REVIEW

By: Prof. Reneta A. Toshkova, MD, PhD, Institute of Experimental Morphology, Pathology and Anthropology with Museum– Bulgarian Academy of Sciences (IEMPAM-BAS),

Member of the Scientific Jury, appointed by Order No. HO-05-05-2 /21.01.2025 of the Director of the IEMPAM - BAS, and elected as an official reviewer at the first meeting of the Scientific Jury, held on 04.02.2025

Regarding: Dissertation (PhD thesis) submitted for the awarding of the educational and scientific degree "Doctor" (PhD) in the Higher Education area: 4. "Natural Sciences, Mathematics and Informatics", Professional Direction 4.3. "Biological Sciences" and Scientific Specialty "Morphology" (01.06.26)
Topic of the dissertation: "Study on the PD-L1 expression in urothelial carcinoma of the urinary bladder as a basis for therapeutic strategy"
Author of the dissertation: Dr. ROSSEN ALEXANDROV SPASOV, part-time doctoral student in the Pathology department, at the Institute of Experimental Morphology, Pathology and Anthropology with a Museum - Bulgarian Academy of Sciences
Supervisor: Prof. Radostina Ivaylova Alexandrova, PhD

The review was prepared in accordance with the requirements of the Law for the Development of the Academic Staff of the Republic of Bulgaria (LDASRB), the Regulations for its Implementation (RI), and the internal regulations of the Bulgarian Academy of Sciences and IEMPAM-BAN. I declare that I have no conflict of interest with the candidate.

Materials on the procedure. The materials submitted on paper and electronic media meet the requirements of the State Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for its implementation and the internal regulations of the Bulgarian Academy of Sciences and IEMPAM-BAS, and include the materials mandatory for the procedure.

The dissertation work was discussed, approved and directed for defense before a Scientific Jury at an extended meeting of the Pathology Section, held by order No. RD 09-70/17.12.2024 of the Director of IEMPAM-BAS.

Biographical data. Dr. Rossen Alexandrov Spasov was born in the town of Sofia. In 1996 he graduated from the Medical University of Sofia with a master degree in Medical doctor. In 2009 he specialized in General and Clinical

Pathology. From 1997 to 2008 he worked as a resident physician, and since 2009 – as a Head of the Department of Clinical Pathology, "Dr. N. Vasiliev" Hospital, in the town Kyustendil. He worked consecutively as a resident physician in the Department of Clinical Pathology, of the Specialized Hospital for Active Treatment and Oncology (SBALO) in Sofia (2012-2019); Assistant at the Faculty of Medicine of the University Hospital "Lozenets" in Sofia (2016-2019). From 2019 to the present, he has been working as a pathologist and Head of the Department of Clinical Pathology, "St. Mina" Hospital for Oncology, Blagoevgrad, as well as a consultant physician in the University Hospital "Lozenets", with the main activities being biopsy, cytological and express intraoperative diagnostics.

Since 2019, he has been a part-time doctoral student at the Department of Pathology, IEMPAM-BAS. He has passed the doctoral minimum exams in the specialized basic subject "Morphology", language and computer technologies and has completed additional specialized courses on the topic of the dissertation. He has successfully completed the educational program and exceeded the required quantitative criteria - he has collected 338 credits out of the required 250. The total scientific output of Dr. Spasov includes 9 scientific publications, of which 6 in publications indexed in WoS/Scopus, 42 participations in national and international scientific forums with posters and reports, participation in 7 projects (1 under the European COST program, 1 bilateral between IEMPAM-BAS and the Lithuanian Academy of Sciences, 2 bilateral between IEMPAM-BAS and the Romanian Academy of Sciences, 1 under the Operational Program "Science and Education for Smart Growth"). The Scopus reference shows 26 citations and h index of 3. Dr. Spasov is a member of the Bulgarian Medical Union, the Bulgarian Society of Pathology, and the Bulgarian Anatomical Society.

Relevance and significance of the dissertation work. The dissertation of Dr. Rossen Alexandrov Spasov is dedicated to a problem of essential importance for human health - therapy of malignant diseases and in particular - immunotherapy of urothelial carcinoma using immune checkpoint inhibitors. The attention is focused on studying the expression of the PD-L1 biomarker as an indicator of the degree of malignancy of urothelial carcinomas and as a target for a personalized therapeutic strategy. The emphasis is on personalizing the therapeutic strategy according to the individual antitumor response of the patient, which distinguishes the dissertation not only with high relevance, but also with a high innovative perspective.

Structure of the dissertation. The dissertation is written on 136 non-standard pages and contains the usual sections - Title page, table of contents - 3 pages, abbreviations used - 1 page, introduction - 2 pages, literature review - 50 pages, goal and objectives 1 - page, materials and methods - 11 pages, results and discussion - 40 pages, summary - 3 pages, conclusions - 1 page and contributions -

1 page, and a list of cited literature - 18 pages. The bibliography includes 224 titles, of which 1 in Cyrillic and 223 in Latin. It is illustrated with 8 tables and 49 figures. All sections are written grammatically correct, with excellent computer literacy, which proves the serious and responsible attitude of the doctoral student towards the overall design of the dissertation

The literature review is contemporary and comprehensive. It is written clearly and tightly, and presents systematized information about bladder cancer, which with the aging population has an increasing socio-economic importance, and about the need for a new personalized immunotherapy approach, based on validated diagnostic and predictive biomarkers. Separate sections present data related to apoptosis as one of the main types of programmed cell death, important in both physiological and pathological conditions; mechanisms of apoptosis and their significance for the development of drugs targeting specific apoptotic genes or pathways; types of antigens and antitumor immune response; immunotherapy and types of immunotherapy in neoplasia. Particular attention is paid to the expression and biological function of the membrane protein receptors PD-1 and PD-L1; the PD-1/PD-L1 signaling pathway and its role as an immune checkpoint signaling system for evading the immune response in neoplasias, and as a basis for the development of a new generation of immunotherapeutic agents (so-called immune checkpoint inhibitors) aimed at blocking the binding of the programmed cell death receptor-1 (PD-1) and its ligand-1 (PD-L1). The information on the modern classification of bladder tumors, epidemiological characteristics, risk factors, prognosis, recurrence and progression of bladder carcinoma, pathomorphological classification and heterogeneity of tumors was fully reflected. The PhD student was able to express the most important achievements of modern therapeutic strategies and immune checkpoint inhibitors in the treatment of malignant urothelial carcinomas, as well as to outline new scientific challenges.

As a logical consequence, the **main goal** has been formulated and **7 specific tasks** have been identified for its achievement.

Evaluation of the materials and methods used. The PhD thesis is professionally completed and an adequate experimental design has been applied. Modern and classical methods have been successfully combined to achieve the goal and the tasks set - histopathological, cytomorphological, immunohistochemical, light microscopic, molecular biological, cell culture, fluorescent. The methods are described in detail, which allows them to be applied in other scientific laboratories. The studies were conducted on samples from 110 patients with a clinically confirmed diagnosis of urothelial carcinoma, 1 sarcomatoid variant of bladder carcinoma, 5 metastatic lymph nodes, 9 bone and soft tissue metastases and in laboratory conditions on model experimental systems of permanent cell tumor lines - human colorectal carcinoma (HT29), human cervical adenocarcinoma (HeLa) and

transplantable rat sarcoma induced with Rous sarcoma virus (RST). Appropriate statistical programs have been applied. The experimental material is well structured and fully sufficient for a dissertation for the scientific and educational degree "Doctor".

The experimental work was carried out in the laboratories of the Institute of Experimental Morphology, Pathology and Anthropology with a Museum - Bulgarian Academy of Sciences and in the Laboratory of General and Clinical Pathology and Forensic Medicine of the University Hospital "Lozenets".

Evaluation of the results obtained. The "Results" section includes the doctoral student's own data, which are presented in a clear manner, in a sequence following the tasks set out in the dissertation. A pathomorphological and clinical analysis of 111 cases of urothelial carcinoma of the urinary bladder was performed in terms of gender, age, presence of local recurrence, differentiation and stage, and the obtained data were statistically processed. The tumors were classified according to the degree of infiltration into the muscle layers. PD-L1 expression has been demonstrated in histological preparations of urothelial carcinoma by immunohistochemical examination with a monoclonal antibody against PD-L1 (clone 73-10). PD-L1 expression was visualized as membrane and/or intracytoplasmic positive staining of the tumor cell population and of the immune (stromal) cells – lymphocytes and tissue macrophages infiltrating the tumor. A correlation was established between the degree of tumor invasion and the levels of PD-L1 expression. PD-L1 status of urothelial carcinomas (histological preparations) was clarified by immunohistochemical examination with a monoclonal antibody against PD-L1.

In a comparative aspect, cytostatic-induced apoptosis in tumor cell lines was studied by double staining with acridine orange and propidium iodide, routine staining with hematoxylin and eosin, and immunohistochemical examination of PD L1 expression. Tumor DNA was extracted from representative and rare histological variants of malignant bladder tumors for future genetic and molecular biological studies.

The conducted research, as well as the obtained results, are logically systematized and illustrated with 3 tables and 40 figures, of which 17 figures are two- and multi-component. The good illustration allows a positive assessment of the conducted research and the obtained results.

The **discussion** is compact and is made simultaneously with the presentation of the obtained results, in the light of the literature data, and the possibilities for their potential use in practice. The **summary** section is a real analysis of all the obtained experimental data and clearly outlines the originality and significance of the scientific work.

The peer-reviewed dissertation is an original scientific study, conducted at a modern level, leading to **7 conclusions**, which I fully accept. They reflect the most significant achievements and regularities regarding the observed effects.

Assessment of the candidate's contributions. The research has achieved **6** contributions of an original, confirmatory and applied nature and can be described as follows:

Original contributions: 1. A collection of tumor tissues from 110 urothelial bladder carcinomas with varying degrees of differentiation and depth of infiltrative growth in the muscle layers, lymph node metastases and bone metastases was created. 2. Statistical data were obtained on the distribution by age, sex, degree of differentiation (G), degree of tumor invasion (T) and stage of the disease of primary and recurrent bladder carcinomas diagnosed in the Department of Clinical Pathology for the period 2016-2020.

Confirmatory contributions: 3. The focal nature of PD-L1 expression in the tumor component and infiltrating immune cells has been confirmed. 4. High expression of PD-L1 in poorly differentiated urothelial carcinomas has been confirmed. 5. High PD-L1 expression has been confirmed in metastatic lymph nodes and distant (bone) metastases

Applied contributions: 6. DNA eluates were extracted from tumor tissues of urothelial bladder carcinomas stratified according to the degree of differentiation, stage and depth of infiltration, which are ready for whole exome (WES) and genomic (WGS) DNA sequencing in future studies in this area.

The contributions are undeniable, enrich science with new data and are of potential importance for medical practice.

Publications related to the dissertation. 3 scientific publications printed in English are presented. In one of them, the doctoral student is the lead author, and in the other two he is in a team. Two of the publications are published in refereed journals and indexed in a global database, including "Asian Journal of Pharmaceutical and Clinical Research", with a quartile of Q4 (12 points) and Farmacia with a quartile of Q2 (20 points) with a total score of 32 points. A list of participations in three scientific forums is enclosed. It is evident that the doctoral candidate meets the required indicators according to the submitted "Report" for the fulfillment of the minimum national requirements and the requirements of the regulations of IEMPAM-BAS for the acquisition of the ONS "Doctor"

Critical notes. I have no critical notes on the substance of the dissertation. I would note minor technical errors and incompletely cited literary sources (No. 115 of 2020, No. 134 of 2019, No. 138 of 2019, No. 139 of 2022, No. 147 of 2020, No. 148 of 2020, written without the title of the article, but with DOI, No. 196 of 2018, No. 204 of 2020. The dissertation work as a whole is written in a very good scientific and literary language.

Evaluation of the Abstract. The abstract is 68 pages long. It is very well designed graphically. It creates a clear and accurate picture of the overall dissertation work, the results achieved, conclusions and contributions.

Acquired competence in accordance with the requirements of the educational and scientific degree "Doctor". Part-time doctoral student Rossen Alexandrov Spasov demonstrates basic theoretical knowledge on the topic of the dissertation, skills for the correct selection of appropriate methods and practical skills for research. He has acquired experience in analyzing, summarizing, and presenting scientific results, and in drafting scientific articles that fully comply with the requirements of the educational and scientific degree "Doctor".

CONCLUSION

The volume and quality of the conducted research define the dissertation work of Rossen Alexandrov Spasov as a completed scientific work with pronounced innovation and significance for the therapy of urothelial carcinoma in clinical medical practice. The volume and quality of the dissertation work and the successful implementation of the educational and scientific program cover the qualitative and quantitative criteria of the ZRAS in the Republic of Bulgaria and the regulations for its application, and the internal regulations of the Bulgarian Academy of Sciences and IEMPAM-BAS. The doctoral student Rossen Alexandrov Spasov possesses knowledge and skills for independent scientific research work.

Based on the above, I give a positive assessment of the dissertation work. I vote "Yes" with conviction and recommend to the members of the esteemed Scientific Jury to award the Educational and Scientific Degree "Doctor" to part-time doctoral student d-r Rossen Alexandrov Spasov in Higher Education area: 4. Natural Sciences, Mathematics and Informatics, Professional Direction 4.3 "Biological Sciences", and the scientific specialty "Morphology" (01.06.26).

20. 03. 2025

Signature:

Sofia

Prof. Reneta A. Toshkova, MD, PhD