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Originals – Prostate cancer

Evaluation of the expression of p22 *phox* subunit of NADPH oxidase (NOX) in prostate cancer and benign prostatic hyperplasia: A comparative study

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Introduction and objective: Recent reports found that prostate cancer is the second most common cancer and the second leading cause of cancer death in men.
Methods and results: Between January 2004 and December 2007, 62 samples were obtained (30 from patients with cancer and 32 from patients with hyperplasia). A clinical, experimental, cross-sectional, comparative, and descriptive trial study was conducted. The inclusion criteria (cancer or hyperplasia diagnosis), exclusion criteria (patients who did not consent to participate in the study or who were not candidates for prostate resection), and withdrawal criteria (damage tissue) were satisfied. The p22 *phox* subunit of NADPH oxidase was detected by immunohistochemistry in patients with prostate cancer and prostatic hyperplasia, from the formation of avidin-biotin complex using diaminobenzidine as a contrast dye. The statistical analysis was determined with Student's t test (Graph Prism 3.0 software); $p<0.05$ was considered a significant statistical difference. The results of the immunoreactivity of p22 *phox* in the prostate stroma and gland were increased in prostate cancer (8.45±3.6 and 25.08±7.5% $p<0.0001$, respectively) compared to the results in prostatic hyperplasia (4.8±2.8 and 6.7±3.1% $p<0.0001$, respectively).
Conclusions: Over-expression of the NADPH oxidase is involved in prostate cancer. Moreover, we suggest that NADPH oxidase in combination with other classic markers could be an indicator for the post-treatment monitoring of the patients diagnosed with hyperplasia and others minor pathologies of the prostate.

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Evaluación de la expresión de la subunidad p22 *phox* de la NADPH oxidasa (NOX) en cáncer de próstata e hiperplasia prostática benigna: estudio comparativo

Palabras clave:
Cáncer de próstata
Hiperplasia prostática benigna
Especies reactivas de oxígeno
p22 *phox*
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RESUMEN

Introducción y objetivo: Recientes reportes ubican que el cáncer de próstata es el segundo cáncer más común y la segunda causa principal de muerte por cáncer en los hombres.

Métodos y resultados: Se obtuvieron 62 muestras (30 de pacientes con cáncer y 32 de pacientes con hiperplasia) colectadas desde enero de 2004 a diciembre de 2007. Se llevó a cabo un estudio clínico, experimental, transversal, comparativo y descriptivo. Se cumplieron los criterios de inclusión (diagnóstico de cáncer o hiperplasia), exclusión (pacientes que no autorizaron a participar en el estudio o no candidatos a la resección prostática) y de eliminación (tejidos dañados). Se detectó por inmunohistoquímica la presencia de la subunidad p22 *phox* de la NADPH oxidasa en pacientes con cáncer de próstata e hiperplasia prostática a partir de la formación del complejo avidina-biotina en presencia de diaminobenzidina como colorante de contraste. El análisis estadístico fue determinado con la prueba t de student (software Graph Prism 3.0) considerando una $p < 0,05$ para diferencias estadísticas. Los resultados de la inmunorreactividad de p22 *phox* en estroma y glándula de la próstata mostraron un incremento en el cáncer de próstata (8,45 ± 3,6 y 25,08 ± 7,5% $p < 0,0001$, respectivamente) en comparación con los resultados encontrados para hiperplasia prostática (4,8 ± 2,8 y 6,7 ± 3,1% $p < 0,0001$, respectivamente).

Conclusiones: La sobreexpresión de NADPH oxidasa se encuentra involucrada en el cáncer de próstata. Además, sugerimos que la NADPH oxidasa, en combinación con otros marcadores clásicos, podría funcionar como un indicador para el monitoreo postoperatorio de pacientes diagnosticados con hiperplasia u otras patologías menores de la próstata.

Introduction

The prostate is the male organ most commonly affected by benign or malignant conditions such as cancer or hyperplasia. As a result of the aging of the population worldwide, over the past two decades there has been an increase in the number of men with prostate cancer (PCa)[1-3].

Oxidative stress is known to be involved in the development of PCa. Oxidative stress is an unbalance between the production of reactive oxygen species (ROS) and the antioxidant defenses; it is the consequence of an excessive production of ROS, decreased antioxidant defenses, or a combination of both[4]. Some ROS, such as superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), are abundant in tumors; at high levels they may induce cell death, apoptosis, and senescence[5].

NADPH oxidase (NOX) consists of a membrane-bound complex (glycosylated protein gp91 *phox* and non-glycosylated protein p22 *phox*) and three cytosolic subunits (p47 *phox*, p67 *phox*, p40 *phox*). This enzyme is one of the main sources of $O_2^{\bullet -}$ and thus has an important role in maintaining the balance of ROS in the cell. In turn, the $O_2^{\bullet -}$ radical can later become other ROS, but for the most part it is dismutated to H_2O_2, mainly through the action of the enzyme myeloperoxidase[5].

On the other hand, several studies have demonstrated the role of NOX in ovarian and colon cancer, and only in PCa cell lines. Xia et al, Brar et al, and Lim et al found that cells from ovarian cancer, colon cancer, and prostate carcinoma cell line DU145 present an overexpression of NOX; they concluded that ROS have an important role in the induction of angiogenesis and tumoral growth[6-8]. Additionally, Arnold et al showed that the overexpression of NOX1 in the DU145 cell line induces the transcription factors c-fos and the cytokine IL-8, with an associated increase in the production of ROS[9].

Currently, the only studies with PCa are those conducted by Chaiswing et al, Kumar et al, and Jajoo et al. They demonstrated that the use of NOX inhibitors (apocynin, diphenyliodonium, and dibenziodolium chloride), and sometimes the induction of overexpression of antioxidant enzymes (Cu/Zn-superoxide dismutase) reduce cell invasion ability, the production of H_2O_2 and $O_2^{\bullet -}$, the activation of mitogens such as ERK1/ERK2, p38, NF-κB, MAP-kinases, and AKT in various PCa cell lines (DU145, PC3, LNCaP, PC3-MM, ALVA, and AT6.1)[10-12].

Based on that, the objective of the present study was to assess, compare, and analyze the protein expression of subunit p22 *phox* of NOX in the prostate stroma and gland of patients diagnosed with cancer and hyperplasia.

Materials and methods

Study subjects: Samples were collected between January 2004 and December 2007. A clinical, experimental, cross-sectional, comparative, and descriptive study was conducted. We selected samples from patients in the surgery records of the Urology Service of the Hospital Central Militar who met the inclusion, exclusion, and withdrawal criteria.

Inclusion criteria: a) patients diagnosed with PCa with low urinary obstructive syndrome and radical prostatectomy; b) patients diagnosed with prostate hyperplasia (BPH) with an indication of prostate transurethral resection (PTUR).

Exclusion criteria: a) patients who did not give their consent to participate; b) patients who were not candidates to PTUR.

Withdrawal criteria: a) patients whose tissue was damaged during transportation.

Patients underwent clinical analyses, digital rectal examination, prostate trans-rectal ultrasound (PTRU), and prostate-specific antigen (PSA) testing.

In patients with a suspected BPH, the digital rectal examination revealed an enlarged gland that was rounded and symmetrical, elastic, with regular surface and borders; in contrast, the glands of patients with suspected PCa, were enlarged, had nodules or areas of induration, stone-like texture, irregular surface, little mobility, and lobe asymmetry. There was no tenderness in either case.

To prepare for the PTRU, patients retained urine and fasted for at least 6 hours; ciprofloxacin 500 mg/12 hours was initiated the day before the procedure and maintained for 4 days after. Patients were positioned in the left lateral decubitus with both knees flexed at 90 degrees; the transducer was covered with two condoms, with a layer of acoustic gel between them. The following sonographic patterns were considered, per Rifkin et al[13].

Normal prostate: homogeneous prostatic echostructure with regular borders, and a volume not exceeding 20 mL.

Nodular prostate: hypoechoic, hyperechoic, or isoechoic nodules with regular and irregular borders, with or without calcifications, with a homogeneous or heterogeneous structure.

Additionally, the following factors were taken into consideration for the diagnosis of prostate cancer:

- Echogenicity and symmetry of the gland
- Assessment of periprostatic tissues
- Assessment of neurovascular bundles
- Assessment of the seminal vesicles
- Assessment of the apex of the prostate gland
- Assessment of the ejaculatory ducts
- Assessment of the periprostatic lymph nodes

In patients with suspected BPH, the most common image was isoechoic; in patients with suspected PCa, the hypoechoic image was the most common, in addition to a heterogeneous, nodular and diffuse prostate with glandular growth and asymmetry and distortion of the capsule; however, no areas of necrosis or infiltration were found in the bladder of seminal vesicles. These observations are consistent with other reports in literature[15-19].

According on the outcomes of the digital rectal exam and the PTRU, all patients underwent a fine needle aspiration biopsy following the method described by Kline[14].

Finally, the biopsies were examined histologically. The specimens were fixed with 10% buffered formaldehyde (pH=7.4) and embedded in paraffin. Slides with 3-µm sections of tissue stained with hematoxylin and eosin (H-E) were prepared for histological analysis to identify areas with tumoral foci or prostate hyperplasia. The following results were obtained:

BPH: We found conglomerated epithelial elements, small glands lined with a regular layer of tall cylindrical or flat cubic epithelium, and an intact basal membrane. These observations are consistent with the nature of the lesions and the histological features reported for BPH[20].

PCa: interspersed stoma and loss of the normal layer of myoepithelial cells that surround the glandular cells, and minimal nuclear anaplasia (well-differentiated: Gleason score 2–4). Disorganization of the glandular elements and more pronounced nuclear anaplasia (moderately differentiated: Gleason score 5–7). Absence of formed glands, and presence of solid masses of individual infiltrative cells, pronounced nuclear anaplasia (poorly-differentiated: Gleason score 8–10). These features are consistent with the histological characteristics reported for PCa[20]. In all cases the tumor was classified as clinically localized.

Based on the digital rectal exam, the sonographic images, and the histological analysis of the biopsies confirming PCa, the urologist indicated a radical prostatectomy; a PTUR was performed in patients with BPH.

The removed prostate and the specimens obtained form the TUR in patients with PCa and BPH, respectively, were prepared for histopathologic examination with H-E stain, to search for tumoral foci or hyperplasia. Three uropathologists did the histopathological analyses independently.

All immunohistochemical tests were done on slides with 3-µm sections of tissue fixed with 10% formalin and embedded in paraffin; these sections were obtained from tumor areas in the radical prostatectomy specimen or from fragments from the area identified as hyperplasia extracted through TUR according to the histopathologic analyses described above.

Immunohistochemistry: The sections in the slides were deparaffinated and warmed to unmask the antigenic sites; peroxidase endogenous activity was blocked with 0.03% H_2O_2 in absolute methanol. The tissue sections were incubated overnight at 4 °C in a 1:100 dilution of monoclonal antibody against p22 *phox*, in a TRIS solution. The primary antibody was removed, and two repeat washes with TRIS were done; the sections were incubated in a 1:500 dilution of rabbit polyclonal antibody as secondary antibody, and two repeat washes with TRIS done. The bound antibodies were detected with the avidin-biotin complex (ABC-kit Vectastain) using diaminobenzidine as the substrate. After repeat washes with TRIS, the sections were stained with hematoxylin. All the sections were incubated in the same conditions and with the same antibody concentration in the same run, so the immunostains are comparable. All the specimens were

examined with an Axiovert 200 M light microscope (Carl Zeiss, Jena, Germany). In the automated morphometric analysis, the percentage of positive cells (brown) were determined using a computed image analyzer KS-300 3.0 (Carl Zeiss, Jena, Germany). This device automatically detects positive cells and determines their percentage per field. Five random fields were analyzed with a magnification of 100 (total area: 1,584,000 μ^2). The results are expressed as a percentage.

Statistical analysis: The statistical significance of the immunoreactivity of the levels of expression of p22 *phox* between the gland or stroma of prostate cancer and hyperplasia groups was determined with Student's t-test using the Graph Prism 3.0 software. The data are expressed as mean±standard deviation. A $p<0.05$ was considered a statistical difference between the two groups.

Table 1 – Expression of p22 *phox* subunit in PCa and BPH

Parameters	Stroma	Gland
BPH	4.8±2.8	6.7±3.1[a]
PCa	8.45±3.6[c]	25.08±7.5[d,b]

PCa: prostate cancer; BPH: prostate hyperplasia.
[a]p=0.0125 vs. BPH stroma.
[b]p<0.0001 vs. PCa stroma.
[c]p<0.0001 vs. BPH stroma.
[d]p<0.0001 vs. BPH gland.
Results are expressed as mean±standard deviation.

Results

Of a total 62 patients, 30 had a histopathologic diagnosis of PCa (48.4%), and 32 had a diagnosis of BPH (51.6%).

In the PCa group, the mean age was 65.3 years, and the PSA 8.6 ng/mL; the Gleason score was 4 in 1 case (3.3%), 6 in 19 cases (63.3%), 7 in 9 cases (30%), and 8 in 1 case (3.3%).

In the BPH group, the mean age was 66.5 years, and the mean pre-operative PSA was 8.7 ng/mL.

Table 1 shows the levels of expression of subunit p22 *phox* of NOX in the BPH group.

As can be observed, the expression levels of NOX were 1.76 and 3.74 times significantly higher in stroma and gland, respectively, in PCa vs. BPH. Furthermore, expression was significantly higher in the glandular part of both study groups (fig. 1).

Discussion

The results from this study show an increased percentage of the area of expression of NOX p22 *phox* subunit in the prostate stroma and gland of patients diagnosed with cancer, compared to those diagnosed with hyperplasia; there was also a significant increase in expression in the glandular part of both study groups.

In prior studies, the production of ROS in the PCa cell line DU 145 was reduced when a flavoprotein inhibitor was used. The expression of the three main NOX subunits (gp91 *phox*, p47 *phox*,

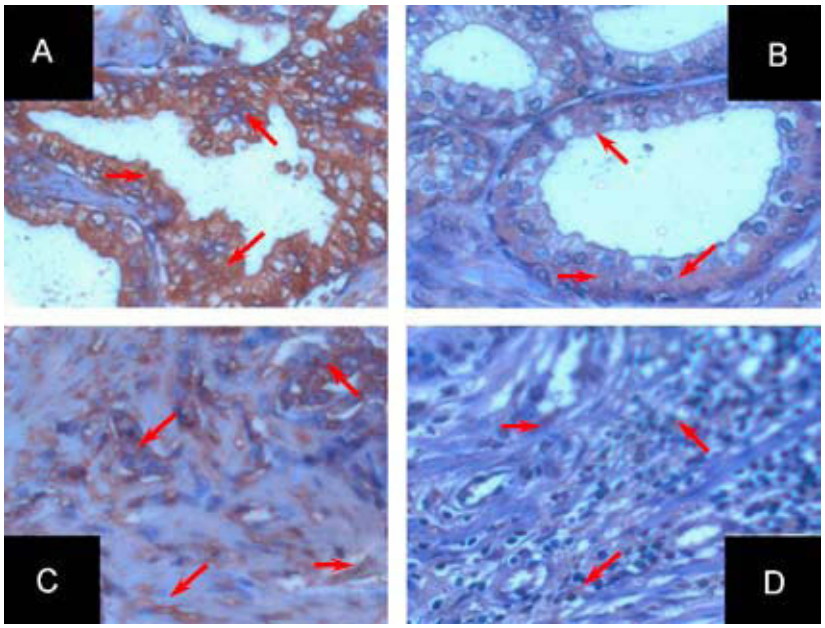

Figure 1 – Immunohistochemistry for p22 phox in gland and stroma of PCa and BPH. A) PCa gland. B) BPH gland. C) PCa stroma. D) BPH stroma, respectively. The percentage of marked area per field (400×) was determined in both groups. A significant increase in p22 *phox* immunoreactivity was found in the PCa group ($p<0.0001$).

and p67 *phox*) was also decreased; consequently, the authors concluded that NOX plays a role as a signaling mechanism that regulates cell growth and apoptosis in PCa[21,22].

On the other hand, it appears that endothelial cells express all the NOX components, including gp91 *phox*, p22 *phox*, p47 *phox*, and p67 *phox*, and that p22 *phox* and p47 *phox* are expressed in the smooth vascular muscle. However, even though gp91 *phox* was identified, p22 *phox* protein was not detected in cell line DU145, while RNAm was[21,23,24]. This observation contrasts with the results of our study, as we did quantify the expression of p22 *phox* in PCa biopsies.

The exact sites of the NOX signaling pathways is still uncertain; they may be tissue-specific, but prior studies suggest that this enzyme may have a signaling role in the regulation of cell growth and apoptosis in PCa[21]. Angiotensin II has been shown to stimulate the activity of NOX in vascular smooth muscle via protein kinase and also in part via NF-κB in the respiratory tract and in melanomas, but it has not yet been studied in patients with PCa[25].

Another study found that high levels of ROS occurred spontaneously in PCa and ovarian cancer. The elevated production of reactive species was blocked by NOX inhibitor diphenylene iodonium and the mitochondrial electron chain inhibitor rotenone[26]. Additionally, isoform NOX1 has been shown to induce vascular endothelial growth factor and the expression of its receptor in several tumors[23]. On the other hand, in ovarian cancer cells, an increased production of ROS was found, which was proportional to the expression of NOX and to the expression of HIF-1 (hypoxia-inducible factor 1)[27]. It has also been observed that in NOX1-transfected cell line DU145, tumoral growth and H_2O_2 production are increased[23].

All this seems to suggest that changes in the redox state when normal cells become aberrant (here, the production of $O_2^{\bullet-}$ would be implicated), as well as differences in prostate architecture, would lead to an invasion of cancer cells in that tissue.

Conclusions

An overexpression of NADPH oxidase is actively involved in PCa. Moreover, we suggest that NADPH oxidase in combination with other classic markers (such as PSA) could function as a marker in post-operative monitoring of patients diagnosed with hyperplasia and others minor pathologies of the prostate.

Conflict of interest

The authors state that they have no conflicts of interest.

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