MOLECULAR MARKERS ASSOCIATED WITH PROSTATE CÁNCER: 3-NITROTYROSINE AND GENETIC AND PROTEIC EXPRESSION OF MN-SUPEROXIDE DISMUTASA (MN-SOD)

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Summary.- OBJECTIVES: Recent reports place prostate cancer (PCa) as third cause of death in the World among males, in Mexico it is the leading cause of male death. In this study, we studied molecular markers and one genetic marker related in tissues with PCa and benign prostatic hyperplasia (BPH): The Mn-superoxide dismutase (Mn-SOD) gen and protein, prostate specific antigen (PSA) and 3-nitrotyrosine (3-NT). It was evaluated if various markers of oxidative stress show altered expression in prostate cancer and BPH.

METHODS: 80 biopsies were obtained. The conditions to amplify and evaluate the immunoreactivity of the genes of interest (Mn-SOD and 3-NT) and the correlation between PSA and Mn-SOD immunoreactivity in prostate cancer and benign prostatic hyperplasia were standardized.

RESULTS: Gene overexpression and Mn-SOD and 3-NT immunoreactivity were greater in prostate cancer with respect to the BPH group. Correlation between levels of PSA and the Mn-SOD immunoreactivity was not observed.

CONCLUSIONS: The above results suggest that the parameters evaluated can be used as tumor markers making the determinations in biopsies of patients suspected of prostate cancer.

Keywords: Prostate cancer. Benign prostatic hyperplasia. Reactive oxygen species. Mn-SOD. 3-nitrotyrosine. Prostate specific antigen.

Resumen.- OBJETIVO: Recientes reportes ubican al cáncer de próstata en el tercer lugar mundial, en México es la principal causa de muerte masculina. En este estudio se evaluaron marcadores genéticos relacionados en cáncer de próstata y en hiperplasia prostática benigna (HPB): Mn-superoxido dismutasa (Mn-SOD), antígeno prostático específico (APE) y 3-nitrotritosina (3-NT).

Se evaluó si existe alguna alteración en la expresión de diversos marcadores de estrés oxidativo en cáncer de próstata e hiperplasia prostática benigna.

MÉTODOS: Se obtuvieron 80 biopsias. Se estandarizaron las condiciones para amplificar y evaluar la in-
most common benign tumor in men and its incidence is related to age. The prevalence of BPH is age-dependent, beginning with its development after the 40 years of age. For 60 years of age, their prevalence is 50% for 85 years and older is 90%.

Like the PCA, is related to the individual’s age (aging) and requires androgen (dihydrotestosterone) to develop and grow. Unlike PCA, BPH is a benign lesion that rarely progresses to a malignancy. Another dissimilarity regarding PCA is that there is no evidence that BPH is a clone was found only a few genetic changes that involve genomic stability. BPH is not detected in morphological degrees in the core that are characteristic of malignant neoplasms. In hyperplastic disease often presents stromal growth variables and it has been suggested that the secretion of growth factors from the mesenchyme could act on adjacent epithelial cells and contribute to the development of hyperplasia of these últimos (4).

It is estimated that the diet can contribute 35% of all human tumors (5). The epidemiological evidence shows that a consistently low intake of antioxidants or low blood levels of antioxidants may increase the risk of cancer.

Has recently been linked to oxidative damage plays a critical role in various clinical conditions such as malignant diseases. Reactive oxygen species (ERO) can cause oxidative damage to DNA and proteins, damage to genes, tumor suppressor and an increase in the expression of proto-oncogenes. The late onset of prostate cancer suggests that a multi-step process is involved in carcinogenesis and the most reasonable candidates for endogenous formation of genotoxins in the later stages of life is the accumulation of ERO (6).

To protect against the toxic effects of the ERO and modulate their physiological effects the cells have developed an intrinsic antioxidant defense system. The antioxidant enzyme system is very complex, being composed of small molecules strong antioxidant (Vitamins E, C, A), primary antioxidant enzymes (manganese, copper, zinc superoxide dismutase, catalase, glutathione peroxidase) and secondary antioxidant enzymes (glutathione reductase and glucose-6-phosphate dehydrogenase). Nitric oxide (NO•) modulates the levels of ERO in part by its reaction with superoxide anion finally proteins involved in cellular response to stress are also important in the modulation of oxidative damage, each component of the antioxidant system is specifically located subcelulares compartments (7).

Because oxidative stress is involved in the pathogenesis of cancer gene expression was evaluated
and the immunoreactivity of Mn-SOD and their relationship with prostate specific antigen (PSA) and 3-nitrotyrosine immunoreactivity (NT-3) in PCa and BPH.

MATERIALS AND METHODS

Subjects of study:
The sample collection was conducted from December 2006 to March 2007, selecting the patient in the book surgery urology service Central Military Hospital and met the criteria inclusion, exclusion and elimination.

Inclusion criteria:

a) diagnosis of PCa patients with lower urinary obstruction syndrome of the urinary tract.

b) Patients diagnosed with BPH, transurethral resection showing prostate (TURP) and radical prostatectomy.

Exclusion criteria:

a) Patients who do not allow to participate in the proceedings.

b) Patients who are not candidates for TURP

Criteria for removal:

a) Patients with RNA extracted and purified had an inappropriate concentration and purity for the study.

b) that the tissue undergoes distortion transfer of genetic material.

The amount of tissue needed for the research was from 1 to 5 g, obtaining samples was done immediately after the completion of the surgery, the transfer time of one hour was up, from the sample collection until his transfer to the laboratory of Molecular Biology, Military Medical School, where he maintained at a temperature of -70 °C in the frozen Revco ® (Legaci Dupont SVVA Refrigerants ULT2186 3-35) to the processing of RNA extraction.

Extraction and quantification of RNA.
The total RNA was extracted with the kit to extract total RNA using the SV Total RNA Isolation System (Promega, Madison, WI, USA), took 150 mg of each of the samples with sterile technique, avoiding contamination RNAsas, harmonized with a politrón (µH Omni International), following the directions of the supplier, and finally to the final elution of total RNA were added 2 µl RNAsas inhibitor and stored at -83 ° C for further analysis. The verification of the integrity of total RNA was performed by electrophoresis on agarose gel 2%, putting 6 µl total RNA extracted sample and the bands were visualized with UV light (KODAK EDAS 290). Quantification of total RNA was carried out using SYBR Green fluorescence with multidetector Synergy HT-I (BIO-TEK Instruments, Inc. Higland Park, Vermont, USA).

Standardization of RT-PCR technique in real time.

For the development of the RT-PCR were designed in real time-specific oligonucleotides (primer) of the gene of interest (Mn-SOD) and reference genes (endogenous candidates) subunit ribosomal 18s, glyceraldehyde-3 phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH) and β-actin (BACT), endogenous genes were validated with software BestKeeper and I like the most stable endogenous control of the burden of total RNA. The sequences were obtained from GenBankTM (Table I). The search was conducted in the sequences (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm their specificity.

Synthesized primers (Invitrogen, Carlsbad, CA) were as follows: gene of interest Mn-SOD; endogenous candidate genes: BACT, 18s, and GAPDH gene G6PDH, which were optimized at a temperature of 57°C (Table I). The conditions for RT-PCR were optimized with a gradient thermocycler (gradient Px2 Thermal cycler Hybaid, Franklin, MA) using the kit SuperScriptTM III Platinum ® SYBR ® One-Step QRT-PCR kit with ROX (Invitrogen, Carlsbad, CA). The footages taken for the records in the GenBankTM under the following number of accessions: Mn-SOD, NM_017051; BACT, NM_001101; GAPDH, NM_002046; subunit 18s, X03205 and G6PDH, NM_004285. The products of amplification by RT-PCR were visualized by gel electrophoresis on a 2% agarose and studied with electrophoresis analysis system EDAS 290 (Kodak, New Haven, CT). The results of the amplifications conditions such as temperature, concentrations of primers, dNTPs (nucleotides) and volumes were transferred to the amplification protocol real-time detection system gene Rotor 6.0 (Corbett Life Science, Sydney City, Australia).

RT-PCR protocol in real-time Rotor-Gene 3000.
The reactions of the RT-PCR were performed in real time with 10 ng RNA extracted from the tissues studied, the dilution for the validation of internal control, absolute quantification and determination of the
efficiencies of all primers were performed using the kit SuperScript™ SYBR® III Platinum® One-Step QRT-PCR kit with ROX (Invitrogen, Carlsbad, CA), which is a reaction mixture containing a complex of Taq DNA polymerase (Platinum) with antibody, which inhibits the activity-temperature environment and the antibody is denatured in the initial phase of the cycle of PCR (95°C for 5 minutes). The following components of the reaction were prepared at a final concentration from the reaction mixture of 6.25μL 2X SYBR® Green I (a buffer in SYBR® Green I dye) 0.4mM per dNTP and 6 mM MgSO_4 (ROX Reference Dye) which is a buffer stabilizer (1X), 4μL total RNA, SYBR® Green 0.25μl One Step Enzyme Mix (in SuperScriptTM RT III Platinum® Taq DNA polymerase and RNase inhibitor (recombinant ribonuclease), 0.4μL MgCl_2 (50 mM), 0.4μL of the first forward (0.4μM), 0.8μL water free RNAsas (12.5 μL the final reaction mixture), and taken to 100 μL tubes. The reaction was carried out initially to 52°C for 10 minutes for the synthesis of DNA and 94°C for 5 minutes for the start of the activity of Taq polymerase (Platinum Taq) and the perdenaturing hybrid RNA-cDNA, followed the cycle of PCR amplification with 40 cycles, each cycle consisting of 95°C for 20 seconds (s), 57°C for 20 s, 72°C for 20 s, which had three points of fluorescence readings to 57, 72 and 83°C, respectively. The passage of the curve had a melting temperature of 60-98°C with a temperature rise of 0.1°C per second and finally a step at 40°C. The concentration was absolute necessary to determine the threshold cycle (Ct) for each transcribed. The expression of both genes as candidates endogenous gene of interest (Mn-SOD) were quantified individually with Gene BestKeeper statistical model for the validation of the model with endogenous Statistical (REST®) for analyzing and quantifying the efficiencies of the genes on endogenous and Mn-SOD.

**Immunohistochemistry:**

For light microscopy, tissue samples of PCa and BPH were fixed by immersion in formalin (pH 7.4) and embedded in paraffin. For histological analysis, tissue sections (3 μm) were stained with hematoxylin and eosin (H & E). Tissue sections were stained with

**Table I. Sequence of Primers That Were Used in the RT-PCR, Position,(96,664),(796,916)
periodic acid Shiff’s (PAS) to show polysaccharides, glycoproteins, and mucopolysaccharides of the cell membrane.

The slides were incubated with periodic acid for 5 min and washed with distilled water. The slides were incubated with Schiff’s reagent for 5 min and counterstained with hematoxylin for 30 s. Histological profile of 5 randomly selected fields were recorded using the software KS-300 (Carl Zeiss, Jena, Germany). The percentage of damaged area with histopathological alterations was obtained (400x magnification). For immunohistochemistry tissue sections (3 μm) was boiled to unmask antigenic sites, the endogenous peroxidase activity was blocked with 0.03% of H2O2 in absolute methanol. Tissue sections were incubated overnight at 4°C 1:100 dilution of a monoclonal antibody against Mn-SOD or 3-NT as appropriate solution TRIS primary antibody was removed and two washes with repetitive TRIS the slides were incubated with a 1:500 dilution of rabbit polyclonal antibody as secondary antibody and two washes with TRIS repetitive. The antibodies were detected together with avidin-biotin complex (ABC-kit Vectastain) and diaminobenzidina as substrate. After repeated washing with TRIS slides were counterstained with hematoxylin. All slides were incubated under the same conditions with the same concentration of antibody, so the immunostaining was comparable. All specimens were examined by light microscopy Axiosver 200M (Carl Zeiss, Jena, Germany). For automated morphometric analysis, the percentage of positive cells (brown) was determined using a computerized image analyzer KS-300 3.0 (Carl Zeiss, Jena, Germany). This equipment automatically detects positive cells determining their percentage field. Five random fields were studied at a magnification of 100 (total area 1,584,000 μm²). The results were expressed as a percentage.

**Data analysis:**

Data from the efficiencies and absolute quantification (Ct) of genes were analyzed using the Rotor-Gene 6.0 software (Corbett Life Science, Sydney City, Australia). The determination of the Ct was performed with the software mentioned above, the data were plotted on the same table and exported to Excel tool by a linear regression similar to the efficiencies of the primers, which compares against the logarithmic concentration (Ct) and data from the calibration curves for Mn-SOD and GAPDH were analyzed by the same software mentioned above.

To determine the slope and Pearson correlation coefficients were used an Excel table. To validate the statistical model was used BestKeeper Gene.

**Statistical analysis of data:**

The results of gene expression were analyzed in an Excel tool where previously imported TK gene Rotor 6.0 software, and used the software to take PRISMA relations representative samples with BPH and PCa.

Was used the Student t-test to compare changes in gene regulation, the immunoreactivity of 3-NT gene and immunoreactivity of Mn-SOD in both tissues. Was considered a p <0.05 as statistically significant difference to the application of statistical tests using the Graph Prism version 3.32.

**RESULTS**

a. Gene expression of MnSOD.

Of the total of 80 patients from whom samples were obtained, 27 patients (33.7%) had a diagnosis of PCa, while 53 patients (66.2%) had a diagnosis of BPH. To determine the most appropriate and endogenous stable RT-PCR was performed in real time by each of three samples of PCa and compared taking the CT (the average of each sample in triplicate); this data is exported to the software REST (Figure 2). After you export and analyze data was taken as an endogenous gene GAPDH, which presented a stability that the other three, with a p <0.005 and Pearson correlation coefficient of 0.953 with the gene of interest Mn-SOD (Table II). Absolute copies of Mn-SOD mRNA in BPH and PCa were compared with t-test with a statistical difference of p = 0.0002, the average absolute copies for PCa was 765.7 ± 185.2, the average was 242.8 HPB ± 18.43. So after analyzing the results of these data, we found that expression is 3.15 times more than overexpression of Mn-SOD gene in PCa compared with BPH (Figure 2).

b. Correlation between PSA levels vs immunoreactivity tissue with PCa.

By Pearson correlation test, no correlation was found between the levels of expression of Mn-SOD and serum levels of PSA (Figure 3).

c. Immunoreactivity of MnSOD.

Of the total of 80 patients were obtained biological samples, 40 patients (50%) had a diagnosis of PCa, while 40 patients (50%) had a diagnosis of BPH. The percentage of immunoreactive area to Mn-SOD were 10.5 ± 24.83% and 14.73 ± 8.7% in BPH. The percentage of immunoreactive area to Mn-SOD (Table II). Absolute copies of Mn-SOD mRNA in BPH and PCa were compared with t-test with a statistical difference to the application of statistical tests using the Graph Prism version 3.32. So after analyzing the results of these data, we found that expression is 3.15 times more than overexpression of Mn-SOD gene in PCa compared with BPH (Figure 2).

d. NT-3 immunoreactivity.

Of the total of 80 patients were obtained biological samples, 46 patients (57.5%) had a diagnosis of PCa, while 34 patients (42.5%) had a diagnosis
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of BPH. The percentage of immunoreactive area to NT-3 were 25.78±15.37% and 4.43±2.2% in PCa and BPH, respectively (Figures 5 and 6).

DISCUSSION

The oxidative damage caused by the ERO is involved in prostate carcinogenesis. The evidence of the literature indicates that Mn-SOD functions as a tumor suppressor, possibly by modulating apoptotic and cell growth and proliferation, as well as a link between certain polymorphisms and PCa. In our study we found an significant increase in this enzyme. It was also noted an increase in the percentage of area marked significantly in tumor tissue. However, several studies have found an increased expression of Mn-SOD in cancer tissue in advanced stages (5).

In this study we found that the gene coding for Mn-SOD is 3.15 times as expressed in PCa tissue than in BPH. On the other hand one can postulate that this increased activity is causing the disruption of cellular functions such as producing cell proliferation responses and programmed cell death (apoptosis), may be one of the causes of the disease, including the degree of malignancy.

There are data that show us that the expression of antioxidant enzymes such as Mn-SOD and glutathione-S-transferase (GST), it is possible that these enzymes or gene product (mRNA) can serve as markers of early premalignant changes that have a high risk of invasive prostate cancer. In other preliminary work is in correlation with our study which has found that the enzyme Mn-SOD is increasing in various cancer (6-11).

The above observations suggest that the development of selective inhibitors for the Mn-SOD is of great interest as a potential therapeutic strategy for certain cancers, including of prostate (12). It is very likely that epigenetic changes or mutations in the gene for Mn-SOD is the cause of the high level found in the expression of Mn-SOD in PCa and that this in turn is the cause of high levels of this protein found in the mitochondria of cells with PCa, other findings of our study (data not shown). This could have potential impact on the survival and proliferation of tumor cells, which has been found in other tumors that behave aggressively and with poor prognosis for the patient. Our results show that the overexpression of this enzyme gene also plays an important role, because this gene product is needed for encoding and translation of the protein.

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<th>TABLE II. DETERMINATION OF MORE STABLE ENDOGENOUS GENE, THE DATA (C&lt;sub&gt;T&lt;/sub&gt;) WERE ANALYZED WITH A RECENT STATISTICAL MODEL BESTKEEPER, WHICH CORRELATES PROPOSED A SET OF GENES (CANDIDATE GENES) AND VALIDATES THE MOST STABLE OF THESE GENES IN OUR STUDY WAS ONE OF THE GAPDH GENES AND MORE STABLE FOR OUR CONVENIENCE, BECAUSE OF ITS AMPLIFICATION THRESHOLD (C&lt;sub&gt;T&lt;/sub&gt;) AND BE AMPLIFIED IN CYCLES SIMILAR OF GENE STUDY (Mn-SOD) AND A PEARSON CORRELATION OF 0.953 AND P = 0.005.</th>
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<td><strong>Correlation coefficient [r]</strong></td>
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Furthermore, chronic inflammation has been linked to the incidence of many cancers, including the prostate (13-15). The inflammation can lead to the persistence of oxidative stress in cancer cells and the reactive oxygen species may represent an advantage for them in supervivencia (6).

Prostate-specific antigen (PSA) is a protein substance of the prostate and sole function of which is the dissolution of the seminal clot. Normal blood levels of prostate-specific antigen in healthy men are very low in the order of millions of times less than semen, and rise in various diseases of prostate (16-17), in this study found that levels of PSA in our patients were concentrations of 0.01 to 41.26.

The serum PSA concentrations are directly proportional to the volume of transition zone, an area well-defined histologically and that the PCa is in most cases increased, so according to this study, we found that the data found in this study are consistent with the literature, as some authors describe increased expression of Mn-SOD in some tumores (8, 10), and on the other hand PSA levels above 4 or even lower levels are a marker for risk of PCa. PSA levels of 10, indicating high risk of incidence of PCa, finding however, that the density measurement of PSA is not a significant predictor of PCa it is still unknown whether

FIGURE 1. Dissociation curves of the products of genes analyzed. With the methodology of SYBR Green I is represented in each Amplicon melting analysis, initially using the Rotor-Gene 6.0 software (Corbett Life Science, Sydney City, Australia). The melting curve analysis is a rapid and accurate method to observe the specificity of PCR. The graphs represent the peak temperatures (ºC) for the Mn-SOD gene, 87.7 ºC, 86 ºC BACT gene, gene subunit 18s, 85.9 ºC, G6DPH gene, and 83.5 ºC gene GADPH, 84.1 ºC which were subject to the protocol dissociation at a temperature of 60-98 ºC.

FIGURE 2. Gene expression of Mn-SOD. Compared the absolute copies of Mn-SOD in both groups of PCa and BPH (p = 0.0002), in “y” shows the absolute number of copies of both groups. For BPH (SEM = 18.43) for PCa (SEM = 18.5).

FIGURE 3. Correlation between PSE and densitometry. In “y” values of the densitometric immunoreactivity of Mn-SOD in PCa tissues, in “x” PSA values of patients with PCa. The graph shows that the PSA had a low correlation with the values of the densitometric immunoreactivity of Mn-SOD in patients with PCa. N = 80, Pearson correlation rate of 0.04182, P = 0.4163.
Our results suggest that there is a change in the balance pro-oxidative-antioxidant in PCa, this imbalance is known to alter the redox processes cellular growth, proliferation and cell cycles, since it is known that some free radicals mediate the activation transduction pathways of cellular transcription factors such as Fos, Jun, and nuclear factor kB and an increase in mitochondrial activity in these cells (19).

On the other hand, has shown that there are differences in morphology and cell growth according to the increase of Mn-SOD at both gene and protein level. Our results suggest that beyond the Mn-SOD protein, the gene alter the intracellular mitochondria or mitochondrial number.

In this study we observed an increase in the 3-NT immunoreactivity in PCa compared to BPH. Alterations have been identified for NO• (species related with reactive protein nitration) and its metabolites in lung cancer and showed that NO•, nitrite and nitrotyrosine are increased in patients with lung cancer, this is consistent with found in our study (20). Moreover, cancer is found that the expression of three isoforms of NO• synthases (inducible: iNOS, endothelial: eNOS and neuronal: nNOS) are taking an increased role in tumor development and angiogenesis. These synthases have not been sufficiently studied in PCa, the data indicates that concentrations of these enzymes are low in benign tissue of the body and they concluded that the epithelial expression of iNOS can be used as a specific marker of PCa, as NO• in part, has an important role in the illness development (21).

The expression of eNOS may have an important role in the carcinogenic process of the prostate tissue. NO• production at the expense of this enzyme may promote the progression to cancer, to regulate the proliferation of tumor cells selectively by angiogenic stimulus in tissues (22). These results support the findings in our study shows differential levels of concentration, because there are more nitration in en-
dothelial cells than in other areas of cancerous tissue and the difference is higher in BPH tissue compared with PCa tissues, the 3-NT concentration is 6-8 times more in PCa.

The results presented in this study suggest that the gene expression of Mn-SOD may have an effect on both processes in mitochondrial function, as in the growth of tumor cells with no correlation with levels of PSA and that the NOS could be involved in tumor growth of PCa, since we have found that iNOS is involved in stimulating angiogenesis, and increased by mutations in the DNA through the direct action of free radicals [23].

REFERENCES AND RECOMMENDED READINGS (*of special interest, **of outstanding interest)


