OPINION

by Assoc. Prof. Ekaterina Boyanova Softova-Zlatarova, MD, PhD,

specialty of Pathology and Cytopathology

SUBJECT: Dissertation of Dr. Lyuben Lyudmilov Stoev,

Doctoral student in full-time form of study in the doctoral program "Pathology and Cytopathology" at the Department of General and Clinical Pathology, Forensic Medicine and Deontology at the Medical University-Varna, for the award of the educational and scientific degree "DOCTOR" in the field of higher education 7. Medicine, Scientific specialty "Pathology and Cytopathology", code 03.01.03.

ON THE TOPIC: "EXPRESSION OF TETRASPANINE MARKERS IN BENIGN HYPERPLASIA AND PROSTATE CANCER"

Scientific supervisor: Prof. Maria Angelova Tsaneva, MD, PhD

With a decision from a meeting of the Faculty Council at the Faculty of Medicine at MU-Varna under protocol № 53 / 28.10.2021 and with order № P-109-469 / 05.11.2021 of the Rector of MU-Varna, I was elected an external member of the Scientific Jury, and on the basis of Protocol № 1 / 11.11.2021 I was appointed to prepare an opinion /in Bulgarian and English/ on the procedure for obtaining an educational and scientific degree "Doctor" with candidate Dr. Lyuben Lyudmilov Stoev at the Medical University of Varna.

As a doctoral student in full-time education in the doctoral program "Pathology and Cytopathology", professional field 7.1. Medicine, Dr. Lyuben Stoev was enrolled by order № P-109-52 / 01.02.2019 by the Rector of Mu-Varna. With report ent. № 102-2649 / 22.10.2021 and by Order of the Rector № R-109-469 / 05.11.2021 Dr. Stoev has the right of defense. The doctoral student has successfully passed the exam for the required doctoral minimum on 28.09.2020, and the foreign language exam on 25.02.2021. For the competition he has submitted on paper and electronic media a set of materials, including all necessary documents in accordance with the requirements of LDACRB, the Regulations for its application and ODAS in MU-Varna.

Brief biographical data and career development of the doctoral student.

Dr. Lyuben Lyudmilov Stoev was born on May 13, 1991 in the town of Dobrich. He completed his secondary education in 2010 at First Language School - Varna. In the same year he was accepted as a student at MU-Varna, where in 2016 he obtained a master's degree in medicine / diploma № 004282 / 11.11.2016 /. For the period of January 2017 to May 2021 he was appointed as a specialist in MHAT "St. Marina" Varna, and in March 2017 after a competition he was selected as an assistant in the Department of General and Clinical pathology at MU-Varna, where he continues to work until now. Since 2018 he has been included as a tissue recovery specialist in "Tissue bank OSTEO-CENTER EAD" - Bulgaria. Since May 2021 he has been a specialist in "Pathology and Cytopathology" / St. № 024896 / 01.07.2021 /.

Dr. Stoev has almost 5 years of experience as an assistant professor in "General and Clinical Pathology". He actively participates in the educational activities of the department, conducting practical classes in general and clinical pathology with medical students – Bulgarian and English program, and in

pathoanatomy with dental students in Bulgarian and English program. As specialist at the Clinic of General and Clinical Pathology at the University Hospital "St. Marina " - Varna Dr. Stoev participates fully and actively in the diagnostic process. He has mastered all the basic methods used in diagnostic biopsy practice, as well as a wide range of methods used in specialized IHC tests. The candidate has participated in several scientific articles, 2 of which are on the topic of the dissertation. With scientific reports, posters and presentations he has participated in 4 national congresses and scientific events with international participation. His professional and scientific interests are focused mainly in the field of oncopathology and maxillofacial pathology. Dr. Stoev is fluent in written and spoken English / C2 / and German / B2 /, and has very good computer literacy. He is a member of the European Society of Pathology and ISUP; member of BMA and BNS of Pathology.

Relevance and significance of the topic

The topic of Dr. Stoev's dissertation, related to the diagnosis and treatment of prostate cancer, continues to be relevant and of great social significance, given the fact that this carcinoma is characterized by unpredictable biological behavior and worldwide it remains one of the leading causes of disability and death in men. Despite the possibilities for early diagnosis, the successes achieved in elucidating some manifestations of this cancer and the applied treatment, the great heterogeneity and combination of morphological findings create a number of difficulties that determine the low 5-year survival. Many of the issues related to the clinical manifestations and morphological diagnosis of PC are still insufficiently studied, and the literature data on the correlation between the results of a number of studies and the prognostic-predictive clinical-morphological parameters of the tumor are incompletely understood, and in some cases contradictory. This requires looking for other diagnostic options, incl. and administering new molecules with potential for tumor markers or with a view to conducting target therapy.

Molecules with such clinical potential are the proteins from the tetraspanin group, which act as mediators in cell migration and interaction with the ECM and are involved in the processes of invasion and metastasis. Of particular interest are two of the representatives in the group - CD9 and CD151, considered as suppressors and promoters of tumor progression, and for which there are still controversies in the literature about their expression in various malignant tumors. Probably the fact of the existing contradictions on the role that these two markers play in the neoplastic process in PC has provoked Dr. Stoev to focus his scientific interest and research on them. The issues and content of the presented dissertation are multifaceted, requiring professional culture, competence and research experience to solve them - qualities that Dr. Stoev undoubtedly possesses, and which have helped him successfully cope with the challenges of the chosen dissertation topic.

Structure and analysis of the dissertation

Dr. Stoev's dissertation is properly structured, written in a sound scientific style and terminology, and is built in compliance with the required proportions. It includes a total of 115 pages, contains all the required sections and is richly illustrated with 38 figures and 48 tables. A total of 194 titles are presented in an 18-page bibliography, of which 4 are in Cyrillic and 190 in Latin, with approx. 64% of the titles have been published in the last 10 years. The literature review impresses with the in-depth analysis of the available literature and shows the very good awareness of Dr. Stoev on the developed topic. The dissertation, acquainted with the achievements in the world science and practice, gives a correct assessment of the unresolved issues concerning the main clinical and morphological indicators, diagnostics, molecular and serum markers, etc., related to PC. The data that is presented in an accessible

and generalized form in the literature review show the author's ability not only to analyze the sources from the literature, but also to pay attention to the existing controversial issues on some points. The review is structured in 7 sections, related to: epidemiology, etiology and risk factors for the development of PC. The morphological characteristics are presented in detail, including the histological variants of the carcinoma according to the WHO classification from 2016; degree of differentiation, stage, prognostic and predictive serum and histological parameters / PSA, histological model of tumor growth, perineural invasion, tumor necrosis /; IHC characteristics of PC, with the most commonly used markers playing a role in disease progression. In the review, the author also presented the types of treatment used in PC. A separate part is devoted to tetraspanins, molecules involved in a number of physiological and pathological processes related to intercellular / cellular signals, stromal remodeling, induction of angiogenesis, and last but not least, participation in the process of tumor metastasis.

In the conclusion, which completes the literature review, Dr. Stoev emphasizes the urgent need for additional studies related to the interactions between the biomarkers CD9 and CD151 with their molecular partners, developing in tumor progression and metastasis. These studies would help to clarify the existing dependencies in prostate cancer with some unfavorable clinical and morphological parameters.

Aim and objectives: The aim of the present study "To analyze the clinical and morphological parameters of patients with non-advanced and advanced prostate cancer in relation to immunohistochemical expression of tetrasponins and clarify their role in tumor progression" is precisely and clearly formulated, and is adequate to topic. It and the 6 tasks arising from it are a logical continuation of the literary review.

Material and methods of research

For the purposes of the dissertation work, a sufficient amount of material was used for statistical processing of the data and determination of their reliability. The base for the realization of the research process are the Department of General and Clinical Pathology, Forensic Medicine and Deontology at MU-Varna, and the Clinic of General and Clinical Pathology at the University Hospital "St. Marina " - Varna. The object of the study are the biopsy materials from the prostate gland of a total of 101 patients - 91 diagnosed with cancer and 10 patients with BPH, forming a control group. The diagnosis and stage of the disease according to the 8th edition of AJCC are determined in the University Hospital "St. Marina" - Varna. Patients were divided into two groups: 50 without evidence of advanced, and 41 patients with advanced cancer. Materials from the 10 control BPH patients were used to assess the expression of the CD9 and CD151 markers. The intensity of cytoplasmic marker expression in glandular epithelial cells was determined in all patients. In the study, Dr. Stoev has selected and included a wide range of adequate methods for the purposes of the study: 1. Routine histological methods on materials only from conventional prostate adenocarcinomas, taken by thick-needle biopsies, biopsies from radical prostate resections and TUR of the prostate. For the purposes of the study, the clinical and morphological parameters were determined, including the detailed description of histological type, cribriform model of tumor growth, TNM stage, degree of differentiation according to Gleason score and ISUP Grade Group; perineural invasion, tumor necrosis, spread inside and outside the prostate and the presence of metastases. 2. Specific test methods, including indirect immunoperoxidase method for immunohistochemical analysis with quantitative assessment, performed using mini KIT high DAKO K 8024. Antibodies, staining reagents and working concentrations are presented in table. 6 and 7. The steps in the

preparation of the biopsy materials for the IHC study, the IHC protocol, the origin of the antibody used and the imaging system are described in detail. The IXX expression of CD9 and CD151 was assessed by H-score, and the intensity of cytoplasmic expression for each tumor and non-tumor gland cell and the percentage of positive cells for each individual intensity were quantified between 0 and 300. When performing the IHC reactions, all elements of the methodology were observed, incl. technological discipline, description of positive and negative controls, etc. The criteria for positivity are precisely formulated when reporting the results, which are processed by a set of modern statistical methods for data analysis, fully meeting the set goals and objectives. The statistical software package IBM SPSS ver.21 was used in the processing, and the graphs were built in Microsoft Excel for Windows.

Evaluation of the obtained results

The results of the research conducted in the dissertation are the most significant part of the work and demonstrate depth and experience for maximum objectivity of the author. In describing them, the sequence of the set tasks is observed. The analysis of the results was conducted at a very good methodological level, which allowed Dr. Stoev to draw the appropriate conclusions from the development.

Biopsy materials from 91 patients with PC were included in the study, 50 of them with non-advanced and 41 with advanced cancer. The average age of patients is 69.39 years, with a minimum age of 45 years and a maximum of 90 years. The most common cancers are in the age group 60-69 years / 37 cases / and 70-79 years / 38 cases /. There are only 6 cases under the age of 60, and there are 10 cases over the age of 80. In 46 of the 50 patients in the non-advanced cancer group, clinical and laboratory PSA data were available prior to histological examination. The analysis of the clinical and morphological characteristics of the patients in this group shows: predominance of cases with low mean PSA values /0-9.9 ng/ml /22 patients/, cribriform tumor growth /in 27 cases/, degree of differentiation, followed via Gleason score - resp. 6 in 12 of the cases, score 7 in 22 cases, and in one case - GS is 10; perineural invasion was found in 35, cases of tumor necrosis were absent; in 58% of the cases the tumor was confined to the gland, 40% were extraprostatic and in one case (2%) surrounding structures were affected. In advanced PCs, patients with high PCA mean values predominate / in 27 patients with more than 50.0 ng/ml, and only in 2 cases the mean value is below 10.0 ng/ml; cribriform growth model was found in 30 cases /73.2%/; high Gleason score 8 is in 15 of the patients, as the largest number of patients /16/ have Gleason score 8 and 9 /16 patients/; PNI was found in 28 of the 41 cases with advanced PC, and tumor necrosis occurred in 4 of the patients. Reporting of local progression showed limitation in the gland in 35 patients, in 3 patients the cancer was extraprostatic, and in 3 cases it affected surrounding structures.

The performed comparative analysis of the data between the clinical and morphological parameters in patients with non-advanced and advanced PC shows: 1. Lack of statistical dependence when comparing the variable age / below and over 70 years / and mean values of PCA in both groups: PSA in patients under 70 years of age is 13.5 ng / ml, and in patients over 70.0 years of age - 67.3 ng / ml, but with a high standard deviation. 2. The analysis between the age variables and the Gleason score shows the presence of statistical dependence only in patients with advanced cancer - with increasing age / 80 + / the Gleason score also increases, as all patients with distant metastases and over 80 years of age had cancer with a Gleason score equal to or greater than 8.

The comparative analysis of the data between the values of PSA and the clinico-morphological characteristics shows: 1. In patients with non-advanced PC: at high values of PSA the cribriform model of growth is more frequent; in the comparative analysis between the mean values of PCA and Gleason score,

a tendency to increase PCA is observed at a correspondingly higher Gleason score. There is a statistically significant difference between the levels of PSA and PNI - the analysis shows that the average value of PSA in cancer with PNI is higher than that for cancer without PNI. In patients with advanced carcinoma, a comparative analysis between PCA values and a cribriform growth pattern showed, although without a statistically significant difference, the presence of higher mean PCA values in cribriform tumor growth; there is a tendency to increase the PCA values compared to the Gleason score, but it is again without statistically significant dependence; there is a similar lack of reliability when comparing the mean PSA values and the presence of PNI.

The comparative analysis between the morphological parameters in patients with non-advanced cancer shows that while out of 15 non-advanced PCs without PNI only 1 has histological data for cribriform growth, in tumors with PNI cribriform structures are found in 22 cases – i.e. in non-advanced PC the cribriform growth pattern is more often associated with PNI. Dependence is also found between PNI and Gleason score - out of 15 cases without PNI, most patients are with Gleason score 6 and 7, and in the group there are no cases with Gleason score 9 and 10. The comparative analysis between Gleason score and T-stage in non-advanced carcinomas shows a statistically significant difference - Gleason score 6 is mainly in cases with T2 / 7 cases/, while Gleason score 8 and 9 is more common in cases with T3 + T4. PNI is more common (in 87% of cases) in more locally advanced carcinomas (T3 and T4).

In a comparative analysis between morphological parameters in patients with advanced PC, no statistically significant difference was found when comparing the two variables "cribriform growth model" and PNI, although the presence of cribriform structures is more common than their absence as in carcinomas without PNI, as well as those with PNI. The comparative analysis between PNI and Gleason score shows that regardless of whether there is PNI or not, the high score is predominant (9 and 10). The comparative analysis between Gleason score and M-stage shows a statistically significant relationship: The majority of M1-stage patients exhibit Gleason score 9.

The main emphasis in the results of the study is the establishment and evaluation of the expression of CD9 and CD151 in PC and BPH. The mean values of cytoplasmic expression of CD9 and CD151 in non-advanced, advanced carcinoma and BPH were analyzed and IHC-expression of markers in relation to clinical and morphological parameters in non-advanced and advanced PC was monitored. Some of the results obtained for the expression of markers at the stages of PC development and their correlation with clinical and morphological characteristics are somewhat divergent, which suggests that it can not be used as a stand-alone prognostic marker, but only in combination with existing indicators such as cribriform model, Gleason score, PNI, and in some cases - age of patients. Based on literature data, Dr. Stoev agrees with other authors that the expression of CD9 has little effect on tumor progression in prostate cancer. As for the marker CD151, given its multifunctional role and interaction with many other factors, it can be assumed that it does not participate in the processes of tumor growth and metastasis alone, but as a component of tetraspanin-integrin complexes in interaction with other molecules. . And this in turn requires the implementation of complex studies among a larger number of patients. Notwithstanding the above, the study of the expression of CD9 and CD151 in relation to the clinical morphological parameters in PC would contribute to the determination of the biological behavior of the tumor, and this may increase the possibilities for the application of adequate target therapy.

In the chapter **"Discussion"** Dr. Stoev compares the results obtained in his study with those published in the literature by other authors for similar studies. The similarities and differences in the

indicated data are considered, and the possible reasons for the existing discrepancies are discussed. The critical analysis of the scientific information on the problem in the context of the results obtained by the dissertation shows the in-depth knowledge of Dr. Stoev on the topic. The presented development is a result of the successful attempt to perform a systematic and in-depth study dedicated to prostate cancer. This is the first scientific study of its kind in Bulgaria, with a complex clinical-morphological and IHC characteristics of the tumor and with such a large volume of indicators and statistical methods for processing the results. The conclusion that Dr. Stoev makes is that the cases used in the research could be of a larger number in order to be able to draw definite conclusions!

Conclusions

The study was conducted on a large scale leads to 11 conclusions, summarizing in detail the results presented and consistent with the purpose and objectives set by the author. Six of them are of a confirmatory nature in relation to the known data from the literature on the connection between some of the studied clinical and morphological indicators. The other 5 conclusions refer to the relationships between age / expression, distant metastases / expression, and the relationship between the expression of the studied tetraspanin markers CD9 and CD151 and the clinical and morphological indicators cribriform growth pattern, Gleason score, perineural invasion and age of patients.

Contributions:

Dr. Stoev has formulated a total of 6 contributions to the dissertation, divided into 2 groups, namely: 1. Original - 2, relating to the complex clinical-morphological and IHC-characteristics of CD9 and CD151 in order to clarify their role in the prognosis of patients with PC. 2. Four scientific contributions of scientific and applied nature, among which 1, 3 and 4 stand out.

Dr. Stoev presented 2 full-text scientific publications related to the topic of the dissertation, published in our journals, and one participation in the National Congress of Pathology, 2017. The publication activity - number of publications, their content and value meet the requirements for presentation of research results.

The **Abstract** is prepared in accordance with the accepted scientific requirements. It is written on 73 pages, illustrated with 25 figures and 43 tables and presents fully, accurately and adequately the dissertation in terms of structure, results and conclusions of the study.

Conclusion:

The dissertation of Dr. Stoev, written in a very well-established scientific and literary language and illustrated in an appropriate way, is a complete, well-structured, with original scientific results and contributions morphological study, contributing to the solution of clinical and morphological problems, related to the diagnosis, treatment and prevention in patients with prostate cancer. The goal of the development has been achieved, the set tasks have been fulfilled. The conclusions are accurate, clearly worded and comprehensive. The contributions have not only scientific but also practical value. The dissertation, as well as the presented publications and scientific communication contain scientific and applied results, meeting the requirements of LDASRB, the Regulations for its application and the terms and conditions for obtaining a scientific degree in MU-Varna. The presented dissertation shows that Dr. Stoev has in-depth theoretical knowledge and skills in the scientific specialty "Pathology and Cytopathology", demonstrating qualities for independent research.

In connection with the above, I confidently give my positive assessment of the proposed dissertation, and vote "FOR" the award of ESD "Doctor" to Dr. Lyuben Lyudmilov Stoev in the scientific specialty "Pathoanatomy and Cytopathology".

03.12.2021   Member of the Scientific Jury: ....

Varna   / Assoc. Prof. E. Softova, MD, PhD /