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REVIEW
from
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REGARDING:

Dissertation on "CYTOGENETIC AND MOLECULAR-CYTOGENETIC MARKERS IN PATIENTS WITH MULTIPLE MYELOMA - PROGNOSTIC SIGNIFICANCE"
Author: Dr. Valentina Dimitrova Miteva, PhD student
Form of education - full-time, Department of "Medical Genetics" Varna University, enrolled in the doctoral program "Genetics" for the acquisition of the ERD "Doctor", Field of higher education: 4. Natural sciences, mathematics and informatics, Professional direction: 4.3. Biological Sciences.
Scientific supervisors: Prof. Dr. Ilina Dimitrova Micheva, d.m. And Assoc. Dr. Trifon Georgiev Chervenkov, Ph.D

Procedure:

According to Order No. R-109-347/ 18.07.2023 of the Rector of the MU - Varna, I was elected as a member of the Scientific Jury, and on the basis of Protocol No. 1/27.07.2023 of the first (on-line) meeting, I was appointed for the chairman of the Scientific Jury and reviewer of the dissertation work of Dr. Valentina Dimitrova Miteva for awarding the educational and scientific degree "Doctor" in the scientific specialty "Genetics".
Dr. Miteva is enrolled as a full-time doctoral student in "Genetics" with a study period of 3 years (Order No. R-109-429 / 16.07.2018 of the Rector of MU-Varna) with the initial topic "Predictive genetic biomarkers in some types of Non-Hodgkin's disease lymphomas", and scientific supervisor Prof. Dr. L.Angelova. Corresponding decision of the Departmental Council (Protocol No. 19/11.12.2020) on the occasion of a Report by the Head of the Department of Medical Genetics (Prof. Dr. L. Angelova, PhD), the topic was modified to "Cytogenetic and molecular-cytogenetic markers in patients with multiple myeloma - prognostic significance' with the scientific leader scientific supervisors Assoc. Dr. Ilina Dimitrova Micheva, PhD and Assoc. Dr. Trifon Georgiev Chervenkov, PhD (Order No. R-109-23/15.01.2021 of the Rector of MU Varna).

The stages of the doctoral studies for admission to the defense have been observed - decision of the Faculty Committee (Report №103-3473 /13.07.2023 by the Dean of the Faculty of Medicine allowed the possibility of extending the regular term by 6 months and interrupting the studies for a period of 1 year for personal reasons of the doctoral student. The doctoral student was expelled with the right of defense (Order No. R-109-347/ 18.07.2023 of the Rector of the MU – Varna).

The dissertation work of Dr. Valentina Miteva was prepared in the Department of Medical Genetics of the Medical University "Prof. Dr. Paraskev Stoyanov" - Varna; Laboratory of Medical Genetics and Clinics of Clinical Hematology at UMBAL "St. Marina" EAD Varna. Dr. Miteva has submitted all the necessary materials according to the requirements of the PRAS of MU-Varna.

This review has been developed and presented in accordance with the requirements of The Law on the Development of the Academic Staff of the Republic of Bulgaria /ZRASRB/, The Regulations for the Implementation of the ZRASRB and the Regulations for Academic Development staff at Medical University /MU/ - Varna. I declare that I have no conflict of interests with the author of the dissertation.

Brief biographical data and professional qualifications

Dr. Valentina Dimitrova Miteva was born on April 23, 1984. in the city of Balchik. She completed "Stefan Karadzha" Secondary School, Natural and Mathematical Profile, Kavarna (Diploma No. 585-55/2003) and University education in Medicine at "Prof. Dr. Paraskev Stoyanov" Medical University, Varna (Diploma No. 001457 / 2011). In the same year, she started working as a medical doctor (substitutive position) in the Laboratory of Medical and Molecular Genetics, at the University Hospital "St. Marina" for 4 months (until March 2012) and continued as a resident doctor in the Neurological Department of "MBAL-Kavarna" EOOD for 9 months (until 2016).
Dr. Miteva began her academic development in October 2016, when, after winning a competition, she became a full-time assistant at the Medical Genetics Department of the University of Varna (until October 2021) and a specialist doctor to the Laboratory of Medical Genetics at the University Hospital "St. Marina" Varna (until July 2020). She became a specialist in Medical Genetics (Diploma No. 025289 /01.01.2022) in this period. He acquired professional skills and competences adequate for teaching medical genetics to "Medicine" students, Bulgarian- and English-language training and to students from the "Midwifery" Faculty in Society Healthcare. The wide range of interests of Dr. Miteva is impressive, participating in a number of qualification courses and specializations, as well as in 3 scientific projects financed by the "Science" Fund of the University of Varna on the problems of infertility and unexplained genetic diseases in children. She is the co-author of 19 full-text publications (10 of which in journals indexed in Web of Science / Scopus) and 29 abstract publications (15 of which in indexed journals).

Structure of the dissertation

The dissertation is written on 123 pages and illustrated with 39 figures and 12 tables. An Appendix is also included. The used borrowings from color figures and photos are with the cited affiliation of the corresponding author.
The dissertation includes: table of contents (3 pages), abbreviations (4 pages), introduction (2 pages), literature review (45 pages), working hypothesis (missing), goal and tasks (2 pages), clinical contingent and methods (16 pages), research results (21 pages), discussion (5 pages), conclusions (1 page), contributions (1 page), bibliography (24 pages), publications related to the dissertation work (1 page) . The bibliography covers 206 literary sources, of which 4 are in Cyrillic, and most of them are from the last 10 years.
The abstract, 69 standard pages, is written in accordance with the dissertation.

Note: The scientific work is structured in the 8 standard sections, in an acceptable ratio; the volume of the literature review is dominant at the brief Discussion.

Actuality of the subject of the dissertation

The dissertation submitted for review concerns a current topic related to the need for improved prognostic value and modernizing the approach to treatment of Multiple Myeloma (MM). It is a debilitating disease with a high incidence in developed countries and a complex genomic phenotype of myeloma cells.
Understanding the behavior of these cells, their biology, are factors of prognostic significance and are of utmost importance for the differentiation of risk groups and for optimizing the applied therapy.
The **literature review** impresses with its volume, illustrated with 20 figures and 9 tables. In it, the author correctly systematizes the scientific information on the developed topic, in places, with unnecessary propaedeutic details. The scientific data is systematized and a good awareness is shown regarding 1) Historical and contemporary review on the biology of Myeloma disease and 2) Clinical and laboratory features and stage, classic and new prognostic biomarkers.
The thesis is supported that in looking for the diagnosis and carrying out therapy, genetic studies are also of fundamental importance, with conventional cytogenetics (CG) and fluorescence in situ hybridization (FISH) being most often used. It is clarified that in the early stages of MM , the karyotype in most cases is normal, and due to the low mitotic activity of myeloma cells, cryptic aberrations remain "hidden". In the late stages of the disease, CG can identify between 30-50% of chromosomal abnormalities. For this reason the offered reporting of the molecular cytogenetic profile by FISH (determining the course of the disease) aims to adapt to the (Revised) International Staging System to detect high-risk patients with MM.
A rich scientific bibliography is presented, which shows the PhD student's good knowledge of significant contributions from world-renowned leading hematologists abroad. There is a lack of developments by Bulgarian authors in the practical and applied field of multiple myeloma.

Note: There is no Summary of the review, from which a Working Hypothesis, giving reasonable arguments for the choice of the developed topic, can be logically constructed.

The aim of the current dissertation is clearly formulated - to establish and analyze the type, frequency and prognostic significance of chromosomal disorders in newly diagnosed patients with a clinical diagnosis of multiple myeloma.
To achieve this goal, 7 **tasks** have been formulated, which are logically connected and in a synthesized form.

1. To select the object of the study, to group it by stages
2. To establish the frequency and structure (type) of the detected chromosomal aberrations
3. To analyze chromosomal aberrations revealed by conventional cytogenetic method and their prognostic significance.
4. To analyze the chromosomal aberrations revealed by the molecular cytogenetic method FISH and their prognostic significance.
5. To analyze the amount of plasma cells in the bone marrow compared to the cytogenetic results.
6. To establish survival against cytogenetic results.
7. To make a comparative analysis of survival curves depending on the staging system.

Particular emphasis is given to the third, fourth and up to the sixth tasks, related to the possibility of deriving a correlation at the cellular and subcellular level of genetic biomarkers with prognosis and survival of patients with MM. *Tasks six and seven need clarification*.

Material and methods - the section is presented in sufficient volume. The study included the prospectively and retrospectively collected data of 110 newly diagnosed patients with multiple myeloma aged 38 to 91. The patient contingent was referred by the University Hematology Clinic of UMBAL "St. Marina" - Varna for genetic analysis in the Laboratory of Medical Genetics of the "St. Marina" UMBAL EAS Varna. Information from the hospital electronic database was also used.

The study period is 5 years (2016-2020), a relatively short period of time to cover, considering the rarity of the disease. Patients were diagnosed according to generally accepted diagnostic criteria and risk stratifications of the International Myeloma Working Committee; the main criterion for inclusion in the study is that patients have undergone conventional cytogenetic and/or molecular cytogenetic analysis by FISH.
The genetic methods learned and applied by the PhD student are described propaedeutically in details and precisely in laboratory protocols, so that they could be reproduced as basic working techniques. A statistical panel with a rich set of statistical methods with specific groups of questions and software products, personal work of the doctoral student, was used.

Note Exclusion criteria are imprecise and need reediting - according to research methods, patients are divided into 2 main groups of genetic studies, untreated with the same methods

The **Results** are described in response to the tasks set and illustrated with 3 tables, 10 figures and 1 Appendix presenting a sample of demonstrative karyograms and FISH signals in patients with pathological results. The attached tables and figures have no duplication of information. According to the type of questions asked for resolution and the studies conducted, the results are grouped into 3 groups and displayed in the Description of patients by demographic indicators (age, gender) and staging system.

- Results of conventional cytogenetic analysis in 97 patients
- Results of application of molecular-cytogenetic research (by fluorescence in situ hybridization) in 30 patients
- Evaluation of the chromosomal finding and stage relative to the average survival of the patients

Cytogenetic testing for prognostically significant chromosomal aberrations over a 5-year period was performed in 97 patients, successful in 83 (86%) of them *(very good methodical success rate for bone marrow culture extraction and analysis in MM patients*). I consider Table 11 here unnecessary (normal chromosomal finding of all examined) and inaccurately titled (a column with results of partially applied FISH is involved).
Interphase FISH analysis was performed once, with selected probes, in a heterogeneous group of 30 patients, incl. 17 covered by both analyses. Thus, the author fell into the trap of the small number of patient groups, which limits the possibilities for comparability of statistical methods. Molecular cytogenetics with different probes for oncohematological markers has been applied in practice in:

- 9 patients - to search for del(17)(p13): found in 6 individuals (67%)
- 15 patients - search for del(17)(p13), translocation t(4;14) and translocation t(14;16): del(17)(p13) found in 3 (20%); translocations have not been identified
- 6 patients - search for a rearrangement in the IGH gene (rear 14q32): not established.

Del(17)(p13) observed in 67% (6/9) - the chromosomal marker, the presence of which is associated with an unfavorable prognosis, is the most represented among the examined patients. I find it appropriate to describe these cases in a table of revealed chromosomal disorders, comparing the two genetic studies (by probe/s used del (17)(p13); t(4;14), t(14;16); rear14q32). This is how the parallel between mutational status and discoverability would emerge. High scientific value would have the possibility of applying a more extended set of molecular genetic markers involving the IGH gene t(6;14), t(11;14), t(14;20) or related to the Mayo stratification criteria to determine of standard and high risk in a wider range of MM patients (listed in Table 7 of the Literature Review). I suspect scientific funding is a matter of fact, and future work in this field requires massive and full-covered application of both analysis with a panel of probes in all patients with MM. For cytogenetically undetected rearrangements in the 14q32 region (IGH gene), a methodologically delicate but highly sensitive molecular genetic DNA analysis by reverse transcriptase of selected cell populations - plasma cells, was introduced and applied.

Attention is drawn to the assessment made by the doctoral student of a statistically significant difference when comparing the average survival of patients with: a) the result of cytogenetic and molecular-cytogenetic research; b) ISS stage of the disease, where mortality by clinical stage is also significant. There was no statistically significant difference between median survival and Revised International Staging System R-ISS. Extrapolation of findings is by comparing Kaplan–Meier curves or regression analysis.

I accept the indicated results and their statistical processing as adequate to the set goals and objectives.

Note - characterization of patients by gender and age is their baseline characteristic and should be in the Material (Patients) and Methods section, not in the Results section.

The **Discussion** is an essential section for any dissertation work, in which the doctoral student analyzes the obtained results in the context of his knowledge of the literature review and looks for a logical analysis in evaluation and comparability.
In population studies similar in volume, similar data are found for the average age of incidence (62 years) and the age of peak incidence 65-69 years (25.45%).
An overall frequency of chromosomal abnormalities in MM patients was sought with multiple studies with similar numbers of patients included, where pathology margins ranged from 31.1% to 66%, against the background revealed in the particular study of 20% needing discussion of the methodological approach for comparison.
The PhD student builds on chromosomal markers and skilfully targets the molecular-cytogenetic level, which has prognostic and predictive marker value for a more precise assessment of the risk of progression. The author tries to evaluate the findings against the clinical stage, albeit in a small group of patients, by going out of genetic laboratory knowledge and seeks an interdisciplinary approach to hematologically affected patients, unused in mass clinical practice.
It is appropriate some other results (deletion in 8q24 as well as 45,XY, –Y) to be comment on. The most common monosomies are on chromosomes 13, 14, 16 and 22, in addition, loss of an X or Y chromosome was found in some of the patients 189.
Note A short interspersed section, albeit on the subject, with elements of a literature review and incompleteness in explanations and analysis.

The **Conclusions** of the PhD Thesis are based on the obtained results and correspond to the set goals and objectives.

1. The applied comparative analysis of the frequency and structure of chromosomal abnormalities in newly diagnosed MM patients shows a high agreement of the data from our study with those described in the literature.
2. The frequency of detected chromosomal aberrations established by conventional CT is 17% (n=14), and they are divided into hyperdiploid and non-hyperdiploid. The incidence of detected pathology by FISH analysis was 30% (n=9).
3. The evaluation of detected chromosomal disorders by CG:
    - In patients with a hyperdiploid karyotype, trisomies predominate
    - Structural disorders predominate in patients with a non-hyperdiploid karyotype
4. The evaluation of detected chromosomal disorders by FISH analysis. Through FISH analysis, in 9 patients (30%), a deletion was found in chromosome 17 - del(17)(p13), in the rest the results were normal.
5. A correlation was established between the amount of plasma cells in an aspiration biopsy of CM and the pathological karyotypes:
    - In all patients (n=14) with a pathological karyotype, the number of plasma cells exceeded 30%.
    - In patients with a normal karyotype, in 51% (n=35) the percentage of plasma cells in the CM exceeded 30%, in 32 (46%) it was less than 30%, and in two (3%) the sternal puncture was dry.
6. A statistically significant difference was found regarding the average survival compared to the cytogenetic finding, in patients with a normal karyotype it was 34 months, and in those with a pathological one - 8 months (p=0.0493).

• No statistically significant difference in median survival was found between patients with hyperdiploid and non-hyperdiploid karyotype probably due to the small number of patients (p=0.63). The same applies to the detected chromosomal abnormalities according to ISS stage (p=0.094).

7. A statistically significant difference was found in survival versus ISS stage (p<0.05) The data from the analysis showed a median survival of 11 months for patients in the third stage. For those in the second stage, it is an average of 60 months, and in the first – 67 months.

• No statistically significant difference in survival was found according to R-ISS stage (p=0, 14). The average survival of patients in the second stages according to R-ISS is 41 months, and in the third – 18 months.

Note. The way of presentation of the majority of conclusions is acceptable. Some of the findings need clarification so they don't sound like findings (1) or explanations (6), others need serious editing (3,4) to meet the task of their prognostic significance.

I accept the author's self-assessment for the **Contributions** of the dissertation work, formed in 2 groups, no such of original type.

Contributions of confirmatory type

1. The importance of classification and risk stratification systems as prognostic factors has been confirmed
2. The importance of the application of conventional cytogenetic method and FISH analysis as routine methods to distinguish low- and high-risk patients with MM has been confirmed
3. The importance of chromosomal disorders as a prognostic factor for survival in the Bulgarian population has been confirmed

Contributions of an practically applied type

1. In all newly diagnosed patients with MM, it is recommended to conduct CG and FISH, which are of utmost importance in the selection of a therapeutic scheme and also for the prognosis.
2. Screening for t(4;14) and del(17p), which have an unfavorable prognosis in newly diagnosed MM patients, is recommended as a mandatory element of the diagnostic panel studies.
3. It is recommended that analysis for t(4;14) and del(17p13) be performed by FISH.

On the contributions of an applied nature, I find inaccuracy (1) regarding the choice of therapeutic scheme (non-binding correlation) and lack of motivation (3) to apply FISH to look for t(4;14) as a result of the present work.

Papers in connection with the dissertation

According to the scientific field of the thesis, Dr. Miteva has presented: 5 publications in full text (co-authored): 3 in refereed journals *Archives of Hellenic medicine* (2023), *Cytology and Genetics* (2023), *General Medicine* (2019) and 2 in the Journal of Medical Referrals (2022) (2021); as well as 2 published announcements from scientific forums *Abstract Book HemaSphere* (2021) and *European Journal of Human Genetics* (2020). It makes a good impression that the PhD student is the first or second author on them; the publications in Archives of Hellenic medicine, Cytology and Genetic and the abstract in European Journal of Human Genetics are refereed journals with an impact factor. Almost all are from the period of the last three years (2021-2023) after the covered period, which explains to some extent the procedural delay to finalization.

Critical notes, commentary and recommendations to the dissertation

I have presented the separate notes and comments in the relevant sections.

My general view of this scientific work essentially represents the doctorial activity in acquisition of laboratory (personal skills, implementation) and analytical experience in molecular cytogenetic testing of patients with myeloma disease in support of clinical oncohematological practice. I fully agree with Dr. Miteva's opinion about the limitations of the study resulting from the small number of patients with applied FISH analysis in a short period of coverage (5 years). We are aware of the financial impact in research investigations at molecular level in medicine (genetics) and understand the restrictions arising from this factor. Large cohort studies with extended genetic analyzes over a

longer period in MM patients are needed. I believe and encourage the PhD student to continue the experience gained to deepen the scientific discussion for the purposes of clinical application.
Some technical and stylistic recommendations for consideration in future projects are related to formulation of Scientific hypothesis, Summary of literature review, detailed writing of cytogenetic markers and FISH signals according to the accepted nomenclature.

On a personal level, I have known PhD student Dr. Miteva since she joined the Department in 2016 until 2022. As an assistant and specialist, she has very good theoretical and practical knowledge in the field of Medical genetics, which she applied daily in her practice as a doctor and teacher. She is a cooperative and tolerant teamworker and possesses high technical skills (certified competencies), in computer programs and platforms.

Conclusion:
The doctoral student demonstrates good *knowledge* in the field of biological, genetic sciences (theoretical and laboratory) and *skills* in using statistical methods and databases in processing for their clinical application. The PhD thesis is contemporary for the Bulgarian patients with MM. The doctoral student meets the scientometric criteria in accordance with the rules for academic development of MU-Varna for awarding the scientific-educational degree "Doctor"and I recommend to the scientific jury to award the scientific and educational degree "doctor" in the specialty "Genetics" to Dr. Valentina Dimitrova Miteva.

25.08. 2023 **Chairman of the Scientific Jury and reviewer:**

PROF. DR LUDMILA ANGELOVA, MD