REVIEW

of the doctoral thesis presented for the acquisition of the scientific degree "Doctor of Sciences" in the field of higher education 4. "Natural Sciences, Mathematics and Informatics ", Professional field 4.3. "Biological Sciences", Scientific specialty: "Molecular Biology".

Author of the thesis: Chief Assistant Professor, Dr. Tihomir Iliev Valchev
Topic: Comparative genomic, transcriptomic and proteomic studies in neurodevelopmental disorders
Reviewer: Academician Prof. Ivan Georgiev Ivanov, Doctor of Biological Sciences,
Bulgarian Academy of Science, Institute of Molecular Biology

1. Subject of review

By order № P33-5262 of 29.10.2020 of the Rector of the University of Plovdiv "Paisii Hilendarski" I am appointed a member of the scientific jury for the defense of a dissertation entitled *„Comparative genomic, transcriptomic and proteomic studies in neurodevelopmental disorders“* for the acquisition of the scientific degree "Doctor of Science" in the field of higher education 4. "Natural Sciences, Mathematics and Informatics ", Professional field 4.3. "Biological Sciences", Scientific specialty: "Molecular Biology". The author of the thesis is Chief Assistant Professor Dr. Tihomir Iliev Vachev from the Department: "Plant Physiology and Molecular Biology", Faculty of Biology, University of Plovdiv "Paisii Hilendarski" (U of P).

The set of materials presented to me on paper and electronic carrier is prepared in accordance with Art. 45 (4) of the Regulations for Development of the Academic Staff of the U of P. It includes the following documents:

–request to the Rector of PU for disclosure of a defense procedure;
- CV (European format);
- copy of diplomas for PhD degree;
- protocols of departmental councils related to the opening procedure;
- thesis;
- thesis abstract;
- list of papers related to the dissertation;
- copies of scientific publications;
- declaration of originality and authenticity of the attached documents;
- reference for compliance with the minimum national requirements;

2. Brief biographical data

Tihomir Vachev was born on January 9, 1981. He is a graduate of the Faculty of Biology, U of P from where he obtained a BS degree in“ Biology” in 2005 and a MS degree in“ Molecular Biology and Biotechnology ” in 2008. Until 2011 he was a full-time PhD student at the Department of Plant Physiology and Molecular Biology, where (under the scientific guidance of Prof. Ivan Minkov) he developed and successfully defended his PhD thesis in "Molecular Biology". Since 2013 he has been a PhD student in Genetics at the Medical University of Plovdiv, from where in 2016 he obtained a second PhD degree in Genetics. At the Dept. Plant Physiology and Molecular Biology, U of P, Dr. Valchev held the positions of biologist, Assistant Professor and Chief Assistant Professor.

3. Relevance of the topic and the thesis goals

The dissertation of Dr. T. Valchev is dedicated to the molecular characterization of a group of widespread neuropsychiatric diseases known as Autism Spectrum Disorders (ASD) and schizophrenia. Until recently, they were of interest only to neurologists and psychiatrists, but during the last two decades, they have also been studied by molecular biologists and geneticists. Thus,

autism and related syndromes have been shown to have a hereditary background. Proof of this is the fact that the probability of developing ASD in identical twins is over 90% in comparison with the controls. All this has motivated Dr. Valchev to dedicate his dissertation to the search for molecular markers for diagnosis and prognosis of ASD and schizophrenia.

The aim of the thesis is formulated briefly: *To conduc comparative genomic, transcriptomic and proteomic studies of neurodevelopmental disorders.* The tasks are set more specifically. They reveal that the author intends to conduct a large-scale study on ASD diagnosed patients in terms of: comparative expression analysis of protein-encoding genes, comparative expression analysis of microRNA (miRNA), comparative proteome analysis (iTRAQ), complete exomatic sequencing, ROC analysis of differentially expressed genes and siRNAs, etc.

Considering the great social significance of the studied diseases, their heterogeneity and difficulties in diagnosis, the relevance of the dissertation topic is beyond doubt.

4. Familiarity with the problem

Judging by the literature review, Dr. Valchev is well acquainted with the literature on his research topic. Much of the review is devoted to the etiology of schizophrenia and ASD, with an emphasis on the molecular basis of their pathology. The presumed factors related to the diseases are considered, such as the age of the parents (particularly the father's age), environmental factors, prenatal and premorbid stress, complications during pregnancy, nutrient factors, use of psychoactive substances, etc.

The main pathogenetic hypotheses for the occurrence of schizophrenia and ASD, such as glutamatergic, dopaminergic and GABA-ergic hypotheses, have been critically analyzed. In a separate section, the molecular genetic characteristics of the studied disorders are considered, namely gene variations, number of gene copies, etc. Along with the genetic factors, some epigenetic factors (DNA methylation and histone modifications, activation of the maternal immune system, etc.) related to schizophrenia and autism are also considered. Special attention is paid to the dysregulation of gene expression under the control of micro RNAs (miRNAs). Here, the author describes their interactions with mRNA, influencing gene expression at transcriptional and translational levels. The relationship between the expression level of cellular and free-circulating miRNAs with neuropsychiatric disorders has also been commented, in terms of their potentials to be used as biomarkers for schizophrenia and autism. In connection with the latter, some methodological and technological aspects of miRNA analysis are considered.

Based on the in-depth and creative analysis of the voluminous literature, Dr. Valchev has found successfully his research niche.

5. Methodology of investigation

The methodological section covers 70 pages and includes a detailed description of the numerous methods used in the thesis: isolation and quantitative analysis of RNA and DNA from peripheral blood; Next generation DNA and RNA sequencing; complete exomatic sequencing; qRT-PCR analysis of the expression profile of miRNA and protein-encoding genes associated with ASD; bioinformatic analysis of sequencing data; analysis of gene coverage and gene expression; identification of differentially expressed genes; gene ontological analysis of differentially expressed genes; KEGG analysis of biological pathways enriched with such genes; quantitative proteomic analysis (ITRAQ), etc. All methods are adequate to the thesis goals and objectives.

6. Characteristics and evaluation of the dissertation

Dr. Valchev's dissertation covers 391 standard pages and contains 89 figures, 17 tables and more than 600 references. It is built in a classic style: Literary Review, Objectives and Tasks, Materials and Methods, Results and Discussion. Unfortunately, I found certain disproportion between the sections. For example, Literary Review + Materials and Methods = 170 pages, while Results + Discussion + 72 voluminous figures and tables + large spaces between them = 140 pages.

The aim of Dr. T. Valchev's thesis is to shed more light on the molecular basis of the autism and schizophrenia. The material substrate of these diseases is the brain, however, because of the impossibility of using brain samples for analysis, he looks for alternative non-invasive options, such as peripheral blood. The compromise with the inaccuracy of this strategy would be justified only in the case that a correlation between any of the marked molecular species and the corresponding mental disorder is to be established. In such a case this molecule would be an invaluable diagnostic and prognostic marker for ASD and/or schizophrenia.

In fulfillment of the set aim and goals, Dr. Valchev and his colleagues from the Medical University of Plovdiv, used a group of 40 children with ASD and 30 healthy individuals (controls), from which they collected peripheral blood samples. The latter are used for isolation of nucleic acids for the following analyses: a) complete exomic sequencing of protein-coding genes; b) comparative quantitative analysis (ITRAQ) of differentially expressed proteins; c) expression analysis of miRNA molecules.

Complete exomic sequencing has been realized by the so-called Next Generation Sequencing (NGS), performed de facto in the laboratories of Oxford Gene Technology, Ltd. As expected, the sequence analysis revealed multiple protein encoding genes associated with ASD, whose mutations might have serious biological consequences for the carriers. The identified mutations fall into four categories: a) Appearance of a new termination codon (affecting the genes MOB3C, REG4, PDE4DIP, RHBG, OR10X1, FMO6P, CAPN8, TSSC1); b) Loss of termination codon (FEZ2, GBA3, ADH1C, MATN2, NANS, ACTN3, NF1); c) Non-synonymous substitutions (SPATA3, SLC22A1, ADCK5, NLRC5, APOBR, NUDT11); d) Reading frame shift (CDCP2, CASP9, HRNR, ATG3, NOP16, FGFR4). In summary, the genomes of the ASD patients contain numerous gene variants of protein-encoding genes, whose in-depth study could help clarify the etiology, pathogenesis, and diagnosis of these diseases.

Quantitative comparative analysis of the differentially expressed proteins in ASD patients was performed by the IITRAQ method. Thus, a total of 351 proteins were identified, 60 of which were differentially expressed in the patient group. Of these, 24 showed increased and 36 decreased level of expression. The latter group includes the genes of serpin-2, interleukin-1, IgJ, angiogenin, fibulin-5, apolipoproteins C2, C4 and F, periostin, myosin-9, plestin-2 and others. Among the differentially expressed proteins stand out those involved in the regulation of the Notch signaling pathway, innate immune response (complement activation), assembly and positioning of the nucleosomes, direction of axons, cholesterol metabolism, transport of lipoproteins and cholesterol cells, etc. Most of the obtained results are affirmative.

Literature data indicate that the ASD and schizophrenia are due to the neuronal plasticity, which in turn is associated with miRNA-mediated post-transcriptional dysregulation in the brain neurons. This is what motivated Dr. Valchev to undertake a large-scale study of the overall expression profile of miRNA molecules isolated from peripheral blood samples. For this purpose, New Generation RNA Sequencing was applied using both individual and pooled miRNA samples. In the pooled samples, 42 miRNAs were identified, of which 29 with decreased, 11 with increased, and 2 with unchanged expression level in ASD children. Of these, 8 miRNAs have a particularly pronounced dysregulation. Their qRT-PCR analysis showed that some of them, such as siRNA-424-5p and miRNA-500a-5p, were associated with ASD. The mRNAs they control have been shown to be involved in various brain processes, and miRNA-500a-5p is also associated with liver cancer.

7. Contributions and significance of development for science and practice

The dissertation of Dr. T. Valchev has a fundamental character, but it has a strong applied orientation. Its ultimate goal is to find molecular markers and to develop a non-invasive method for diagnosing autism and schizophrenia. Although at this stage his results are affirmative, they enrich the knowledge of molecular basis of the two mental illnesses. Dr. Valchev has not currently defined any reliable biomarker but we have the reason to expect that such marker to be identified soon

among the hundreds of dysregulated biomolecules (genes, proteins and miRNAs) described in his thesis.

8. Evaluation of dissertation publications

Nine research papers have been published in connection with the Dr. Valchev's thesis. Of them 6 are in national and 3 in international scientific journals. In all papers Dr. Valchev is leading (first or last) author. Of the articles in national journals 4 are in Folia Medica (Q3, SJR 2017 = 0.21) and 2 in Compt. Rend. Bulg. Acad. Sci. (Q2, SJR 2017 = 0.21). Of those in international journals 2 are in Balkan J Med. Genetics (Q4, SJR 2017 = 0.2) and one in Biodiscovery (without Q and SJR). The total SJR score of all articles is 1.66. According to Scopus, the listed papers have been cited so far 56 times. My opinion is that the papers related to the thesis are published in low-ranking journals, which makes the author invisible for the international scientific community.

9. Personal participation of the author

Judging by the leadership position in the published articles, I accept that the predominant part of the dissertation's achievements are personal work of Dr. Valchev.

10. Abstract

The abstract of Dr. Valchev's thesis adequately reflects its essence and achievements.

11. Critical remarks and recommendations

I have the following remarks related to the peer-reviewed dissertation:

- The volume of the thesis has been greatly increased at the expense of the sections Literature Review and Materials and Methods. While the second section is relevant to the PhD theses, in a D.Sc. Dissertations it should contain mostly citations of the methods rather than a detailed discription.
- The "Conclusion" section is missing. In my many years of practice as a reviewer, this is the first case of a dissertation without formulated conclusions.
- "Contributions" are poorly and inadequately formulated. I cannot accept the "New generation sequencing technology or Whole-Exome Sequencing has been successfully applied" and "New generation sequencing technology RNA-Sequencing has been successfully applied" as a scientific contribution.
- Most of the results are confirmatory.
- The papers published in relation with the dissertation are not enough as number (9 only). They do not fit also the volume of the thesis (almost 400 pages). Another weakness is their low SJR score (1.66 only).
- The list of citations is not prepared according to the rules. It contains 56 links that can not be open from a computers without IP in Scopus.
- The dissertation contains many typographical, grammatical and stylistic errors, which creates the impression of negligence and a feeling that the text is at the penultimate stage of preparation.

12. Personal impressions

I have known Tihomir Valchev since the time of his doctoral studies, when I reviewed his first PhD thesis (2011). Since then, I have been observing his professional development and now I am pleased to congratulate him on his successful professional growth.

13. Recommendations for future use of the dissertation results

Dr. Valchev describes numerous genes associated with ASD, whose structure (sequence), transcriptional or translational activity is different in comparison with the control group. At this stage none of these molecules cannot be used as a reliable diagnostic marker. I would advise the

author to continue his research by selecting a smaller group of biomolecules on the basis of their extremes in the deviation from the corresponding controls and their parameters must be determined at individual (rather than pool) level. The most reliable of them must be normalized and compared with the already used in practice biochemical indicators.

14. Summary assessment of the fulfilment of the national minimal criteria

The summarized assessment for covering the minimal national criteria for the scientific degree "Doctor of Sciences" in the field of "Natural Sciences" is presented in the Table 1:

Table 1

Indicator	Minimum required	Actual score
A: Indicator 1	50	100
B: Indicator 2	100	100
C: Indicators 3 и 4	-	-
D: Indicators 5-10	100	124
E: Indicator 11	100	112
G: Indicators 12-	-	-
Total	**350**	**436**

CONCLUSION

The D.Sc. thesis of Dr. T. Valchev is dedicated to the molecular characterization and search for molecular markers for diagnosis of autism spectrum disorders (ASD) and schizophrenia. To this end, a patient group of children with ASD has been examined. Peripheral blood samples are used to isolate nucleic acids for whole exomic sequencing of protein-coding genes, comparative analysis of differentially expressed proteins and miRNAs. Multiple genes and miRNA molecules associated with ASD have been identified, whose expression level differs from that of the control group. The obtained results are mostly confirmative but they shed new light on the molecular etiopathogenesis of the investigated diseases. The results related with the thesis are published in 9 scientific articles with a total SJR = 1.66 and they are cited 56 times. To my understanding this publication activity is too low, but since the overall activity of Dr. Valchev satisfies the minimal national criteria (436 against 350 requested points), I recommend the esteemed Scientific Jury to award him the scientific degree "Doctor of Science" in the field of higher education 4. Natural sciences, mathematics and informatics, professional field 4.3. Biological sciences, scientific specialty "Molecular Biology".

11.12.2020 г.

Reviewer:

/Prof. Ivan Ivanov/