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RESEARCH

Periacinar Retraction Clefting in Nonneoplastic and Neoplastic Prostatic Glands: Artifact or Molecular Involvement

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Abstract A space between neoplastic acini and prostatic stroma is not rare and studies have interpreted this as an artifact, considering the absence of endothelial cells indicating vascular invasion. Thus, the aims of this work were to characterize and correlate the occurrence and extent of retraction clefting with the reactivities of α and β dystroglycan (αDG, βDG), laminin, matrix metalloproteinase 2 (MMP-2), p63, insulin-like growth factor 1(IGF-1), vimentin, and fibroblast growth factor 2 (FGF-2). The study was based on nonneoplastic and neoplastic prostatic tissues obtained from necropsies and retropubic radical prostatectomies. The results showed that periacinar retraction clefting was significantly more frequent in prostatic carcinoma samples than in normal prostatic acini. Most of the neoplastic acini (72.0%) showed retraction clefting of more than 50% of circumference, which were significantly more frequent in Gleason score 7 and 6. Decreased collagen and reticular and elastic fibers were verified in the stroma around neoplastic acini. Weak and discontinuous αDG, βDG, and laminin immunoreactivities and intensified MMP-2, vimentin, IGF-1 and FGF-2 immunoreactivities were verified in the neoplastic acini; p63 immunoreactivity was negative in all carcinomas. Thus, these findings showed that the lack of epithelial basal cells, DGs, and laminin and increased MMP-2, IGF-1, and FGF-7 could be considered important pathways in periacinar retraction occurrence. This study demonstrated the origin of and the biological mechanisms responsible for periacinar retraction clefting in prostatic carcinoma.

Keywords Prostatic cancer · Histological criteria · Retraction clefting · Dystroglycans · Matrix metalloproteinases · Insulin-like growth factor · Fibroblast growth factor, Vimentin · Laminin · p63

Abbreviations

DG	dystroglycan
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
FGF	fibroblast growth factor
IGF	insulin-like growth factor
MMP	matrix metalloproteinase

Introduction

The diagnosis of prostatic carcinoma is based on three major histological criteria; infiltrative growth pattern, the absence of a basal cell layer; and the presence of macronucleoli [1–3]. Several supportive diagnostic criteria have

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been proposed, but only a few are specific for tumor. Proposed additional criteria are: marginated nucleoli; multiple nucleoli; wispy, bluish mucinous secretions; intraluminal crystalloids; intraluminal amorphous eosinophilic material; collagenous micronodules; glomerulations; periacinar cleft-like spaces; and others, but many of these supportive criteria can also be present in benign glands or some non-neoplastic proliferative acinar conditions [2–4].

Few authors have paid attention to the so-called periacinar halos, retraction clefting, or cleft-like spaces within neoplastic tissue [2, 3, 5, 6]. Prostatic cancer neoplastic cells often appear pulled away from the surrounding stroma, leaving halos around the acini [2, 3, 7]. Only a limited number of reports have compared and validated retraction clefting as supportive diagnostic tumor tissue [2, 3, 6].

The prostate stroma is made up of a complex arrangement of stromal cells and extracellular matrix (ECM) associated to growth factors; regulatory molecules; and restructuring enzymes which lead to general biological signs and have mechanical influences on the epithelial cells, apart from being considered an important morphogenesis and maturation regulator for the gland [8]. Structural components such as collagen fibers and reticular fibers provide mechanical rigidity and flexibility to the tissue [9]. Dystroglycan (DG) is a major non-integrin adhesion molecule expressed in a wide variety of tissues at the interface between the basement membrane and the cell membrane [10, 11]. Decreased DG expression was observed in high-grade prostatic cancer, showing abnormal interaction between prostatic cell and extracellular matrix causing metastasis [10–12].

In addition, proteins such as insulin-like growth factor (IGF) and fibroblast growth factor (FGF) are important mitogenic factors for maintaining prostatic function [13, 14]. IGF-1 and its associated signaling pathway is one of the most significant positive growth-promoting signal transduction pathways, while the fibroblast growth factor (FGF) family plays the role of both growth and angiogenic factors.

Degradation of ECM-surrounding tumours is a critical step in the invasion and metastasis of malignant epithelial cells [14]. The degradation process is mainly mediated by zinc-dependent matrix metalloproteinases (MMPs) produced by stromal cells. An increasing amount of evidence suggests that cancer cells can stimulate MMP production in a paracrine manner [14].

Thus, the primary cause or causes and the molecular mechanisms underlying the development and progression of periacinar retraction clefting are poorly understood. Also, the relation between epithelium-ECM and pathogenesis of periacinar retraction clefting is unclear. Thus, the aims of this study were to correlate the presence and extent of retraction clefting with the reactivities of p63, adhesion molecules, growth factors, and matrix metalloproteinase in nonneoplastic and neoplastic prostatic glands, as well as, to estimate diagnostic importance and to define the criteria for retraction clefting determination.

Materials and Methods

Human Samples

Fifty prostatic samples were obtained from 60 to 80-year-old patients (mean 71 years) with and without prostatic lesions hospitalized in Campinas University (UNICAMP) School of Medicine's teaching Hospital. The prostatic samples were divided into two groups (25 samples per group): Normal Group (no lesions) and Prostatic Carcinoma Group.

The Normal Group included 25 prostatic samples, which were obtained from necropsied patients without diagnosis of prostatic or other urological diseases. The interval between death and necropsy examination ranged from 1 to 3 h.

The Prostatic Carcinoma Group included the other 25 prostatic samples, which were taken from patients submitted to radical retropubic prostatectomy between February and November 2010. The surgical specimens were step-sectioned and totally embedded. The diagnosis of adenocarcinoma was confirmed histologically in all cases, according to morphological criteria [15] by a senior uropathologist (AB) and graded using the Gleason grading system.

All samples from Normal and Prostatic Carcinoma groups were obtained from the parasagittal midline of the posterior surface of the prostatic peripheral zone, and submitted to histopathological and immunohistochemical analyses.

Approval was obtained from UNICAMP School of Medicine Research Ethics Committee (No: 0094.0.146.000-08).

Histopathological Analysis

All prostatic samples from Normal and Prostatic Carcinoma groups were fixed by immersion in 10% buffered formaldehyde, embedded in paraplast (Paraplast Plus, Brazil), cut into 6 μm thick sections and submitted to the following staining procedures: Hematoxylin-Eosin, Masson's trichrome, and Ammoniacal silver. For each case, 8–30 slides were analyzed and photographed with a *Zeiss Axiophot* photomicroscope (Zeiss, Hamburg, Germany).

In the Prostatic Carcinoma Group, all tumors consisted of at least 30 or more neoplastic glands. The areas of periacinar retraction clefting were analyzed on high power field (400x) and graded as a percentage of gland circumference separated from the stroma in three categories:

glands without clefts, glands with clefts up to 50% of the circumference, and glands with clefts which affected more than 50% of the circumference [3]. Cases with ten or more glands with clefts affecting more than 50% of gland circumferences were considered as positive. The areas were measured using the *Axio Vision* computerized image analysis system (Zeiss, Hamburg, Germany).

Immunolabelled αDG, βDG, Laminin, MMP-2, p63, IGF-1, Vimentin and FGF-2

All prostatic samples, the same as used for histopathological analysis, were taken and cut into 6 μm thick sections. Different protocols were used for antigen retrieval. Sections were incubated in 0.3% H_2O_2 to block endogenous peroxidase and nonspecific binding was blocked by incubating the sections in blocking solution at room temperature. Primary rabbit polyclonal antibody H-300 (sc-28534; Santa Cruz Biotchenollogy, USA) for αDG, rabbit polyclonal antibody H-242 (sc-28535; Santa Cruz Biotchenollogy, USA) for βDG, rabbit polyclonal antibody H-187 (sc-20143; Santa Cruz Biotchenollogy, USA) for laminin, mouse monoclonal antibody IM53 (Calbiochem, USA) for MMP-2, mouse monoclonal p63 (sc-8431; Santa Cruz Biotchenollogy, USA) for p63, rabbit polyclonal N-20 (sc-720; Santa Cruz Biotechnology) for IGF-1, mouse monoclonal ab8069 (abcam, USA) for vimentin, and rabbit polyclonal (sc-79; Santa Cruz Biotchenollogy, USA) for FGF-2 were diluted in 1% BSA (1:150) applied to the sections overnight at 4°C. Bound antibody was detected with an Envision HRP kit (Dako Cytomation Inc., USA). Secondary labeled polymer (Envision HRP kit) was applied for 40 min at room temperature. Peroxidase activity was detected using a diaminobenzidine chromogen mixture (Envision HRP kit). Sections were lightly counterstained with methyl green and Harris hematoxylin and photographed with a *Zeiss Axiophot* photomicroscope (Zeiss, Hamburg, Germany).

To evaluate the intensity of antigen immunoreactivity, the percentage of positive-staining epithelial and/or stromal cells was examined in ten fields for each antibody under high magnification (400x). Staining intensity was graded on a scale of 0–3, with 0 (no immunoreactivity), 0% positive epithelial and/or stromal cells; 1(weak immunoreactivity), 1–35% positive epithelial and/or stromal cells; 2 (moderate immunoreactivity), 36–70% positive epithelial and/or stromal cells; and 3 (intense immunoreactivity), >70% positive epithelial and/or stromal cells [16].

Statistical Analysis

The differences between groups were tested using the proportion test. For all analyses, a type-I error of 5% was considered statistically significant.

Results

Histopathological Analysis

Periacinar retraction clefting was significantly more frequent in prostatic carcinoma samples (92.0%) than in normal prostatic (12.0%) acini (Table 1). Glands with clefts that affected more than 50% of circumference were significantly more frequent in the Prostatic Carcinoma Group (72.0%) than in the Normal Group (Table 1). Also, glands with clefts that affected up to 50% of the circumference were found in the Normal group (12.0%), but this was significantly lower than in the Prostatic Carcinoma (20.0%) Group (Table 1).

The most common Gleason pattern observed in the prostatic carcinomas was 3 (Table 2). Fourteen prostatic carcinoma samples were of Gleason score 6, eight Gleason score 7, and three Gleason score 5 (Table 2). In the prostatic carcinoma samples, periacinar retraction clefting was significantly more frequent in Gleason scores 7 and 6 (Table 2).

The Normal group showed different sizes and folded mucosa of the acini (Fig. 1a,b,c). The secretory epithelium presented a secretory layer of columnar cells and another of basal cells (Fig. 1a,b). The prostatic stroma showed thin collagen fibers underlying the secretory epithelium and reticular fibers underlying the epithelium around the acini (Fig. 1a,b,c).

In relation to the Prostatic Carcinoma group, samples showed infiltrative adenocarcinoma characterized by peaked neoplastic acini in addition to a lack of basal layer and occurrence of periacinar retraction clefting, affecting more than 50% of acini circumference (Fig. 1d,e,f). The cell nuclei which covered the neoplastic acini were voluminous, showing an oval shape and prominent nucleoli (Fig. 1d,e). Moderate stromal hypertrophy was observed with a quantity of collagen and reticular fibers; these fibrilar elements were decreased around neoplastic acini (Fig. 1d,e,f).

Table 1 Distribution of periacinar retraction clefting in nonneoplastic and neoplastic glands

Periacinar Retraction Clefting	Groups	
	Normal (n=25)	Prostatic Carcinoma (n=25)
Clefting affecting up to 50% of gland circumference (%)	3 (12.0%)	5 (20.0%)*
Clefting affecting more than 50% of gland circumference (%)	0 (0%)	18 (72.0%)*
Without Clefts (%)	22 (88.0%)*	2 (8.0%)

* Statistical significance (test of proportion, P<0.0001)

Table 2 Distribution of Gleason score and periacinar retraction clefting in glands with prostatic carcinoma

Gleason pattern with scores	Number of cases (%)	Clefting affecting up to 50% of gland circumference (%)	Clefting affecting more than 50% of gland circumference (%)	Without Clefts (%)
Gleason 5 (3+2)	3 (12.0%)	0 (0%)	1 (33.3%)	2 (66.7%)*
Gleason 6 (3+3)	14 (56.0%)	4 (28.0%)	10 (72.0%)*	0 (0%)
Gleason 7 (3+4)	6 (24.0%)	1 (16.0%)	5 (84.0%)*	0 (0%)
Gleason 7 (4+3)	2 (8.0%)	0 (0%)	2 (100%)*	0 (0%)
Total	25 (100%)	5 (20.0%)	18 (72.0%)	2 (8.0%)

* Statistical significance (test of proportion, $P<0.0001$)

Immunolabelled αDG, βDG, Laminin, MMP-2, p63, IGF-1, Vimentin and FGF-2

Antigen immunoreactivity was analyzed in both nonneoplastic and neoplastic glands. Also, clefts that affected more than 50% of circumferences were analyzed in Gleason scores 7 and 6 only, which were more frequent for this criterion.

Intensified αDG immunoreactivity was seen in the prostatic epithelium of the Normal Group (92.1%), and was weak and discontinuous (2.2%) in the neoplastic acini (Fig. 2a,e, and Table 3). Also, βDG immunoreactivity was intense around the whole circumference in all normal acini (89.6%) and absent in the Prostatic Carcinoma Group (Fig. 2b,f, and Table 3). Intense immunoreactivity for laminin was observed around the whole circumference in all normal acini (82.5%) wheras the Prostatic Carcinoma group showed weak and discontinuous immunoreactivity (1.5%) for this antigen (Fig. 2c,g, and Table 3).

In contrast, intensified MMP-2, vimentin, IGF-1 and FGF-2 immunoreactivities were verified around the whole neoplastic acini when compared to the Normal Group, representing 83.4%, 92.3%, 78.4%, and 87.5% of immunoreactivity (Figs. 2d,h, 3b,c,d,f,g,h, and Table 3).

Only basal cells were stained by p63. Intense p63 immunoreactivity was seen around the whole circumference in all nonneoplastic acini (96.2%) but was negative in all carcinomas (Fig. 3a,e, and Table 3).

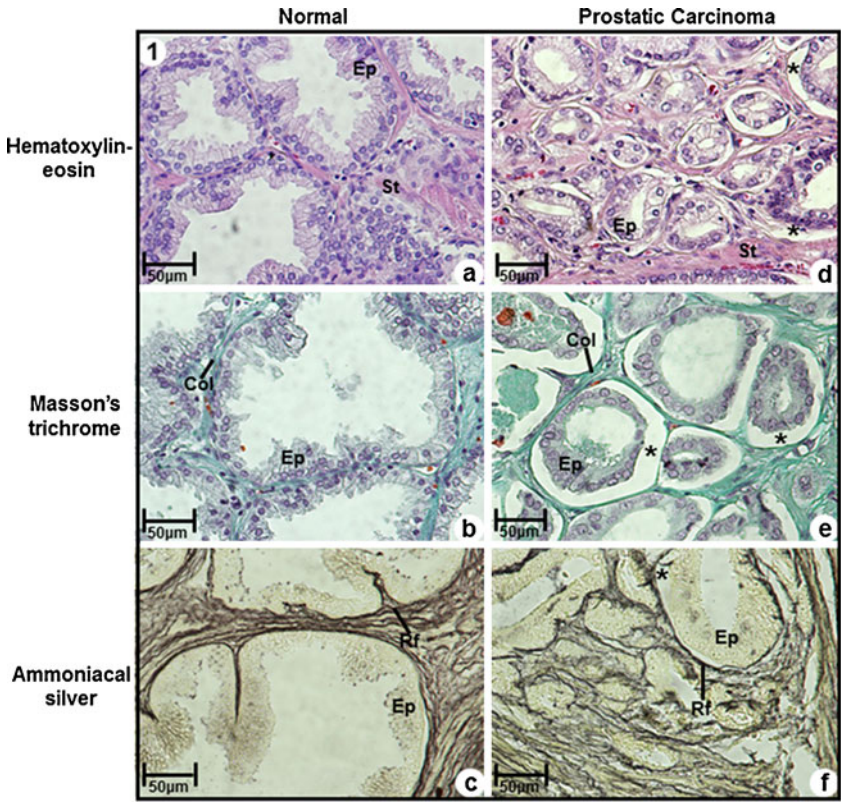

Fig. 1 Photomicrographs of the prostatic peripheral zone from Normal (**a**, **b**, **c**) and Prostatic Carcinoma (**d**, **e**, **f**) groups. **a** Acini without retraction clefting and epithelium with secretory columnar and basal cells. **b**, **c** Thin collagen and reticular fibers underlying the epithelium. **d** Neoplastic acini with clefts that affected more than 50% of their circumference (*asterisks*). **e**, **f** Moderate stromal hypertrophy and collagen and reticular fibers were decreased and discontinuous around neoplastic acini, characterizing periacinar retraction clefting (*asterisks*). **a**–**f**: **Ep**–epithelium, **St**–stroma, **Col**–collagen fibers and **Rf**–reticular fibers

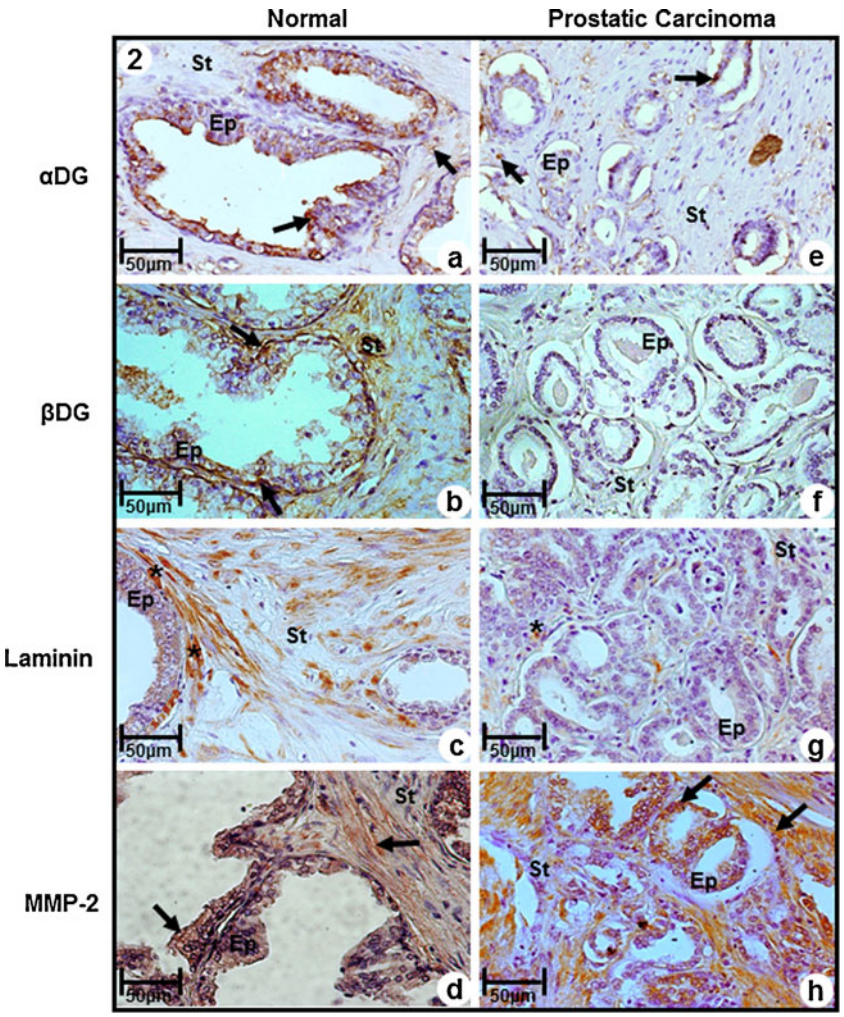

Fig. 2 Immunohistochemistry of the prostatic peripheral zone from Normal (**a**, **b**, **c**, **d**) and Prostatic Carcinoma (**e**, **f**, **g**, **h**) groups. **a** Intense αDG immunoreactivity (*arrows*) in the prostatic secretory epithelium and stroma. **b** Intense βDG immunoreactivity (*arrows*) in the periacinar prostatic stroma. **c** Intense laminin immunoreactivity (*asterisks*) in periacinar stroma. **d** Weak MMP-2 immunoreactivity (*arrows*) in the prostatic secretory epithelium and stroma. **e** Weak αDG immunoreactivity (*arrows*) in acini with periacinar retraction clefting and stroma. **f** Absent βDG immunoreactivity in the periacinar prostatic stroma of acini with periacinar retraction clefting. **g** Weak laminin immunoreactivity (*asterisk*) in the stroma periacinar around acini with periacinar retraction clefting. **h** Intense MMP-2 immunoreactivity (*arrows*) in acini with periacinar retraction clefting and stroma. **a**–**h**: **Ep**–epithelium and **St**–stroma

Table 3 Immunolabelled antigen intensities of epithelial and stromal cells in prostatic carcinoma with periacinar retraction clefting and normal prostatic tissue

Antigens	Groups	
	Normal (n=25)	Prostatic Carcinoma (n=25)
αDG	3 (92.1%)	1 (2.2%)
βDG	3 (89.6%)	0 (0.0%)
Laminin	3 (82.5%)	1 (1.5%)
MMP-2	1 (19.7%)	3 (83.4%)
p63	3 (96.2%)	0 (0.0%)
Vimentin	1 (25.5%)	3 (92.3%)
IGF-1	2 (36.4%)	3 (78.4%)
FGF-2	1 (5.4%)	3 (87.5%)

0, no immunoreactivity; 1, weak immunoreactivity (1%–35% positive epithelial and/or stromal cells); 2, moderate immunoreactivity (36%–70% positive epithelial and/or stromal cells); 3, intense immunoreactivity (>70% positive epithelial and/or stromal cells)

Discussion and Conclusion

This study demonstrated that periacinar retraction clefting seems to be a feature of neoplastic acini and not normal acini especially when clefts are seen in more than 50% of the acinar circumference. Clefts that affected more than 50% of the acinar circumference were present in 92% of examined prostatic carcinomas, which were also most pronounced in Gleason scores 7 and 6. Furthermore, positive immunoreactivities for the different molecules suggest that this microscopic feature is not an artifact. Increased MMP-2, vimentin, IGF-1, and FGF-2 and the loss of laminin and dystroglycan positive immunolocalizations indicated the extent of retraction clefting which was a possible benefit for disease progression. Also results invariably showed negative immunoreactivity for p63 in all carcinomas with periacinar retraction clefting and positive immunoreactivity in normal cases, suggesting that this phenomenon was related to the lack of basal cells.

The presence of retraction clefting around neoplastic glands is an additional criterion favouring prostatic adeno-

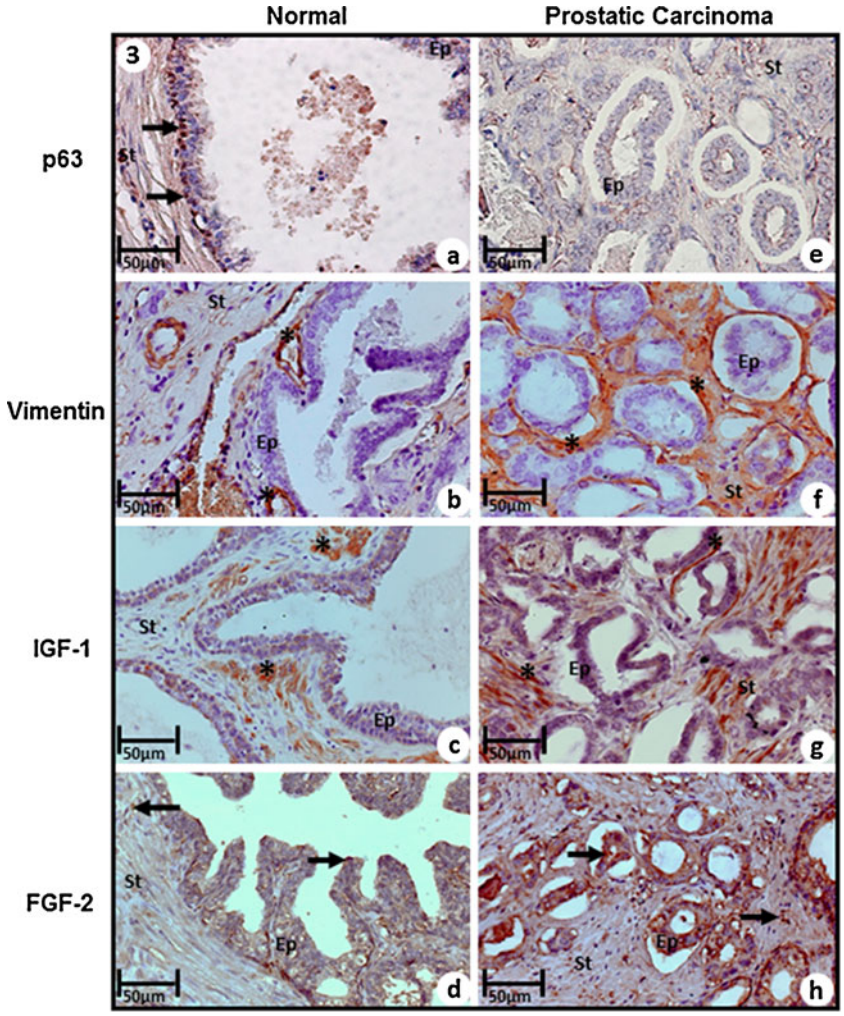

Fig. 3 Immunohistochemistry of the prostatic peripheral zone from Normal (**a**, **b**, **c**, **d**) and Prostatic Carcinoma (**e**, **f**, **g**, **h**) groups. **a** Intense p63 immunoreactivity (*arrows*) in prostatic epithelial basal cells. **b** Weak vimentin immunoreactivity (*asterisks*) in prostatic stroma. **c** Moderate IGF-1 immunoreactivity (*asterisks*) in periacinar stroma. **d** Weak FGF-2 immunoreactivity (*arrows*) in the prostatic secretory epithelium and stroma. **e** Absent p63 immunoreactivity in prostatic epithelial basal cells. **f**, **g** Intense vimentin and IGF-1 immunoreactivities (*asterisks*) in the prostatic stroma around acini with periacinar retraction clefting. **h** Intense FGF-2 immunoreactivity (*arrows*) in acini with periacinar retraction clefting and stroma. **a**–**h**: **Ep**–epithelium and **St**–stroma

carcinoma. Halpert et al. (1963) [17], in autopsy studies, were the first to briefly describe prostatic adenocarcinoma halos around neoplastic acini. Other studies have shown that clefting affecting more than 50% of circumference in more than 50% of analyzed glands was only found in neoplastic glands, suggesting its potential for use in routine diagnostics [3, 18]. Young et al. (1998) [19] mentioned the association between Gleason pattern 3 adenocarcinoma and prominent periacinar clefts, suggesting that this phenomenon is probably an artifact. Conversely, Tomas & Krušlin (2004) [16] and Tomas et al. (2006) [20] attributed periacinar clefting to the lack of basal cells and changes in stroma that are present in prostatic adenocarcinoma, and did not consider the clefts as a simple artifact. These same authors showed that the stromal reaction in prostatic carcinoma, as well as in retraction clefts, was more pronounced in Gleason pattern 3. The presence of extensive retraction artifact in prostatic carcinoma correlated with tumour characteristics signifying aggressive behaviour and indicated poor biochemical recurrence-free survival [21].

The origin of clefting in tumor specimens is unknown but may be related to an abnormality in interactions between prostatic epithelial cells and ECM. As malignant glands are known to lack basal cells, this also may be one of the reasons for cleft appearance [7].

Biochemically, prostate cancer progression is associated with the deregulation of specific growth factors and their respective signaling pathways [22, 23]. IGF-1, which is produced by prostatic stromal cells in response to androgen stimulation, works in a paracrine manner by stimulating the surrounding prostatic epithelial cells, resulting in increased proliferation [14, 24]. Proliferation of prostate cancer cells is stimulated by an activated IGF-1 signaling pathway [25]. FGFs are expressed in almost all tissues and play important roles in a variety of normal and pathological processes, including development, wound healing, and neoplastic transformation [26]. FGFs are bound in the ECM and can be released by the activity of degradative enzymes such as proteases [27]. FGF-2 is expressed in many human malignancies, including prostate cancer [26]. Giri et al.

(1999) [28] showed that FGF-2 was present at significantly higher concentrations in clinically localized cancer tissue (almost 2.5-fold) compared to normal prostate.

ECM is a substrate for MMPs which are extremely important for the cellular function mechanism [29]. MMP expression has been characterized as weak or not detectable in the great majority of benign tissue however it increases in malignancy [30]. According to Martin & Matrisian (2007) [31] and Reynolds & Kyprianou (2006) [14], MMPs could benefit the tumoral growth by direct or indirect ways, signaling growth factors such as IGF and FGF. MMPs are capable of digesting ECM and basement membrane components [29]. Increased expression of MMPs in prostate cancer leads to proteolytic breakdown of the basement membrane and ECM structures leading to release of FGFs [26, 32]. In addition to their role in extracellular matrix degradation, MMPs are involved in IGF liberation and in growth factor inhibition (IGF-binding proteins). MMP-2 has been involved in laminin rupture leading to cellular motility [33]. Other authors have indicated that the cleaved fragment of laminin is found in remodeled tumoral areas, suggesting another mechanism in which MMPs may promote cellular migration and invasion [29].

It is also known that DG is a target of the MMP family. Yamada et al. (2001) [34] showed that degradation of two DG subunits (α-extracellular and β-transmembrane) by MMP disintegrated the DG complex and disrupted the link between epithelial cells and the ECM via the DG complex. Abnormal DG expression not only leads to the rupture of structural interaction between the extracellular matrix, the cellular membrane, and the intracellular cytoskeleton, but also acts as a guide to cellular surface changes and adhesion loss, affecting paracrine signaling [35]. Also, Tomas & Krušlin, (2004) [16] showed that stromal components in prostatic cancer significantly differ from normal prostate. According to these authors, normal prostate stroma was predominantly composed of smooth muscle cells with very few fibroblasts, myofibroblasts, and collagen fibers opposite to stroma in prostatic cancer that was enriched by fibroblasts and myofibroblasts, and showed a decrease in number of smooth muscle cells. The vimentin expressed in mesenchymal cells, is a well-known marker for epithelial-mesenchymal transition [36–38]. Previous studies have demonstrated that vimentin is expressed in epithelial cells that undergo tumor invasion [38].

The current study demonstrated the origin of and the biological mechanisms responsible for periacinar retraction clefting in prostatic carcinoma. The periacinar retraction was not simply a technical artifact without significance, but instead represented the consequence of processes that affected neoplastic acini and surrounding stroma. The lack of epithelial basal cells, dystroglycans, and laminin led to a rupture of the structural interaction between the ECM, the cellular membrane, and the intracellular cytoskeleton, affecting paracrine signaling, suggesting an important pathway in periacinar retraction occurrence. Increased IGF-1 and FGF-2 and their interaction with MMPs characterized the molecular disorder of retraction clefting around prostatic carcinoma. Thus, periacinar retraction clefting might be used as a reliable criterion in prostatic carcinoma diagnosis.

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