The -786T>C promoter polymorphism of the NOS3 gene is associated with prostate cancer progression

Karina Marangoni, Thaíse G Araújo, Adriana F Neves and Luiz R Goulart*

Address: Federal University of Uberlândia, Institute of Genetics and Biochemistry, Molecular Genetics Laboratory, Campus Umuarama, Block 2E, Room 24, 38400-902, Uberlândia, MG, Brazil

Email: Karina Marangoni - kmarangoni@yahoo.com.br; Thaíse G Araújo - thaise_araujo@yahoo.com.br; Adriana F Neves - neves.af@gmail.com; Luiz R Goulart* - lrgoulart@ufu.br

* Corresponding author

Abstract

Background: There is no biological or epidemiological data on the association between NOS3 promoter polymorphisms and prostate cancer. The polymorphisms in the promoter region of NOS3 gene may be responsible for variations in the plasma NO, which may promote cancer progression by providing a selective growth advantage to tumor cells by angiogenic stimulus and by direct DNA damage.

Methods: This study aimed evaluating the NOS3 promoter polymorphisms by PCR-SSCP and sequencing, associating genotypes and haplotypes with NOS3 expression levels through semi-quantitative RT-PCR, and with PCA3 mRNA detection, a specific tumor biomarker, in the peripheral blood of pre-surgical samples from 177 patients; 83 PCa and 94 BPH.

Results: Three novel SNPs were identified -764A>G, -714G>T and -649G>A in the NOS3 gene promoter region, which together with the -786T>C generated four haplotypes (N, T, C, A). NOS3 gene expression levels were affected by the -786T>C polymorphism, and there was a 2-fold increase in NOS3 levels favored by the incorporation of each C allele. NOS3 levels higher than 80% of the constitutive gene expression level (B2M) presented a 4-fold increase in PCa occurrence.

Conclusion: The -786T>C polymorphism was the most important promoter alteration of the NOS3 gene that may affect the PCa progression, but not its occurrence, and the incorporation of the C allele is associated with increased levels of NOS3 transcripts. The NOS3 transcript levels presented a bimodal behavior in tumor development and may be used as a biomarker together with the PCA3 marker for molecular staging of the prostate cancer.

Background

Enzymes responsible for nitric oxide (NO) synthesis constitute a family with at least three distinct isoforms, inducible, neuronal and endothelial [1]. The nitric oxide synthase 3 (NOS3) is located at the 7q35-q36 chromosome locus and the characterization of the 5'-flanking genomic region indicates that the NOS3 promoter is 'TATA-less'. This feature has been described for genes such as housekeeping genes and is usually associated with multiple transcription start sites. The NO synthase mRNA does not correspond to these criteria and the presence of specific potential transcription factor binding sites in the promoter could account for the cell specificity of its transcription [2].
The NOS3 seems to have an important role in vascular development, maintenance of the vascular tone and tumor growth in human prostate cancer (PCa) [3]. The net effects of NO depend on its available concentration, target cell, and interactions with reactive oxygen species (ROS), metal ions, and proteins [4,5].

The tumor-associated NO production may promote cancer progression by providing a selective growth advantage to tumor cells, by the angiogenic stimulus [6,7] and by the raise on mutations due to direct action of free radicals in DNA [8], and may also stimulate hyperplasia in normal tissue [8-10].

Genetic polymorphisms in the promoter region of NOS3 gene (Figure 1) may be responsible for variations in the genetic control of plasma NO [11,12]. Although the promoter region is not part of the mRNA, it is directly related to the exons, and might be intervening at gene expression, decreasing or increasing it. Moreover, mutation points present in 5’-flanking region may confer a higher instability to the mRNA. To date ten variant NOS3 alleles have been identified in this region, these are, or may be, associated with decreased enzyme activity [13].

In this study, we have performed a specific analysis of a 236 base pair (bp) fragment of the NOS3 gene promoter region flanking the polymorphisms -786T>C and -690C>T, through DNA conformational assays (SSCP – single strand conformation polymorphism) and sequencing, determining genotypes and haplotypes, and estimating their possible association with PCa and benign prostatic hyperplasia (BPH) diseases. Three novel single nucleotide polymorphisms (SNPs) were characterized. Additionally, we have evaluated the effect of these five NOS3 SNPs on the mRNA expression levels of the peripheral blood of PCa and BPH patients, and their association with circulating tumor cells in the blood.

**Methods**

**Patients, sample collection and preparation**

This work was developed at the Laboratory of Nanobiotechnology of the Federal University of Uberlandia (UFU) together with the Urology Service of the University Clinics' Hospital. All the peripheral blood samples were...
obtained from patients that live in Uberlandia – MG (Brazil) and were enrolled during 2003 and 2004. The ethnic background was not recorded since the Brazilian population is highly heterogeneous and miscegenated, so it cannot be determined. The investigation was approved by the UFU Research Ethics Committee under the number 005/2001. Peripheral blood samples were collected before surgery in a vacutainer™ tube containing K3 EDTA 7.2 mg, and maintained at 4°C. To search for possible mutations in the promoter region of the NOS3 gene, we have performed the PCR-SSCP analysis in blood samples from 177 patients, which were grouped into two classes: 83 PCA patients (mean age, 69 years; range, 50 to 87 years) and 94 BPH (mean age, 68 years; range, 49 to 87 years), according to histological classification of tissues. BPH patients were submitted to TURP, except five patients that have undergone open prostatectomy. All PCA patients were submitted to radical prostatectomy, and were selected by using the following criteria: negative X-rays and bone scan analyses, and rectal examination compatible with organ confined cancer. DNA was extracted from leukocytes according to protocol previously published [14]. The mRNA was isolated by the Guanidine Isothiocyanate extraction method [15] with minor modifications. DNA and mRNA concentrations and quality were obtained by spectrophotometric absorbance readings at 260 and 280 nm.

Amplification and genotyping of the -786T>C polymorphism

The presence of the -786T>C polymorphism in the 5’-flanking region of the NOS3 gene was determined by PCR amplification with the primers 5’-ATG CTG CCA CCA GGG CAT CA- 3’ and 3’-GTC CIT GAC TCT GAC ATT AGG G- 5’ [16]. A volume of 30 μL was used for each PCR reaction, which contained 5 μmoles of each primer, 200 μM of each dNTP (desoxynucleotide triphosphate), 1.5 mmol/L MgCl2, 50 mmol/L KCl, 10 mmol/L Tris-HCl at pH 8.3 and 1 U Taq DNA polymerase (Phonestia) with 4 μL of genomic DNA. The conditions of amplification were denaturing at 95°C-5 min, 35 cycles at 94°C-1 min, 62°C-1 min, 72°C-1 min, and a finally termination at 72°C-10 min. The amplified fragments were separated on 1.5% agarose gel electrophoresis and stained with ethidium bromide.

PCR-SSCP analysis

A PCR-SSCP analysis, described elsewhere [17], was performed to detect mutations within the NOS3 promoter region. Two microliters of PCR products were mixed with 18 μL of a low ionic strength buffer (10% sucrose, 0.01% bromophenol blue, and 0.01% xylene cyanol) and heated at 10 min at 97°C. Products were separated on 15% polyacrylamide gel electrophoresis (49:1 – acrylamide:bisacrylamide) with 200 volts for 19 hours at room temperature, and detected by silver nitrate staining [18,19] with minor modifications.

Purification of SSCP fragment bands and sequencing

During genotyping of the -786T>C polymorphism of the NOS3 gene promoter region, three novel SNPs have been discovered [-764A>G, GenBank: RefSeq NM_EF042808/-714G>T, GenBank: RefSeq NM_EF042809/-649G>A, GenBank: RefSeq NM_EF042810]. Additionally, the two SNPs previously described [-786T>C, GenBank: RefSeq NM_2070744/-690C>T, GenBank: RefSeq NM_3918225] were also characterized. Seven SSCP conformations were observed in the polyacrylamide gel electrophoresis (Figure 2), although only three conformations were expected.

Each DNA fragment band originated from the PCR-SSCP technique was scrapped and individually reamplified using the same PCR reaction condition.

The purification of reamplified fragments was performed by DNA precipitation with ammonium acetate (7.5 M) and the pellet was resuspended in 10 μL of water. DNA concentration and quality were obtained by using the spectrophotometric absorbance readings at 260 and 280 nm.

The purified products were submitted to a capillary sequencer (Megabace 1000), using the DYEEnamic ET Dye Terminator Cycle Sequencing kit (GE Healthcare). Three sequencing reactions had been carried for each fragment and injected twice to minimize sequencing artifacts. Sequences were processed and edited in the software DNASTAR Lasergene – SeqMan and EditSeq – (version 7.0, 2006). Sequences were aligned with the corresponding NOS3 sequence (GenBank: NM_348236) using the MegAlign procedure (DNASTAR Lasergene). The consensus sequences for each SSCP fragment band was established and comparisons were performed based on the original NOS3 gene sequence.

RNA extraction and RT-PCR

Two micrograms of total RNA from blood, 10 U of RNase inhibitor (Invitrogen), 40 U of the Murine Moloney Leukemia Virus Reverse Transcriptase (MMLV-RT) (Amer-Sham Biosciences), 1X MMLV-RT Buffer, 200 μM of each dNTP and 6 μM of hexamer random primers were incubated at 37°C for 1 hour and heated at 95°C-5 min. The 20 μL final volume of each reaction was completed with DEPC (diethylpyrocarbonate)-treated water. For normalization of amplification reactions, the internal positive control gene, the constitutive B2M gene (B2M: 5’-AGA GAA TGG AAA GTC AAA- 3’ and 5’-TGT TGA TGT TGG ATA AGA- 3’), generating a 534-bp fragment, was used to validate reactions and to further characterize RNA quality of each sample.
Nested RT-PCR for the PCA3 transcript detection

A sensitive nested PCR assay for the detection of PCA3 mRNA was performed as previously reported [20] with minor modifications. A total of 137 patients were analyzed for the expression of circulating tumor cells in the peripheral blood, as evidenced by the PCA3 tumor biomarker positivity (Figure 3A and 3B).

Semi-quantitative RT-PCR of the NOS3 gene

A representative sample of the genotyped population was selected, consisting of 38 patients (18 PCa and 20 BPH cases), and were analyzed in three replicates for the NOS3 mRNA relative expression levels. PCR reactions consisted of: 4 μL of cDNA, 1 U of Platinum Taq DNA Polymerase (Invitrogen), 50 mmol/L KCl, 10 mmol/L Tris-HCl at pH 8.3, 200 μM of each dNTP, 8 pmol of each oligonucleotide, 2 mmol/L MgCl2. The reaction was incubated for 48
cycles at 94°C-40 s, 55°C-40 s, 72°C-50 s, preceded by an initial denaturation at 95°C-2 min and a final extension cycle at 72°C-10 min. The designed primers sequence for the NOS3 were: sense 5’-CCT CAG GTT CTG TGT GTT GAT CAG ACCT GGC AGC AAC C-3’ and antisense 5’- GAT CAG ACCT GGC AGC AAC C-3’, generating a 322-bp fragment. The B2M constitutive gene was concomitantly amplified in the same reaction as described before.

**Densitometric readings to estimate relative gene expression levels**

The amplicons obtained for both NOS3 and B2M genes were analyzed and quantified according to their agarose signal intensities by using the ImageMaster VDS Software program version 2.0 (Amersham Biosciences). The densitometric readings were normalized by using the target NOS3 mRNA:B2M mRNA ratio and relative levels were estimated for each sample (Figure 3C and 3D).

**Statistical analysis**

Chi-square analyses were performed to compare genotypic and haplotypic frequencies for the average of clinical parameters, such as: age, PSA serum levels. Multiple regression analysis and Pearsons’ correlations were used to verify the association among genotypes, PCA3 detection and clinical data. The Shapiro-Wilk test was used to verify normality of the relative levels of NOS3 gene expression, and mean comparisons of the mRNA relative levels were performed between PCa and BPH patients’ groups through the Mann-Whitney test. Probability levels below 5% (p < 0.05) were considered significant. A cut-off value was determined for the NOS3 transcript relative levels and the OR were estimated to verify the chance of PCa occurrence in the presence of high concentrations of NOS3 transcripts. Pearsons’ correlation analysis was performed among NOS3 relative expression levels of PCa and BPH patients in association with patients’ age, serum PSA levels, the TNM adenocarcinoma histopathological staging, Gleason score and polymorphisms.

**Results**

**Molecular characterization of the SSCP conformations and the haplotypes models of the promoter region**

Sequences of the SSCP electrophoretic bands were obtained and the consensus sequence was established (Figure 4). Sequence alignments were compared with the original sequence of the NOS3 gene [GenBank: NM348236], and all SNPs were positioned in the sequence, and their mutation classifications were assigned (Table 1).

Based on consensus sequences, it was possible to construct four specific haplotypes N (-786T>C; -690A>G; -649G>A), C (-786T>C; -714G>T; -690C>T), A (-786 T>C; -764A>G; -690C>T) and T (no mutations). Chromosome models were also generated to explain the seven SSCP electrophoretic conformations: TT (no mutations), TC (-786T>C + [=], -714G>T + [=], -690C>T + [=]), CC (-786T>C + -786T>C, -714G>T + -714G>T, -690C>T + -690C>T), TA (-786T>C + [=], -714G>T + [=], -690C>T + [=]), AA (-786T>C + -786T>C, -714G>T + -714G>T, -690C>T + -690C>T), CA (-786T>C + -786T>C, -764A>G + [=], -714G>T + [=], -690C>T + -690C>T and TN (-786T>C + [=], -690C>T + [=], -690C>T + [=], -690C>T + [A] + [=]) (Figure 2).

**SSCP conformations and haplotypic frequencies of the five mutations in promoter region of NOS3 gene**

Table 2 shows SSCP conformations with their respective genotypes and haplotypic frequencies for all five characterized polymorphisms performed in 177 patients, from which 83 (46.9%) were histologically diagnosed as PCa and 94 (53.1%) as BPH. The -786T>C polymorphism presented the highest frequencies in both patients’ groups, with higher frequencies for the TT and TC SSCP conformations. For the PCAa group, all the other conformations (TA, CA, TN, and AA) did not present frequencies higher than 6%; however, the same four genotypic conformations presented higher frequencies in the BPH group, with 13.8% (13 out of 94) for the TA SSCP conformation, and 9.6% (9 out of 94) for the CA SSCP conformation.
Figure 4
Sequences alignment using DNASTAR Lasergene (SEQMAN and EDITSEQ), version 7.0 (2006). The point mutations are represented in a black box. A) -786T>C polymorphism. B) -764A>G polymorphism. C) -714G>T polymorphism. D) -690C>T polymorphism. E) -649G>A. Coding DNA reference sequence of promoter NOS3 gene [GenBank: NM_348236]. SSCP Conformations: AA, CC, TN, TA and TT.
No significant differences for SSCP conformations frequencies between patients’ groups were observed \((p = 0.10)\). However, the comparison of the SSCP conformations frequencies within the PCa patients \((p = 0.0082)\) and within BPH patients \((p = 0.0011)\) were significant different.

There was no haplotypic frequencies difference between groups \((p > 0.05)\). However, inspection of haplotypes revealed that the C \((-786T>C; -714G>T; -690C>T)\) and T (no mutation) haplotype was the most common one in both patients’ groups. One rare haplotype N \((-786T>C; -690A>G; -649G>A)\) has been observed in PCa patients \((1.2\%)\). Although not significant, the haplotype A \((-786T>C; -764A>G; -690C>T)\) was more frequent in BPH patients \((12\%)\) than in PCa \((8\%)\). Both haplotypes and SSCP genotypic conformation frequencies distribution were in Hardy-Weinberg equilibrium.

The estimated chance for cancer occurrence, considering the seven SSCP genotypic conformations among groups (PCa and BPH), were: TT + TC + CC + CA + AA \textit{versus} \((\text{vrs})\) TA (odds ratio \((\text{OR}) = 3.13, \text{CI}_{95\%} = 0.98 - 10.01)\), TT + CA \textit{vrs} TA (\text{OR} = 2.99, \text{CI}_{95\%} = 0.89 - 10.05), CC \textit{vrs} CA + TA (OR = 3.38, \text{CI}_{95\%} = 1.00 - 11.37), TT \textit{vrs} CA + TA (OR = 3.10, \text{CI}_{95\%} = 1.25 - 7.72). All combinations of SSCP conformations were tested for the OR, but only the most significant ones were demonstrated. It was observed a 3.38-fold higher chance of having cancer when the CC SSCP conformation is considered in relation to CA + TA. A 3.10-fold higher chance of having cancer was obtained for the TT SSCP conformation in relation to CA + TA. The TT + TC + CC + CA + AA \textit{vrs} TA and TT + CA \textit{vrs} TA associations were close to significance \((p < 0.10)\), and presented a relative risk of 3.13 and 2.99-fold higher chance of having cancer, respectively.

Table 3 shows the estimated chance for cancer occurrence, considering haplotypes and their number of copies between groups (PCa and BPH), and no significant ORs were obtained. The sample size was quite small for some haplotypes, but we presented the true haplotypic frequencies of copy numbers in the population investigated for the polymorphisms within the 236-bp region, and although the haplotypes were not significantly associated with prostate cancer, this is the first description of such variations in the \(NOS3\) promoter.

In agreement with the Kruskal-Wallis test, significant differences between patients’ groups for mean prostate specific antigen (PSA) were observed \((p = 0.0048)\). Pearson’s correlation coefficients for genotypes \textit{vrs} PSA (\(p = 0.02\)), genotypes \textit{vrs} gleason score \((p = 0.0195)\), age \textit{vrs} PSA \((p = 0.0412)\), age \textit{vrs} gleason score \((p = 0.0421)\), gleason score \textit{vrs} Tumor-Node-Metastasis (TNM) score \((p = 0.0021)\) and PSA \textit{vrs} TNM score \((p = 0.0189)\) within the PCa patients were significantly different. No significant differences among BPH patients were observed (Table 2).

**Analysis of the PCA3 transcript detection and association with polymorphisms**

The prostate cancer antigen 3 (PCA3) transcript detection (Figure 3A) was performed in 149 patients \((149 \text{ out of 177})\), from which 109 \((73.2\%)\) were negative and 40 \((26.8\%)\) were positive. Among PCA3 positive patients, 62.5% \((25 \text{ out of 40})\) were histologically diagnosed as PCa and 37.5% \((15 \text{ out of 40})\) as BPH. Among PCA3 negative patients, 42.2% \((46 \text{ out of 109})\) were PCa and 57.8% \((63 \text{ out of 109})\) were BPH (Table 4). Although patients'
Table 2: SSCP conformation and haplotypic frequencies, clinical parameters and laboratory data of the NOS3 gene in peripheral blood of patients with prostate cancer and benign prostatic hyperplasia.

| SSCP Conformation | Genotypes | PCa (N = 83) | BPH (N = 94) |
|-------------------|-----------|-------------|-------------|
|                   | N (%)     | * Age (ng/ml) | * PSA (ng/ml) | * TNM score | * Gleason score | % positive PCA3 | * [NOS3] | % (N) | * Age (ng/ml) | * PSA (ng/ml) | % positive PCA3 | * [NOS3] |
| TN                | 1 (1.2)   | 77          | 101.0       | --          | 9              | 0              | 0         |       |               |              |               |          |
| c. [-786T>C] + [-714G>T] | 9 (10.8) | 67.5 (7.3)  | 41.4 (78.8) | 1           | 8              | 42.9           | 8 (8.5)   | 66.8 (7.8) | 15.3 (11.7) | 12.5        | 0.5 (0.2)   |
| c. [-690C>T] + [-649G>A] | 30 (36.2) | 68.5 (8.4)  | 24.0 (28.4) | 2           | 7              | 28.0           | 1.1 (1.8) | 29 (30.9) | 66.7 (9.2)  | 9.8 (10.3)  | 18.5        | 0.5 (0.3) |
| TT                |           |             |             |             |                |                |           |       |               |              |               |          |
| No mutations      | 32 (38.6) | 69.7 (7.8)  | 17.3 (18.7) | 1           | 6              | 35.7           | 1.1 (1.5) | 35 (37.2) | 68.9 (8.4)  | 11.9 (12.3) | 15.4        | 0.6 (0.4) |
| c. [-786T>C] + [-714G>T] | 4 (4.8)  | 64.5 (10.0) | 11.6 (7.2)  | 2           | 6              | 66.7           | 4.7 (4.3) | 13 (13.8) | 67.2 (10.1) | 9.5 (7.3)   | 20.0        | 0.4 (0.3) |
| c. [-690C>T] + [-649G>A] | 5 (6.0)  | 63.8 (7.4)  | 9.4 (3.4)   | 1           | 6              | 40.0           | 6.0 (0.3) | 9 (9.6)   | 66.7 (8.6)  | 11.9 (7.9)  | 42.9        | 0.6       |
| TA                |           |             |             |             |                |                |           |       |               |              |               |          |
| c. [-786T>C] + [-714G>T] | 2 (2.4)  | 70.5 (7.8)  | 12.1 (12.1) | --           | 7              | 50.0           | --        | 0        | --            |              | --          |          |
| c. [-690C>T] + [-649G>A] |          |             |             |             |                |                |           |       |               |              |               |          |
| Total             | 83        |             |             |             |                |                |           |       |               |              |               |          |
|                   |           | 43.1        | 94           |             |                |                |           |       |               |              |               | 19.0      |
|                   |           | (1 out of 2)| (25 out of 71)|          |                |                |           |       |               |              |               |          |

p(1) = 0.0082
p(2) = 0.10
p = 0.0011

| Haplotypes | %       | * Age (ng/ml) | * PSA (ng/ml) | * TNM score | * Gleason score | % positive PCA3 | * [NOS3] | % (N) | * Age (ng/ml) | * PSA (ng/ml) | % positive PCA3 | * [NOS3] |
|------------|---------|---------------|---------------|-------------|----------------|----------------|---------|       |               |              |               |          |
| N          | 1.0     | 77            | 101.0         | --          | 9              | 0              | 0       |       |               |              |               |          |
| T          | 58.0    | 69.9          | 38.5          | 1           | 6              | 56.0           | 1.1     | 56.0   | 67.6         | 10.4         | 53.2        | 0.4      |
| C          | 33.0    | 66.7          | 22.7          | 1           | 7              | 32.0           | 1.1     | 32.0   | 67.5         | 11.0         | 30.1        | 0.3      |
| A          | 8.0     | 66.3          | 11.0          | 2           | 6              | 12.0           | 2.6     | 12.0   | 66.9         | 10.7         | 16.7        | 0.2      |
| Total      | 100.0   | 100.0         | 100.0         | 100.0       | 100.0          | 100.0          | 100.0   | 100.0  | 100.0        | 100.0        | 100.0       | 100.0    |

(1) representative sampling of the population; (*) mean (± SD); (--) inapplicable data
(1) Comparison (genotypes) in the groups with probability levels obtained by chi-square test
(2) Comparison (genotypes) between groups with probability levels obtained by Kruskal-Wallis test
(a) Genotypes correspond to the promoter NOS3 polymorphisms
histological classification (PCa and BPH) were carefully analyzed, it is possible that transurethral resection of prostate (TURP) biopsies of BPH patients have been misdiagnosed, once biopsy procedures may not have reached tumor specific sites.

PCA3 positivity in all SSCP conformations and haplotypes were also shown in Table 4. It was expected a higher frequency of PCA3 positivity in the PCa group and a lower frequency of positivity in the BPH group, as observed, although not significant. It is interesting to mention that the PCA3 positivity in the PCa group for the TC + CC conformations was 2.6 times higher than the frequency observed in the BPH group (32.5% vs 12.5%, P = 0.02). On the other hand, CC + TC conformations frequencies were not significantly different between patients’ groups (PCa = 20.2% vs BPH = 26.6%) within the negative PCA3 detection class (Table 4). The TA + CA conformations presented significantly higher frequencies in the BPH (11%) than in the PCa group (3.6%) within the negative PCA3 class (P = 0.03).

In the overall, the PCA3 clinical parameters for PCa cell detection in the peripheral blood were: 35.0% of sensitivity (25 out of 71), and 81.0% of specificity (63 out of 78); however, it is important to emphasize that a positive result for PCa patients is not an indication of metastasis, and the PCA3 detection in BPH patients may indicate that they may have been misdiagnosed.

For the negative PCA3 detection (Table 4), all genotypic frequencies were higher among BPH patients, except for the TN SSCP conformation that presented only a negative result for one PCa patient.

Considering the haplotypic frequencies for the positive and negative PCA3 detection classes, there were no differences between patients’ groups. However, the T haplotype (no mutations) was the most frequent one followed by the C (-786T>C; -714G>T; -690C>T) and A (-786 T>C; -764A>G; -690C>T) haplotypes. The A haplotype was more frequent in BPH than in PCa patients, for both positive and negative PCA3 classes, although it was not significant.

### Analysis of the NOS3 gene expression levels in association with polymorphisms

The semi-quantitative analyses of the NOS3 transcript levels and their association with polymorphisms and haplotypes were performed by selecting a representative sample of the population based on the genotypic frequencies and clinical parameters. The 38 patients used in this study presented the same genotypic frequencies and average values for the clinical parameters observed in the patients’ population (177 patients).

The NOS3 relative levels related to the beta-2-microglobulin (B2M) in the peripheral blood did not follow a normal distribution (n = 38, p = 0.33). Although not significant, the mean NOS3 relative levels was four times higher in PCa in relation to BPH patients (ratio NOS3 mRNA/B2M mRNA: 2.23 and 0.50, respectively) (Figure 3B).

A cut-off value for the relative levels of NOS3 transcripts were established based on the maximum average levels observed for the BPH group. The cut-off value for negative results was below 0.8 for the NOS3 mRNA/B2M mRNA ratio, which means 80% of the observed value for the B2M transcript levels. Considering the cut-off value, it was

---

**Table 3: NOS3 gene promoter haplotypes, number of copies, and prostate cancer risk.**

| Haplotypes<sup>a</sup> (SSCP Conformation) | Units | Zero Copies | One Copy | p-Value | Two copies | p-Value |
|------------------------------------------|-------|-------------|----------|---------|------------|---------|
| No mutations (T)                         | Controls/cases | 17/16 | 48/36 | 0.73 | 29/30 | 1.0 |
| [-786T>C; -714G>T; -690C>T] (C)          | OR (95% CI) | 1.00 (ref) | 0.80 (0.36 – 1.79) | 0.92 | 1.10 (0.47 – 2.58) | 0.81 |
| [-786T>C; -764A>G; -690C>T] (A)          | OR (95% CI) | 1.00 (ref) | 0.98 (0.52–1.83) | 0.13 | 1.31 (0.46 – 3.76) | -- |
| [-786T>C; -690A>G; -649G>A]<sup>b</sup> (N) | OR (95% CI) | 1.00 (ref) | 0.51 (0.23 – 1.12) | -- | 0/0 | -- |

<sup>a</sup> Alleles listed for dbSNP ID (GenBank) in 5’ to 3’ order: NM_2070744, NM_EF042808, NM_EF042809, NM_3918225, NM_EF042810.

<sup>b</sup> Haplotype that appear only once between groups (PCa and BPH).

CI: confidence interval/OR: odds ratio

(-- inapplicable data)
observed a 4.0-fold higher chance (CI95%, 0.95 – 16.77; p = 0.12) of having cancer when the NOS3 expression levels were equal or higher than 0.8.

Among PCa patients, only the Pearson's correlation coefficients obtained among the NOS3 gene expression and PCA3 data (p = 0.038) were significant. For BPH patients, the NOS3 levels, serum PSA, patients' age on diagnosis and PCA3 detection were not correlated among each other. However, the average NOS3 levels presented a bimodal behavior in PCa patients classified according to their tumor stages, with higher levels in the pT2 stage (Figure 5A).

No significant differences among NOS3 gene expression levels and haplotypes were observed between groups (Table 2). However, there was a significant association of NOS3 genotypes and gene expression levels for PCa patients (p = 0.011), but not within the BPH group. It was observed increased levels of NOS3 transcripts within the PCa group, especially for patients with TA and CA SSCP conformations, with NOS3 mRNA/B2M mRNA ratios of 4.7 and 6.0, respectively.

The average NOS3 levels were 3.52-fold higher in PCA3 positive patients (NOS3 mRNA/B2M mRNA = 3.17) in comparison to the average observed in PCA3 negative patients (NOS3 mRNA/B2M mRNA = 0.90), although not significant, due to the low number of PCA3 positive samples analyzed.

Analysis of the NOS3 expression levels across the -786T>C polymorphism on all patients showed a linear behavior with an additive component for each C allele incorporated into the genotype, with higher levels (2.27) observed for the (-786T>C + -786T>C) mutant homozygous patients, followed by medium levels (1.47)

| SSCP | Genotypes | Positive PCA3 mRNA (N = 40) | Negative PCA3 mRNA (N = 109) |
|------|-----------|-----------------------------|-----------------------------|
|      |           | PCa N (%) | BPH N (%) | Total | PCa N (%) | BPH N (%) | Total |
| TN   | [-786T>C] + [:=] | 0 | 0 | 0/0 | 1 | 0 | 1/109 |
|      | [-690C>T] + [:=] | (0.9) | (0.9) | 1/109 |
|      | [-649G>A] + [:=] | (0.9) | (0.9) | 1/109 |
| CC   | [-786T>C] + [-714G>T] + [-690C>T] | 3 | 1 | 4/40 | 4 | 7 | 11/109 |
|      | (7.5) | (2.5) | (10) | (3.7) | (6.4) | (10.1) |
| TT   | No mutations | 7 | 5 | 12/40 | 18 | 22 | 40/109 |
|      | (17.5) | (12.5) | (30) | (16.5) | (20.2) | (36.7) |
| TC   | [-786T>C] + [:=] | 10 | 4 | 14/40 | 18 | 22 | 40/109 |
|      | [-714G>T] + [:=] | (25) | (10) | (35) | (16.5) | (20.2) | (36.7) |
|      | [-690C>T] + [:=] | (25) | (10) | (35) | (16.5) | (20.2) | (36.7) |
| TA   | [-786T>C] + [:=] | 2 | 2 | 4/40 | 1 | 8 | 9/109 |
|      | [-764A>G] + [:=] | (5) | (5) | (10) | (0.9) | (7.3) | (8.3) |
|      | [-690C>T] + [:=] | (5) | (5) | (10) | (0.9) | (7.3) | (8.3) |
| CA   | [-764A>G] + [:=] | 2 | 3 | 5/40 | 3 | 4 | 7/109 |
|      | [-714G>T] + [:=] | (5) | (7.5) | (12.5) | (2.7) | (3.7) | (6.4) |
|      | [-690C>T] + [-690C>T] | (5) | (7.5) | (12.5) | (2.7) | (3.7) | (6.4) |
| AA   | [-786T>C] + [-714G>T] + [-690C>T] | 1 | 0 | 1/40 | 1 | 0 | 1/109 |
|      | [-764A>G] + [-714G>T] | (2.5) | (2.5) | (2.5) | (0.9) | (0.9) | (0.9) |
|      | [-690C>T] + [-690C>T] | (2.5) | (2.5) | (2.5) | (0.9) | (0.9) | (0.9) |
| Total | 25/40 | 15/40 | 40/149 | 46/109 | 63/109 | 109/149 |
|      | (62.5) | (37.5) | (26.8) | (42.2) | (57.8) | (57.8) |

Table 4: PCA3 mRNA detection in the peripheral blood of PCa and BPH patients across genotypic SSCP conformations and their haplotypes.

| SSCP | Haplotypes | PCa % | BPH % | PCa % | BPH % |
|------|------------|-------|-------|-------|-------|
| N    | [-786T>C; -690A>G; -649G>A] | 0 | 0 | 1 | 0 |
| T    | No mutations | 56 | 53 | 61 | 59 |
| C    | [-786T>C; -714G>T; -690C>T] | 32 | 30 | 31 | 32 |
| A    | [-786T>C; -764A>G; -690C>T] | 12 | 17 | 7 | 9 |

* PCA3 gene expression (PCa = 71/BPH = 78)
Figure 5
Graphics representations. A) The average NOS3 relative levels of PCa patients classified according to tumor stages and a predicted tendency line. B) Regression analysis for the average NOS3 relative levels in association with the -786T>C polymorphism genotypes for all patients and for the group of patients with positive PCA3 detection.
Discussion

During the genotyping of the -786T>C polymorphism of the NOS3 promoter [16], we have discovered and characterized three novel mutations [-764A>G, GenBank: NM_00242808/-714G>T, GenBank: NM_00242809/649G>A, GenBank: NM_00242810] in the PCA and BPH patients. The association between these three mutations together with two SNPs previously described [-786T>C, GenBank: NM_2070744/-690C>T, GenBank: NM_3918225] and prostate cancer was further examined.

The five polymorphisms have generated seven genotypic profiles in the polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) gel electrophoresis and four haplotypes, suggesting that some mutations are closely linked; therefore, they have evolved together, originating a restricted number of haplotypes. It is interesting to observe that when the -786T>C mutation was present at least two other mutations were linked. But, when the normal -786T allele was present, no additional polymorphisms were detected, explaining the four possible haplotypes.

This is the first publication that associates the promoter polymorphisms of the NOS3 gene with prostate cancer risk; however, other polymorphisms, such as the 894G>T (Glu298Asp) and the intron 4 have also been evaluated [21-24].

The possibility of linkage between the two previously described NOS3 gene polymorphisms and the promoter polymorphisms has not been investigated, and some controversies may occur in the literature probably due to the incorrect population stratification based on genotyping, which could be generating linkage disequilibrium among polymorphisms in the population that may present a functional association with the NOS3 expression and activity. However, there is an evidence that the 894G>T polymorphism had no influence on NOS3 transcriptional levels, but it was associated with PCA3 detection [24].

The strategy of determining genotypic conformations of the NOS3 promoter region through the SSCP technique demonstrated to be very effective in classifying all patients. The significant relative risks of cancer occurrence related to CC and TT SSCP conformation (3.38 and 3.10, respectively) in comparison to TA + CA suggest that the -786T>C polymorphism is the most important promoter alteration that may affect the PCa progression. It is also important to observe that patients with the CC SSCP conformation, in both PCa and BPH groups, presented the highest serum PSA averages.

In the present work, there is a good evidence that the -786T>C polymorphism affects the NOS3 expression, with the (-786T>C + -786T>C) mutant homozygous condition presenting the highest levels of NOS3 transcripts, and acting in an additive manner, as the C allele is incorporated into the genotype. This was also observed within the positive PCA3 detection class, although positive PCA3 patients present higher levels of NOS3 transcripts. However, some authors have associated the (-786T>C + -786T>C) genotype with low levels of NOS3 mRNA, which would be contributing to a higher risk of cardiovascular diseases [25-27].

The association of the -786T>C promoter polymorphism with NOS3 transcriptional levels and the highly significant odds ratio (4-fold) for elevated NOS3 levels (NOS3:B2M > 0.8) and the risk of PCa occurrence are important evidences of the NOS3 gene promoter role in the PCa progression. This is further supported by the 2-fold increase in NOS3 mRNA levels in the positive PCA3 detection class, as demonstrated in Figure 5B. The NOS3 transcriptional activity was also highly correlated with disease staging (Figure 5A), once its highest level is reached at the pT2 stage, when the angiogenic stimulus is required for tumor cell dissemination and metastasis.

Recent studies have described the NO involvement in many biological processes affecting carcinogenesis [28-31]. Our results are also supported by previous works [24], which have also indicated that NOS3 levels may present a bimodal behavior during cancer development.

The fine mapping of the NOS3 promoter region, flanking the region -820 to -583, has allowed us to construct four haplotypes and seven genotypic combinations. We have found that none of the haplotypes did affect significantly the NOS3 levels; in fact, a unique polymorphism (-786T>C) may be responsible for the NOS3 transcription regulation, and this polymorphism is observed only in haplotypes N, C and A.

The increasing NOS3 expression levels associated with the -786T>C polymorphism may contribute to cancer progression by providing a selective growth advantage of tumor cells, by the angiogenic stimulus [6,7] and by caus-

for the (-786T>C + [=]) heterozygous and lower levels (0.67) for normal homozygous patients (Figure 5B).

Similarly, the NOS3 levels within the positive PCA3 class (grouping BPH and PCA patients) presented a linear behavior considering the -786T>C polymorphism, with higher levels (3.1) for the (-786T>C + -786T>C) mutant homozygous patients, followed by medium levels (2.3) for the (-786T>C + [=]) heterozygous and lower levels (1.5) for normal homozygous patients (Figure 5B)

The significant relative risks of cancer occurrence related to CC and TT SSCP conformation (3.38 and 3.10, respectively) in comparison to TA + CA suggest that the -786T>C polymorphism is the most important promoter alteration that may affect the PCa progression. It is also important to observe that patients with the CC SSCP conformation, in both PCa and BPH groups, presented the highest serum PSA averages.

In the present work, there is a good evidence that the -786T>C polymorphism affects the NOS3 expression, with the (-786T>C + -786T>C) mutant homozygous condition presenting the highest levels of NOS3 transcripts, and acting in an additive manner, as the C allele is incorporated into the genotype. This was also observed within the positive PCA3 detection class, although positive PCA3 patients present higher levels of NOS3 transcripts. However, some authors have associated the (-786T>C + -786T>C) genotype with low levels of NOS3 mRNA, which would be contributing to a higher risk of cardiovascular diseases [25-27].

The association of the -786T>C promoter polymorphism with NOS3 transcriptional levels and the highly significant odds ratio (4-fold) for elevated NOS3 levels (NOS3:B2M > 0.8) and the risk of PCa occurrence are important evidences of the NOS3 gene promoter role in the PCa progression. This is further supported by the 2-fold increase in NOS3 mRNA levels in the positive PCA3 detection class, as demonstrated in Figure 5B. The NOS3 transcriptional activity was also highly correlated with disease staging (Figure 5A), once its highest level is reached at the pT2 stage, when the angiogenic stimulus is required for tumor cell dissemination and metastasis.

Recent studies have described the NO involvement in many biological processes affecting carcinogenesis [28-31]. Our results are also supported by previous works [24], which have also indicated that NOS3 levels may present a bimodal behavior during cancer development.

The fine mapping of the NOS3 promoter region, flanking the region -820 to -583, has allowed us to construct four haplotypes and seven genotypic combinations. We have found that none of the haplotypes did affect significantly the NOS3 levels; in fact, a unique polymorphism (-786T>C) may be responsible for the NOS3 transcription regulation, and this polymorphism is observed only in haplotypes N, C and A.

The increasing NOS3 expression levels associated with the -786T>C polymorphism may contribute to cancer progression by providing a selective growth advantage of tumor cells, by the angiogenic stimulus [6,7] and by caus-
ing DNA damage due to the direct action of O$_2$ free radicals, as an effect of the excess NO production [8].

The suggestion that decreasing NOS3 expression levels, which consequently reduce the NO production, would have an anti-apoptotic role, and may promote tumor growth [32] may be explained in part by the bimodal behavior of the NOS3 levels across the stages as shown in this investigation and in a previous work [24], once lower levels is mainly seen in advanced tumor stages (pT3 and pT4).

The first evidence of the association of NOS3 polymorphisms with circulating tumor cells was demonstrated between the intron 4 polymorphism and the folate hydrolase – prostate-specific membrane antigen (FOLH1) expression in the peripheral blood [23]. According to these authors, patients with the ‘a’ allele have low plasmatic NO levels, and therefore are more inclined to have viable circulating tumor cells.

In this investigation, we did not find association between the NOS3 gene promoter polymorphisms with circulating tumor cells PCA3 detection in the peripheral blood, as shown elsewhere with the 894G>T polymorphism and the PCA3 detection [24].

The PCA3 gene is a highly specific prostate tumor biomarker that is not found in other kinds of cells and tissues, whereas its detection on peripheral blood may indicate a possible metastasis [20]. We believe that the PCA3 is more specific for tumor detection than the FOLH1 marker, which presents a high variation on gene expression among PCa and BPH patients and its utilization as a biomarker is highly controversial (unpublished data).

In the overall, the PCA3 clinical parameters for PCa cell detection in the peripheral blood were: 35.0% of sensitivity (25 out of 71), and 81.0% of specificity (63 out of 78); however, it is important to observe that a positive result is not an indication of metastasis and that some BPH cases may have been misdiagnosed, reducing its true clinical value.

In fact, the PCA3 detection may become a potential biomarker for blood diagnosis, once it is exclusively detected in prostate cancer cells; therefore, patients historically diagnosed as BPH may probably be tumor confined disease that was missed during biopsy sampling or by pathological examination. On the other hand, a negative PCA3 detection in cancer patients may also suggest that the tumor is organ confined. However, it is important to emphasize that positive PCA3 detection is not an indication of faster tumor development or invasiveness.

Prostate cancer is a complex disease due to multifactorial and multifocal events caused by many biological mechanisms. These mechanisms are represented by differential gene expression profiles, generating disease developmental stages that vary from latent to aggressive forms. Therefore, detection of key genetic alterations at the molecular level may be a useful tool as a prognostic indicator. Despite the complexity of events that the NO participates in many biological processes, its association with carcinogenesis is critical once NO levels modulate tumor development. Part of this differential NO expression is regulated by NOS3 promoter polymorphisms, specifically the -786T>C, which may have an influence on the progression of the disease and not on its occurrence.

There are at least five regulatory elements within the amplified promoter region (-820 to -583) and their interaction with all polymorphisms have not been investigated. Therefore, it is possible that other external and internal factors may independently influence NOS3 gene expression masking the true effect of polymorphisms, which may explain the high variability of NOS3 transcript levels observed within PCa and BPH groups.

In the present work, higher NOS3 transcript levels in peripheral blood of positive PCA3 patients than in negative ones suggest that tumor may be in a medium-late (pT2) stage of the disease and corroborates with the molecular approach for disease staging. Therefore, we propose that patients with differential expression/detection, such as high NOS3 and negative PCA3 may indicate pT1 stage, while low NOS3 and positive PCA3 may indicate pT3 or pT4 stages.

Conclusion

In conclusion, this is the first publication that demonstrates an association of the NOS3 promoter region polymorphisms with prostate cancer progression. The strategy of determining genotypic conformations of the NOS3 promoter region through the SSCP technique demonstrated to be very effective in genotyping all patients, which were classified into four haplotypes and seven genotypic conformations. The C allele of the -786T>C polymorphism was always associated with at least two other mutations (haplotypes C, A and N), while the T haplotype (containing the T allele) had no mutations. Significant relative risks (> 3 fold) of cancer occurrence were related to the -786T>C polymorphism, and it is the most important promoter alteration that may affect the PCA progression, but not its occurrence. NOS3 transcript relative levels (NOS3:B2M > 0.8) were 4-fold higher in PCa than in BPH, and due to its bimodal behavior, the NOS3 levels may be used as a biomarker together with the PCA3 marker for molecular staging of the disease.
Abbreviations
NO: nitric oxide; NOS3: nitric oxide synthase 3; PCA: prostate cancer; ROS: reactive oxygen species; bp: base pair; SSCP: single strand conformation polymorphism; BPH: benign prostatic hyperplasia; SNP: single nucleotide polymorphism; OR: odds ratio; vs: versus; PSA: prostate specific antigen; TNM: tumor-node-metastasis; PCA3: prostate cancer antigen 3; T1URP: transurethral resection of prostate; B2M: beta-2-microglobulin, PCR: polymerase chain reaction; FOLH1: folate hydrolase – prostate-specific membrane antigen; UFI: Federal University of Uberlandia; dNTP: deoxyribonucleotide triphosphate; MMV-RT: murine moloney leukemia virus reverse transcriptase; DEPC: diethylpyrocarbonate; CNPq: National Counsel of Technological and Scientific Development; CAPES: Coordination of Perfectioning of Staff of Superior Level; FAPEMIG: Foundation of Support to the Research of the State of Minas Gerais.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
KM, TGA and AFN have equally contributed for the present study (research design, sample collection, processing, and analysis). LRG is senior author and wrote the paper with KM. The authors read and approved the final manuscript.

Acknowledgements
The authors would like to thank the medical staff from the Urology Division of the University Hospital for providing the biological samples and the clinical parameters and the financial support from CNPq, CAPES and FAPEMIG.

References
1. Forstermann U, Boissel JP, Kleinitzer H: Expression control of the ’constitutive’ isoforms of nitric oxide synthase (NOS I and NOS III). FASEB J 1998, 12:773-790.
2. Faiss S, Meyer S: Compilation of vertebrate-encoded transcription factors. Nucleic Acids Res 1992, 20:3-26.
3. Grande M, Carlstrom K, Stege R, Pousette A, Faxen M: Estrogens increases the endothelial nitric oxide synthase (NOS3) mRNA level in LNPCa human prostate carcinoma cells. Prostate 2000, 45:232-237.
4. Tamir S, Burney S, Tannenbaum SR: DNA damage by nitric oxide (NO). Chem Res Toxicol 1996, 9:821-827.
5. Zhuang JC, Wright TL, deRojas-Walker T, Tannenbaum SR, Wogan GN: Nitric oxide-induced mutations in the HPRT gene of human lymphoblastoid T6K cells and in Salmo nella typhimurium. Environ Mol Mutagen 2000, 35:39-47.
6. Sandau KB, Zhou J, Kietzmann T, Brune B: Regulation of the hypoxia-inducible factor I alpha by inflammatory mediators' nitric oxide and tumor necrosis factor-alpha in contrast to desferoxamine and phenylhydrazine oxide. J Biol Chem 2001, 276:39805-39811.
7. Sharp PA: RNA Interference – 2001. Genes Dev 2001, 15:485-490.
8. Vanvakas S, Schmidt HH: Just say NO to cancer? J Natl Cancer Inst 1997, 89:406-407.
9. Gallo O, Masino E, Morbidelli L, Franchi A, Fini-Storchi I, Vergari WA, Ziche M: Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. J Natl Cancer Inst 1998, 90:587-596.
10. García-Cardenas G, Folkman J: Is there a role for nitric oxide in tumor angiogenesis? J Natl Cancer Inst 1998, 90:560-561.
11. Miyahara K, Kawamoto T, Sase K, Yui Y, Toda K, Yang LX, Hatiori R, Aoyama T, Yamamoto Y, Doi Y, Ogoshi S, Hashimoto K, Kawai C, Sasayama S, Shizuta Y: Cloning and structural characterization of the human endothelial nitric oxide synthase gene. Eur J Biochem 1994, 227:19-726.
12. Tsuchida T, Yokoyama K, Arat T, Takemoto F, Hara S, Yamada A, Kawaguchi Y, Hosoya T, Igar J: Evidence of association of the eNOS gene polymorphism with plasma NO metabolite levels in humans. Biochem Biophys Res Commun 1998, 245:190-193.
13. Nadaud S, Bonardeaux A, Latrhop M, Soubrier F: Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. Biochem Biophys Res Commun 1994, 198(3):1027-1033.
14. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1989.
15. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156-159.
16. Nagayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, Nakamura S, Ito T, Saito Y, Ogawa Y, Nakao K: T-786→C mutation in the 5’-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. Circulation 1999, 99:2864-2870.
17. Orita M, Iwahana H, Kanazawa H: Detection of polymorphism of human DNA by gel electrophoresis as single strand conformation polymorphism. Proc Natl Acad Sci U S A 1989, 86:2766-2770.
18. Blum H, Beirg H, Gross HI: Improved silver staining of proteins, RNA and DNA in polyacrilamide gels. Deterofos 1987, 8:93-99.
19. Bassam BJ, Caetano-Anolles G, Gresshoff PM: Fast and sensitive silver staining of DNA in polyacrilamide gels. Anal Biochem 1991, 196:80-83.
20. Bussemakers MJ, van-Bokhoven A, Verhaegh GW, Smit FP, Karthaus HF, Schalken JA, Debruyne FM, Ru N, Isaacs WB: DD3: A new prostate-specific gene, highly overexpressed in prostate cancer. Cancer Res 1999, 59:5975-5979.
21. Medeiros R, Morais A, Vasconcelos A, Costa S, Pinto D, Oliveira J, Ferreira P, Lopes C: Outcome in prostate cancer: association with the endothelial nitric oxide synthase gene polymorphism at exon 7. Clin Cancer Res 2002, 8:3433-3437.
22. Medeiros R, Morais A, Vasconcelos A, Costa S, Pinto D, Oliveira J, Lopes C: Endothelial nitric oxide synthase gene polymorphisms and genetics susceptibility to prostate cancer. Eur J Cancer Prev 2002, 11:343-350.
23. Medeiros R, Morais A, Vasconcelos A, Costa S, Carrilho S, Oliveira J, Lopes C: Endothelial nitric oxide synthase gene polymorphism and the shedding of circulating tumor cells in the blood of prostate cancer patients. Cancer Lett 2003, 189:85-90.
24. Maragoni K, Neves AF, Cardoso AM, Santos WK, Goulart LR: The endothelial nitric oxide synthase Glu298Asp polymorphism and its mRNA expression in the peripheral blood of patients with prostate cancer and benign prostatic hyperplasia. Cancer Detect Prev 2006, 30:7-13.
25. Nakayama M, Yasse H, Yoshimura M, Shimasaki Y, Ogawa H, Kugiyama K, Mizuno Y, Harada E, Nakamura S, Ito T, Saito Y, Miyamoto Y, Ogawa Y, Nakao K: T-786→C mutation in the 5’-flanking region of the endothelial nitric oxide synthase gene is associated with myocardial infarction, especially without coronary organic stenosis. Am J Cardiol 2000, 86:628-634.
26. Wang XL, Wang J: Endothelial nitric oxide synthase gene sequence variations and vascular disease. Mol Genet Metab 2000, 70:241-251.
27. Senthil D, Raveendran M, Shen YH, Utama B, Dudley D, Wang J, Wang L, Wang XL: Genotype-dependent expression of endothelial nitric oxide synthase (eNOS) and its regulatory proteins in cultured endothelial cells. DNA Cell Biol 2005, 24(4):218-224.
28. Fellely-Bosco E, Yokoyama K: Role of nitric oxide in genotoxicity: implication for carcinogenesis. Cancer Metastasis Rev 1998, 17:25-37.
29. Fukumura D, Jain RK: Role of nitric oxide in angiogenesis and microcirculation in tumors. Cancer Metastasis Rev 1998, 17:77-89.
30. Geller DA, Billiar TR: Molecular biology of nitric oxide synthases. Cancer Metastasis Rev 1998, 17:7-23.
31. Orucovic A, Lala PK: Role of nitric oxide in IL-2 therapy induced capillary leak syndrome. Cancer Metastasis Rev 1998, 17:127-142.
32. Dimmeler S, Haendeler J, Nehls M, Zeiher AM: Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CCP)-32-like proteases. J Exp Med 1997, 185:601-607.

Pre-publication history
The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/8/273/prepub