REVIEW

COMPETITION FOR DOCTORAL DEGREE Protocol No. 1/04.11.2024

MEDICAL UNIVERSITY - VARNA; FACULTY OF MEDICINE; DEPARTMENT OF GENERAL AND CLINICAL PATHOLOGY, FORENSIC MEDICINE AND DEONTOLOGY

by Assoc. Prof. Silvia Nikolaeva Genova, MD, PhD

Department of General and Clinical Pathology

Medical University - Plovdiv

of a dissertation for the acquisition of the educational and scientific degree "Doctor"

Order No. R-109-357/ 25.10.2024 concerning the procedure for acquiring the ESD "Doctor";
Scientific specialty 7. Healthcare and sports;
Professional field 7.1. Medicine;
Doctoral program: *Pathology and cytopathology, code 03.01.03*
Author: Dr. Nevena Zhelyazkova Yanulova

Form of doctoral study: regular

Department: General and clinical pathology, forensic medicine and deontology, MU-Varna

Subject: ***APOPTOSIS AND NECROPTOSIS IN RENAL CELL CARCINOMA***

Scientific supervisor: Prof. Maria Angelova Tsaneva, MD, PhD
General and clinical pathology, forensic medicine and deontology, MU-Varna

1. General presentation of the procedure and doctoral student

The presented set of materials on paper/electronic medium **was** in accordance with Art. 70 (1) of the Procedure for the acquisition of the ESD "Doctor" at MU-Varna; Regulations for the Development of the Academic Staff at MU-Varna from 07.08.2024 and included all necessary documents.

The doctoral student has presented two full-text publications in peer-reviewed journals where she is the first author.

2. Brief biographical data

Dr. Nevena Zhelyazkova Yanulova obtained MD in 2013 at MU-Varna. From 2020 she has been a full-time assistant at the Department of General and Clinical Pathology, Forensic Medicine and Deontology, MU-Varna. From 2020, she has been a full-time doctoral student.

Dr. Yanulova acquired a specialty in General and Clinical Pathology in 2019. She participated in three scientific projects with significant scientific contributions. She is fluent in English.

3. Relevance of the topic and set goal and tasks.

The topic of the dissertation is current and does not repeat other studies in our country on apoptosis (Apoptosis Inducing Factor- AIF) and necroptosis (RIPK3) marker expression in renal cell carcinoma (RCC).

The dissertation contains 169 standard pages and is illustrated with 80 tables and 65 figures. The literary references include 266 literary sources, 5 in Cyrillic.

RCC accounts for more than 80% of upper urinary tract malignancies and 3% of all malignant tumors. There are two age peaks of increased morbidity - around 40 years of age and in the interval between 50 and 70 years. Approximately 40% of patients with this carcinoma die due to disease progression, and necroptosis plays a key role in multiple aspects of tumor biology, oncogenesis, tumor metastasis, and tumor immunity. RCC is characterized by uncontrolled cell proliferation, lack of cell death and strong resistance to conventional chemotherapy. In this regard, I believe that the chosen topic is relevant and can provide basic results for tumor therapy and effective regulation of necroptosis in RCC.

The doctoral student examines the different forms of cell death with morphological signs of necrosis, which is regulated in a programmed manner through certain signaling pathways. To understand the molecular mechanism of necroptosis, most studies have focused on investigating tumor necrosis factor alpha (TNFα), receptor-interacting protein kinase 3 (RIPK3) and caspase-8, as well as the molecular pathways of activation and deactivation and interaction between the components of necroptosis in the cell. Transduction of necroptotic signaling components through the nucleus is a mechanism for regulating cytosolic necrosome formation and a form of regulation of necroptotic cell death, and inhibition of cell death is a hallmark of malignant tumors.

In the present dissertation, the doctoral student studies for the first time in our country apoptosis and necroptosis, nuclear and cytoplasmic expression of AIF and RIPK3 in kidney carcinoma and performs an extensive analysis of the expression of the necroptosis marker in tumor cells according to the histological type of the tumor, T-stage of tumors, TIL, metastases, clinical data and survival of RCC patients.

4. Knowledge of the problem

The introduction presents the issues related to RCC, etiology, pathogenesis and the main therapeutic methods in patients with kidney carcinomas, skillfully pointing to the unresolved scientific, diagnostic and prognostic problems on the subject. The literature review is extremely detailed (50 pages long), illustrated with 12 adapted figures, presents a rich literature reference and shows in-depth knowledge of the issue.

The literature review begins with epidemiology, etiology, and risk factors for the development of RCC. Family burdens, individual characteristics and genetic predisposition are addressed by presenting the latest data on hereditary syndromes in which kidney tumors develop at an earlier age and the need for molecular genetic analysis. Novel genetically linked syndromes with the BRCA1-associated protein-1 (BAP1) gene predisposing to early-onset familial RCC, Hereditary leiomyomatosis syndrome and RCC, Hereditary papillary carcinoma of the kidney, Hamartoma tumor syndrome PTEN have been derived.

Dr. Yanulova utilizes the new histopathological classification of kidney tumors, which was updated in 2022. From the World Health Organization (WHO), the diagnosis of which is based on light microscopy, immunohistochemical studies and possible, associated, underlying molecular changes. The classifications are illustrated with tables, which helps for accurate and quick orientation in the subject. She uses the new classification approaches to develop her doctoral work.

Prognostic and predictive factors, as well as degree of differentiation in accordance with *2022 World Health Organization/International Society of Urological Pathology (WHO/ISUP) Classification of Tumors* are discussed on 15 pages, including the aggressive variants: such as RCC with sarcomatoid and rhabdoid differentiations. TNM staging is presented in detail, after which the doctoral student moves on to tumor necrosis and apoptosis, noting that the presence or absence of tumor necrosis in RCC must necessarily be included in the pathologist's routine response as a prognostic factor.

On 13 pages, the main pathways of apoptosis, extrinsic and intrinsic pathways of apoptosis, morphological characterization of apoptosis, apoptosis-inducing factor, evidence for the existence of alternative signaling pathways that initiate programmed cell death-associated events are reviewed. One of these pathways is caspase-independent and is mediated by apoptosis-inducing factor (AIF).

In RCC, immunohistochemical expression of AIF was found to be downregulated in tumor tissue in 84% of cases, and this downregulation was associated with deletion of AIF and methylation of its promoter.

To explain the mechanism of TNFα-induced necroptosis and the receptor-interacting proteins and cellular pathways involving necroptosis, the explanations in the text are illustrated with 5 detailed figures that help to understand the role of RIPK3 in oncogenesis. RIPK3 is also involved in the processes of neoplasm growth and progression. Four major mechanisms by which RIPK3 can promote tumor growth have been identified. RIPK3 is also involved in the processes of neoplasm growth and progression. The doctoral student reviewed the manifestations of RIPK1, RIPK3 and MLKL in various tumour tissues such as: colorectal carcinoma, pancreatic carcinoma, prostate carcinoma, non-small cell lung carcinoma, breast carcinoma, malignant melanoma and acute myeloid leukaemia, evaluating the results individually and skilfully moving on to set specific goals for this thesis.

The analysis of the literature review is discussed as a conclusion and highlights that data on the impact of necroptosis in the development and progression of RCC are scarce and there is a growing need for new and different therapeutic strategies for RCC cases not responsive to conventional therapy. Renal cell carcinoma tumor cells are resistant to the intrinsic and extrinsic pathways of apoptosis, which can occur by another, alternative pathway that is caspase-independent and involves AIF. The role of AIF in RCC is poorly understood and further studies are needed, which is why it is the subject of the present study. The doctoral student notes the need for further in-depth studies on the role of RIPK3 in tumor development, progression, metastasis, recurrence, and anti-tumor immunity in BCC, as well as its correlation with clinic-morphological parameters.

Goal and tasks: The goal was clearly and precisely formulated, namely to study the immunohistochemical expression of the apoptosis-inducing factor (AIF) marker and the necroptosis marker, receptor-interacting protein kinase 3 (RIPK3) in patients with renal cell carcinoma and to determine their prognostic value.

The identified 6 tasks significantly exceed the set goal in terms of volume. The doctoral student aims to study AIF apoptosis and RIPK3 necroptosis both in tumor and adjacent non-tumor tissue, in metastatic lesions and to make a comparative analysis of the obtained results. Tasks 5 and 6 aim to compare AIF and RIPK3 markers with clinicopathological parameters: sex, age, tumor stage,

histological appearance, degree of differentiation, tumor necrosis, tumor-infiltrating lymphocytes, vascular invasion, patient survival, as well as to determine their prognostic role in renal carcinoma.

5. Research methodology

A total of 80 patients were retrospectively included, divided into 3 groups by histological type (pRCC-papillary carcinoma, chCC-chromophobe carcinoma and ccRCC-clear cell carcinoma). The material used in the study was sufficient to obtain statistically reliable results. The patients from the selected cases were biopsied and/or operated on in Saint Marina University Hospital, Varna. The research methods are modern, and their reporting, description and comments show that the dissertation student uses them skillfully.

Staging criteria, degree of differentiation, area of necrosis, intensity of TILs, and staging criteria in tumors were clearly and precisely defined. The chosen research methodology allowed to achieve the set goal and to obtain an adequate answer to the tasks solved in the thesis. The immunohistochemical expression of AIF was assessed semi-quantitatively using H-score (histo-score) on tissue sections and evaluation of nuclear expression of RIPK3. The percentage of positive cells for each individual intensity was calculated, and the H-score was calculated using a formula.

Statistical methods: data processing and analysis were performed with the statistical package IBM SPSS ver. 21 and graphs were constructed in Microsoft Excel. I should note the extremely good and detailed statistical processing of the materials, which gives credibility to the results obtained. The results are presented in summary form in tables and are illustrated with appropriate graphs: box-plot type, bar graphs, and survival curves are also calculated. The chosen research methodology allows achieving the set goal and obtaining an adequate answer to the tasks solved in the thesis.

Results and discussion: the Results section is presented on 79 standard pages, illustrated with 52 figures and 70 tables. One is immediately impressed by the extremely detailed and thorough presentation and illustration of the research.

The results obtained by Dr Yanulova are well explained and illustrated, correctly described, logically arranged and accompanied by well-structured tables, figures and photographs. The quality of the photographic material in terms of routine microscopic and immunohistochemical examination I can define as excellent. The cytoplasmic and nuclear expressions of AIF and RIPK3 in tumor and normal tissues of all 80 patients were determined according to the objectives of the thesis. The mean cytoplasmic expression of RIPK3 was determined by H-score.

According to some of Dr. Yanulova's statistically significant results, a correlation was found between T stage and tumor-infiltrating lymphocytes. The doctoral student found a statistically significant correlation between the extent of tumor necrosis factor (TN) and the histological variant of RCC. In the present study, SCCs and PCs with TN predominated. The presence of TN was an independent predictor of overall survival in patients with SCC and PC. Dr Yanulova found that about one fifth of patients without regional lymph node metastasis (N0) had necrosis greater than 30%. The right kidney had more cases with necrosis, over 30%, compared to the left. In relation to the sex of the patients, almost 92% of the tumors in the female sex did not have TILs, while in the male sex this proportion was 50%.

Dr. Yanulova found that in advanced renal cell carcinoma (T3 and T4 stage), tumors showed a lower degree of differentiation, a larger area of necrosis, a higher intensity of TILs and hade a higher risk of death compared to earlier stages of carcinoma.

Tumor necrosis was more common in papillary renal cell carcinoma than in its chromophobe variant. From the clinico-morphological indices, the large area of tumor necrosis correlates with lymphovascular invasion.

In the absence of vascular invasion, the survival rate of patients with renal cell carcinoma was higher compared to patients in whom it was present.

Survival of renal cell carcinoma patients decreased with increasing patient age and area of tumor necrosis. It was more prolonged in males compared to females.

For tasks 2 and 3 regarding AIF expression, the doctoral student found no statistically significant difference in the cytoplasmic intensity of AIF in the tumor and non-tumor tissues of renal cell carcinoma, and AIF expression in the cytoplasm of tumor cells was higher compared to nuclear expression.

Dr. Yanulova found no correlation of AIF nuclear expression in renal cell carcinoma with any of the clinical and morphological parameters examined: age, sex, histological variant, degree of differentiation of the carcinoma, tumor necrosis, TILs, vascular invasion, T, N and M stage, tumor localization and patient survival. In the cytoplasm of papillary renal cell carcinoma, apoptosis-inducing factor expression was higher than that of the clear cell variant, and the difference was statistically significant. The expression of AIF in the cytoplasm of tumor cells was higher in T1 stage compared to T3 stage, which also appears to be a prognostic factor.

Regarding RIPK3 in the cytoplasm of tumor cells, Dr. Janulova found that it decreased compared to adjacent non-tumor tissue. The cytoplasmic level of RIPK3 in tumor cells is higher compared to nuclear content.

The correct choice of the statistical methods used contributes to the correctness of the conclusions drawn. Each result is accompanied by an explanation and statistical conclusions.

Dr. Yanulova did not establish a correlation of the nuclear expression of AIF in renal cell carcinoma with any of the examined clinico-morphological indicators: age, sex, histological variant, degree of carcinoma differentiation, tumor necrosis, TILs, vascular invasion, T, N and M stage, tumor location and patient survival. In the cytoplasm of papillary renal cell carcinoma, the expression of apoptosis-inducing factor was higher than that of the clear cell variant, the difference being statistically significant. The expression of AIF in the cytoplasm of tumor cells in T1 stage is higher compared to T3 stage, which is also a prognostic factor.

Regarding RIPK3 in the cytoplasm of tumor cells, Dr. Yanulova found that it decreased compared to adjacent non-tumor tissue. The cytoplasmic level of RIPK3 in tumor cells was higher compared to the nuclear content.

The correct choice of the used statistical methods contributes to the correctness of the conclusions drawn. Each result is accompanied by an explanation and statistical inferences.

6. Characteristics and evaluation of the dissertation work

The doctoral student makes a detailed comparative analysis of the cytoplasmic and nuclear expression of RIPK3 AIF and necroptosis RIPK3 in both tumor and adjacent non-tumor tissue, in metastatic lesions and makes a comparative analysis of the obtained results depending on the age of the patients, the histological type of the tumor, T - stage of tumors, metastases, and survival of patients with BCC. The research conducted and the results achieved fully correspond to the tasks set. A parallel is drawn between the data in the literary sources, the world experience and the achieved own results. The discussion is of a very good standard, with clear and precise scientific and statistical language and style. 266 literary sources were used, of which 5 were from Bulgarian sources, and the experience of our studies in the field of necroptosis in breast, prostate and colorectal carcinomas was also reflected.

7. Contributions and significance of the development for science and practice.

18 conclusions, 2 original scientific contributions and 5 practical-applied scientific contributions were formulated. The conclusions are based on the statistical correlation of the studied cases and the practical interpretation of the results according to the tasks set. Regarding the contributions, the doctoral student determined the prognostic and predictive value of AIF and RIPK3 in patients with RCC for the first time in Bulgaria. The processes of apoptosis and necroptosis as prognostic and predictive markers were evaluated immunohistochemically by AIF and RIPK3.

For the first time, a complex clinico-morphological and immunohistochemical analysis of AIF and RIPK3 was performed, in relation to the main clinico-morphological indicators such as age, area of tumor necrosis and vascular invasion as prognostic factors for reduced survival in patients with RCC.

The morphological profile of advanced renal cell carcinoma was confirmed in terms of the degree of differentiation, tumor necrosis, infiltration of TILs, and the risk of death was assessed. The doctoral student proved that the cytoplasmic expression of AIF in renal cell carcinoma was related to lymphovascular invasion and tumor progression.

One of the significant original contributions of the study is the complex clinico-morphological and immunohistochemical analysis of renal cell carcinoma in relation to patient survival.

The main achievement of the scientific work is the formulation and justification of a new hypothesis regarding the role of apoptosis and necroptosis in RCC. Dr. Yanulova proved by new means essential new aspects in the existing scientific problem. The obtained new facts prove the role of AIF and RIPK3 in the progression and survival of BCC and provide perspectives for the development of research in this field.

8. Evaluation of publications connected to the dissertation

Dr. Yanulova presented two full-text publications in peer-reviewed journals, where she is the first author.

9. Thesis summary

The thesis summary is 67 pages long and is prepared according to the requirements, it includes the main parts of the dissertation, the necessary volume of tables, figures and photographs. It provides an excellent overview of the overall dissertation work and reflects the main results, conclusions and contributions.

10. Critical remarks and recommendations

I have no critical remarks on the layout of the dissertation, the research conduction and the set of materials provided.

11. Personal impressions

From the presented dissertation, I believe that Dr. Yanulova has independently developed the topic, and the results shown define her as a responsible, diligent and consistent young scientist who is very good at handling scientific sources and facts, is able to analyze, compare and summarize the results obtained, has analytical and practical thinking. It is evident that Dr. Yanulova shows enviable

consistency and ambition to work in the field of science and to develop in her chosen professional field.

12. Recommendations for future use of dissertation contributions and results.

I recommend that the large number of results in the present work be published in journals and presented at national and international forums, as well as use of the data, results and literature summary in a monographic work.

CONCLUSION

In conclusion, I want to say that a tremendous amount of research has been done, even exceeding the standard requirements for a doctoral thesis. The dissertation contains scientific, scientific-applied and applied results, which represent an original contribution to science and meet all the requirements of the Act on the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for its Implementation and the Regulations of MU-Varna. The submitted materials and dissertation results fully comply with the specific requirements adopted in connection with the Regulations of the MU-Varna for the application of the mentioned Act.

The dissertation shows that the doctoral student Dr. Yanulova **has** in-depth theoretical knowledge and professional skills in a scientific specialty 7. Healthcare and sports, professional field 7.1. Medicine; and **demonstrates** qualities and skills for independent conduct of scientific research. Based on the above, I confidently give my ***positive assessment*** for the conducted research presented by the above-reviewed dissertation work, thesis summary, achieved results and contributions, and ***I propose to the honorable scientific jury to award the educational and scientific degree 'doctor'*** to Dr. Yanulova in the doctoral program "Pathology and cytopathology" and I urge **the other members of the honorable scientific jury to vote positively.**

December 04th, 2024

Reviewer: ..

Assoc. Prof. Sylvia Genova, MD, PhD