion (panel B). Asterisks: cancerous glands. Bars: 50 µm. Panel C: quantitation of CD34-positive vasa nervorum endothelial cells in nerves with perineural invasion (+PNI) or without perineural invasion (-PNI). No differences are noticed between the pT3a (blue bars) and pT3b tumors (red bars).

FIGURE 3. Vasa nervorum remodeling in patients diagnosed with locally advanced prostate cancer. Standard histochemical methods with haematoxylin-eosin-saffron staining of cell inclusions and nuclei (panels A and B), periodic acid-Schiff reaction for glycogen, glycoproteins, glycolipids, and proteoglycans (panel C), and Masson's trichrome technique for collagen (panel D). In 22% of patients the subendothelial layer (asterisks) of vasa nervorum contains collagen fibers and is characterized by the absence of pericytes. For most vasa nervorum (panel B) the intima consists of

endothelial cells and their basal membrane. In context of cancer, the vasa nervorum endothelial cells (arrows) and Schwann cells (arrowheads) of prostatic nerve ganglions showed strong presence of FSHR (panel E and inset i1). L, capillary lumen; NC, nerve cells; Bars: 50 µm.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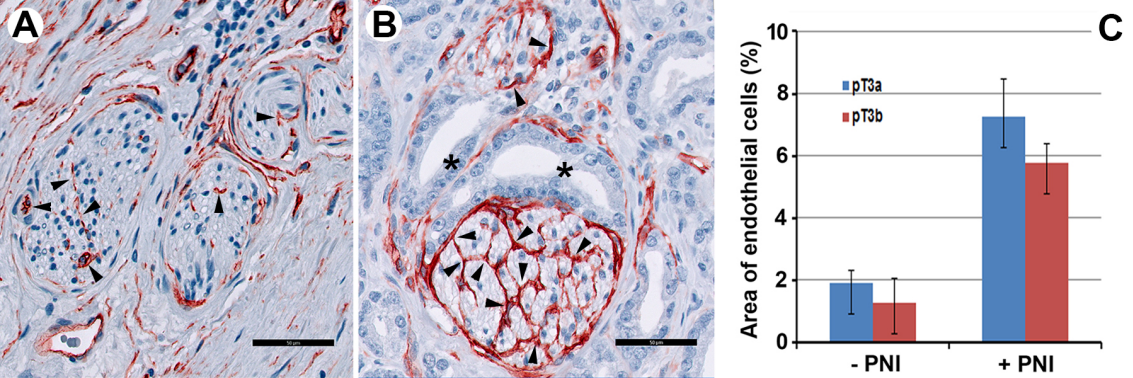
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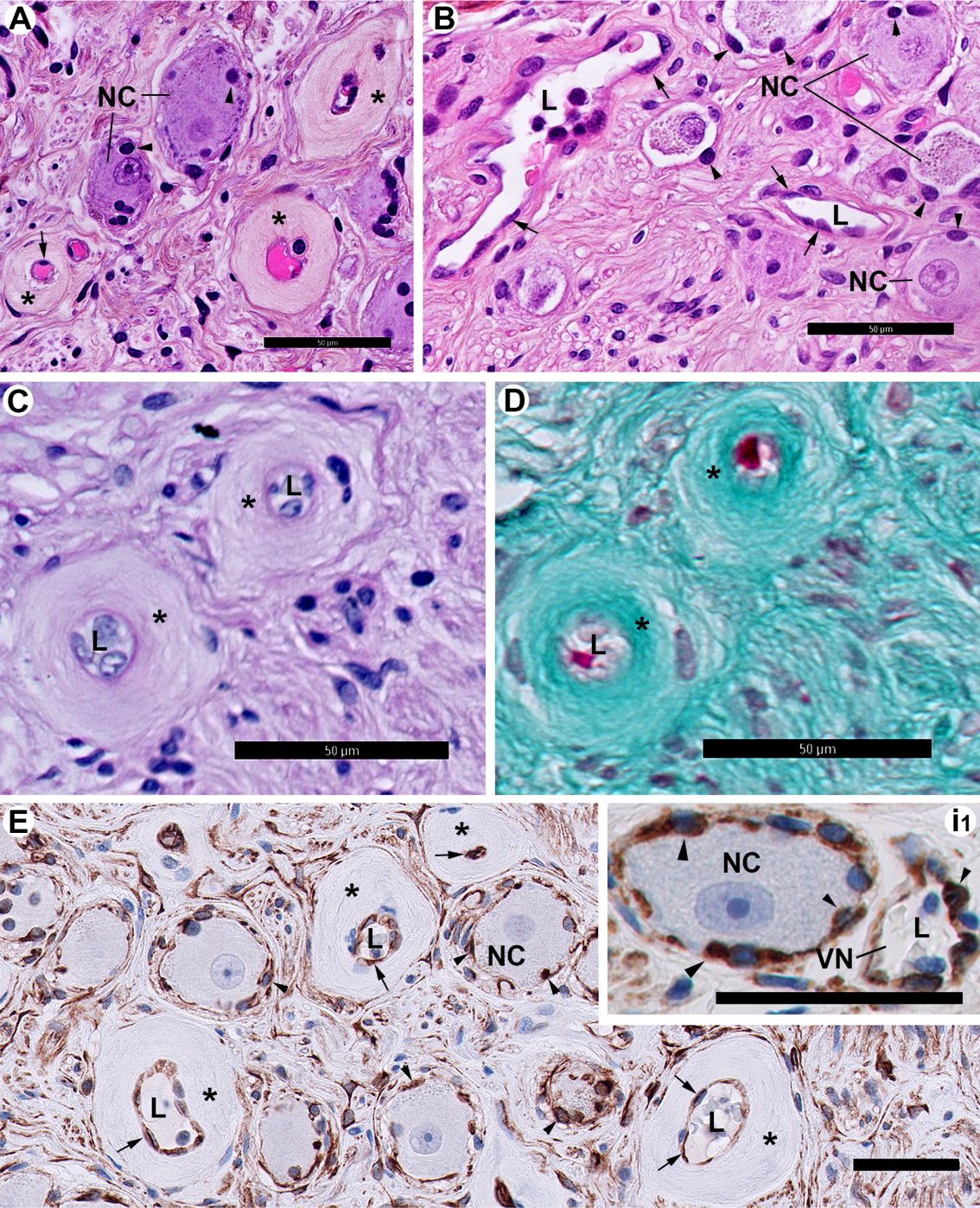
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TABLE 1. Clinical characteristics of the study patients

Clinical characteristics of patients	**All cases** (n = 85)	**pT3a** (n = 70)	**pT3b** (n = 15)
Age at prostatectomy [median (range)]	67.3 (58 - 78)	68 (58 - 78)	67 (62 -70)
PSA at diagnosis [median (range)]	7.3 (3.6 - 26)	7 (3.6 - 26)	7.4 (6 - 18.6)
Gleason score			
< 7	22	17	5
≥ 7	63	53	10
Seminal vesicle extension			
No	70	70	0
Yes	15	0	15

TABLE 2. Primary antibodies used in present the immunohistochemical present study

Antibody	Specificity	Description	Isotype	Antigen retrieval	Incubation time	Dilution	Supplier
Qbend-10	CD34	mouse monoclonal	IgG1	Tris-EDTA pH9	30 min	1/200	DAKO
RB-9031	VEGF	rabbit polyclonal		Citrate pH6	60 min	1/100	Thermo / LabVision
11B55	VEGFR-2	rabbit polyclonal		Tris EDTA pH9	60 min	1/100	Cell Signaling
bFM-2	FGF-2	mouse monoclonal	IgG1	Citrate pH6	30 min	1/100	MERCK
D2-40	Podoplanin	mouse monoclonal	IgG1	Citrate pH6	30 min	1/500	DAKO
S-2644	S100	rabbit polyclonal		Citrate pH6	30 min	1/3000	Sigma
FSHRA02	FSHR	mouse monoclonal	IgG2a	Citrate pH6	30 min	50 ng/ml	Institut Curie