Manganese superoxide dismutase (Mn-SOD) genic expression in tissue with prostate cancer and benign prostatic hyperplasia

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ABSTRACT

Background: Recent reports have placed prostate cancer (CaP) as the third most common cancer worldwide and in Mexico it is the principal cause of death from cancer in men with more than 70% of cases presenting in advanced stages. Several genetic markers are being studied in CaP. Relatively few studies have been reported in the medical literature on the manganese superoxide dismutase (Mn-SOD) gene. The present study evaluated whether there was any alteration in Mn-SOD expression in CaP and benign prostatic hyperplasia (BPH).

Materials and methods: Forty BPH samples and 20 CaP samples were obtained and conditions were standardized to amplify the Mn-SOD gene. Amplification conditions were carried over to real time RT-PCR amplification protocol in “Rotor-Gene 3000” equipment and the number of absolute copies was determined for each sample.

Results: There was 3.15 times more Mn-SOD gene overexpression in the CaP group than in the BPH group. Mean absolute copy expression for BPH was 242.8 and for CaP was 765.7.

RESUMEN

Antecedentes: Recientes reportes ubican al cáncer de próstata en el tercer lugar mundial. En México, es la principal causa de muerte masculina y más de 70% se presenta en estadios avanzados. Se están estudiando varios marcadores genéticos en cáncer de próstata; para el caso del gen de la Mn-superóxido dismutasa, hay pocos estudios en la literatura, por lo tanto en este estudio evaluamos si existe alguna alteración en su expresión en cáncer de próstata e hiperplasia prostática benigna.

Material y métodos: Se obtuvieron 40 y 20 muestras de casos con hiperplasia prostática benigna y de cáncer de próstata, respectivamente, y se estandizaron las condiciones para amplificar el gen de interés. Las condiciones de amplificación se llevaron al protocolo de amplificación por RT-PCR en tiempo real en el equipo “Rotor-Gene 3000”, y se determinaron el número de copias absolutas de cada muestra.

Resultados: El gen de Mn-superóxido dismutasa se sobreeexpresa en cáncer de próstata en promedio 3.15 veces más con respecto al grupo con hiperplasia prostática benigna, con una expresión media de copias absolutas para

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Conclusions: Mn-SOD gene expression in CaP had a greater statistical significance than in BPH, suggesting that this gene could be used as a tumor marker in biopsies of patients suspected of presenting with CaP.

Key words: prostate cancer, benign prostatic hyperplasia, real time RT-PCR, Mn-superoxide dismutase, Mexico.

INTRODUCTION

Prostate cancer (CaP) is the second most common neoplasia after skin cancer in men and the second cause of death by cancer after lung cancer. Numerous factors are attributed to the increase in CaP. More than 200,000 men in the United States are diagnosed with CaP annually and 30,000 men die from the disease each year. After the age of 50 years, CaP incidence increases 3 or 4 times every 10 years. Afro Americans have the highest CaP incidence rate with an overall 102% incidence increase from 124 cases per 100,000 individuals to 250 per 100,000. Hispanic Americans have an intermediate incidence rate of 104 cases per 100,000 individuals. Asian countries, especially Japan and China have one of the lowest incidence and mortality rates for CaP in the world. Mortality incidence of CaP in Japan between 1992 and 1995 was 4 cases per 100,000 individuals. In Mexico CaP was second in 1998 with respect to neoplasia in men, after skin cancer. It also was second in mortality for all neoplasia after cervical carcinoma.

CaP etiology is not fully understood. Up to the present, aspects considered to be risk factors are age, androgen production and metabolism, geographic area/ethnicity, dietary habits and family history.

Currently there are two clinical staging systems, the tumor-nodule-metastasis (TNM) system that is widely used in Europe and the American Modified Whitmore-Jewitt Staging System. The difference between these two systems is that the TNM system has more subdivisions.

In 1978 the TNM staging system was proposed and published by the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre le Cancer (UICC). The 3 aspects that this system evaluates are local tumor stage, lymph node affection and presence of metastasis.

The Gleason system is the most widely used method for analyzing histological differentiation grade. It was proposed by the Veterans Administration Cooperative Urological Research Group (VACURG). Classification is based on the degree of glandular differentiation and the pattern of tumor growth in reference to prostate stroma. This pattern varies from well-differentiated (Grade I) to non-differentiated (Grade V). Due to the fact that the majority of prostate carcinomas contain more than one histological pattern, this system also takes heterogeneity into account in relation to different tumor areas, assigning one histological grade to the predominant or primary pattern and another to the prevalent secondary pattern. The combined score of the primary and secondary grades is clearly related to the biological conduct of the tumor.

Benign prostatic hyperplasia (BPH) is histologically defined as a disease characterized by an increase in epithelial and stromal cells in the periurethral area of the prostate. It is a pathological process that contributes to the development of lower urinary tract symptoms in elderly men. The principal complication in BPH patients are the common lower urinary tract complaints of nicturia, tenesmus, weakened urine stream and incomplete bladder voiding sensation.

BPH is the most common benign tumor in men and is age-related. It begins after the age of 40 years,
with a 50% prevalence in 60-year-old men and a 90% prevalence in 85-year-old men. Although clinical evidence of the disease is infrequent, lower urinary tract symptoms are also age-related. Approximately half of all men histologically diagnosed with BPH present with moderate to severe lower urinary tract symptoms.7

Like CaP, BPH is related to the age of the individual and requires androgens to develop and grow. In contrast to CaP, BPH is a benign lesion that rarely progresses to a malignant tumor. Another difference from CaP is that there is no evidence that BPH is clonal. Only some genetic changes have been found that implicate genomic stability. No degrees of morphologic characteristics of malignant neoplasia have been detected in the nucleus of BPH cells. Hyperplastic pathology often presents with variable stroma growth and it has been suggested that growth factor secretion by the mesenchyme could act on adjacent epithelial cells and contribute to their hyperplasia.8

Clinical and laboratory studies have identified factors that are necessary for BPH development: dihydrotestosterone (DHT) and aging. It has been demonstrated that the prostate becomes more sensitive to androgens with age. Various theories have been suggested such as: 1) stromal-epithelial interaction (stromal cells can regulate epithelial or other stromal cell growth by means of a paracrine or autocrine mechanism secreting growth factors such as basic fibroblast growth factor or transformer growth factor, 2) aging acts on primordial cells that undergo a block in the maturation process that prevents them from entering into programmed apoptosis and they become immortalized.7

It is estimated that diet contributes to 35% of all human cancers.9 Epidemiological evidence consistently reveals that a diet low in antioxidants or a low level of antioxidants in the blood can increase cancer risk. Smoking as well as chronic inflammation - two of the principal causes of cancer - have a strong free radical component in their action mechanisms.

Oxidative damage has recently been related to various clinical conditions as having a critical role and these conditions include malignant diseases. Reactive oxygen species (ROS) can cause DNA oxidation and damage to proteins, damage to tumor suppressor genes and an increase in proto-oncogenic expression. Cancer shows a pro-oxidative change in the redox state.

ROS are potential carcinogens due to the fact that they facilitate mutagenesis in addition to tumor promotion and progression. Even normal cells show an increased proliferation and expression of growth-related genes if they are exposed to hydrogen peroxide or superoxide.

The majority of ROS-induced mutations seem to envelop guanine, causing G-T transversions. If this is related to critical genes such as oncogenes or tumor suppressor genes it can result in cancer initiation or progression. Chronic prostatic hyperplasia is diagnosed in the majority of men around the age of 40 years. But the late appearance of CaP suggests that a multiple-step process is involved in carcinogenesis and the most reasonable candidates for the endogenous formation of genotoxins in late stages of life are accumulated ROS.10

To protect against ROS toxic effects and to modulate their physiological effects, cells have developed an intrinsic antioxidant defense system. The antioxidant enzymatic system is very complex and is composed of small molecules with great antioxidant weight (vitamins E, C, A) primary antioxidant enzymes (manganese, copper, zinc superoxide dismutase, catalase, glutathione peroxidase) and secondary antioxidant enzymes (glutathione reductase, glucose-6-phosphate dehydrogenase). DNA repairing proteins and enzymes are considered to be part of the antioxidant system just as metal kidnapping proteins are important in antioxidant cell state modulation. Nitric oxide modulates ROS levels partly by its reaction with the superoxide anion and finally the proteins involved in cellular stress response are also important in oxidative damage modulation. Each component of the antioxidant system is specifically located in subcellular compartments.11

Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen which in turn can be eliminated by catalase or glutathione peroxidase.

The role of Mn-SOD in carcinogenesis is still uncertain. SOD2 induction in tumors may be due to stress and genotoxicity induced by oxidant and inflammation exposure. This enzyme has also been observed to be implicated in the resistance of tumor cells to cytotoxic drugs and radiation.

Many studies have suggested that the Mn-SOD gene could be a tumor suppressor gene and that malignant tumors have lower Mn-SOD activity. Nevertheless, many types of tumor cells have been found to contain high levels of Mn-SOD compared with their non-malignant counterpart.12 Increased SOD expression in tumor cells in cancers of the esophagus, stomach, ovaries, lungs and in leukemia and decreased SOD expression in pancreatic cancer, colorectal cancer and in melanoma have been described in the literature.13-15
## MATERIALS AND METHODS

**Study subjects:** Samples were collected within the time frame of December 2006 to March 2007 from patients listed in the surgery registrar of the Urology Service of the Hospital Central Militar that met all inclusion, exclusion and elimination criteria. **Inclusion criteria:** (a) patients diagnosed with CaP with lower urinary tract obstructive syndrome, (b) patients diagnosed with BPH with indication for transurethral resection of the prostate (TURP) and radical prostatectomy.

**Exclusion criteria:** (a) patients who did not give their authorization to participate in the procedure, (b) patients who were not TURP candidates.

**Elimination criteria:** (a) patients whose extracted and purified RNA was inadequate for the study, (b) tissue that suffered from genetic material degradation during transport.

The quantity of tissue necessary for the study was from 1-5 g. Samples were obtained immediately after surgical intervention and transportation time was a maximum of 1 hour from sample collection to its transport and storage at -70°C in the Revco® ultrafreezer (Legaci ULT2186 3-35 Dupont SVVA Refrigerants) at the Molecular Biology Laboratory of the Escuela Médico Militar up to its RNA extraction processing.

RNA extraction and quantification. Total RNA was extracted with the SV Total RNA Isolation System (Promega, Madison, WI, USA) extraction kit. Samples of 150 mg each were taken using a sterile technique, avoiding RNAase contamination and homogenizing them with a polytron (Omni µH International) following the manufacturer's instructions. Two µL of RNAase inhibitor was then added to the final total RNA elusion and they were stored at -83°C for later analysis. Total RNA integrity verification was carried out by means of electrophoresis in a 2% agarose gel, with 6 µL of extracted total RNA per sample. Bands were viewed under UV light (EDAS 290 KODAK). Total RNA quantification was carried out by means of fluorescence with SYBR Green, with the Synergy HT-I multidetector (BIO-TEK Instruments, Inc. Highland Park, Vermont, USA).

Real-time RT-PCR technique standardization. Specific oligonucleotides (primers) of the Mn-SOD gene and for the reference genes (endogenous candidates) were designed for real-time reverse transcription-polymerase chain reaction (RT-PCR) development: ribosomal subunit 18s, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose 6 phosphate dehydrogenase (G6PDH) and ß-actin (BACT); endogenous genes were validated using BestKeeper software and the most stable one was used as the endogenous control for total RNA load. Sequences were obtained from the GenBankTM (Table 1). Sequence search was carried out at http://www.ncbi.nlm.nih.gov/BLAST/ to confirm specificity.

The primers (Invitrogen, Carlsbad, CA) of the following genes were synthesized: Mn-SOD gene; endogenous candidate genes: BACT, 18s, GAPDH and the G6PDH gene, which were optimized at a temperature of 57°C (Table 1).

RT-PCR conditions were optimized with a gradient thermal cycler (gradient Px2 Thermal Cycler Hybaid, Franklin, MA) using the SuperScript™ III Platinum® SYBR® One-Step qRT-PCR Kit with ROX (Invitrogen, Carlsbad, CA). The sequences taken correspond to registers in the GenBankTM under the following access numbers: Mn-SOD, BACT, NM_001101; GAPDH, NM_002046; subunit 18s, X03205 and G6PDH, NM_004285. Amplification products by RT-PCR were viewed by electrophoresis in a 2% agarose gel and studied with the EDAS 290, (KODAK, New Haven, CT) electrophoresis analysis system (Image 1). Amplification results such as temperature conditions and primer, dNTP (nucleotide) and volume concentrations were transferred to the amplification protocol in real time with the Rotor gene 6.0 (Corbett Life Science, Sydney City, Australia) detection system.

**Rotorgene 3000 real-time RT-PCR protocol.** Real-time RT-PCR reactions were carried out in 10 ng of extracted RNA from the tissues studied. Dilutions for internal control, absolute quantification and efficiency determination validation of all primers were carried out with the SuperScript™ III Platinum® SYBR® One-Step qRT-PCR Kit with ROX (Invitrogen, Carlsbad, CA). It is a reaction mixture containing Taq DNA polymerase (Platinum) complex with antibody that inhibits activity at room temperature and that antibody is degraded in the initial PCR cycle (95°C at 5 minutes). The following reaction components were prepared at a final concentration: the reaction mixture of 6.25 µL 2X SYBR® Green 1 (a buffer contained in SYBR® Green 1 dye) 0.4 mM for each dNTP and 6 mM MgSO4 (ROX Reference Dye) which is a buffer stabilizer (1X), 4 µL total RNA, 0.25 µL SYBR® Green One Step Enzyme Mix (contained in SuperScript™ III RT Platinum® Taq DNA polymerase, and RNase Inhibitor (Ribonuclease recombinder), 0.4 µL MgCl2 (50 mM), 0.4 µL of forward primer (0.4 µM), 0.4 µL of reverse primer (0.4 µM), and 0.8 µL of RNAase-free water (12.5 µL of the final reaction mixture) and put in 100 µL tubes. Reaction initially took place at 52°C for 10 minutes for DNA synthesis and at 94°C for 5 minutes for initial
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The course of the melting curve had a temperature interval of 60–98°C with a temperature increase of 0.1°C per second and a final step at 40°C. Cycle threshold (Ct) for each transcript had to be determined in order to obtain the absolute quantification. Endogenous candidate gene expression as well as Mn-SOD gene expression were individually quantified with the BestKeeper Gene statistic model for endogenous gene validation and with the REST© statistic model for efficiency analysis and the relative quantification of endogenous genes and Mn-SOD.

**Data analysis:** Gene efficiency and absolute quantification (Ct) data were analyzed using Rotor-Gene 6.0 software (Corbett Life Science, Sydney City, Australia). Ct was determined and data were graphed with the same software and exported to an Excel table resulting in similar lineal regressions for primer efficiency. Logarithmic concentration was compared with Ct and the data of the Mn-SOD and GAPDH calibration curves were also analyzed with the same software.

**Table 1. Primer sequence used in RT-PCR.**

<table>
<thead>
<tr>
<th>Targets</th>
<th>Positions</th>
<th>Nucleotide sequence</th>
<th>Extension length</th>
<th>GenBankTM Sequence</th>
<th>Extension efficiency</th>
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<td>NM_004285</td>
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</tbody>
</table>

**Image 1.** Amplification products by real-time RT-PCR with total RNA CaP and BPH samples. Electrophoresis is observed in 2.0% agarose gel. Lane 1 is the BACT gene (143pb) extension product, lane 2 GAPDH (175pb), lane 3 123pb Marker, lane 4 18s subunit (151pb), lane 5 G6PDH (159pb).
Slope determination and Pearson coefficient correlation were used to determine the most stable and adequate endogenous gene, real-time RT-PCR was carried out in triplicate for each of the CaP samples and was compared with C_{r} (mean triplicated result of each sample). These data were exported to the REST software (Image 2). After data export and analysis GAPDH was taken as the endogenous gene. It presented with greater stability than the other 3 with a P < 0.005 and with a 0.953 Pearson correlation coefficient with the Mn-SOD gene of interest (Table 2).

Absolute copies of CaP and BPH Mn-SOD mRNA (Image 3) were compared using the Student t test producing a statistically significant difference of P = 0.0002. Mean absolute copy for CaP was 765.7 with mean standard deviation (SD) of 185.2. Mean absolute copy for BPH was 242.8 with mean SD of 18.43. Result analysis of these expression data showed there was 3.15 times more genic Mn-SOD overexpression in CaP than in BPH (Image 4).

RESULTS
From the total of 60 patients that samples were taken from, 20 patients (33.3%) had established CaP diagnosis while 40 patients (66.7%) had established BPH diagnosis. Within the CaP group mean age was 70.4 years (55-90 year range), mean preoperative prostate specific antigen (PSA) concentration was 39.75 ng/mL (0.005 - 416 ng/mL range), 8 patients (40%) had PSA concentration < 4 ng/mL, 2 patients (10%) had PSA concentration between 4 and 10 ng/mL and 10 patients (50%) had PSA concentration > 10 ng/mL. Gleason score was 5 in one case (5%), 6 in one case (5%), 7 in six cases (30%), 8 in nine cases (45%) and 9 in three cases (15%). TURP was performed in 18 patients (90%) and radical prostatectomy was carried out in 2 patients (10%). In the BPH group (40 patients) mean age was 68.3 years (47-86 year range), mean preoperative PSA concentration was 7.21 ng/mL (0.26–58.6 ng/mL range), 20 patients (50%) had a PSA concentration < 4 ng/ml, 13 patients (32.5%) had a PSA concentration between 4 and 10 ng/mL and 7 patients (17.5%) had a PSA concentration > 10 ng/mL. All 40 patients underwent TURP.

To determine the most stable and adequate endogenous gene, real-time RT-PCR was carried out in triplicate for each of the CaP samples and was compared with C_{r} (mean triplicated result of each sample). These data were exported to the REST software (Image 2). After data export and analysis GAPDH was taken as the endogenous gene. It presented with greater stability than the other 3 with a P < 0.005 and with a 0.953 Pearson correlation coefficient with the Mn-SOD gene of interest (Table 2).

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DISCUSSION
The present study demonstrates that the codifying gene for Mn-SOD is expressed 3.15 times more in CaP tissue than in BPH tissue. It is the authors’ opinion that this increase may indicate that Mn-SOD enzyme activity is elevated because another study of this enzyme demonstrated the same thing (immunohistochemical data not shown). This increase may also be part of the defense mechanisms against oxygen metabolites produced during oxidative stress. On the other hand, it can be postulated that this increased activity provokes alteration in cell functions producing responses such as cellular proliferation and programmed apoptosis, thus being one of the causes of disease progression and even malignancy degree.

There has been much information about the relation between ROS and proteins, enzymes and cofactors that can be transformed by molecules modified by free radicals and that can induce carcinogenesis. It is known that Mn-SOD overexpression and glutathione-transferase (GST) together with oxidative imbalance induce cellular membrane damage that appears in various human tumors.

Data exist that show there is increased expression of antioxidants such as Mn-SOD and GST in certain dysplasia and this increase has been observed in the serum of patients with carcinoma of the esophagus, stomach, lung and ovary. It is possible that these enzymes or their genic product (mRNA) may serve as early markers for the premalignant changes that have a high risk for invasive CaP.

These observations suggest that the selective development of Mn-SOD inhibitors will be of great interest as a potential therapeutic strategy for certain cancers, includidt it is probable that epigenetic changes or some mutation in the Mn-SOD gene is the cause of the high level of Mn-SOD expression in CaP found in the present study and the one prior to it (data not shown). This could have potential effects on the survival and proliferation of tumor cells, an event that has been found in other aggressive tumors with poor prognosis for the patient.

The Mn-SOD enzyme probably plays a very important role in treatment resistance of various tumors. Overexpression of antioxidant enzymes has been observed to simultaneously participate in invasive tumor progression and the present study shows that
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Manganese superoxide dismutase (Mn-SOD) genic overexpression of this enzyme also plays an important role since this genic product is necessary for the codification and translation of said protein.

To evaluate changes in Mn-SOD gene expression, PCR methodology combined with reverse transcription (RT) and real time was used (see methodology). Mn-SOD gene level expression was analyzed and its overexpression was found to be 3.15 times higher in CaP tissue when compared with BPH tissue. This may be the cause of the concentration increase in its genic product (mitochondrial Mn-SOD) in CaP patients. However, further studies are necessary to be able to explain the specific cause of the high immunoreactivity of MnSOD in this tissue.14 There is some controversy, given that other studies have suggested that an increase in Mn-SOD gene expression can induce phenotypic transformation reversion in tumor cells.13

On the other hand, the results of the present study suggest that there is an alteration in the pro-oxidative-antioxidant balance in CaP and this imbalance has been observed to alter cellular oxidoreduction processes, growth, proliferation and cellular cycles and it is known that certain free radicals mediate cellular transduction activation pathways of transcription factors such as Fos,

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**Table 2.** Determination of the most stable endogenous gene. Data (C.T) were analyzed with a recent BestKeeper statistical model which correlates a set of proposed genes (gene candidates) and validates the most stable of them. In the present study GAPDH was one of the most stable genes due to its amplification threshold (C.T) since it amplified in very similar cycles to the study Mn-SOD gene, with a Pearson correlation of 0.953 and a P=0.005.

<table>
<thead>
<tr>
<th></th>
<th>BACT vs BK</th>
<th>18s vs BK</th>
<th>GAPDH vs BK</th>
<th>G6PDH vs BK</th>
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<tr>
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<tr>
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</tr>
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<td>Power [x- times]</td>
<td>2.87</td>
<td>2.12</td>
<td>2.14</td>
<td>1.03</td>
</tr>
</tbody>
</table>

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**Image 2.** Dissociation curves of analyzed gene products. With SYBR Green I methodology, the melting curve of each analyzed amplicon is represented, initially using Rotor-Gene 6.0 software (Corbett Life Science, Sidney City, Australia). Melting curve analysis is a fast and exact method for observing PCR specificity. Graph peaks represent temperatures in °C for the Mn-SOD gene, 87.7°C, BACT gene 86°C, subunit gene 18s, 85.9°C, G6DPH gene, 83.5°C and GAPDH gene, 84.1 °C which underwent dissociation protocol at a temperature of 60–98°C.

**Image 3.** Electrophoresis representative of RNA extracted from CaP and BPH tissues (150 mg) in 2.0% agarose gel. Bands characteristic of 18s and 28s subunits are observed, indicating there is no evidence of sample degradation and in turn, confirming the presence of the rest of the extracted total RNA due to band integrity carried out with the SV Total RNA Isolation System protocol kit (Promega®, Madison, WI, USA).

**Image 4.** Mn-SOD genic expression. Mn-SOD absolute copies from the CaP and BPH groups were compared (P=0.0002). The number of absolute copies of both groups is shown on the y axes. For BPH (mean SD=18.43), for CaP (mean SD=185).

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Jun and nuclear KB factor and an increase in mitochondrial activity in these cells.17

On the other hand, it has been demonstrated that there are differences in morphology, growth and cellular tumorigenicity in relation to Mn-SOD increase at the genic level as well as at the protein level. The results of this study suggest that beyond the Mn-SOD protein, the overexpressed gene alters the intracellular situation in the mitochondrion or in mitochondrial number. Various studies have suggested that this overexpression plays an important role in the alteration of mitochondrial number, size, form and distribution. The results of the present study suggest that the Mn-SOD expressing gene may have an effect on both processes - on mitochondrial function as well as on tumor cell growth. Oberley et al. made the analysis that the decrease in Mn-SOD activity, just like reduced genic expression, plays a critical role in malignant transformation because experimental increase in Mn-SOD activity can induce tumor cell differentiation.11

- CONCLUSIONS

By means of real-time RT-PCR analysis, genic overexpression was found to be 3.15 times greater in CaP tissue when compared with BPH tissue.

The results show us that there is no relation between PSA concentrations and Mn-SOD genic overexpression.

Genic expression determination of antioxidant enzymes closely related to cancer, as is the case with Mn-SOD, may be used in the future as a tumor marker for early disease diagnosis, taking into consideration the low cost of this technique.