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# Investigating the Prognostic Value of DD3 and AMACR Expression in Prostate Cancer

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## Abstract

**Introduction:** Prostate cancer (PCa) is a major public health issue in men older than 40 years old. New genetic diagnostic markers like DD3 and racemase (AMACR) have been the subject of several studies in prostate cancer. Still, the real value of these markers in terms of prognosis has not been studied yet. Objectives: To describe and correlate DD3 and AMACR gene expression in PCa patients who underwent radical retropubic prostatectomy (RRP) and to compare their expression in patients with localized and advanced tumors. **Methods:** Forty-two prostate samples were collected. DD3 and AMACR gene expression were measured by Reverse Transcription Polymerase Chain Reaction (RT-PCR). Clinical and pathological data were obtained from the patient's registry. Results: DD3 and AMACR did not correlate with the pathological stage. Spearman's correlation coefficient was $\rho = -0.15$ ($p = 0.39$) and $\rho = 0.17$ ($p = 0.49$) for DD3 and AMACR, respectively. The mean difference in DD3 expression between localized and advanced disease was 0.74 (CI: −0.70 - 0.21; $p = 0.30$); the mean difference in AMACR between groups was 0.11 (CI: −0.32 - 0.10; $p = 0.28$). **Conclusions:** Comparison of gene expression between localized and advanced tumors has not shown significant differences. Correlation among different pathological groups was not important. DD3 and AMACR, although expressed in prostate cancer tissue, cannot be used as prognostic markers based on pathological staging.

## Keywords

Prostate Cancer, Tumor Markers, Staging

## 1. Introduction

Prostate cancer has an overall incidence of 52 cases per 100,000 inhabitants in Brazil. Only in Rio Grande do Sul, we have estimated 80.63 cases per 100,000 inhabitants in 2008 [1]. This is the second most common cause of malignant neoplastic diseases, only behind non-melanoma cutaneous cancer. At the same time, prostate cancer is the second leading cause of death among neoplastic diseases in men, with pulmonary cancer the number one. The impact of men with this disease makes prostate cancer a public health problem, with a very large number of studies [2].

Screening investigation for prostate cancer is made by measuring blood levels of Prostate-Specific Antigen (PSA), plus digital rectal examination. If any of these exams is considered abnormal, one should perform a biopsy of the prostate to confirm the cancer diagnosis [3]. Despite PSA being an excellent tumoral marker, it has low specificity for malignant prostate disease. PSA levels can be elevated in many conditions, like benign prostatic hyperplasia and prostatic infections [4].

Heredity is a controversial theme in prostate cancer. There is evidence of Mendelian inheritance in familial cases. Steinberg *et al.* related a two or threefold increase in cancer risk when first-degree relatives had a diagnosis of prostate cancer [5]. According to Carter *et al.*, the agglomeration of cases among relatives of the same family would be related to autosomal dominant inheritance of a rare allele (populational frequency = 0.003). People with this allele could have a lifetime cumulative risk of prostate cancer of 88%, compared with 5% of non-carriers [6]. Although only 10% of prostate cancer occurrences are due to the inheritance of high-penetrance genes, the study of these cases is very important, as one could identify these high-risk patients and, therefore, perform better screening and prevention. Epidemiologic studies discovered a large association between genetic polymorphism and the risk of prostate cancer development [7] [8]. Some genes and cellular receptors are also subjects of studies, including their correlation with patients' clinical outcomes and therapeutic responses. The presence of specific markers in prostate cancer is a very promising field in terms of diagnosis, treatment, and prognosis [9].

The treatment of prostate cancer is guided according to the stage of the disease. In localized cancer, the treatment is usually surgery or radiotherapy. In advanced prostate cancer, there is no definitive therapy, although palliation can be achieved in most cases with hormone therapy, chemotherapy, and, more recently, immunotherapy. It's widely accepted that the most important predictor of cancer outcome is the patient's stage (**Table 1**) [10]. Although the complete TNM system has several divisions, we can divide all the patients into localized cancer (including stages T1 and T2—confined to the prostate) and advanced cancer (stages T3, T4, N+, M+, all of these outside the prostate capsule). Any new specific marker targeting diagnosis and prognosis would be very important data [11].

In recent years, a lot of research has been conducted to demonstrate several genes somewhat linked to prostatic diseases. DD3 is a new marker that has been

**Table 1.** Pathological staging of prostate cancer.

| | |
|---|---|
| **T0** | No evidence of a primary tumor |
| **T1a** | Tumor found in tissue after benign surgery, 5% or less is cancerous and Gleason score < 7 |
| **T1b** | Tumor found in tissue after benign surgery, >5% is cancerous, and Gleason score 7 or higher |
| **T2a** | Tumor involves less than half of one lobe |
| **T2b** | Tumor involves more than half of one lobe, but not both lobes |
| **T2c** | Tumor involves more than one lobe |
| **T3a** | Unilateral extracapsular extension |
| **T3b** | Bilateral extracapsular extension |
| **T3c** | Tumor invades seminal vesicle(s) |
| **T4a** | Tumor invades bladder, neck, external sphincter, and/or rectum |
| **T4b** | Tumor invades levator muscle and/or fixed to pelvic wall |
| **N+** | Metastasis in regional lymph nodes |
| **M+** | Distant metastatic spread |

identified and found in modified prostate cancer cells. Some authors consider DD3 the most specific marker for carcinoma of the prostate. Hessels *et al.* have shown a negative predictive value of 90% and sensibility of 67% in prostate cancer biopsies, compared with normal cells [12]. DD3 is being studied as a promising marker with some better characteristics than the low-specific PSA [9] [13] [14].

AMACR (Alpha-Methylacyl Coenzyme A Racemase) codifies an enzyme that performs the catalytic racemization of carboxylic ramified coenzyme A thioesters. AMACR is located in peroxisomes and mitochondria [15]. Racemase has an important function in biliary acid biosynthesis and beta-oxidation of ramified chain fatty acids. A mutation of the AMACR gene occurs in some motor-sensory neuropathy in adults. Studies are demonstrating AMACR overexpression in malignant prostate cells, acting as a sensitive and specific marker of cancer, even in early cases, occurring in 80% to 100% of all cases [11] [16]. Besides, AMACR is also being studied as a serologic marker and as an auxiliary factor in the diagnosis of prostate cancer in biopsy samples. The evaluation of prostatic biopsy specimens with immunohistochemical staining was related to prostate tumors, regardless of the Gleason score [17]. No correlation between AMACR levels and PSA levels was found after surgery in a 3-year follow-up study carried out with 120 patients who underwent radical prostatectomy [18]. AMACR's high sensibility and specificity in malignant tumors indicate a potential new marker in the diagnosis confirmation in patients who undergo doubtful biopsy of the prostate [19] [20].

Despite all the progress toward new markers, the complete role of DD3 and AMACR is still under study. The expression of these genes in patients with prostate cancer and the relationship with neoplasia staging has not been established

yet. Knowledge of these molecular markers in prostate cancer cells in patients who undergo radical retropubic prostatectomy in Hospital de Clínicas de Porto Alegre (HCPA) will provide a better assessment of the studied population and may, in the future, help to distinguish patients with localized or advanced prostate cancer.

The purposes of this study are to evaluate the gene expression of DD3 and AMACR in patients with prostate cancer who undergo radical retropubic prostatectomy RRP), to correlate the gene expression with pathologic stage findings, and to measure the gene patterns between localized and advanced cancer.

## 2. Methods

**Study Design:** This is a cross-sectional study performed in Hospital de Clínicas de Porto Alegre with surgically treated prostate cancer patients. All the patients were recruited according to their scheduled surgery in chronological order and the availability of the collecting team. The tissue sample was collected as soon as possible after the extraction of the prostate. Approximately 1 - 2 cc was properly collected, stored in liquid nitrogen, and carried to the laboratory. Preoperative PSA levels, biopsy of the prostate data, and pathologic results were collected from the patient's registry. Pathologic findings were summarized according to the TNM classification (Table 1).

**Inclusion criteria:** Patients with prostate cancer who undergo RRP surgery.

**Exclusion criteria:** Hormonotherapy before surgical treatment.

**Total RNA extraction:** The tissue's total RNA was extracted with TRIzol® reagent using the manufacturer's protocol (Life Technologies, Inc., Breda, Netherlands). RNA was spectrophotometrically quantified at 260 nm using 1 µl aliquot of RNA in the samples diluted in 499 µl of water, read in duplicate. RNA's concentration in the original solution was calculated by this formula (considering the correspondence of one absorbance unit at 260 nm to 40 µl of RNA per ml of solution):

$$[\text{RNA}] = A_{260} \times D \times 40\ \mu g/mL$$

where A = absorbance and D = aliquot dilution to quantify.

**RT-PCR gene expression evaluation:** DD3 and AMACR mRNA expression was evaluated indirectly by the reverse transcription polymerase chain reaction (RT-PCR) technique. cDNA synthesis was made initially with 2 µg of total RNA, using the Super-Script First-Strand Synthesis System for RT-PCR kit (Invitrogen, Life Technologies®), following the manufacturer's indicated steps. cDNA was kept frozen in −20˚C (−4˚F) until the PCR amplification. PCR reactions were made using specific primers for each studied gene. All the genes' PCR reactions had their conditions patterned (temperature, cycle numbers, primers quantity, cDNA quantity) to optimize the reaction conditions and avoid data analysis in the plateau. The results of the amplifications were visualized by agarose gel electrophoresis, and the quantification of mRNA was performed by analyzing the density of the bands using the ImageMaster VDS image caption system.

**Statistics:** Distribution of DD3 and AMACR were characterized in terms of mean, median, standard deviation, and extreme values. These data were correlated using Spearman's rho coefficient ($\rho$). Afterward, the gene expression had a logarithmic transformation to achieve a normalized distribution. The pathological stage was divided into two groups: localized (T1 and T2) and advanced (T3, T4, N+, M+). A two-sample t-test was used to analyze the gene distribution between the groups. Significance was considered as $p < 0.05$, with a confidence interval (CI) of 95%.

**Ethical aspects:** This study was submitted to the Ethics Committee of Hospital de Clínicas de Porto Alegre and the Brazilian National Committee of Ethics in Research (CONEP). All the patients were interviewed and signed a term agreeing to the collection of a prostate sample.

## 3. Results

Forty-two patients had prostate tissue collected (October 2006 to March 2008). The data are summarized in Table 2. The mean age was (62.60 ± 6.86) years (range 47 to 72). PSA levels range from 1.70 to 73.30 ng/ml, mean of 10.66 ± 13.76. The biopsy of the prostate (Figure 1) was positive in the left lobe in 17.1% (7 cases), positive in the right lobe in 34.1% (14 cases), and bilateral in 20 patients (48.8%). One patient had PSA and biopsy data missing.

Table 2. Clinical and laboratory data.

| Variable | Mean | Median | Min | Max | N |
|---|---|---|---|---|---|
| **Age** | 62.60 | - | 47 | 72 | 42 |
| **PSA** | 10.66 | 6.87 | 1.70 | 73.30 | 41 |
| **DD3** | 1.23 | 1.02 | 0.41 | 2.90 | 35 |
| **AMACR** | 0.74 | 0.59 | 0.30 | 2.40 | 19 |

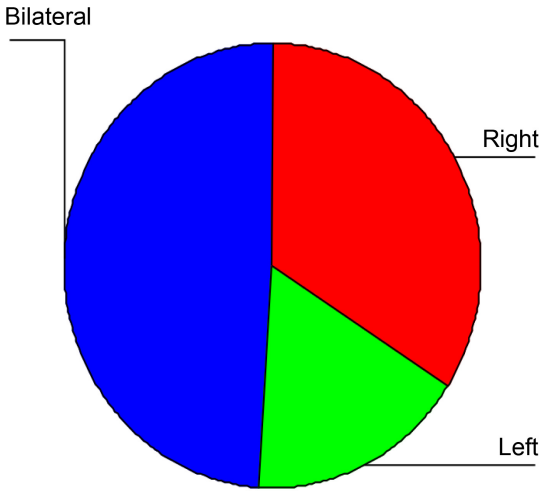


Figure 1. Biopsy of the prostate. Bilateral: neoplasia in both lobes; Right: right lobe cancer; Left: left lobe cancer.

Pathological aspects: Twenty-three patients (54.8%) had localized disease, all of them in the T2 stage. Advanced disease was found in 19 patients (45.2%), 15 in the T3 stage, and four in the T4 stage. The pathologic summary can be viewed in Table 3.

Table 3. Pathologic stage findings.

| Stage | Frequency | Percentual | Cumulative Perc | Localized/ Advanced |
|---|---|---|---|---|
| **T1a** | 0 | 0 | 0 | Localized |
| **T1b** | 0 | 0 | 0 | Localized |
| **T2a** | 7 | 16.7 | 16.7 | Localized |
| **T2b** | 3 | 7.1 | 23.8 | Localized |
| **T2c** | 13 | 31.0 | 54.8 | Localized |
| **T3a** | 9 | 21.4 | 76.2 | Advanced |
| **T3b** | 2 | 4.8 | 81.0 | Advanced |
| **T3c** | 4 | 9.5 | 90.5 | Advanced |
| **T4a** | 4 | 9.5 | 100.0 | Advanced |
| **T4b** | 0 | 0 | 100.0 | Advanced |
| **N+** | 0 | 0 | 100.0 | Advanced |
| **M+** | 0 | 0 | 100.0 | Advanced |

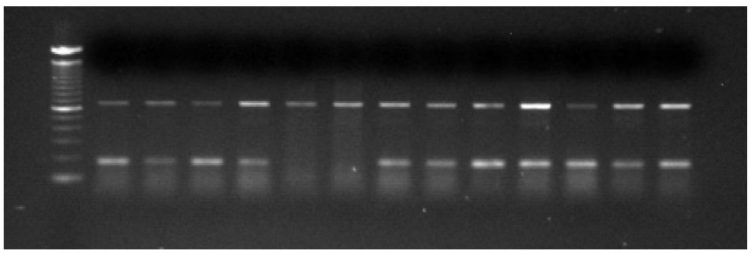


Figure 2. Agarose 2% gel representing DD3 bands (179 bp) and β2m (623 bp) in PCa samples.

DD3: Gene expression could be asserted in 35 patients (Figure 2), with a mean of 1.23 ± 0.60 Arbitrary Units (UA), range 0.41 - 2.90 (Table 2). The correlation between DD3 and pathological staging was not significant ($\rho = -0.149$, p = 0.39) (Figure 3).

DD3 mean expression in the localized neoplasia group was 1.36 ± 0.71. In the advanced group, the mean was 1.07 ± 0.42. Logarithmic transformation was performed, and the t-test was used to compare means between the groups (Figure 4). This analysis has not shown a significant difference between the groups, with a mean difference of 0.74 (CI: −0.70 - 0.21, p = 0.30).

AMACR: Results were asserted in 19 patients (Figure 5), with a mean of (0.74 ± 0.46) AU (range 0.30 - 2.40). Correlation between AMACR and pathologic stage

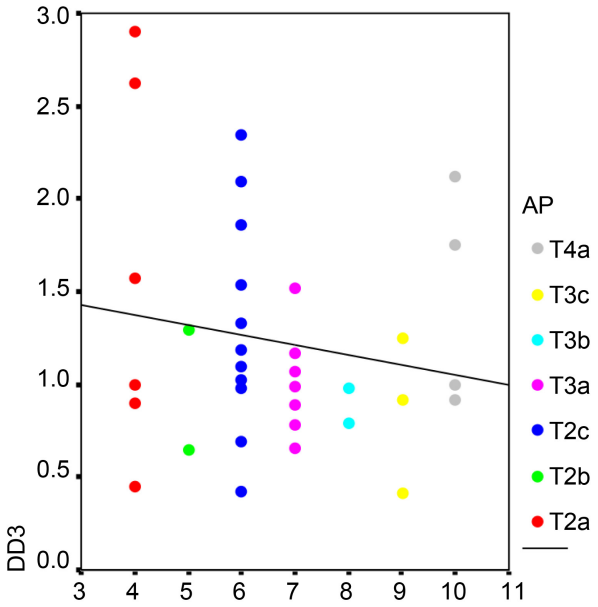


**Figure 3.** The scatter of gene expression. DD3: gene expression of DD3 in Arbitrary Units (AU). Homogeneous dispersion is seen among the pathologic stages.

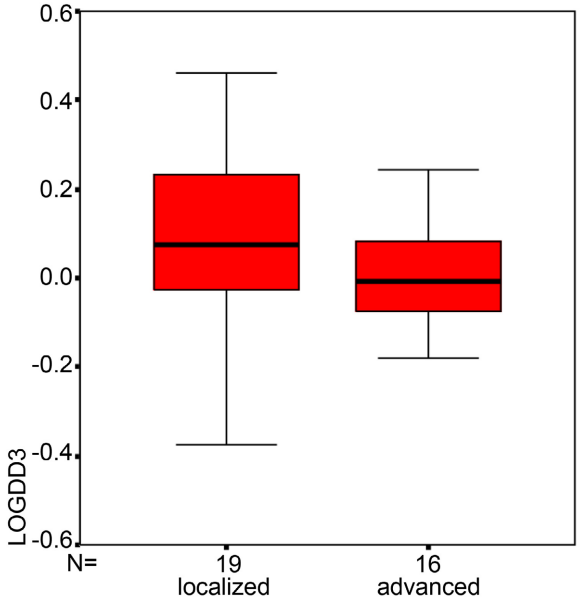


**Figure 4.** DD3 distribution between localized and advanced cancer. LOGDD3: Logarithmic expression of DD3 in AU. Negative values are seen due to the logarithmic transformation.

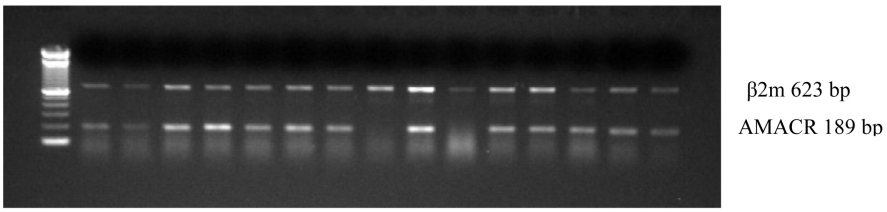


**Figure 5.** Agarose 2% gel representing AMACR bands (179 bp) and β2m (623 bp) in PCa samples.

again was not significant ($\rho = 0.16$, p = 0.49) (Figure 6).

AMACR gene expression in localized cancer patients had a mean of 0.61 ± 0.30. The mean in advanced disease was 0.81 ± 0.54. Statistical difference between the groups was not significant (mean difference 0.11, CI: −0.32 - 0.10, p = 0.28) (Figure 7).

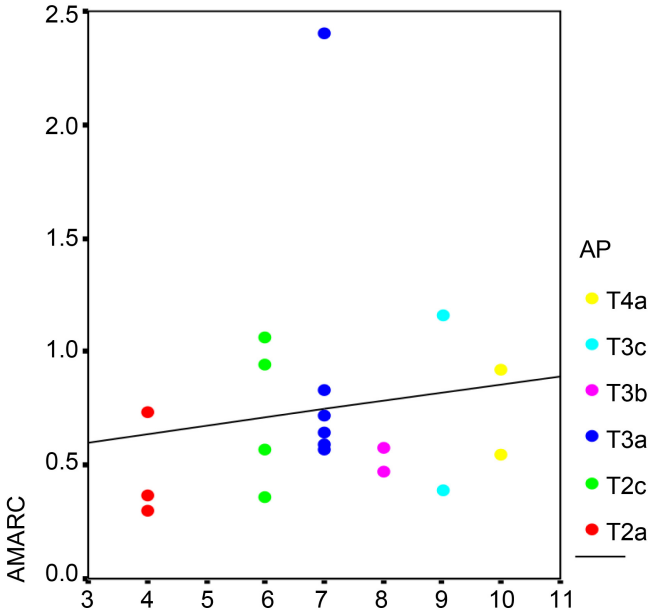


Figure 6. The scatter of gene expression. AMARC: gene expression of racemase in AU. Again, there is homogeneous dispersion among the pathological stages.

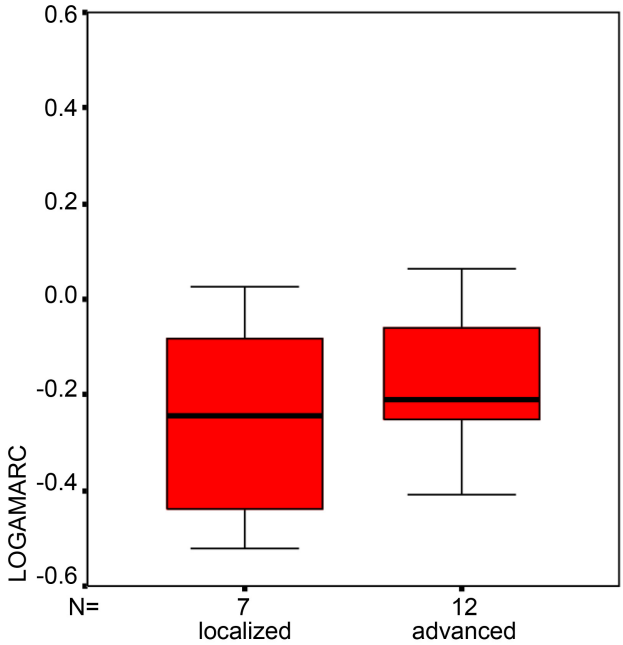


Figure 7. AMACR distribution between localized and advanced cancer. LOGAMARC: Logarithmic expression of racemase in AU. Negative values are seen due to the logarithmic transformation.

## 4. Conclusions

Many studies are being conducted on new markers of prostatic diseases. Due to its high incidence, any progress in PCa could have an important population impact in terms of survival, quality of life, and costs to the public health system. There are very few studies that compare new markers' gene expression with pathological staging. Our sample correlated DD3 and AMACR expression among PCa stages and compared the distributions of the genes in localized and advanced PCa groups.

In our study, the PSA levels were < 10.00 ng/ml in most cases. However, two patients had levels higher than 60.00 ng/ml, deviating the mean to 10.66 ng/ml. The advanced prostate cancer stages were higher than reported in the literature, and this may be in part due to the difficulty for the patient to access the public health system in Brazil, leading to a time delay in treatment. The high level of cases of bilateral cancer in the biopsy supports these findings. Amling *et al.* described a 2782-patient analysis with 68% localized prostate cancer and 32% advanced cancer [21]. Pettus *et al.* reported 800 RPP cases, 62.25% being localized [22].

Recent advances in molecular biology are promising, as genetics and clinical medicine are increasingly interrelated. The DD3 is being studied as a new marker with greater specificity, but few papers have compared its expression among the prostate cancer pathologic stages. Our sample has shown an equal distribution of DD3 in the different stages of prostate cancer. Taskén *et al.* cite DD3 and AMACR as new potential markers for prostate cancer, but they didn't describe their clinical use [23]. Schemk-Braat *et al.* also performed several new markers (DD3 among them), but they didn't evaluate their clinical use [24]. Tao *et al.* have shown a higher DD3 expression in prostate cancer compared to non-prostate cancer patients, yet DD3 was not compared with the pathologic stage [25]. Bialkowska-Hobranska *et al.* analyzed retrospectively DD3 expression in 26 patients without pathological stage correlation [26].

AMACR also does not correlate with pathologic staging, as its distribution is similar in several stages. The gene expression of AMACR between localized versus advanced cancer also showed no significant difference. Zielie *et al.* described the AMARC's potential role in using it with PSA in prostatic secretions as an auxiliary method in diagnosing prostate cancer [27]. Stewart *et al.* analyzed the utility of AMACR in further biopsies of intra-epithelial neoplasia of the prostate, not describing AMACR as a prognostic marker [28]. A 4.8-year cohort (Rubin *et al.*) has shown higher cancer recurrence when the patients had low expression of AMACR, suggesting that the higher expression of the gene could be linked to a better prognosis, although this study does not report stage-based differences [29].

Our study has described the expression of DD3 and AMACR in patients submitted to RRP in Hospital de Clínicas de Porto Alegre. The results showed that there was no difference in gene expression in the pathologic stages. According to this study, both DD3 and AMACR have no association with tumor stage.

In our final conclusions, we would like to address the fact that our sample was relatively small, which could affect statistical power and generalizability. This is a

limitation of this particular study.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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