

OPINION

by Assoc. Prof. EKATERINA BOYANOVA SOFTOVA-ZLATAROVA, MD, PhD
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Regarding the defense

of the dissertation of Dr. Nevena Zhelyazkova Yanulova, a doctoral candidate in the full-time doctoral program Pathoanatomy and Cytopathology at the Department of General and Clinical Pathology, Forensic Medicine, and Deontology, Medical University of Varna, for the awarding of the educational and scientific degree "Doctor" in the professional field 7.1. Medicine, scientific specialty Pathoanatomy and Cytopathology

Title: Apoptosis and Necroptosis in Renal Cell Carcinoma

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

Pursuant to Order No. R-109-357/25.10.2024 from the Rector of Medical University - Varna (MU-Varna), Prof. Dimitar Raykov, MD, DSc, based on a report with ref. No. 102-2657/04.10.2024 from Assoc. Prof. Deyan L. Dzhenkov, MD, PhD, Head of the Department of General and Clinical Pathology, Forensic Medicine, and Deontology, as per decision of the Faculty Council under Protocol No. 28/14.10.2024 and a report with ref. No. 103/6909 from 22.10.2024 from Prof. Yoto Yotov, MD, DSc, Dean of the Faculty of Medicine at MU-Varna, and in accordance with Article 24, paragraph 6 and Article 30, paragraph 3 of the Regulations for the Implementation of the Act on the Development of Academic Staff in the Republic of Bulgaria (ADASRB), Article 68, paragraph 1 of the Regulations for the Development of the Academic Staff at MU-Varna, Dr. Yanulova, enrolled as a full-time PhD student under Order No. R-109-444/28.10.2020 of the Rector of Medical University - Varna, was granted permission for defense. (on the proposal of Assoc. Prof. Deyan L. Dzhenkov, MD, PhD, Head of Department, sample from protocol No. 425/03.10.2024. from the Departmental Council of the Department of "General and Clinical Pathology" at MU-Varna).

Pursuant to Protocol No. 1/04.11.2024, I was tasked with preparing an opinion (in both Bulgarian and English) regarding the procedure for awarding the educational and scientific degree "**DOCTOR**" to Dr. Nevena Zhelyazkova Yanulova at MU-Varna.

The doctoral student successfully passed the minimum doctoral exams on 25.02.2022 and the foreign language exam (on 01-02.10.2020 level B1 CEFR). For the competition, Dr. Yanulova presented all necessary materials on paper and electronic media in accordance with the requirements of ADASRB, the Regulations for its Implementation and Regulations of MU-Varna.

Brief biographical data and career development of the doctoral student

Dr. Nevena Zhelyazkova Yanulova was born on February 21, 1987 in Dobrich. She graduated from the Ivan Vazov High School of Science and Mathematics - Dobrich (September, 2001-May, 2006) with a Biology and Chemistry profile.. In 2007 she was accepted as a student at MU-Varna and graduated in 2013. With protocol No. 622/10.09.2013 of the State Examination

Commission, Dr. Yanulova acquired a professional MD qualification (diploma series MUV, No. 001588, reg. No. 002.386/06.11.2013). From July 2014 to June 2018 Dr. Yanulova specialized in General and Clinical Pathology at the specialization center at St. Marina University Hospital - Varna. As of January 1, 2019, after successfully passing the state exam, Dr. Yanulova was recognized as a specialist in General and Clinical Pathology. (reg. No. 022568/March 1, 2019, series MUV-2019, No. 4090). From December 2013 to June 2014. Dr. Yanulova worked as a resident doctor at the Department of Pathology at the Dobrich Hospital, and from 09.2015 to 09.2018 was a part-time assistant at the Department of General and Clinical Pathology. From April 2019 she worked as an assistant doctor at the Clinic for General and Clinical Pathology, and during the period 09.2019-03.2020 as a substitute assistant at the Department of Pathology. In March, 2020 after a competition, Dr. Yanulova was appointed full-time assistant at the Department of General and Clinical Pathology, Forensic Medicine and Deontology at MU-Varna.

As a doctoral student and specialist, Dr. Yanulova has been taking an active part in both research and diagnostic-biopsy and autopsy activities of the Clinic and the Department of Pathology at the Medical University and University Hospital in Varna. She has a total of 9 years of teaching experience as a full- and part-time assistant and a substitute. Her scientific interests are mainly focused on endocrine and renal tumor pathology. In an effort to continuously improve her qualifications, Dr. Yanulova has participated in a number of prestigious scientific events: congresses, conferences, etc., held in our country. From 2017 to 2023 she participated as an author in two national congresses and one national conference held in our country, presenting a total of 5 posters and proceedings on topics in the field of tumor pathology, including renal, pigment and other. tumor lesions. She has 2 publications in journals with IF: one in Cureus from 2021, and one in Folia Medica from 2018.

Dr. Yanulova is a member of the Bulgarian Medical Association and Bulgarian Society of Pathology. She has a very good command of written and spoken English, good computer literacy and communication skills, acquired during her work as a resident and assistant in pathology. She has well-coordinated organizational skills, allowing her to quickly adapt, presentation and teamwork skills, command of an office suite.

Dr. Yanulova actively participated in the development of three projects sponsored by the Science Fund at MU-Varna on following topics:

1. Predictive and prognostic role of IHC-expression of apoptosis-inducing factor (AIF) and RIPK3- marker for necroptosis in RCC, MU-Varna, Science Fund, 2021;
2. Investigation of expression levels of IHC-marker for apoptosis and necroptosis in basocellular and spinocellular carcinoma; MU-Varna, Science Fund, 2021;
3. "Ph(-) myeloproliferative neoplasms - morphological and immunohistochemical characteristics; MU-Varna, Science Fund, 2016.

Relevance and importance of the topic

The topic of the dissertation work of Dr. Yanulova, concerning the possibilities for diagnosis and treatment of renal cell carcinoma (RCC), does not cease to be topical and of great social significance, given that this particular etiology and course of cancer continues to be one of the main causes of disability and death, especially in male patients. RCC is not among the common tumours in man. However, there is now a continuous upward trend in the number of cases, with

a more than 2-fold increase in incidence over the last 50 years. According to GLOBOCAN, in 2020, RCC was the cause of death in about 180,000 cases worldwide. The trend of increasing incidence is particularly pronounced among residents of European countries and among younger individuals. In Bulgaria, according to the BNCR-Sofia, in 2016-2017, renal tumors ranked eighth among men. In 2017, 474 new cases were diagnosed, and 91 of the patients were aged 65-69 years. In females, 244 cases were registered, of which 46 were in the age group 70-74 years. For Varna district, 55 new cases were diagnosed in both sexes in the same year. There is a trend of increased incidence of RCC per 100,000 people for the period 1980-2016, with an increase from 2.1 in 1980 to 8.4 in 2017 for men; and from 1.4 to 3.8 for women.

The presence of still understudied issues related to the morphological characterization and biological behavior of RCC necessitates systematic clinicomorphological and ICH studies that could help determine the therapeutic approach in each case. In this aspect, elucidation of the prognostic and predictive role of RIPK3 and AIF (markers of cell death necroptosis and apoptosis) could contribute to the discovery of new signaling pathways and development of new therapeutic strategies, especially considering that there is only scarce data in the literature to study necroptosis in experimental settings, and none that studies it in humans. Many of the issues related to the clinical manifestations and morphological diagnosis of RCC are not fully elucidated, even controversial in some cases, and this calls for search for other diagnostic options and application of newly developed molecules with potential as tumor markers and the conduct of targeted therapy. In this aspect, the topic of the presented scientific work is topical and dissertable, and the problems and content - multifaceted - a fact that requires competence of the researcher and sufficient, extensive research experience in solving the set tasks - a problem with which Dr. Yanulova has coped brilliantly.

Structure of the scientific work

The submitted dissertation comprises 169 pages and meets the requirements of ADASRB and the Regulations of MU-Varna. The thesis is illustrated with 80 tables and 65 figures and includes the following parts: Title page (1 page); Contents (3 pages); Introduction (2 pages), Abbreviations used (1 page); Literature review (49 pages), Goal and tasks (1 page), Materials and Methods (8 p.), Results and Discussion (80 p); Summary (2 p.), Conclusion (2 p.), Contributions (1 p.), Publications related to the thesis (1 p.), and References (20 p.). The bibliographic references include 266 sources, 5 in Cyrillic and 261 in Latin.

The literature review impresses with the thorough analysis of the available literature and shows very good knowledge of the topic. The data presented in an accessible and summarized form of the review show the author's ability not only to analyze the literature sources, but also to pay attention to the existing controversial issues on some points. In structuring the review, Dr. Yanulova has focused her attention on issues related to epidemiology, etiology (with genetic predisposition) and especially on the three risk factors: obesity, hypertension and smoking. Prior kidney disease, most commonly polycystic kidney disease, is also accepted as a risk factor; the morphological-macroscopic characterization of RCC is presented, as well as the new updated WHO histopathological classification of 2022, which has the greatest relevance for practice; prognostic factors in RCC are reviewed; types of differentiation grade, TNM staging; tumor necrosis with emphasis on its prognostic role; microvascular invasion are presented; great attention is paid when considering forms of cell death: apoptosis-signaling pathways and its morphological characterization; apoptosis-inducing factor (AIF) and its expression in malignant tumors; necroptosis-signaling pathways and its mechanisms; role of necroptosis-related marker RIPK3 in oncogenesis; conclusion.

In conclusion, at the end of the literature review, Dr. Yanulova convincingly emphasizes the urgent need for further studies concerning the interactions between the AIF and RIPK3 markers and some of the environmental factors developing during tumor progression and metastasis. The author emphasizes the significant role that these studies would play in clarifying the existing dependencies in RCC with some unfavorable clinico-morphological parameters.

Goal and tasks: The goal of the present study was to investigate the immunohistochemical expression of apoptosis marker (AIF) and necroptosis marker (RIPK3) in patients with renal cell carcinoma and to determine their prognostic value. In order to achieve this goal, the dissertator has set 6 tasks concerning: 1. Investigating and comparing the clinic-morphological characteristics in relation to survival of selected RCC patients; 2. Determining the IHC expression of AIF in RCC tumor tissue and comparing it with that of adjacent non-tumor tissue; 3. Performing a semi-quantitative evaluation of IHC expression of RIPK3 in RCC tumor tissue and comparing it with adjacent non-tumor tissue; 4. Evaluation of IHC expression of AIF and RIPK3 in metastatic lesions of selected cases with histologically verified metastases and comparison with their expression in the primary tumor; 5. Investigation of IHC expression of AIF and RIPK3 in relation to clinicopathological characteristics of patients with RCC: gender, age, tumor stage, histological type; degree of differentiation, tumor necrosis; TIL and vascular invasion; 6. Analysis of apoptosis marker AIF and necroptosis marker RIPK3 in relation to patient survival and determination of their prognostic role in RCC.

MATERIALS AND METHODS:

The present study included 80 RCC patients, divided into 3 groups: group I: 20 patients diagnosed with pRCC; group II: 21 patients diagnosed with chRCC and group III: 39 patients diagnosed with ccRCC. All patients were operated on at St. Marina University Hospital. The following histological parameters were evaluated: histological type, presence and area of TN, TILs, vascular invasion and degree of differentiation in ccRCC and pRCC. TNM stage was determined, and 15 patients had histologically verified distant metastases. IHC expression levels of AIF and RIPK3 were analyzed in the three patient groups and in 14 histologically verified distant metastases. The material basis for the realization of the dissertation work was the Department of General and Clinical Pathology, Forensic Medicine and Deontology at MU-Varna, and information from the MultiLab electronic database of the University Hospital.

Dr. Yanulova has selected and included:

1. Routine histological methods: 3-4 specimens from each tumor resection were examined, containing tumor parenchyma, necrosis section and adjacent non-tumor tissue. The materials were processed using the standard paraffin-embedded method, and 5 micrometer-thick sections were stained with hemalacon-eosin to assess changes in the primary tumor and metastases. Relevant clinicomorphological parameters were determined for the purposes of the study.

2. Specific research methods, including indirect immunoperoxidase method for immunohistochemical analysis with quantitative evaluation performed using mini KIT high Ph DAKO K 8024. The antibodies used, staining reagents and working concentrations are presented in Table 1. The steps involved in the preparation of biopsy materials for IHC examination- IHC protocol, origin of antibody used and imaging system are described in detail. The evaluation of IHC expression was performed by examining 10 fields at the highest magnification (x400) for each case. IHC expression of AIF/RIPK3 was assessed semiquantitatively using H-scores on tissue sections, with a range of 0 to 300, and the intensity of cytoplasmic, respectively nuclear, expression was determined for each cell from tumor and non-tumor tissue, and the percentage of positive cells for each intensity. Finally, the H-score

was calculated using the appropriate formula for this purpose. All elements of the methodology, including technological discipline, were followed when performing the IHC reactions. The positivity criteria were precisely formulated in reporting the results, which were processed using a set of modern statistical methods for data analysis, fully meeting the stated goal and tasks. The statistical software package IBM SPSS ver.21 was used to process data and graphs were constructed in Microsoft Excel for Windows.

Results. The mean age of the patients was 62.8 years, the youngest being 28 years and the oldest 81 years old. For statistical processing, patients were divided into three groups: group I <44: 4 cases; group II 45-64: 40 cases and group III >65: 36 cases. Histological findings in 39 cases were of ccRCC type/48.8%/; 20 /25%/ were pRCC and 21 /26.2%/ were chRCC. Mainly men were affected: 56men and 24 women; m:f ratio 2:1. A detailed table presents the clinical and morphological characteristics of the 80 patients studied according to relevant indices. The degree of differentiation was determined using the ISUP system of 2022 in ccRCC and pRCC with a variation from G1 to G4; the degree of differentiation in chRCC was not determined due to lack of prognostic value (WHO 2022) in this variant. In terms of tumor localization: in 42 patients it was in the left kidney and in 38- in the right kidney. When TILs in the tumor tissue were taken into account, they were found to be absent in 50 cases, mildly expressed in 21 cases, moderately expressed in 9 cases and absent in (0%). LVI was found in 33 patients in heterogeneous vessels and absent in 47 patients. In terms of T stage, the cases were distributed as follows: 35 cases in T1 stage; 14 cases in T2 stage, 26 cases in T3 stage and 5 cases in T4 stage. The analysis of the N-stage showed that 52 cases had no histological lymph nodes, 19 cases had no metastases /N0/ and 9 cases had metastases /N1/; according to the M-stage there were three cases with Mo, 15 cases with M1 and 62 cases with Mx.

The results of the research carried out in the presented dissertation are the most significant part of Dr. Yanulova's work, and demonstrate the author's thoroughness and attempt for maximum objectivity. Their analysis has been conducted at a very good methodological level, which has allowed Dr. Yanulova to draw the appropriate conclusions and implications from the discussion. The results obtained in the study are presented in an appropriate form, illustrated by numerous figures and tables of extremely good quality. In the discussion, Dr. Janulova consistently presents and comments on the results obtained from the dissertation's own research, along with a comparison with results published in the literature by other authors for similar studies. Along with comparing her own results with the results obtained from the studies of other, foreign and Bulgarian authors, Dr. Janulova makes a thorough analysis of the similarities and differences in the data, and discusses the possible reasons for any discrepancies between them. In this aspect, the critical analysis of the scientific information on the problem, in the context of the results obtained by Dr. Yanulova, shows the profound knowledge of the student on the developed topic. The 18 conclusions that emerged from the study, which was conducted on a considerable scale, meet the stated goal and tasks, are very well formulated and represent the logical conclusion of the results obtained; I accept them without remarks in the form in which they are presented. Among the conclusions of particular significance are No: 1, 3, 5, 6, 7, 10, 11, 13, 15, 17 and 18. Dr. Yanulova presented the **contributions** of the dissertation work in two groups: Contributions of an original nature - these are the two scientific contributions relating to: 1. A comprehensive clinical-morphological and IHC analysis of RCC in relation to patient survival, and 2. Immunohistochemically assessed by AIF and RIPK3 processes of apoptosis and necroptosis as prognostic and predictive markers. Five scientific contributions are of a practical-applied nature, with particularly significant among them being those with No. 1, 2, 4 and 5, relating to: 1. Assessed significance of the main clinical-morphological indicators: age, area of tumor necrosis and vascular invasion as prognostic factors for reduced survival in

patients with RCC; 2. Confirmed morphological profile of advanced RCC by relative degree of differentiation, tumor necrosis, infiltration with TIL, and estimated risk of death; 4. Existing relationship between cytoplasmic expression of AIF in RCC and LVI by relative tumor advancement; 5. The role of cytoplasmic expression of RIPK3 in RCC for tumor differentiation and occurrence of LVI was assessed. The volume of data that Dr. Yanulova interpreted in the detailed analysis conducted, relating to the established dependencies between clinical and morphological indicators, cytoplasmic and nuclear expression of the studied markers (RIPK3 and AIF), and patient survival is impressive.

Publications related to the topic of the dissertation:

Dr. Yanulova has submitted two full-text scientific publications related to the topic of her thesis in Bulgarian journals. The research output: number of publications, their content and quality meet the requirements for the presentation of research results.

The thesis summary is prepared in accordance with the accepted scientific requirements. It comprises 67 pages, illustrated with 31 figures and 15 tables, and presents the dissertation completely, accurately and adequately in terms of structure, results and conclusions..

In conclusion, the presented dissertation is a thorough study that "sheds light" on the issues related to the development of the RCC. The results obtained and conclusions drawn are not only useful for clinical practice, but also have significant scientific and theoretical value. The presented work shows that Dr. Yanulova possesses in-depth theoretical knowledge and skills in the scientific specialty Pathoanatomy and Cytopathology, demonstrating qualities for independent scientific research. The results obtained and the contributions made in the dissertation fully meet all the requirements of ADASRB, its Regulations on Implementation and the Regulations of MU-Varna. Taking into account the way the dissertation topic is developed, the conclusions and contributions made, I confidently give my positive evaluation of the presented work, recommending the members of the esteemed Jury to award the educational and scientific degree "Doctor" to Dr. Nevena Zheliazkova Yanulova in the scientific specialty Pathoanatomy and Cytopathology".

December, 19th, 2024

Varna

Member of the Scientific Jury

Assoc. Prof. Ekaterina Softova, MD, PhD

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