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**CLINICAL SCIENCE**


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**PROSTASIN, A POTENTIAL TUMOR MARKER IN OVARIAN CANCER- A PILOT STUDY**

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doi: 10.1590/S1807-59322009000700006

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Costa FP, Batista Junior EL, Zelmanowicz A, Svedman C, Devenz G, Alves S, da Silva ASM, Garicochea B. Prostasin, a potential tumor marker in ovarian cancer- a pilot study. Clinics. 2009;64(7): 641-4.

**INTRODUCTION:** Ovarian cancer is generally diagnosed at advanced stages of the disease; therefore, poor prognoses are typical. The development of tumor markers is thus of utmost importance. Prostasin is a protease that in normal tissues is highly expressed only in the prostate gland and seminal fluid. A previous study showed that prostasin is highly overexpressed in ovarian cancer cell lines. This study sought to evaluate the expression of prostasin in ovarian cancer.

**METHODS:** Fresh tumor samples of ovarian epithelial cancer (n: 12) were analyzed for expression of prostasin mRNA (messenger ribonucleic acid) by conventional and real time quantitative PCR (polymerase chain reaction). As a standard control, a normal prostate sample was analyzed.

**RESULTS:** Using conventional PCR, prostasin was detected in all but one sample. Using quantitative PCR, prostasin was overexpressed in all but one of the samples as compared to the control (prostate).

**CONCLUSIONS:** These findings indicate that prostasin is overexpressed in many epithelial ovarian cancers. Further studies of prostasin as a potential biomarker for this disease are warranted.

**KEYWORDS:** Biomarker; Screening; Diagnostic; PRSS8; Detection.

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**INTRODUCTION**

Ovarian cancer is the third most common cause of cancer-related death in women worldwide, and in many parts of the world ovarian cancer is the most common cause of gynecological cancer-related mortality.<sup>1,2</sup>

The dismal prognosis associated with ovarian cancer is mainly due to the fact that the majority of patients are diagnosed in the advanced stages of the disease. In earlier

stages, the 5-year overall survival rate is around 90%, whereas in more advanced stages it is below 30%.<sup>3</sup> There is thus a need for early detection strategies in order to increase the number of patients who can be diagnosed during the curable stages of the disease.

At present, tumor markers for early ovarian cancer with sufficient sensitivity and specificity for screening are not available.<sup>4-6</sup> However, during the last few years, several promising candidate proteins have been identified.<sup>7-10</sup> Although many proteins, alone or in combination, show promise as potential tumor markers, much work remains in order to validate such findings. Prostasin has been demonstrated to be overexpressed in ovarian cancer cell lines and has been detected in the serum of ovarian cancer patients at levels significantly higher than that of normal controls.<sup>11</sup> In this study, we investigated the expression of prostasin mRNA in ovarian cancer in order to further corroborate these findings and to generate more data on prostasin as a potential biomarker in ovarian cancer.

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Received for publication on February 16, 2009

Accepted for publication on April 13, 2009

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## METHODS

From September 2003 to September 2005, fresh tumor samples were collected from 30 patients undergoing abdominal surgery for ovarian tumor diagnosis and treatment in the following hospitals located in Porto Alegre, Brazil: Hospital São Lucas, Hospital Fêmina and Hospital Conceição. Eligibility criteria included no previous cancer diagnosis or treatment. All patients signed informed consent before the surgery. This study was approved by the Ethics Committees of each hospital mentioned above.

The specimens were collected during the surgical procedures for ovarian resection or biopsy and stored at  $-70^{\circ}\text{C}$ . Samples from benign cysts and non-epithelial ovarian cancers were not used. Normal prostatic tissue was used as a control and as a physiological reference for prostasin expression. The study was carried out in accordance with the ethical standards of the local ethics and scientific committees and the Helsinki Declaration.

### Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using TRIZOL<sup>®</sup> reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

The cDNA was generated by using SuperScript II Rnase H<sup>-</sup> reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA). The total mixture contained RNA, oligo dT,  $\text{MgCl}_2$ , enzyme buffer, dithiothreitol, dideoxynucleotides, SuperScript II and RNase inhibitor (RNase Out<sup>™</sup> Ribonuclease Inhibitor; Invitrogen Life Technologies, Carlsbad, CA, USA). One tube containing RNA, dideoxynucleotides and oligo dT was initially incubated at  $65^{\circ}\text{C}$  for 5 minutes in a thermocycler (iCycler, BioRad, Hercules, CA, USA) and placed on ice for one minute. After that, the reverse transcription reaction components cited above were added and incubated at  $42^{\circ}\text{C}$  for 50 minutes. The mixture was heated at  $70^{\circ}\text{C}$  for 15 minutes and denatured in ice for 1 minute. Finally, RNase H was added to the mixture, which was incubated at  $37^{\circ}\text{C}$  for 20 minutes. The cDNA was stored at  $-20^{\circ}\text{C}$ .

The RT-PCR reaction was performed by using primer sets specific for prostasin (forward primer: 5'-ACTTGAGCCACTCCTTCCTTCAG-3'; reverse primer: 5'-CTGATGGTCCCAAAAAGCACAC-3')<sup>11</sup> and the housekeeping gene  $\beta$ -globin (forward primer: 5'-GAAGAGCCAAGGACAGGTAC-3'; reverse primer 5'-CAACTTCATCCACTGTCACC-3' (268 base pair). The total mixture for the PCR contained 2.5  $\mu\text{l}$  of cDNA, 10 pmol of each primer, 200 pmol of dideoxynucleosides, 1.5

mM of  $\text{MgCl}_2$ , 10X enzyme buffer, 2 U of Taq Polymerase and deionized water. PCR amplification in an automatic thermocycler (MJ Research PT150CA, USA) was performed with 31 cycles at  $96^{\circ}\text{C}$  for 1 minute,  $60^{\circ}\text{C}$  for 1 minute and  $72^{\circ}\text{C}$  for 10 minutes. A mixture without DNA was used as a negative control to rule out contamination, and the amplification result was verified using 3.5% agarose gel electrophoresis and an ultraviolet transilluminator (201 Macrovue Transluminator LKB, Bromma, Sweden).

### Real-Time RT-PCR

To evaluate the relative expression of the prostasin gene in the tumor samples, the quantitative RT-PCR technique was performed. Reverse transcription of the total RNA was carried out with random hexamers, reverse transcriptase,  $\text{MgCl}_2$ , and dideoxynucleotides. For real-time assessment of prostasin gene expression, Taqman chemistry was employed; an amplification reaction mix was generated that contained Taq enzyme buffer, dideoxynucleotides, Hot-Start Taq DNA polymerase (AmpliTaq, Applied Biosystems, Foster, CA, USA) and  $\text{MgCl}_2$ , according to the manufacturer's instructions. Fifty nanograms of cDNA was added to this mixture in addition to the specific human prostasin or  $\beta$ -actin primers/probes assembled in separate wells (Assays-on-Demand<sup>®</sup>, Applied Biosystems, Foster, CA, USA). The probes were labeled either with FAM or VIC fluorophores. The cDNAs were then amplified by denaturation for 10 minutes at  $95^{\circ}\text{C}$ , followed by 50 two-step cycles of denaturation at  $95^{\circ}\text{C}$  for 15 seconds and annealing-extension at  $60^{\circ}\text{C}$  for 60 seconds. The PCR efficiency was examined by serially diluting the template cDNA (100 ng, 50 ng, 25 ng, 12.5 ng and 0 ng). The  $C_t$  values and concentrations of the serial dilutions were plotted, and linear regression analysis was used to obtain the determination coefficient ( $r^2$ ) of the reaction; the latter should be at least 98% to enable relative expression analysis using the  $\Delta\Delta C_t$  Method. Each cDNA was amplified in triplicate, and a no-RT mRNA sample was included in every plate for background correction.<sup>12</sup> For each test sample, prostasin and  $\beta$ -actin were amplified in parallel.  $\beta$ -actin was used to normalize the quantity of RNA and to adjust the efficiency of the RT reaction. The changes in fluorescence for every cycle were monitored in a thermocycler (ABI7500, Applied Biosystems, Foster, CA, USA), and the relative expression was calculated as described by Livak et al.<sup>13</sup> The relative amount of PCR product generated from each primer/probe set was determined on the basis of the  $C_t$  value. The  $C_t$  value was then subtracted from that of each target gene to obtain a  $\Delta C_t$  value. The difference between the  $\Delta C_t$  values of the samples for the target gene and the  $C_t$  value of a calibrator

(prostate) was determined. The relative quantitative value was expressed as  $2^{-\Delta\Delta CT}$ . Therefore, the prostasin expression for each sample was presented as the fold change relative to the calibrator tissue, in this case prostate.

## RESULTS

### Patient characteristics

In total, 30 patients were screened, and 12 were included. Among the 18 excluded samples, 10 were benign cysts, 3 were non-epithelial ovarian cancers (thecoma, metastatic melanoma and Krukenberg tumor) and the quality of the RNA in 5 of the samples was not sufficient for analysis. Of the 12 patients included, 90% had advanced disease (clinical stage III and IV). The mean age was 61.2 years (29-83).

### RT-PCR

Positive RT-PCR results for prostasin were obtained in 11 of the 12 samples.

### Real-time RT-PCR. Efficiency and validation calculation

As explained above, the PCR efficiency was examined by serial dilution by a factor of 2. In this case, a difference of 1 amplification cycle ( $C_T$ ) should be observed. This was exactly the result we obtained, generating determination coefficients of 0.9902 and 0.9985.

### Real-time RT-PCR

The prostate gland is the human tissue with the highest prostasin expression. Therefore, prostate tissue was used as a control and as a physiological reference. All but one of the epithelial samples showed a significant relative increase in prostasin expression. The highest level was 3.30-fold, higher and the mean was about 2-fold higher than the control.

## DISCUSSION

Prostasin was first identified in 1994 when Yu et al. isolated it from seminal fluid and demonstrated localization in epithelial cells and the ducts of the prostate using radioimmunoassay and immunohistochemical techniques.<sup>14</sup>

Prostasin is a 40-kDa trypsin-like proteinase.<sup>14</sup> The catalytic triad that is essential for the enzymatic activity of prostasin is a histidine, aspartic acid, and serine sequence. The sequences around the active sites of serine proteinases are highly conserved.<sup>15</sup>

Although the biological and physiological roles of prostasin are not known at present, the high levels of prostasin in the prostate and seminal fluid (over 20-fold higher than any other tissue examined) suggest it may play an important physiological role in these locations. Other data also show that prostasin is present at low levels in other tissues, such as the lung, kidney, liver, bronchi, colon and salivary glands, indicating that it may have roles in other biological processes as well.<sup>14</sup> In normal ovarian tissue, prostasin is not highly expressed.<sup>11,15</sup>

Prostasin's role in cancer is unclear, but there is evidence indicating that it may be related to tumor promoter mechanisms,<sup>16</sup> as well as tumor suppressor mechanisms.<sup>17-19</sup> Recently, other data has also shown that prostasin is involved in proteolytic cleavage of the extracellular domain of EGFR (epithelial growth factor receptor), causing a constitutively phosphorylated receptor<sup>20</sup> that could potentially participate in fueling tumor growth.

Prostasin was first studied as a potential marker for prostate cancer by Laribi et al. They found that all 86 of their blood samples from normal controls failed to amplify prostasin PCR products, whereas blood samples from 35 of 96 (36%) prostate cancer patients were positive. Of the metastatic patients, 63% (17/27) were positive, but only 26% (18/69) of patients with local tumors were positive.<sup>20</sup>

The potential use of prostasin as a marker of ovarian cancer has been suggested in a study demonstrating that prostasin mRNA expression ranged from 120- to 410-fold higher in ovarian cancer cell lines than normal ovarian cell lines. The abnormal expression of prostasin in these cell lines was further confirmed at the protein level by immunostaining. Since the expression of prostasin was so markedly different between normal and malignant ovarian cells, the serum prostasin levels were investigated in patients with and without ovarian cancer. The prostasin levels were higher in the serum of ovarian cancer patients than in controls. Moreover, the serum prostasin levels were significantly reduced after surgery in the vast majority of the cases studied.<sup>11</sup>

Our study is the first to perform quantification of mRNA prostasin expression in fresh-frozen ovarian cancer tissue samples. The data corroborate that prostasin is overexpressed in many ovarian cancers and that expression is significantly higher in malignant than normal tissue (control). Thus, further studies exploring the use of prostasin as a potential biomarker in ovarian cancer are warranted.

## ACKNOWLEDGEMENTS

We thank Conselho Nacional de Pesquisa (CNPq) for supporting this experiment.

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