Hepsin overexpression predictive factor in evolution of prostate cancer

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Abstract

Purpose: Other c DNA microarray studies have shown that hepsin is one of the highly over expressed genes in prostate cancer tissue compared with normal and benign prostatic hyperplasia tissue. We quantitatively analysed hepsin gene expression with real-time polymerase chain reaction and calculated its relationships with clinical and pathological parameters in a large cohort of samples.

Materials and Method: Matched prostate tissue samples from the cancerous and non cancerous parts of the same prostates were obtained from 38 patients with prostate cancer who underwent transurethral prostate resection. Quantitative reverse transcriptase-polymerase chain reaction was performed using Light Cycler Fast Start DNA Master SYBR Green I on a Light Cycler system. The ratio of hepsin /beta-actin (a housekeeping gene) was used to normalize data.

Results: Hepsin over expression in cancerous compared with non cancerous tissue was found in 28 of the 30 patient samples (90%, p =0.001). In 28 patients hepsin over expression was 10 to 100 times in cancerous tissue than in benign tissue. Absolute values of hepsin, without ratio hepsin/beta-actine, have shown in 23 cases enrolled overexpression of hepsin in cancer tissue and also lower value in 8 of them (p<0.001). For the prognosis a cut off at the 75th percentile provided a significant difference between patients at lower risk (pT2 and Gleason score less than 7) and higher risk (pT3/4 and Gleason score 7 or greater).

Conclusions: Hepsin expression may be in our opinion used for assessing prostate cancer aggressiveness and so for improvement or generating predictive models.

Key words: prostate cancer, hepsin, serine, tumour markers, prognostic, predictive models

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Introduction

Prostate cancer is currently recognized as one of the major health problems affecting the male population. In Europe are present estimated, about 260,000 new cases annually diagnosed. Prostate cancer constitutes about 11% of all male cancers in Europe and is responsible for 9% of all cancer deaths among men in the EU \[6, 10\].

The involvement of genetic factors in the occurrence of prostate cancer is supported by several observations. Thus, men in Nigeria also present an incidence of this disease similar to African Americans not just from the United States but from the entire American continent (even from countries with a less good economical situation as Jamaica, for example, where the incidence of prostate cancer rises to 300/100.000 \[5,8,10\].

The appearance of bioumoral diagnosis of prostate cancer with use of PSA has increased the number of men diagnosed but it also led to the reduction of the average age at the time of diagnosis. This meant that, during these years, prostate cancer becomes a disease of middle-aged men \[3.10\]. Another advantage of PSA was assessing the possibility of early diagnosis. Thus, looking to overall incidence of this disease, an increasingly important place is occupied by local and regional stages instead of metastatic stages. In the same matter the number of those who could be treated radically increased, thus increasing the number of radical prostatectomies in the United States, from 7/100,000 in 1983 to 32/100,000 in 1992, reported to patients with localized stage of the disease \[2, 4, 5, 9\].

PSA is the best available tumor marker. However, this marker has no specificity due to the increase levels in benign prostate tumors. Molecular forms of PSA2, 3 and another member of the human tissue belonging to the kalikreine family of human glandular tissue can increase specificity \[6.9\]. Although in recent years there have been proposed sets of biomarkers/gene none of them can predict the possible progression of the disease \[1,10\]. The pathological stage, tumor volum, resection margin status, and Gleason score, are important factors of prognosis for biochemical failure after radical prostatectomy but adding the database diagrams of new biomarkers can accurately preclassify the condition leading to an optimisation of the financial and human allocated resources \[7,12\]. Recent studies that have used as method of working DNA microarray have identified the over expression of certain compounds by proteomic and genomic level in prostate cancer tissue compared to benign prostate tissue \[13\].

Some of these studies have shown that hepsin is a cell surface serine protease which is highly expressed in human prostate cancer; however, the functional significance of this phenomenon is unknown and this over expression in prostate epithelium in vivo causes disorganization of the basement membrane \[14\].

One of these studies, which focused mainly on the degree of tumor aggressiveness Gleason 4 and 5 revealed an hepsin over expression in tumor tissues of 3-4 times higher \[1,10\]. This observation implies hepsin possible role in prostate cancer development and progression. We investigated the hepsin expression in malignant and benign prostate tissues by measuring (RT-PCR) reaction. We also measured the hepsin expression to examine possible relationships with stage, Gleason score and other clinical parameters.

Materials and method

A. Population study.

During March 2012-September 2012 were prospectively included in the study a group of 49 patients aged between 47 and 78 years. Patients were admitted in Center for Uronephrology and Renal Transplantation Fundeni for clinical suspicion, biochemical and /or ultrasound of a prostate tumor assessed and complete / incomplete retention of urine. After signed the informed consent, the patients followed standard procedure, meaning transrectal ultrasound evaluation and biopsy with standard collection of peripheral 12 cores. For those 49 patients were generated the following inclusion and exclusion criteria:

Inclusion criteria

1. Patient age less than 80 years
2. The patient signed informed consent before any procedure
3. Total prostate volume measured through transrectal ultrasound is under 80cm3
4. Complete or incomplete urine retention with residue over 100ml
5. Prostate adenocarcinoma, classified Gleason
6. Identifiable lesion TRUS / MRI with contrast
7. Clinical staging and imaging
8. Normal ranges of coagulation samples

Exclusion criteria

1. Impure adenocarcinoma
2. Other histological types of prostatic tumor
3. Haematological disorders
4. Immunological disorders
5. Acute hepatitis, chronic hepatitis B, C, HIV and syphilis
6. Allergies, atopic area
7. Symptoms of urethritis or cystitis
8. Systemic bacterial infections or urinary tract materialized through bacteriuria, bacteremia or through increases of PCR values
9. Patients with neuro psychiatric disorders
10. Patients with cardiac disease leading to surgical contraindication
11. The patients are not able to understand and follow the procedures
12. Patients who in the opinion of anesthetic and surgical team are not eligible
13. Patients physician addicted, institution, or institutionalized patients
14. Treatment with 5-alpha reductase inhibitors

According to the mentioned criteria, from the proposed study group, were selected 38 patients. For the documented obstructive syndrome, was performed according to standard procedures, (TURP) transurethral resection of prostate / monopolar / 3 principles / same surgeon / spinal anesthesia. We collected fragments containing neoplastic prostatic tissue and benign prostatic tissue. Each fragment of neoplastic tissue was sent to be prepared for PCR after histological verification of eliminating the impure fragments (fragments containing more than 20% benign tissue). In the same manner were selected the chips distributed on the arm with benign tissue. In this way, 7 patients were excluded. Evaluation of patients on tumor stage was based on information obtained through clinical and imagistic examination, MRI pelvic and TRUS.

B. Temperature steps of standard PCR reaction

Initial distortion

For a PCR reaction be carried out efficiently, it is very important that mold DNA molecules to be initial completely denatured. This can be achieved by heating the reaction mixture to 94–95°C for 2 minutes. Usually a distortion of 20-30 sec at 94-95°C is sufficient, but this one must be adapted according to the used tubes and thermocycler (for example if working in 500 µl 500 ml tubes longer times are required for, than if working in 200 µl tubes). However, for GC-rich matrix are recommended longer distortion times.

Primers attachment

In the vast majority of experiments, the temperature of primers attachment must be determined and optimized empirically. Choosing this temperature is one of the critical factors to ensure high specificity of a PCR reaction. If the attachment temperature is too high, there is no attachment of the primers, and if the temperature is too low, it increases the likelihood of non-specific attachment.

The Polymerization

Taq DNA polymerizes about 60 bases per second at 72°C. In each cycle, a period of 45 seconds is more than sufficient for some fragments amplification of up to 1 kbp. For the amplification of fragments larger than 1 kbp, time is calculated in multiples of 45 seconds, followed by adjustments for various matrix.

In order to get a higher amount of amplicon, the time for polymerization may vary as follows: for the first 10 cycles it will be use a constant time of polymerization (for example, 45 seconds for 1 kbp), and for the following 20 cycles the time for polymerization increases with 2-5 seconds per cycle (for example, 50 seconds for 11 cycle, 55 seconds for 12 cycle and so on.). Such a progressive increase of the polymerization’ time gives enzyme over time for polymerize, because while the PCR reaction progresses, in the reaction mixture it will be more matrix and the less active enzyme (enzyme yield decreases due to prolonged exposure to high temperatures).

Number of cycles

In a typical PCR reaction, less then 10 matrix molecules can be amplified in less than 40 cycles to give a product (amplicon) detectable in agarose gel electrophoresis. To an high increase of cycles number it may be accumulated on-specific reaction products.

Final Polymerization (extension)

In more reactions, after the last amplification cycle, the reaction tubes are kept at 72°C for 5 to 15 minutes to achieve complete polymerization of the partial products, as well as the renaturaration of single-stranded molecules.

Amplification plateau

A PCR reaction of DNA amplification in vitro is not infinite so that after a certain number of cycles, the amplified sequence is no longer accumulating exponentially, and the reaction enter in a linear phase (stationary) called the amplification plateau.
**Graphical representation of the PCR generated cycles**


**C. Preparation of tissue / primary design.**

Fresh prostate tissue samples were obtained from tumor and non-tumor tissue of the resected specimens. Small pieces of tissue were dissected by a pathologist immediately after prostate removal, then frozen and stored in liquid nitrogen until analysis. Only tumors that were fully surrounded by malignant tissue as described by analyzer were used in this study. We have not included any sample in which benign prostatic glands have made up more than 10% from the total mass. In this way contamination of tumor samples with benign glands was reduced to a minimum. Most of the tumors were located in dorsal and lateral zone of prostate.

The tissue, characterized as normal have usually been taken from the inside of the contralateral lobe. The tissues were pulverized with a hammer and placed in liquid nitrogen. RNA was extracted using an RNeasy kit according to the manufacturer’s instructions.

RNA concentration was determined spectrophotometrically. Total RNA (2 microg) was reverse transcribed into cDNA using the first component of Superscript II, a system used for pre-amplification (Gibco BRL, Gaithersburg, Maryland). Primers used for hepsin gene were 5'-GGACCCCAACAGCGAGGAGAAC-3' antisense respectively 5'-ACAATGAGCTGCTGCGTGTGGCT-3'.

Primers for beta-actin were 5'-ACAATGAGCTGCTGCGTGTGGCT-3' antisense respectively 5'-TCCTCCTTAATGTCACGCACGA-3'.

For each sample, the target value and endogenous control (beta-actin) were determined using a calibration curve. Molecular target quantity (hepsin) was then divided to the amount of beta-actin to obtain a normal value. All determinations were performed in duplicate.

Since each value of hepsin has been reported to control value (beta-actin), this report has been used as an aggregate of hepsin expression. Standard calibration curves separated for beta-actin and hepsin, were built using repeated dilutions of cDNA collected from prostatic tissue for beta-actin or dilutions in plasmids series for hepsin. Plasmid for hepsin was prepared in accordance with the manufacturer’s instructions, with a TOPOTA Cloning kit (Invitrogen, Carlsbad, California). Standard curves of dilutions have been included in each term. Light Cycler Software have automatically calculated the standard curve by graphical representation of the dilution start for each standard sample of the cycle. Samples concentrations were then calculated as consequence. Standards for hepsin and beta-actin RNA were destined to follow an arbitrary starting concentration and 5 dilutions in 10 series, each of them with definitely concentrations depending on the dilution factor. They were used to build the standard curve.

**D. Statistical evaluation.**

Statistical analyses were performed using the MedCalc version 12.5.0

**Results**

There have been studied the associations between clinical and pathological parameters, grading tumor stage, Gleason score, PSA, free PSA and hepsin expression. Hepsin was overexpressed in approximately 90% of neoplastic tissue. In 28 cases hepsin expression was between 10 and 100 times greater in tumor tissue compared to the non-tumoral tissue. Absolute values of hepsin without correcting with beta-actin showed similarly hepsin overexpression resulted in tumor side at 23 patients included in the study and lower expression in 8 cases (p < 0.001).

We considered all patients as a single group. Those 31 patients had an average age of 63 years, an average of preoperative PSA of 17.9 ng/ml, an average of free PSA of 7.7% and a medium prostate volume of 39 ml. PSA serum (Spearman rank correlation coefficient rs = 0.074, p = 0.28), the percentage of free PSA (rs = 0.76, p = 0.003), prostate volume (rs = 0p = 0.35), age (rs = 0.019, p = 0.78) and Gleason score (rs = 0.114, p = 0.33); they were not correlated with hepsin expression.

In case of evaluating tumor stage on note that hepsin expression was higher at patients in the stages pT3 and PT4 versus pT2, showing a trend towards significance. (p = 0.13). Hepsin expression was higher in patients with adenocarcinoma score Gleason 7 or higher compared to those with Gleason score under 7 but there was not still significant (p = 0.20).

Seeking a proper way to separate patients with high risk of those with low risk, we considered the threshold of positivity equal with percentage of 75 (hepsin expression threshold = 28.2). At this all parameters (stage, and Gleason score) were significantly dif-
different regarding high risk groups and low risk groups (table 3). Together, these data have demonstrated hepsin gene overexpression resulted in prostatic tumor tissue compared to the normal tissue of the same gland. Overexpression resulted has been associated with high grade tumors and stage tumors and was independent of PSA serum value.

**Table 1. Hepsin expression in prostate cancer – tumor stages assessment**

<table>
<thead>
<tr>
<th>Tumoral stage</th>
<th>Number of patients</th>
<th>Ratio hepsin/β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>12</td>
<td>8.3 p=0.13</td>
</tr>
<tr>
<td>T3-T4</td>
<td>19</td>
<td>16.2</td>
</tr>
</tbody>
</table>

**Table 2. Hepsin expression in prostate cancer – reporting to the Gleason score**

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>Number of patients</th>
<th>Ratio hepsin/β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 7</td>
<td>10</td>
<td>7.6 p=0.21</td>
</tr>
<tr>
<td>Over 7</td>
<td>21</td>
<td>15.1</td>
</tr>
</tbody>
</table>

**Table 3. Hepsin expression (as threshold) reported to tumor stage and Gleason score**

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Positive hepsin</th>
<th>Negative hepsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>T2</td>
<td>3</td>
</tr>
<tr>
<td>Stage</td>
<td>T3-T4</td>
<td>12</td>
</tr>
<tr>
<td>Gleason Score</td>
<td>Under 7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Over 7</td>
<td>13</td>
</tr>
</tbody>
</table>

**Discussion**

Hepsin is a type II cell surface serine protease, initially discovered as a DNA clone in the human liver. Hepsin gene encodes 417 monocatenare, proteins, with a molecular mass of 50 kDa [1, 2]. In vitro experiments have demonstrated hepsin involvement in maintaining cellular morphology, liver cell growth and blood clotting by activating the human factor VII [10,11]. The primary amino acid sequence of hepsinei is trypsin.

Hepsin as serin protease is present in different amounts in different tissues. It is abundantly expressed in liver, while lower levels are observed in the pancreas, testes, prostate, lung, thyroid, and pituitary gland. In immunohistochemical investigations using Polyclonal Antibodies, pure antihepsin and hepsin specific coloration, has been observed in 7 cases of renal cell with carcinoma, exclusively in the membranes of tumor cells, while in normal kidney tissue and other tissues, the coloration was negative. Hepsin overexpression was associated with ovarian cancer appearing in 27 of the 32 cases of ovarian carcinoma (84%) and in 7 of the 12 potential malignancies, while in normal ovarian tissue, never appeared [1, 3, 4, 9]. Hepsin overexpression in tumor cells is, according to the results of this study, in 90% of cases of prostate cancer. Our study used the quantitative qRT-PCR, which is quality superior, compared to techniques used in previous studies.

Searching for new potential tumor markers for prostate cancer has been accelerated using gene expression profile by DNA-microarray. In 2001, five studies have been published on this subject. The first study analyzed 4712 genes and showed that hepsin was the only gene overexpressed in all 11 cases malignant and unexpressed in 4 nonmalignant samples. Hepsin was also proven to be overexpressed in prostate intraepithelial neoplasia, compared with prostate benign hyperplasia tissue. It has shown a correlation of hepsin overexpression with prostate neoplastic transformations. [3,10] Structure and homology with other serin proteases involved a strong role for hepsin not only for promoting the growth of the tumor, but also for cancer therapy. Welsh et al. have evaluated 23 primary cancerous tissues and 9 nonmalignant tissues and have selected from 8920 genes, 400 genes with overexpression and specific expression in prostate cancer [1, 5, 11].

In addition to MIC -1, a member of the transformed growth factor superfamily, overexpressed in 21 from 24 samples, hepsin was overexpressed in all primary tumors. In another study, more than 5520 known genes and 4464 uncharacterized genes, were analyzed in 56 tissue specimens of copies distributed as follows: 13 benign prostatic tissue, 9 normal prostatic tissue and 36 localized and advanced prostate cancer and 3 cell lines. Immunohistochemical studies revealed hepsin overexpression resulted in prostate intraepithelial neoplastic lesions, followed by primary prostate cancer, hormono-resistant prostate cancer and benign prostatic tissue of the lowest degree [2, 8,14]. These remarks are in contrast to our results related to the hepsin expression.

We have found that, generally, hepsin overexpression has been associated with over 7 Gleason scores, advanced stages T3-T4, (tables 2 and 3), indicating a positive correlation between the hepsin expression and the probability of cancer progression.
From 6800 genes examined by Stamey et al. it was found a subset of 22 up-regulated genes and 64 down-regulated genes in benign prostatic tissue and in all cancerous prostate tissue samples. Because with Gleason score 4/5, the tumor was the strongest predictor of postoperative progression, and PSA was insufficient, the investigation focused solely on the Gleason score. Also in that study, the amount of hepsin was overexpressed with the 34-times greater in tumor tissue compared to benign tissue. Also, Stamey et al. have classified differential expression of genes on functional category and chromosomal localization [1, 10, 12, 13].

Hepsin over expression in prostate epithelium in vivo occurs basal membrane disruption. Hepsin over expression in a mouse model with induced non-metastasized prostate cancer, has no impact on cell proliferation, but induce the basal membrane disruption and promotes primary tumor progression and metastasis to the liver, lung and bone. There are evidence in vivo that intensification of hepsin activity, this serine protease in prostate primary tumor, promote cancer progression and metastasis [1]. RNA hepsinic growth was accompanied by an increase in the protein expression level.

It is unclear how hepsinic mRNA level is correlating with the various stages/levels of prostate cancer. Although initial studies have shown that the highest hepsin levels are found in prostate intraepithelial neoplasia (PIN) and they decrease with prostate cancer progression, recent studies have demonstrated that hepsinic mRNA levels increase with cancer progression and reach a maximum in the most aggressive prostate carcinomas (Gleason grade 4 and 5) [2, 9, 10]. The knowledge of central mechanism responsible for producing metastasis is important in developing effective therapies to combat cancer.

Our preliminary results show that hepsin overexpression produces basal membrane disorganization, so it probably acts in the initial stages of metastatic pathway. Future experiments will help us to determine hepsin proteolytic activity substrate which might be responsible for this function in the basal membrane disorganization. Type II serin proteases have been implicated in cancer progression. However, the evidence has been more likely circumstantial. Many of these proteins are overexpressed in prostate cancer. Because hepsin promotes prostate cancer progression, specific inhibition of hepsin proteolytic activity could be effective in blocking the progression of prostate cancer [1, 2, 3, 5].

Our study has a large number of investigated tumor samples, generated on this operative way and this allowed us to estimate the relationship between aggressive tumors and non-aggressive ones, as well as between pT2 and pT3 tumors. Final results confirm those earlier, demonstrating the hepsin expression in malign prostate tissues, to nearby nonmalignant tissues of the same glands. It is also noted that there is a hepsin overexpression in advanced tumors of stage T3-T4 and aggressive as Gleason score Gleason-7 or greater than 7.

Conclusions
The deficiencies of current serum markers for prostate cancer include their inability to predict the evolution of prostate cancer and biochemical PSA relapse rate. It is important to remember that studies that use DNA microarray technique (based on the gene expression), provides us with a lot of data. Light Cycler technology seems to be an excellent tool to investigate and confirm the differences in genes expression.

We believe that hepsin gene has important potential in diagnosis and prognostic and is a marker for prostate cancer. Hepsin expression can be an important biomarker to generate predictive models of prostate cancer evolution after the imposition of the radical visa treatment or paleativ, which can lead to an anticipation of the human and financial resources which are designed to be used.

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