DOCTORAL THESIS

ABSTRACT

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The role of angiogenesis and hormonal status in the progression of prostate adenocarcinomas

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## CONTENT

1. **INTRODUCTION**  

2. **THE CURRENT STATE OF KNOWLEDGE**  
   2.1. Epidemiology and risk factors in prostate adenocarcinomas  
   2.2. Pathogenic mechanisms in prostate carcinogenesis  
   2.3. Tumor angiogenesis in prostate carcinogenesis  
   2.4. Prostate adenocarcinoma and hormonal dependence  

3. **PERSONAL CONTRIBUTION**  
   3.1. WORKING HYPOTHESIS AND GENERAL OBJECTIVES  
   3.2. RESEARCH METHODOLOGY  
      3.2.1. Approaching the topic  
      3.2.2. Material and Method  
   3.3. RESULTS  
      3.3.1. Clinico-epidemiological study of prostate adenocarcinomas  
      3.3.2. Histopathological study of prostate adenocarcinomas  
      3.3.3. Immunohistochemical study of prostate adenocarcinomas  

4. **DISCUSSIONS**  
   4.1. DISCUSSIONS ON CLINICO-EPIDEMIOLOGICAL STUDY  
   4.2. DISCUSSIONS ON THE HISTOPATHOLOGICAL STUDY  
   4.3. DISCUSSIONS ON IMMUNOHISTOCHEMICAL STUDY  

5. **CONCLUSIONS**  

6. **BIBLIOGRAPHY**  

7. **ANNEX**  

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**KEY WORDS:** prostate adenocarcinoma, histopathology, immunohistochemistry, hormone receptors, angiogenesis, proliferation
INTRODUCTION

Prostate adenocarcinoma (PA) is one of the most common malignant neoplasms of men after the 5th decade of life. Although the lesions have a low mortality rate compared to other malignancies, this is especially true in the case of prostate localized disease. In the case of aggressive, metastatic or hormone-resistant tumors, the rates of morbidity and mortality increase and rank the lesions on the 5th place as the cause of death in men due to cancer. The introduction of prostate adenocarcinoma screening decreased the mortality rate, but had a negative impact on the overdiagnosis of lesions. Thus, the identification of aggressive prostate adenocarcinomas for effective antineoplastic therapy may attenuate the prognosis of patients.

Due to the hormonal dependence of PA, as well as the associated risk factors, incidence and prognosis of lesions, prostate tumors are an unique model for investigating carcinogenesis. The remodeling of the glandular architecture and the prostate stroma under the action of steroid hormones exerted through receptors seems to influence the proliferation rate. At the same time there are data indicating the dependence of prostate angiogenesis on tissue hormonal status. These biomolecular aspects of PA progression are relatively rarely in the literature.

The study proposes the complex integrated clinico-epidemiological, histopathological and immunohistochemical analysis of PA, by investigating the expression of proteins that are representative for angiogenesis, steroid receptor status and tumor proliferation, in relation to the aggressiveness parameters of the lesion. The results of the study may contribute to the identification of PA with aggressive biological behavior and to the improvement of the patients stratification criteria for the targeted and effective therapy.
1. THE CURRENT STATE OF KNOWLEDGE

The subchapter 2.1 summarizes the latest information on the epidemiological data of prostate adenocarcinomas (PA) regarding the incidence of lesions in relation to age, race, geographical distribution, mortality and survival rates. Data were also provided on the risk factors involved in age-related prostate carcinogenesis, steroid hormones, inflammation, genetics, or the environment.

In the subchapter 2.2, the pathogenic mechanisms involved in the genomic and phenotypic heterogeneity of PA have been described, as well as the mechanisms by which androgens, along with other transcription factors, altered tumor suppressor genes and epigenetic changes are involved in the initiation and progression of lesions.

In the subchapter 2.3, are described the classic aspects of angiogenesis regarding the role in tumor development, the types of molecular mechanisms and proangiogenic factors involved, the possible therapeutic implications, with the description of the particular aspects of the prostate.

In the subchapter 2.4, are provided information on the dependence of PA on the status of hormones and implied receptors, as well as the mechanisms by which the lesions become hormone-resistant.

3. PERSONAL CONTRIBUTION

3.1. WORKING HYPOTHESIS AND GENERAL OBJECTIVES

The working hypothesis of the study were:

- the existence of a complex associated clinico-epidemiological and histopathological profile of PA, which are decisive for the biological behavior of the lesions,
• the angiogenesis is involved in the progression of PA in a manner dependent on steroid receptor status,

• the presence of an angiogenic and hormonal immunohistochemical profile of PA, which can be associated with the clinico-pathological aggressiveness parameters of the lesions.

To test the first proposed hypothesis, a descriptive retrospective study was performed over a period of 4 years (2016-2019) and a prospective descriptive study over one year (2020), studies that were used for clinico-epidemiological and histopathological analysis. To test the proangiogenic and hormonal immunohistochemical profile of PA, we performed a semiquantitative and qualitative (descriptive) immunohistochemical analytical study on a selected group of cases.

The general objectives of the study were:

• Extending the knowledge related to clinical-epidemiological, histopathological, immunohistochemical factors involved in the prostate carcinogenesis, in order to deepen their mechanisms;

• Completion of morphological parameters to evaluate the diagnosis of prostate adenocarcinomas by possible identified molecular targets;

• Identification of angiogenic mechanisms that in a hormone-dependent context are involved in the acquisition of an aggressive phenotype of prostate adenocarcinomas;

• Identification of specific markers associated with hormone-dependent angiogenesis to quantify the aggressiveness of prostate adenocarcinomas;

3.2. RESEARCH METHODOLOGY

3.2.1. Approaching the topic

Prostate adenocarcinoma (PA) ranks second among malignant neoplasms worldwide in men, most cases being diagnosed after the age of 70 [3,16,17].
PA influences the quality of life of patients and the biological behavior of tumors is relatively difficult to assess, given that aggressive, metastatic and hormone-independent PA places lesions in 5th place as the cause of death in men [11,13]. In this context, the study of the biomolecular mechanisms involved in the progression of PA is a permanent concern.

3.2.2 Material and Method

The study is descriptive retrospective and prospective, with an analytical component on selected cases and used human material from 344 patients hospitalized, investigated and operated in the Urology Clinic of the Craiova County Emergency Clinical Hospital for a period of 5 years (2016 -2020). In order to carry out the study, the informed consent of the patients was obtained, and the ethical norms of scientific research were observed, with the agreement of the Research Ethics Commission within UMF Craiova (no. 63/ 14.07.2020).

*The clinical-epidemiological study* followed the age group, the associated risk factors, the symptomatology, the serum level of PSA and clinical imaging aspects.

*The histopathological study* (HP) used Hematoxylin-Eosin (HE) slides from the archives of the Pathology Laboratory of the Craiova County Emergency Clinical Hospital. The surgical pieces were processed by the usual paraffin embedding method and HE staining. In the HP study we looked at the aggressiveness parameters of the lesions represented by the tumor type, growth pattern, classic and simplified (grading groups) Gleason scores, the presence of perineural and vascular invasion. Also, the tumor extension represented by the pTNM (clinico-pathological) tumor stage and the prognostic groups were followed for the cases in which the prostatectomy was performed.

*The immunohistochemical* (IHC) study was performed on 61 cases of patients diagnosed with PA who underwent prostatectomy. The biological material was processed in the laboratory of the Morphopathology discipline
within UMF Craiova. The EnVision™ FLEX polymer amplification working system was used for the IHC study using the antibodies in the table below:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>External positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>AR441/Dako (mouse antihuman)</td>
<td>1:50</td>
<td>Tris/EDTA, pH 9</td>
<td>Normal prostate</td>
</tr>
<tr>
<td>ER (α)</td>
<td>1D5/Dako (mouse antihuman)</td>
<td>1:50</td>
<td>Tris/EDTA, pH 9</td>
<td>Mammary gland</td>
</tr>
<tr>
<td>PR (A/B)</td>
<td>PgR636/ Dako (mouse antihuman)</td>
<td>1:50</td>
<td>Tris/EDTA, pH 9</td>
<td>Mammary gland</td>
</tr>
<tr>
<td>VEGF (A)</td>
<td>VG1,Dako (mouse antihuman)</td>
<td>1:50</td>
<td>Tris/EDTA, pH 9</td>
<td>Kidney</td>
</tr>
<tr>
<td>CD105 (endoglin)</td>
<td>EP274/ABcam (rabbit antihuman)</td>
<td>1:100</td>
<td>Tris/EDTA, pH 9</td>
<td>Kidney</td>
</tr>
<tr>
<td>Ki67</td>
<td>MIB1/ Dako (mouse antihuman)</td>
<td>1:70</td>
<td>Citrate, pH 6.1</td>
<td>Lymph node</td>
</tr>
</tbody>
</table>

The panel of antibodies used

The quantification of the reactions was performed according to the data from the literature. Clinical-epidemiological, HP and IHC results were introduced into an electronic database and statistically analyzed using tests from the SPSS 10 software for social science statistics.

3.3. RESULTS

3.3.1. Clinico-epidemiological study of prostate adenocarcinomas

Most PAs were diagnosed in the last 3 years of the study (242 cases, 70.3%), with a maximum variation in the number of cases of 14.5%. In this study, the diagnostic age of PA was between 44-91 years, with a mean value of 72.7 ± 10.3 years, most cases belonged to patients> 70 years and the range of 70-79 years was the most affected. Risk factors for PA investigated were observed for 173 cases (50.3%), being represented by family history (7.8%), abnormal serum testosterone levels (3.8%), chronic inflammation (22.1%) or
associations (16.6%). Symptoms of patient presentation were frequently represented by pollakiuria / nocturia (24.7%), dysuria (13.7%), hematuria (8.1%) and pelvic discomfort (11.6%). Serum PSA levels most frequently showed values between 20-50 ng / ml (173 patients, 50.3%), followed by the group of associated serum PSA values of 11-19 ng / ml (25.3%), ≤ 10ng / ml (17.4%) and> 50 ng / ml (7%). The imaging aspects characteristic of patients with PA were represented by guided ultrasounds and multiparametric prostate nuclear magnetic resonance (MRI) examinations which were available in 70 cases and which allowed the PI-RADS 5 score to be established in 35 cases, PI-RADS 4 in 26 cases and PI-RADS 3 in 9 cases, the imaging-histopathological concordance being 60%.

3.3.2. Histopathological study of prostate adenocarcinomas

Architectural and cytological criteria for the diagnosis of PA were represented by the infiltrative pattern, rigid glandular lumens, absence of the basal layer, nucleoli prominence, cytoplasmic amphophilia, amorphous secretions. In most cases, various tumor types have associated areas of conventional acinar adenocarcinoma. Acinar (conventional) adenocarcinoma was present in 248 cases (72.1%) of the cases, which were followed in order of frequency by foamy cell type (9.9%), ductal (6.1 %), mucinous (4.4%), atrophic (2.3%), pseudohyperplastic (2%), sarcomatoid (2%) and with signet ring cells (1.2%) types. Pure tumor growth patterns were present in 208 cases (60.5%), while mixed patterns were identified in 136 cases (39.5%). The most common Gleason score was score 6, present in 107 cases (31.1%), the growth pattern being type 3. Gleason score 7 was identified in 66 cases (19.2%), Gleason score 8 in 78 cases (22.7%), Gleason score 9 in 36 cases (10.5%) and Gleason score 10 in 57 cases (16.6%). Compared to the simplified grading groups of PA, most cases belonged to group 1 (Gleson scores 6) present in 107 cases (31.1%). Analysis of growth patterns related to tumor type indicated statistically significant association of conventional, atrophic,
pseudohyperplastic and foamy cell types with pattern 3, ductal type with pattern 4 and mucinous, sarcomatoid and signet ring cell types with pattern 5. There were also statistically significant associations of ductal, mucinous, signet ring cell and sarcomatoid tumor types with increased Gleason grading and grading groups. *Perineural invasion* was present in 38.4% of cases and *vascular invasion* in 10.2% of cases, being associated in ductal, mucinous and sarcomatoid PA and grading groups 4 and 5. Most cases belonged to *stage II* (27 cases, 44.2%), followed by stage III (25 cases, 41%) and stage I (9 cases, 14.8%). Most PAs were included in *prognostic group* IIB (49.2%), followed frequently by group III (41%), and groups I / IIA (4.9%). Stage III and prognostic groups IIB / III were more frequently associated with ductal, mucinous, and sarcomatoid types, increased Gleason scores / grading groups, vascular, and perineural invasion.

### 3.3.3. Immunohistochemical study of prostate adenocarcinomas

*AR (androgen receptor)* immunoexpression was identified in all cases analyzed in the nucleus of the luminal cells of the tumor glands, the signal being present in the non-tumor glands and in stromal elements. The expression AR in relation to age indicated higher values in the groups <60 years and ≥ 80 years. Analysis of PSA values relative to AR expression indicated a tandem increase in tissue and serum mark values. We observed the frequent association of ductal, signet ring cells and sarcomatoid adenocarcinomas with AR scores 7-8. AR scores varied in relation to tumor growth patterns, pattern 3 being associated with scores 3-5 and patterns 4, 5 and mixed scores 6-8, statistically significant aspects. Gleason scores and increased grading groups also associated AR scores of 6-8, statistically significant issues. There were also significantly higher differences in AR scores with perineural invasion, vascular invasion, in stages II / III and in prognostic groups IIB / III.

*ER (estrogen receptor)* immunoexpression was absent in most cases in the tumor glandular epithelium. In 2 cases (3.3%) of ductal adenocarcinoma we
found focal nuclear staining in tumor cells. In 4 cases of acinar adenocarcinoma with growth patterns 4 and 5 we found a weak aberrant cytoplasmic expression of the marker. In 15 cases (24.6%), we found stromal nuclear staining. Benign / reactive glandular epithelia retained ER expression especially if they associated the presence of well-differentiated PA, with growth patterns 3 or 4. Both in the nontumoral glands and in the ducts, the ER expression was located in the luminal cells.

*PR (progesterone receptor)* expression was negative in most cases, with 3 cases of acinar PA with poor nuclear focal expression in rare tumor epithelial cells being identified. PR staining from the non-tumor glands and prostate ducts were focal. The PR reaction was also present in the stromal elements represented by lymphocytes and fibroblasts.

Analysis of *VEGF immunoexpression* (*vascular-endothelial growth factor*) indicated the presence of cytoplasmic reaction in 55 cases, which accounted for 90.1% of cases, the reaction being present in the tumor and inconsistent in vascular endothelium and stromal elements. We found high values of final staining score FSS VEGF in all categories being exclusive for the group <50 years, 80-89 years and ≥90 years. In the PSA groups ≥20ng / ml, the high values of FSS VEGF predominated, the aspects being statistically significant. In relation to the tumor type, the differences in FSS VEGF were at the limit of statistical significance. Analysis of tumor growth patterns indicated a significant association of high FSS VEGF with patterns 4, 5, and mixed, increased Gleason scores / grading groups, perineural and vascular invasion, stages II / III, and prognostic groups IIB and III.

Analysis of CD105 (endoglin) expression was identified in all cases analyzed in the cytoplasm of endothelial cells of neoformation tumor vessels. Regarding age, the mean values of MVD CD105 were higher for the categories <50 years, 80-89 years and ≥90 years. We found higher MVD values in the group with PSA 20-50 ng / ml and > 50ng / ml. MVD CD105 was different in
relation to the PA type, the highest values being identified in the case of ductal and sarcomatoid types. Compared to the tumor growth patterns, we found significantly higher values in the case of patterns 5, mixed and 4, Gleason scores 9-10 and grading groups 4 and 5. PAs that showed perineural invasion and vascular invasion associated significantly higher MVD values CD105. In the case of the tumor stage, we found significantly higher values of MVD CD105 in the case of advanced lesions, in stages II / III. Compared to the prognostic groups, the CD105 MVD values were significantly higher in the case of groups IIB / III, compared to groups I / IIA.

Ki67 immunolabels were identified in all cases analyzed in the nucleus of tumor cells and in rare stromal lymphocytes. The highest mean Ki67 PIs (proliferation index) were observed for the extreme age groups, respectively <50 years and ≥90 years, as well as in the case of PSA values of 20-50 ng / ml or> 50 ng / ml. The highest PI values were identified in the case of ductal, with signet ring cells and sarcomatoid types. Ki67 PI was superior in the case of patterns 4, mixed and 5, Gleason scores 9-10 and grading group 5. Comparative analysis of Ki67 immunoexpression for PAs that showed perineural and vascular invasion indicated a significantly higher mean PI value. Ki67 markings were higher in stage II / III tumors. Also, in terms of prognostic groups, the mean values of PI Ki67 were significantly higher for groups IIB / III.

Analysis of the mean percentage values of AR staining and mean values of MVD CD105 and PI Ki67 indicated a positive linear correlation of the three markers analyzed (p <0.001, Pearson test). Also, the percentage values of FSS VEGF were linearly positively correlated with the percentage values of AR and MVD CD105 values (p <0.001, Pearson test), as well as with PI Ki67 (p = 0.002, Pearson test).
4. DISCUSSIONS

4.1. DISCUSSIONS ON CLINICO-EPIEMIOLOGICAL STUDY

The rate of diagnosis of PA increases with age, especially after 50 years and with maximum incidences after 70 years, with an average age of diagnosis around 66 years [12]. Risk factors associated with PA are age, ethnicity, family history, hormonal disorders, and chronic inflammation [7,12]. PSA is a glycoprotein secreted by the prostatic epithelium and seminal vesicles, which is released under normal conditions in plasma in small quantities, growing both in malignant tumors and in many non-tumor conditions such as benign prostatic hyperplasia, prostatitis, perineal trauma, sexual activity, old age [6]. The symptoms of localized AP are reduced or absent, but occur in advanced and metastatic disease [8]. Currently, the multiparametric nuclear magnetic resonance examination, mpRMN, can identify malignant neoplastic lesions, through the PI-RADS score.

4.2. DISCUSSIONS ON HISTOPATHOLOGICAL STUDY

Histological types of prostate adenocarcinomas have prognostic and therapeutic significance. There are studies that have shown an excellent prognosis for atrophic, pseudohyperplastic, foamy cell and microcystic cell types, while signet ring cells, pleomorphic, sarcomatoid cell types are associated with a reserved prognosis [10]. The Gleason grading system is one of the oldest and most effective pathological and clinical tools successfully in clinical practice and in relation to patient prognosis [15]. In 2013, five simplified (prognostic) grading groups were proposed, which were subsequently recommended by the WHO and the American Joint Committee on Cancer (AJCC) [4]. An incidence of perineural invasion of 7-44% in untreated PA groups is indicated and is associated with increased Gleason scores / grading groups [18]. Vascular invasion is identified in broad ranges of 5-53% and is an important predictor for prognosis, although some studies indicate conflicting data [10]. By grade, tumor stage is the most important parameter for specific
recurrence and mortality of PA. The usefulness of *prognostic groups* for AP assessment is proven in studies that have shown positive effects of therapy associated with different stages of prognosis, the system being helpful to oncologists [10].

### 4.3. DISCUSSIONS ON IMMUNOHISTOCHEMICAL STUDY

**Hormone receptors.** There are some studies that have indicated the association of increased AR expression with imaging or histological aggressiveness markers such as Gleason score or tumor stage [9]. The involvement of estrogen receptors in vascular function remains controversial, but some studies indicate that ERα in endothelial cells has an inhibitory role in angiogenesis [1]. Regarding the expression of PR at the prostate level, there are studies in which the negativity of PR was found in most PAs, and the superiority of staining in benign prostatic epithelia [2].

**Angiogenesis** is a complex multistage process involved in the progression and survival of malignant tumors [14]. *VEGF*, respectively VEGF-A type, is one of the most important proangiogenic factors. At the prostate level, VEGF expression is also mediated by the androgen receptor and may involve the activation of oncogenes [14]. *Vascular microdensity (MVD)* is used to quantify the process of angiogenesis in general. Some studies have indicated that MVD is superior in prostate cancer compared to normal tissue or benign lesions and correlates with the prognostic histological parameters of PA [14].

**Tumor proliferation.** Ki67 was used in this study for the analysis of tumor proliferation, in the literature Ki67 immunoexpression being associated with tumor grade and / or tumor stage, some of these studies being performed on large groups of over 500 prostatectomies [5].
5. CONCLUSIONS

The study allowed the following conclusions:

- the mean age of diagnosis was 72.7 ± 10.3 years, 72.7% of patients being over ≥70 years.
- in 50.3% of cases risk factors were identified, and the most common symptomatology for PA was polakiuria / nocturia (24.7%).
- PSA serum levels were more commonly between 20-50 ng / ml (50.3%) and HP / PI-RADS concordance was 60%.
- HP analysis indicated the predominance of acinar PA type (72.1%), other types identified being with foamy cells, ductal, mucinous, atrophic, pseudohyperplastic, sarcomatoid, with signet ring cells.
- pure tumor growth pattern was observed in 60.5% of cases, and mixed pattern in 39.5%, the most common being pattern 3 in pure form (31.1%) or mixed (29%), followed by pattern 4 pure (12.8%) or mixed (29.7%) and pattern 5 pure (16.6%) or mixed (20.3%).
- the most common Gleason score was score 6 and compared to simplified grading groups most cases belonged to group 1 (31.1%).
- perineural invasion was present in 38.4% of cases and vascular invasion in 10.2% of cases.
- most PAs were classified as stage II (44.2%) disease, followed by PAs with extraprostatic extension stage III (41%); most PAs belonged to prognostic groups IIB (49.2%) or III (41%).
- AR immunoexpression was identified in all cases at the tumor level and in some stromal elements, with high AR scores being associated with ductal, signet ring cells and sarcomatoids PAs, increased PSA values, complex or mixed growth patterns, increased Gleason scores / grading groups with advanced stages and reserved prognosis groups.
- ER and PR reactions were absent in most investigated PAs, positive focal staining being present in 2 cases of ductal PA (ER) and 3 cases of acinar PA (PR); in 6.5% of cases ER stainings were present in the cytoplasm of tumor cells belonging to acinar PAs; both types of stainings were present in the non-tumor glands and prostate ducts and sometimes in the stromal elements.
- VEGF immunoexpression was identified in 90.1% of PA cases, being observed in vascular endothelium and stromal elements.
- high FSS VEGF values were associated with extreme ages, elevated PSA values, and HP aggressiveness parameters of PA.
- CD105 (endogline) expression was present in all cases in the cytoplasm of tumor vessel endothelial cells, with high mean MVD values associated with extreme ages, elevated PSA values, ductal and sarcomatoid types, and HP aggressiveness parameters of PA.
- Ki67 immunoexpression was identified in all cases in the nucleus of tumor cells and rare stromal lymphocytes, high PI Ki67 being associated with extreme ages, elevated values, ductal, with signet ring cells and sarcomatoid types and HP aggressiveness parameters of PA.
- the statistical analysis indicated significant correlations of AR, VEGF, CD105 and Ki67 expression, indicating the involvement and synergistic dependence of RA status, angiogenesis and tumor proliferation in the progression of PA.
- in our study most PAs presented an AR + / ER- / PR immunophenotype, the stromal staining suggesting the involvement of steroid hormones in stromal remodeling associated with angiogenic and tumor invasion processes.
- the statistical relations of the investigated markers with the HP aggressiveness parameters of PA, suggests their usefulness in identifying lesions with reserved evolutionary potential and in stratifying tumors to optimize the specific therapy.

13
6. BIBLIOGRAPHY


7. ANNEX

LIST OF PUBLICATIONS

