Tissue Immunostaining of Candidate Prognostic Proteins in Metastatic and Non-metastatic Prostate Cancer

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Abstract

Prostate cancer (PCa) lacks specific markers capable of distinguishing aggressive tumors from those with indolent behavior. Therefore, the aim of this study was to evaluate the immunostaining of four candidate proteins (PTEN, AKT, TRPM8, and NKX3.1) through the immunohistochemistry technique (IHC), in tissues from patients with metastatic and non-metastatic PCa. Tissues from 60 patients were divided into three groups categorized according to prognostic parameters: better prognosis (n=21), worse prognosis (n=22), and metastatic (n=17). Immunostaining was analyzed by a pathologist and staining classifications were considered according to signal intensity: (0) no staining, (+) weak, and (++ and +++) intermediate to strong. AKT protein was independently associated with the presence of extraprostatic extension (p=0.012; OR=0.050; 95% CI=0.005-0.524). The immunostaining for TRPM8 (p=0.005) and NKX3.1 proteins (p<0.001) differed between malignant tumor and adjacent tissue as well as for proteins cellular location (nucleus and cytoplasm). NKX3.1 showed positive and predominantly strong immunostaining in all patients, in both tumoral and adjacent tissues. The staining in the prognostic groups showed that all metastatic samples had a positive immunostaining, with strong intensity for NKX3.1 (p=0.035). In the non-metastatic group, this strong protein staining was not observed in any patients. This study confirmed that the NKX3.1 protein is highly specific for prostate tissue and indicated that NKX3.1, AKT and TRPM8 may be prognostic markers for prostate cancer.

Introduction

According to the World Health Organization (WHO), prostate cancer (PCa) is the fourth most incident cancer, with approximately 1.4 million new cases worldwide. In men, it is the second most common type after lung cancer (Global Cancer Observatory 2020; Culp et al. 2020; Sung et al. 2021) and the fifth leading cause of death (Bray et al. 2018; INCA 2019; Sung et al. 2021). For each year of the 2020–2022 triennium, 625,000 new cases of cancer are estimated in Brazil (INCA 2019), with prostate cancer being the second most common type (Global Cancer Observatory 2020; INCA 2019).

Currently, in Brazil, digital rectal examinations and prostate-specific antigen (PSA) measurements are used as screening methodologies for PCa, and patients with abnormalities in the exam and/or PSA dosages above 10ng/mL are referred for a transrectal ultrasound-guided needle biopsy (Sociedade Brasileira de Urologia 2018; Porcaro et al. 2019; Vendrami et al. 2019).

Additionally, it is known that PSA is an excellent marker for identifying prostatic alterations, however, it is not specific and exclusive for malignant alterations (Vendrami et al. 2019; Lomas and Ahmed 2020). In this context, the search for specific biomarkers that could become potential molecular markers for PCa, capable of predicting clinical and pathological complications in the patient, is important and is under study. Immunohistochemistry (IHC) is a widely used tool in clinical routine to confirm diagnoses with tissue markers (Giannico et al. 2017; Orakpoghenor et al. 2018; Comperát 2018).

Some molecules are already being studied by IHC as they are considered potential candidates as markers of PCa, among them those involved in the cell survival pathway, phosphatidylinositol-3-kinase/serine-threonine kinase/mammalian target of the rapamycin complex (PI3K/AKT/mTOR), in addition to transient melastatin 8 (TRPM8) and NK3 homeobox 1 (NKX3.1) stand out, as they play an important role in prostate carcinogenesis.

It is known that the PI3K/AKT/mTOR signaling pathway is one of the pathways that is most dysregulated in cancer (Koundouros and Poulogiannis 2018), and an aberrant expression of this pathway has already been demonstrated in studies in the early and late phases of PCa (Taylor et al. 2010; Sreenivasulu et al. 2018). Studies also show that deletion of the *PTEN* tumor suppressor gene in PCa is very common, being very present in metastatic and castration-resistant tumors (Robinson et al. 2015; Wozniak et al. 2017; Jamaspishvili et al. 2018).

The TRPM8 channel is a homotetramer formed by subunits, showing 8 putative glycosylation sites and an immunogenic epitope. It is highly expressed in the prostate, as its ion channel functions as a testosterone receptor, suggesting a role in the regulation of androgenic responses (Asuthkar et al. 2015b). Furthermore, evidence demonstrates that it has important role in the development and progression of neoplasms, especially in PCa, being overexpressed in malignant tumor tissue compared to non-malignant tissue. This protein is present in PCa refractory hormone and with a high Gleason score (Yee 2015).

The *NKX3.1* gene is a tumor suppressor member of the NK family of homeobox genes, participating in cell specification and organogenesis processes in several species. In humans, this gene is primarily related to normal prostate development. Its loss of expression leads to defects in prostate protein secretion and ductal morphogenesis and contributes to prostate carcinogenesis (Abate-Shen et al. 2008).

Therefore, the current study aimed to evaluate tissue immunostaining of the tumor suppressor proteins PTEN and NKX3.1, of AKT protein, involved in a cell survival pathway, in addition to the testosterone receptor TRPM8, in samples from patients with metastatic and non-metastatic PCa, in the search for candidate markers for malignant prostate cancer.

Material And Methods

Study Group and Sample Characterization

In this retrospective longitudinal study, it was evaluated samples of prostatic malignant and adjacent non-tumor tissues embedded in paraffin from 60 male patients with a confirmed diagnosis of PCa after radical prostatectomy, at the Hospital do Cancer de Londrina (HCL). The study was approved by the Research Ethics Committee Involving Human Beings of the State University of Londrina- Brazil, under number 176/2013. Patients participated voluntarily and signed a free and informed consent form and answered a modified personal questionnaire based on Carrano and Natarajan (1988).

Clinical and pathological data were obtained from medical records, which were used, together with the guidelines of the National Comprehensive Cancer Network (NCCN version 4.2019), for classification of patients into three experimental groups: 1) PCa with a better prognosis (n = 21); 2) PCa with a worse prognosis (n = 22); and 3) metastatic PCa (n = 17). Patients with a Gleason score ≤ 7 (3 + 4), staging ≤ T2b, and PSA ≤ 10 ng/mL were considered to have

better prognosis PCa. Patients with a Gleason score ≥ 7 (3 + 4), staging ≥ T3a, and PSA ≥ 20 ng/mL were considered to have worse prognosis PCa. Patients with metastasis were classified according to the presence of lymph node invasion and/or distant metastasis.

The sample's protein profiles were compared between the metastatic *versus* non-metastatic group (with better and worse prognosis), as well as their malignant and adjacent non-tumor tissues. All samples in the present study were from biopsy and radical prostatectomy, without neoadjuvant chemotherapy.

Histopathological Analysis

Tissues obtained from the biopsy were stained with hematoxylin and eosin to confirm the clinical diagnosis of PCa and to verify the presence of tumor and adjacent non-tumor tissue for further analysis and comparison of immunostaining of proteins in the tissues. This step was performed by pathologists from the HCL. The histopathological classification used was based on international standards established by the WHO, such as Gleason score and clinical staging determined by the Tumor/Node/Metastasis (TNM) system, following the recommendations of the AJCC (American Joint Committee on Cancer).

Immunohistochemistry

Experiments were carried out according to a protocol based on Guembarovski et al. (2018) with modifications, regarding antigenic recovery. Formalin-fixed paraffin-embedded tissue samples of metastatic and non-metastatic malignant and adjacent non-tumor tissues were obtained. Cuts were made, 5–6 µm thick, and fixed on silanized StarFrost® slides (Knittel glass, ALE).

Samples were washed in absolute xylol and rehydrated with absolute alcohol and distilled water. Antigen recovery was performed in a Panasonic Junior smart microwave oven (Panasonic da Amazônia S/A, Manaus, AM, BRA) using three cycles of time and power; the first cycle being 4 minutes at 80w, and the second and third cycles of 5 minutes at 60 w each, using a 250mL aqueous medium solution containing citric acid buffers (4.5 mL to 21 g/L) and sodium citrate (20.5 mL to 29.4 g/L), in addition to 130 µL of Tween 20 detergent (Synth, Diadema, SP, BRA). Next, the endogenous peroxidase blocker available in the mouse/rabbit HRP/DAB ABC detection kit (Abcam, Cambridge, MA, USA) was used, one drop per cut in each reaction, for 30 minutes, in the dark, and at room temperature. SuperBlock™ (Thermo Fisher Scientific, Rockford, IL, USA) was also added to minimize binding to secondary structures, using one drop per cut in each reaction, for 1 hour, at room temperature.

Subsequently, *overnight* incubation of the four primary antibodies was carried out in the refrigerator: 1) *rabbit* pan-AKT (polyclonal clone EPR9941-2, Abcam, Cambridge, MA, USA) 1:1000 dilution; 2) *rabbit* PTEN (monoclonal clone, Abcam, Cambridge, MA, USA) dilution 1:50; 3) *rabbit* TRPM8 (polyclonal clone, Abcam, Cambridge, MA, USA) dilution 1:300; and 4) *rabbit* NKX3.1 (EPR16653 monoclonal clone, Abcam, Cambridge, MA, USA) dilution 1:500. Antibody dilutions were tested following the manufacturer's instructions on positive control tissues: colon tumor (AKT), breast cancer (PTEN), normal prostate (TRPM8), and benign prostatic hyperplasia (NKX3.1). Negative controls were performed to verify the specificity of the primary antibody in all slide batteries, where it was replaced by phosphate-buffered saline (PBS).

The secondary antibody kit (mouse/rabbit detection kit HRP/DAB ABC, Abcam, Cambridge, MA, USA) was used according to the manufacturer's instructions, and as a chromogen, the Pierce™ DAB substrate kit (Thermo Fisher Scientific, Rockford, IL, USA), using concentrated DAB ([2x]) for NKX3.1 and ([4x]) for the other antibodies (AKT, PTEN, and TRPM8), based on the manufacturer's protocol, for 5 minutes of incubation at room temperature. The slides were then washed with running water and counterstained with Harris hematoxylin, and mounted with Entellan® Novo (Merck, Billerica, MA, USA,).

Immunostaining for protein profiles in experimental groups was analyzed by an experienced pathologist. The classifications were considered according to staining signal strength: (0) no staining, (+) weak, and (+ + and +++) strong, according to Figs. 1 and 2.

Statistical Analysis

The comparison of the mean ages of the experimental groups was performed using the *Student's* t test. To compare the staining in tumor and adjacent non-tumor tissues for each protein evaluated, the McNemar test for related samples was used.

Kendall's Tau test was used to analyze the correlations between the proteins immunostaining by IHC and clinicopathological parameters, and logistic regression was performed for the variables that showed significance, to verify whether they were independently associated with protein staining. To analyze the interaction between protein tags, the Kendall Tau correlation test was also performed.

All statistical analyses were carried out using IBM® SPSS® software *Statistics for Windows, version* 20.0 (IBM® Corp., Armonk, N.Y., USA), considering a significance level (α) of 5%.

Results

The mean age of the total sample was 67.6 ± 7.4 years. The mean age according to prognostic group was 64.4 ± 4.6 (better prognosis), 67.6 ± 5.7 (worse prognosis), and 71.4 ± 10.1 years (metastatic), with a significant difference between the better prognosis and metastatic groups (p = 0.008). In addition, a significant difference was also observed between the mean age of the metastatic versus non-metastatic group (better and worse prognosis together) (p = 0.010).

As a general overview, the proteins PTEN, AKT, and TRPM8 were not expressed or presented a weak immunostaining in practically all samples, in both the malignant tissue, PTEN: 28/55 (50.9%); AKT: 30/55 (54.5%); TRPM8: 42/59 (71.9%) and in the adjacent non-tumor tissue, PTEN: 23/54 (42.6%); AKT: 34/54

(63.0%); TRPM8: 38/58 (65.5%) (Online Resource 1–6). The NKX3.1 protein, on the other hand, showed a more prominent immunostaining in both tissues, with strong intensity in malignant tumor tissue 48/57 (84.2%) and in adjacent non-tumor tissue 29/57 (50.9%) (Online Resource 4–6). Representative images of the protein's immunostainings are shown in Figs. 1 (PTEN, AKT, and TRPM8) and 2 (NKX3.1).

Kendall's correlation analysis showed that the prognosis groups (better and worse prognosis and metastatic) were positively correlated with Gleason score ($p < 0.001$; Tau = 0.437), TNM ($p < 0.001$; Tau = 0.670), PSA ($p < 0.001$; Tau = 0.552), seminal vesicle invasion ($p < 0.001$; Tau = 0.499), extraprostatic extension ($p < 0.001$; Tau = 0.697), and perineural invasion ($p < 0.001$; Tau = 0.547).

Photomicrograph of weak intensity immunostaining using the immunohistochemistry technique for PTEN, AKT, and TRPM8 proteins, evaluated in tumor tissue samples and adjacent non-tumor tissue from patients with PCa. Letters represent the evaluated proteins, being a (negative control), b (PTEN), c (AKT), and d (TRPM8). 40x magnification. Source: author himself.

Photomicrograph of strong intensity immunostaining using the protein immunohistochemistry technique for NKX3.1, evaluated in tumor tissue samples and adjacent non-tumor tissue from patients with PCa. Letters represent the evaluated proteins, being a (negative control) and b (NKX3.1). 40x magnification. Source: author himself

PTEN

PTEN protein did not show differences in immunostaining between tumor and adjacent non-tumor tissues ($p = 0.513$) or in the cellular locations (cytoplasm: $p = 0.100$) and (nucleus: $p = 0.587$). PTEN immunostaining did not demonstrate any significative association with the prognostic groups, the prognostic parameters or biochemical recurrence and metastasis (Online Resource 7).

AKT

AKT protein was also not expressed differently in the tissues of the same patients ($p = 0.515$) or in relation to cellular locations: cytoplasm ($p = 0.092$) and nucleus ($p = 0.264$) (Table 1). Furthermore, it was associated with the prognosis groups (better and worse prognosis and metastasis) ($p = 0.005$) and with the extraprostatic extension parameter ($p = 0.011$) (Online Resource 8).

Table 1
Comparative proteins immunostaining between tumor and adjacent non-tumor tissues in PCa patients

Protein evaluated	Staining in tumor tissue x adjacent tissue	p value of McNemar
PTEN		
	Tumor x Adjacent non-tumor	0.513
	Cytoplasm x Cytoplasm	0.100
	Nucleus x Nucleus	0.587
AKT		
	Tumor x Adjacent non-tumor	0.515
	Cytoplasm x Cytoplasm	0.092
	Nucleus x Nucleus	0.264
TRPM8		
	Tumor x Adjacent non-tumor	**0.005***
	Cytoplasm x Cytoplasm	**0.036***
	Nucleus x Nucleus	**0.012***
NKX3.1		
	Tumor x Adjacent non-tumor	**< 0.001***
	Cytoplasm x Cytoplasm	**< 0.001***
	Nucleus x Nucleus	**< 0.001***
McNemar's Test. * Significance level of $p < 0.05$.		

TRPM8

TRPM8 protein was expressed differently in tumor and adjacent non-tumor tissue ($p = 0.005$), with higher immunostaining in malignant tumor, even if of low intensity; the same result was observed for cellular locations of the immunostaining: cytoplasm ($p = 0.036$) and nucleus ($p = 0.012$), in both tissues (Table 1). In addition, protein immunostaining was associated with the Gleason score parameter ($p = 0.035$) (Online Resource 9).

NKX3.1

NKX3.1 protein was expressed differently between tumor and adjacent non-tumor tissue ($p < 0.001$), with higher immunostaining and strong intensity in malignant tumor tissue when compared to the adjacent non-tumor tissue; the same result was observed for cellular locations of the immunostaining: cytoplasm ($p < 0.001$) and nucleus ($p < 0.001$), in both tissues (Table 1).

There was no association between NKX3.1 immunostaining with prognostic parameters or biochemical recurrence and metastasis (Table 2). When NKX3.1 tumor immunostaining was associated with the prognostic groups (non-metastatic and metastatic), it was observed that all patients in the metastatic group (17/17, 100%) presented positive and strong immunostaining, while in the non-metastatic group although predominantly strong immunostaining was also verified ($p = 0.035$; $\chi^2 = 0.033$), this result was not observed in all patients (31/40, 77.5%). Furthermore, in the Kendall correlation analysis, NKX3.1 immunostaining was positively correlated with metastasis ($p = 0.035$; $Tau = 0.282$).

Table 2
Comparison analysis of patient ages and clinicopathological data in relation to NKX3.1 protein immunostaining in tumor tissue of PCa patients

Clinical-pathological data	Absence of staining (%)	Weak staining (%)	Strong staining (%)	p value of χ^2	p value of Kendall (value of Tau)
Experimental groups					
Non-metastatic	0 (00.0%)	9 (100%)	31 (64.6%)	**0.033***	**0.035*** (0.282)
Metastatic	0 (00.0%)	0 (00.0%)	17 (35.4%)		
Experimental groups					
Better prognosis	0 (00.0%)	4 (44.4%)	15 (31.2%)	0.100	0.099 (0.208)
Worse prognosis	0 (00.0%)	5 (55.6%)	16 (33.3%)		
Metastatic	0 (00.0%)	0 (00.0%)	17 (35.4%)		
Age					
< 65 years	0 (00.0%)	4 (44.4%)	16 (33.3%)	0.522	0.525 (0.085)
> 65 years	0 (00.0%)	5 (55.6%)	32 (66.7%)		
Gleason score					
6	0 (00.0%)	1 (11.1%)	14 (29.8%)	0.423	0.195 (-0.166)
7	0 (00.0%)	5 (55.6%)	24 (51.1%)		
> 8	0 (00.0%)	3 (33.3%)	9 (19.1%)		
TNM					
T1 to T2a	0 (00.0%)	2 (22.2%)	9 (23.1%)	0.805	0.728 (0.048)
T2b-T2c	0 (00.0%)	3 (33.3%)	9 (23.1%)		
>T3	0 (00.0%)	4 (44.4%)	21 (53.8%)		
PSA (ng/mL)					
< 10	0 (00.0%)	5 (55.6%)	16 (34.0%)	0.468	0.250 (0.146)
10–20	0 (00.0%)	2 (22.2%)	14 (29.8%)		
> 20	0 (00.0%)	2 (22.2%)	17 (36.2%)		
Seminal vesicle invasion					
No	0 (00.0%)	7 (77.8%)	29 (76.3%)	0.926	0.927 (0.014)
Yes	0 (00.0%)	2 (22.2%)	9 (23.7%)		
Extraprostatic extension					
No	0 (00.0%)	5 (55.6%)	19 (51.4%)	0.821	0.823 (0.033)
Yes	0 (00.0%)	4 (44.4%)	18 (48.6%)		
Perineural invasion					
No	0 (00.0%)	7 (87.5%)	26 (74.3%)	0.425	0.430 (0.122)
Yes	0 (00.0%)	1 (12.5%)	9 (25.7%)		
Relapse					
No	0 (00.0%)	4 (66.7%)	13 (41.9%)	0.266	0.272 (0.183)
Yes	0 (00.0%)	2 (33.3%)	18 (58.1%)		

Kendall's Tau correlation and Chi-square test. *Significance level of $p < 0.05$. Due to lack of data, some variables did not include the total of 60 patients.

Multinomial Logistic Regression

To verify whether the clinical-pathological variables that showed statistical significance were independently associated with tumor staining of the AKT, TRPM8, and NKX3.1 proteins, a multinomial logistic regression analysis was used.

It was found that the AKT protein was independently associated with the presence of extraprostatic extension ($p = 0.012$), with weak immunostaining being a protective factor against the presence of extraprostatic extension when compared to strong immunostaining (OR = 0.050; 95%CI = 0.005–0.524). In the

prognostic groups (better and worse prognosis and metastatic), AKT immunostaining was associated, but not independently. TRPM8 and NKX3.1 proteins, on the other hand, were associated with the Gleason score parameters and prognostic groups (non-metastatic and metastatic), respectively, but not independently, as shown in Table 3.

Table 3
Association between AKT, TRPM8, and NKX3.1 proteins staining in tumor tissue with clinical-pathological parameters by multinomial logistic regression

Clinical-pathological data	Tumor immunostaining	χ^2 Wald	OR (95%CI)	p value
Experimental Groups (Better and Worse Prognosis and Metastatic)	**AKT**			
	Weak	1.026	0.608 (0.232–1.592)	0.311
	Strong	0.001	1.016 (0.357–2.893)	0.976
Extraprostatic extension	**AKT**			
	Weak	6.256	0.050 (0.005–0.524)	**0.012***
	Strong	1.984	0.171 (0.015–1.994)	0.159
Gleason score	**TRPM8**			
	Weak	0.734	0.624 (0.212–1.837)	0.392
	Strong	3.382	4.740 (0.903–24.893)	0.066
Experimental Groups (Non-metastatic and Metastatic)	**NKX3.1**			
	Strong	2.405	2.254 (0.807–6.295)	0.121
Age-adjusted multinomial logistic regression analysis, considering the staining of AKT, TRPM8, and NKX3.1 proteins in tumor tissue. *Significance level of $p < 0.05$.				

Protein Interaction

To assess whether the immunostaining results are related to each other, an interaction analysis was performed, considering the staining only in the tumor tissue for all possible protein combinations. Significant interactions were observed between PTEN and AKT ($p < 0.001$; $\chi^2 < 0.001$) and PTEN and TRPM8 proteins ($p = 0.010$; $\chi^2 = 0.086$) (Table 4).

Table 4
Comparison of the interaction between protein immunostaining in tumor tissue of PCa patients

Protein interaction	p value of χ^2	p value of Kendall (value of Tau)
PTEN X AKT	**< 0.001***	**< 0.001*** (0.566)
PTEN X NKX3.1	0.792	0.529 (0.084)
PTEN X TRPM8	0.086	**0.010*** (0.326)
AKT x NKX3.1	0.434	0.255 (0.152)
AKT x TRPM8	0.126	0.133 (0.189)
NKX3.1 x TRPM8	0.759	0.556 (0.077)
Kendall's Tau correlation and Chi-square test. *Significance level of $p < 0.05$.		

Discussion

The evaluation of four proteins immunostaining by IHC (PTEN, AKT, NKX3.1, and TRPM8) in malignant tumor and adjacent non-tumor tissues of patients with prostate cancer indicated that TRPM8 protein was differentially expressed, with higher immunostaining in malignant tumor tissue. However, our most prominent result was the fact that NKX3.1 immunostaining was observed in all samples in the present study, both in tumor tissue and in the adjacent tissue non-tumor, indicating high specificity for prostate tissue. In addition, there was a strong tumor immunostaining for NKX3.1 in all patients of the metastatic group.

Certain results obtained from biopsies may be inconclusive, as the biological material collected is insufficient, with few atypical glands for analysis, and a repeat examination may be necessary. Therefore, the use of complementary techniques and the use of new biomarkers are extremely important. A widely used and highly relevant tool is the IHC technique (Kristiansen 2018; Orakpoghenor et al. 2018), which allows identification of the presence or absence of certain proteins in specific tissues, and the staining intensity is generally used as the gold standard (Jamaspishvili 2018).

It is known that age is one of the main risk factors linked to PCa (Vaidyanathan et al. 2016; Junior et al. 2016; Tse et al. 2018; INCA 2019), therefore, aging increases the risk of developing the disease and its possible aggravation. The significative results obtained in this study comparing the mean ages between patients in the better prognosis and metastatic groups (p = 0.008), in addition to the result of the comparison between the metastatic *versus* non-metastatic groups (better and worse prognosis) (p = 0.010), confirm that PCa is a disease of advanced age and that late diagnosis may be associated with a more severe condition of the disease, such as the development of metastases.

The genomic deletion of *PTEN* is very common in PCa, as it is the most lost tumor suppressor in the early stages of the disease (Lotan et al. 2011; 2017; Jamaspishvili et al. 2018; Hamid et al. 2019). Consequently, an increase in AKT expression would possibly occur, as these proteins participate in the same signaling pathway (Kurose et al. 2021). Low or absent expression of PTEN was expected in the worse prognosis and metastatic groups; on the other hand, the AKT protein should be more expressed in tumor tissue in these groups of patients. However, we verified that the immunostaining intensities of PTEN and AKT were similar, showing a similar pattern of immunostaining in many of the samples, in the tumor tissue PTEN: 28/55 (50.9%) and AKT: 30/55 (54.5%) and in the adjacent non-tumor tissue PTEN: 23/54 (42.6%) and AKT: 34/54 (63.0%). Our data are not distinct from the literature, as according to the database The Human Protein Atlas (2021c), the PTEN protein has predominantly cytoplasmic immunostaining, and the prostate tissue has low expression of this protein (The Human Protein Atlas 2021a). The AKT protein, on the other hand, presents nuclear immunostaining and in normal prostate tissue, its expression is median (The Human Protein Atlas 2021a).

Tumor immunostaining of PTEN protein did not show any significant association with the prognostic groups, prognostic parameters, or recurrence and metastasis. AKT protein immunostaining, on the other hand, was associated with groups of prognosis (better and worse prognosis and metastatic) and extraprostatic extension, being independently associated with the latter mentioned parameter. AKT is an oncogenic protein, so the presence of immunostaining may favor tumor growth. Extraprostatic extension is a parameter indicative of tumor aggressiveness, which can lead to invasion of adjacent tissues and, consequently, to metastases.

Literature data demonstrate that PTEN and AKT are part of the same cell survival pathway, PI3K/AKT/mTOR, where PI3K acts as an agonist of the pathway converting PIP2 into PIP3, favoring the binding of AKT to PIP3, which it will mediate, through activation of proteins, cell growth, proliferation, survival, and migration (Gonçalves et al. 2018). PTEN protein, on the other hand, acts as a direct antagonist of the pathway, converting PIP3 into PIP2, therefore having a role as a lipid phosphatase (Gonçalves et al 2018; Jamaspishvili et al. 2018). This role of antagonist (PTEN) and agonist (AKT) of the cell survival pathway was confirmed in our protein interaction analysis, in which it was observed that strong AKT intensity immunostaining was present when absent or weak intensity immunostaining of PTEN was observed.

Asuthkar et al. (2015a, b, c, 2017) suggested that TRPM8 is a key element in the testosterone-induced response pathway, and that its activity can significantly contribute as an anti-tumor defense mechanism, serving as a new therapeutic target. The immunostaining of the TRPM8 protein, in general, was quite weak in the samples of the present study, in the tumor tissue: 42/59 (71.9%) and in the adjacent non-tumor tissue TRPM8: 38/58 (65.5%). In addition, it was more present in tumor tissue compared to adjacent non-tumor tissue and a significant difference was observed in immunostaining of this protein in the cellular location. According to literature data, this protein is highly expressed in the prostate, both in tumor-free individuals and in patients with PCa (Asuthkar 2017). Our results do not support this study, but the fact that this protein was more marked in the tumor tissue than in the adjacent tissue non-tumor, suggests the need for future studies with new sample groups, to verify whether it may have any correlation with the malignant change.

Asuthkar et al. (2015b) found that TRPM8 protein and testosterone are directly involved in localized interactions in the plasma membrane of cells in the periphery of the prostate and in the plasma membrane of the endoplasmic reticulum of cells in the lumen. Furthermore, the authors suggested that testosterone-induced TRPM8 might be an important regulator of Ca^2 homeostasis and the cell cycle in prostate cells. Although TRPM8 mRNA levels increase during prostate tumor progression (Tsaveler et al. 2001), protein levels are not proportionally equal. Asuthkar et al. (2015c) found that TRPM8 is redirected to degradation in PCa, while protein recovery effectively suppresses tumor cell growth. This fact could explain the low expression of this protein in the samples of the present study.

One of the main prognostic factors described in the literature is the histological grade, with the Gleason score being the most used (Cambruzzi et al. 2010). This is a very important prognostic parameter in the assessment of tumor progression and aggressiveness (Löbler et al. 2012). TRPM8 was not associated with the prognostic groups or biochemical recurrence and metastasis, however, it was associated with the Gleason score parameter, but not independently. Yu et al. (2014) and Yee et al. (2015) demonstrated an association of TRPM8 with Gleason score, in which TRPM8 immunostaining was correlated with a high Gleason score.

Another interesting result was the interaction observed between PTEN and TRPM8 proteins, in which it was verified that the presence of one protein is associated with the presence of the other. However, when analyzing the interaction through the String Database (2021), we found no direct interaction between these proteins, but that TRPM8 can interact with other peripheral proteins of the PI3K/AKT/mTOR and PTEN signaling pathway, such as PPP1CA, PPP1CB, and PPP1CC, for example, which are catalytic serine/threonine-protein phosphatase subunits, and associate with several regulatory proteins to form highly specific holoenzymes, aiming to dephosphorylate hundreds of biological targets.

NKX3.1 protein presented the most intense immunostaining in the tissues of the patients with PCa in the present study, with a significant difference being observed in the tumor tissue (48/57; 84,2%) *versus* adjacent non-tumor tissue (29/57; 50.9%). Differences in immunostaining regarding cellular locations (nucleus and cytoplasm) were also observed. These results corroborate the study of Gurel et al. (2010), that clearly showed the nuclear immunostaining of NKX3.1 present in practically all analyzed samples, presenting a high pattern of nuclear staining and a high rate of positivity in metastases. According to the database The Human Protein Atlas (2021b), NKX3.1 presents immunostaining both in the nucleus and in the cytoplasm, with nuclear staining being the most evident. In addition, this protein has a high expression rate in the prostate tissue.

It's already well established that clinicopathological parameters used when studying neoplasms are extremely important, as the data help to confirm and classify patients, and can help guide more effective therapy. The prognosis of PCa is fundamentally related to some histopathological data, such as topography/laterality, tumor volume/size, histological type, degree of differentiation, presence of capsular to extraprostatic neoplastic invasion, state of the surgical margins and the presence of metastases in regional or distant lymph nodes (Cambruzzi et al. 2010). Within this context, PTEN, AKT, and TRPM8 proteins were not significantly correlated with prognostic parameters or biochemical recidive and metastases evaluated in our samples.

NKX3.1 immunostaining was positively correlated and significantly associated, but not independently, with the metastatic group, where all patients had intense immunostaining. Previous studies found that NKX3.1 expression was strongly present in metastatic PCa samples, showing high sensitivity for the prostate, and even when associated with PSA (Kristiansen 2017), PSMA (Huang et al. 2018), or HOXB13 (Abouhashem and Salah 2020), they were considered good markers for detecting metastases of prostatic origin. In addition to these studies, the International Society of Urological Pathology (ISUP) (Epstein et al. 2016) has already indicated the NKX3.1 protein as an excellent biomarker of prostate origin in PCa metastases, being highly specific for this tissue. Our results confirm that NKX3.1 is a specific marker of prostate tissue and indicate that it can be used to identify a primary site of metastasis, but also that it could be an early predictor of this phenomenon, given its immunostaining profile in metastatic patients.

Conclusions

NKX3.1 protein is highly specific for prostate tissue, as it showed positive immunostaining in all samples in the present study, both in tumor and in adjacent non-tumor tissues. Furthermore, the fact that it showed a significant difference in staining between tumor and non-tumor tissue, as well as being expressed in all patients in the metastatic group and with strong intensity, raises the possibility that NKX3.1 can be a candidate biomarker for metastasis and prognosis, besides identification of the primary site in prostate cancer. Also, AKT and TRPM8 may be prognostic markers for prostate cancer, that deserve future research.

Abbreviations

WHO: World Health Organization; PCa: Prostate Cancer; PSA: Prostate-Specific Antigen; IHC: Immunohistochemistry; PI3K: Phosphatidylinositol-3-kinase; AKT: Serine/Threonine Kinase; mTOR: Mammalian target of the rapamycin complex; TRPM8: Transient receptor potential for melastatin 8; NKX3.1: NK3 homeobox 1; HCL: Londrina Cancer Hospital; CEP/UEL: Research Ethics Committee Involving Human Beings of the State University of Londrina; NCCN: National Comprehensive Cancer Network; TNM: Tumor/Node/Metastasis; AJCC: American Joint Committee on Cancer; PBS: Phosphate-Buffered Saline; ISUP: International Society of Urological Pathology.

Declarations

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Competing interests

The authors declare that they have no competing interests.

Author contributions

Érica Romão Pereira participated in study design and acquisition of data, experimental procedures, performed statistical analysis and interpretation, and drafted the manuscript. Amanda Letícia Francelino, Laís Capelasso Lucas Pinheiro participated in the collection of samples and medical records, participated in the study design and immunohistochemical reactions. Carlos Alberto Miqueloto participated in study design and experimental procedures. Alda Fiorina Maria Losi Guembarovski participated the histopathological assays for selection of tumor tissues and adjacent non-tumor tissues and performed the immunohistochemical analysis of the samples. Karen Brajão de Oliveira participated in the statistical analysis and data interpretation. Paulo Emílio Fuganti made the sample collection possible. Ilce Mara de Syllos Cólus participated in the design of the study and reviewed the manuscript for important intellectual content. Roberta Losi Guembarovski participated in the design of the study, interpretation of data and gave final approval of the version to be published. All authors read and approved the final manuscript.

Data Availability

All data generated or analyzed during the current study are included in this published article.

Ethics approval

This study was approved by the Institutional Ethics Committee Involving Humans at State University of Londrina, Londrina – Paraná (PR), Brazil (CEP/UEL 176/2013; CAAE 19769913.0.0000.5231, in accordance with Resolution 466/12 of the National Research Ethics Commission).

Consent to participate

Study purpose and procedures were explained to all patients and written informed consent was obtained from all individual participants included in the study.

Consent to publish

Not applicable.

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Figures

Figure 1

Immunostaining profile of PTEN, AKT and TRPM8 proteins using immunohistochemistry technique

Figure 2

Immunostaining profile of NKX3.1 protein using immunohistochemistry technique

Supplementary Files

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