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REVIEW OF THE DOCTORAL THESIS

Review of the doctoral thesis of M.Sc. Badr Saif Mohsen Qasem entitled „Metabolomics analysis of time and oxygen effect on Fibrosarcoma cell line (HT1080) – model studies" performed at the Department of Biochemistry, Molecular Biology and Biotechnology, Faculty of Chemistry, Wroclaw University of Science and Technology under the supervision of Professor Piotr Młynarz.

Despite a huge number of studies and financial outlays, cancerous diseases still are the leading cause of worldwide death. Also worrying are the forecasts, which indicate that in the next two decades the number of deaths per year due to cancer may even double. Therefore, a fuller understanding of cancer and its causes in order to prevent and reduce the occurrence of these diseases is an important and significant challenge for the world of science in the coming years. Understating the multiple stages of tumorigenesis at the cellular level as consequences of molecular change accumulation, allowing cancer cells to manifest autonomous proliferation, apoptosis resistance, invasiveness, immune system evasiveness, immortality, and metastasis may facilitate and accelerate research aimed at limiting the development of these diseases. In addition, it seems very important to define the influence of a wide range of factors on the tumor microenvironment, shaping the condition for cancer development. One such factor, which has been of interest for several decades, is the effect of molecular oxygen level (e.g. hypoxia) on the metabolism of cancer cells. Over the past decades, scientists performed different studies trying to understand complexity of these phenomena. However, still is a lot of questions unanswered. On the other hand, it is promising the recent emergence of new techniques and analytical methods, including metabolomics and proteomics, which, due to their complexity, range and innovativeness of approach, may make it possible to explain the phenomena related to carcinogenesis.

Thus, the doctoral thesis submitted for review fits well into the trends of searching for explanations of phenomena related to cancer and the influence of various factors on the tumor microenvironment. The more so that among the modern approaches such as metabolomics, which using advanced analytical tools including mass spectrometry, nuclear magnetic resonance spectroscopy and chemometrics, the greatest hopes are placed in the identification of specific markers of the development of various types of cancer. Therefore, it is assumed that thanks to these techniques, it will be possible to broaden the scope of knowledge about the reasons why cancer cells under hypoxic conditions select a less efficient way of producing ATP and losing carbon atoms in the form of lactate, which is required for the biosynthetic process, what are the exact proportions of cancer cell activity between glycolytic and the Krebs cycle, and what is the role of stromal components in the tumor microenvironment? In addition, by analyzing the large number of metabolites present in cancer cell line samples, more information is expected to be obtained regarding the identification of pathological biochemical pathways.

The assessed doctoral dissertation of 195 pages includes three main chapters, each ended with a list of references (Chapter 1 - Introduction; Chapter 2 - Quantitative 1H NMR analysis of intracellular and extracellular metabolome of HT1080 cell line under hypoxia, normoxia, and hyperoxia; Chapter 3 - The effect of hypoxia-reoxygenation and normoxia-deoxygenation in HT1080 cell line metabolome by 1H NMR analysis), two of which are in preparation for sending to scientific journals (chapter 2 and 3). In both manuscripts in preparation, the PhD student is the first author.

In addition to the three main chapters mentioned (1, 2 and 3; pp. 14-192) and the list of manuscripts of theses in preparation for sending to scientific journals (List of publications A; pp. 193), the dissertation contains a list of all publications of the PhD student (List of publications B; pp. 193-194), a list of conferences and webinars (pp. 194-195), a foreword and preface (pp. 2-3), an abstract in English (pp. 4-6) and Polish (pp. 6-8) and a list of abbreviations used (pp. 9-13). At this point, it should be noted that the list of abbreviations used would be more helpful in reading the dissertation if this list was presented alphabetically.

In the twenty-four-page chapter 1 (pp. 15-39) entitled "Introduction" the author presented in a synthetic way the current state of knowledge about cancer biology as well as the metabolism and progression process of these diseases. In the initial part of this chapter, the PhD Student emphasizes that despite many endeavors cancerous diseases still are the leading cause of worldwide death and require further in-depth studies. Further, the Author discussed

the properties of human tumorigenesis, the transformation of normal human cells into cancer cells, the multistage nature of carcinogenesis, and the tumor microenvironment and cancer progression. In this chapter, the PhD Student also indicates the latest considerations on the Warburg effect related to metabolism reprogramming and cancer progression. In this passage, based on literature data the PhD Student points out to the important issue that even in tumors where pyruvate oxidation is suppressed and lactate is produced, the cells can still rewire their mitochondrial metabolism to produce other metabolic byproducts and intermediates that are important for biosynthesis. In the next subchapter of the introduction on very important issue related to the metabolism of cancer cells, the author outlines the relationship between cellular glucose metabolism and tumor progression and the role of some essential and nonessential amino acids in the metabolism of cancer cells. As the PhD Student points out, to find a strategy for survival in a stress condition (e.g. under nutrients deprivation), the cancer cell undergoes a complex metabolic mode, which may changed depending on how severe the these conditions. Moreover, in this subchapter, the PhD Student indicates that several recent studies focusing on the roles of nonessential amino acids on cancer progression, defined glutamine as a second nutrient and fuel for cancer cells. Later in this subchapter, the Author described important issues regarding the role of serine and aspartate in the metabolism of cancer cells. The Author describes fundamental information related to the importance of oxygen molecules to cancer cell metabolism and its concentrations *in vitro* and *in vivo* terminology. In the final fragment of the introduction, the Author presented in a concise manner the *in vitro* models for metabolomics research as well as the contributions of NMR-based in metabolomics and chemometric analysis as powerful and advantageous technology to investigate cultured cell line metabolome. Points out that currently large-scale metabolomic studies provide the greatest cognitive opportunities in understanding the ongoing processes of carcinogenesis. In my opinion, the issues presented in the introduction are closely related to the subject of the doctoral dissertation, have been developed with the use of extensive literature (99 items) and indicate a very good and broad view of the Author on the research topic undertaken.

In the next two chapters, consisting of 5 subchapters each (Introduction, Materials and Methods, Results, Discussion, Summary and References), the PhD Student presented the scope of experimental and analytical work, the results obtained and an adequate discussion summarized with conclusions related to two very important and interesting research problems.

The 77-page chapter 2, entitled "Quantitative 1H NMR analysis of intracellular and extracellular metabolome of HT1080 cell line under hypoxia, normoxia and hyperoxia", consists of 5 mentioned subchapters with 25 figures and 10 tables. At this point, an incorrect description of the numbering of the last figure and the last two tables should be indicated, and thus also incorrect references to figures and tables in the text. The last figure of chapter 2 (page 102) should be number 25 while is number 23. The consequence of this is the reference on page 101 of the dissertation to figure 23, and it should be to figure 25. A similar mistake occurs in the numbering of the last two tables of this chapter, i.e. the numbering of the tables on pages 97-98 should be Table 9 and Table 10 while there is Table 7 and Table 8. In addition, on pages 97-98, the text incorrectly refers to Table 7 and Table 8 when should be Table 9 and Table 10.

In a short but factual introduction to this chapter, the PhD Student discusses the role of different oxygen levels in the environment of cancer cells as well as *in vitro* techniques and the NMR method that can be used to study their metabolism after exposure to different oxygen conditions. Then, in the subchapter "Materials and methods" consisting of nine parts, the Author describes evaluation cell culturing process and experimental design, cell culturing medium preparation, culture media extraction, cells extraction, cell growth measurement, NMR data acquisition, metabolites identification, processing for data analysis and statistical analysis. At this point, it should be emphasized that the *in vitro* experiments, the techniques used to prepare samples for analysis, as well as analytical and statistical methods were complex and time-consuming and required a lot of work to fully achieve the assumed research goal. However, thanks to this, it was possible to obtain a lot of new information about the intracellular and extracellular metabolome of the HT1080 cell line exposed to hypoxia 1%, normoxia 6% and hyperoxia 21% condition. Summing up this part of chapter 2, I can say that a well-thought-out and refined methodology is its strong point.

At this point, an incorrect references to figures in the text should be indicated. On page 83 of the dissertation it is incorrect refer to Figure 12 and Figures 13b, 13d and 13f when should be Figure 13 and Figures 14b, 14d and 14f.

On the basis of numerical summaries, which are the result of organizational, experimental and analytical work, the PhD Student presented a description of his own achievements in individual parts of the subchapter "Results". As part of the adopted research organization, the Author first discussed the research results related to the experimental methodology evaluation. In accordance with the applicable rules, the Author

determined/checked the conditions for proper cell growth and viability and the most optimal number of cells that provide a reasonable spectrum for NMR quantification, i.e. 1 x 10^7 cells. Another important stage of the research was to determine the effect of oxygen concentration on the growth of HT1080 cells line and to demonstrate changes in their growth under various oxygen conditions, i.e.: hypoxia, normoxia and hyperoxia.

In a further part of this subchapter, the PhD Student presented a time-dependent effect of various oxygen concentrations on the intracellular and extracellular metabolome of the HT1080 cell line. The significant achievement of the PhD Student is the comparative analysis of the metabolome based on 1H NMR using advanced statistical tools between the HT1080 cell line exposed to different levels of oxygen at different points in time and the demonstration of metabolic regulation dependent on sensitivity to oxygen concentration and time. It should also be emphasized that the data obtained from these analyzes on the number of identified metabolites (over thirty in each of the tested systems) and the positive results of the verification of the applied statistical models prove the great analytical skills and precision of the PhD Student and the appropriate approach to the research problem undertaken. In conclusion, the identification of a number of metabolites and the proposition of metabolic regulation dependent on time and sensitivity to oxygen concentration are an important achievement of the dissertation.

The next part of chapter 2 of the dissertation is an eleven-page discussion in which the PhD Student assessed the results obtained against the background of the available scientific literature. In the first sentences of the discussion, the author reminds that *in vitro* metabolomics studies typically performed at standard oxygen concentration of 21%, which is extremely high when compared to normal peripheral tissues. Therefore, in the work, the Author used an oxygen concentration of 21% as hyperoxia and 6% as normoxia compared to 1% for hypoxia. In my opinion, the proposed solution is justified and allows to explain a number of phenomena related to the processes of cancer. Then the PhD Student presented a general summary of his research showing that there was a metabolic profile differences at 1%, 6%, and 21% oxygen level and nutritional stress through cultivation time and changes in a metabolite-sensitivity variations for extracellular and intracellular metabolome leading to produce metabolic phenotype of fibrosarcoma (HT1080) cell line. Moreover, the Author indicated, that presented studies deliver more sufficient model of using a proper oxygen concentration as normoxia and a potential therapeutic approach by hyperoxia and starvation against cancer. Then, in the further part of the discussion, the PhD Student discussed in detail

the most important results of the first part of his research related to the metabolic changes of intracellular and extracellular metabolome at hypoxia, normoxia and hyperoxia through cultivation time and the sensitivity of the HT1080 cell's metabolome to hypoxia, normoxia and hyperoxia at each interval time point. Based on the data obtained, the PhD Student could indicate, among other things, that in his study for the first time was observed an increased levels of accumulation of 3-hydroxybutyrate, 3-methyl-2-oxovalerate and 2-oxoisocaproate a at the cellular space along with induced the efflux of these metabolites to extracellular milieu at hyperoxia 21% after 36h of incubation. Another interesting finding of PhD Student is fact that in HT1080 cells line the level of extracellular pyruvate under hypoxia 1% was lowest compared to normoxia 6% and hyperoxia 21% and showed high sensitivity in terms of impact of an oxygen concentration and the time. Moreover, the Author found that the monocarboxylate transporter plays a crucial role in regulating extracellular pyruvate levels under different oxygen conditions, acting as both a transporter and sensor for oxygen concentration.

In several places in this chapter there is a various notation for the oxygen level for "hyperoxia" (e.g. page 45), the Author used the notation "20.9%" while in most of the work he used the notation "21%".

The chapter 2 is summarized by correct conclusions that are well documented and result from the conducted experiments and correspond to the assumed goals. The last element of this part of this chapter is a bibliography containing 99 items presenting issues related to the subject of research.

The 74-page chapter 3, entitled "The effect of hypoxia-reoxygenation and normoxia-deoxygenation in HT1080 cell line metabolome by 1H NMR analysis", consists of 5 subchapters (Introduction, Materials and Methods, Results, Discussion, Summary and References) with 13 figures and 6 tables.

In the introduction, the PhD Student very concisely describes the phenomenon of tumor hypoxia resulting from the rapid and uncontrolled proliferation of tumor cells with abnormal mass and vascular dysfunction of the tumor, as well as various approaches of tumors to facilitate oxygen delivery. Hence, in this part of dissertation, the PhD Student aimed to establish a very interesting *in vitro* model of hypoxia and normoxia transitions by inducing hypoxia-reoxygenation and normoxia-deoxygenation to investigate the intracellular and extracellular metabolic profiling on the HT1080 cell line.

Then, in the subchapter "Materials and methods", similarly to chapter 2, consisting of nine parts, the Author describes evaluation cell culturing process and experimental design, cell culturing medium preparation, culture media extraction, cells extraction, cell growth measurement, NMR data acquisition, metabolites identification, processing for data analysis and statistical analysis.

As with experiments related to quantitative analysis of intracellular and extracellular metabolome of HT1080 cell line under hypoxia, normoxia and hyperoxia, it should be emphasized that the *in vitro* experiments, the techniques used to prepare samples for analysis, as well as analytical and statistical methods used in the study presented in chapter 3 were complex and time-consuming and required a lot of work to fully achieve the assumed research goal. However, thanks to this, it was possible to obtain a lot of new information about the effect of hypoxia-reoxygenation and normoxia-deoxygenation on metabolome of HT1080 cell line using in 1H NMR analysis. Summing up this part of chapter 3, I can say that a well-thought-out and refined methodology is its strong point.

On the basis of numerical summaries, which are the result of organizational, experimental and analytical work, the PhD Student presented a description of his own achievements in individual parts of the subchapter "Results". As part of the adopted research organization, the PhD Student first presented the results of study related to the cell growth curves and indicated that the direct counting assay performed revealed different variations in the growth of HT1080 cells under deoxygenation and reoxygenation conditions. In a further part of this subchapter, the PhD Student presented the results related to effect of the reverse oxygen concentrations changes and time on HT1080 cell line metabolome. After analyzing by a 1H NMR and a statistical processing the samples (the intracellular and extracellular) collected from each cell incubation time point in the model of hypoxia-reoxygenation and normoxia-deoxygenation the PhD Student identified as much as 41 metabolites for the intracellular system and 36 metabolites for the extracellular system while 3 metabolites were not assigned. Further statistical analysis made it possible to determine different states and regulations for individual identified metabolites.

As in chapter 2, the next part of chapter 3 of the dissertation is a discussion counted forty-five pages in which the PhD Student assessed the results obtained against the background of the available scientific literature. At the beginning of this part of the dissertation, the PhD Student rightly emphasizes that the tumor microenvironment involves a complex interplay of oxygen diffusion gradients and nutrient availability that shape the

metabolic reprogramming and adaptation of cancer cells. Thus, the experiments conducted by the doctoral student, which are the first in this field, and the results obtained are a very important achievement and contribution to cancer research.

The discussion of the obtained results began with a description of the post normoxia deoxygenation impact on of HT1080 cells metabolome. Based on the data obtained, the PhD Student could pointed out that after 12h and 24h of incubation for the schema normoxia-deoxygenation at hypoxia 1%, there was a significant increase of various intracellular and extracellular metabolites compared to normoxic cells. Moreover, the Author indicated, that these findings showed even short-term oxygen deprivation can have a significant impact on cellular metabolism and the accumulation of metabolites inside and outside of the cells. Thus, these observations have important implications for understanding the metabolic changes that occur in response to oxygen deprivation and availability and the potential impact on cellular function and survival. It is also important to demonstrate by the PhD Student that nicotinamide adenine dinucleotide plays a crucial role in cancer cell metabolism and survival, particularly under hypoxic conditions and the suggestion that there are further research is needed to fully understand the mechanisms underlying the effects of nicotinamide adenine dinucleotide on cancer cell metabolism.

In the area of research on choline the PhD Student indicated interesting observation that the upregulation of sn-glycero-3-phosphocholine in schema of normoxia-deoxygenation under hypoxic conditions may indicate altered choline metabolism that could be relevant for cancer diagnosis and treatment. However, in the field of research on creatine, the PhD Student informs that his discoveries are consistent with other studies implicating SLC6A8 activity and showing that, the upregulation of intracellular creatine levels in hypoxic triple negative breast cancer cells *in vitro* is caused by the transcriptional activation of the SLC6A8 gene by p65/NF-κB. The PhD Student also discovered an increase of extracellular fumarate in the schema normoxia-deoxygenation compared to normoxic cell after 12h and 24h of incubation at hypoxia and suggest that the cancer cells release fumarate to the extracellular space during hypoxia might be as a survival mechanism. The PhD Student explains that under low oxygen conditions (hypoxia), cancer cells are unable to produce energy through oxidative phosphorylation and must switch to anaerobic metabolism. This leads to the accumulation of metabolic intermediates, including fumarate, which can accumulate to toxic levels within the cell. To prevent cellular damage, cancer cells may release excess fumarate into the extracellular space, thereby reducing its intracellular concentration. This process may

contribute to the growth and progression of the cancer cells, as well as the surrounding tissue. Another important extracellular metabolite described by the Author was acetate which showed the extracellular upregulation what may indicate that this metabolite may play a significant role in providing acetyl-CoA for lipid biosynthesis in hypoxic cells. The PhD Student suggests that cancer cells may rely on locally produced acetate as a major source to support cell-cell communication during hypoxia, potentially due to slower metabolism in these cells and highlight the complex interplay between cancer cell metabolism and communication pathways, which may contribute to tumor progression. This part of the dissertation is summarized by correct nineteen conclusions that are well documented and result from the conducted experiments and correspond to the assumed goals. The second part of the discussion concerns the post hypoxia reoxygenation impact on the metabolic phenotype of HT1080 cells. The presented results provide a huge number of novel insights into the impact of oxygen concentration on HT1080 cells and transition in the system of hypoxia-reoxygenation, shedding light on the processes occurred. As the PhD Student indicated the intracellular and extracellular metabolome of hypoxic cells and reoxygenized hypoxic cells at normoxia after 12h and 24h showed an increase of the catabolic capacity and induced amino acids influx into cells to sustain energy production and building blocks of intermediaries amino acids for biosynthesis process through various mechanisms. In this area, within the detailed results the PhD Student showed, among other things, that showed the limitations of intracellular and extracellular of glucose and downregulation of glutamine on the system of hypoxia-reoxygenation after 12h and 24h incubation at normoxia what was consistent with another authors findings. This part of the dissertation is summarized by correct conclusions that are well documented and result from the conducted experiments and correspond to the assumed goals. The last element of this part of the chapter is a bibliography containing 219 items presenting issues related to the subject of research.

To sum up, the findings presented in this dissertation provide crucial insights into understanding the both terms physiological and pathological implications of various oxygen level in HT1080 cells, highlighting the importance of oxygen transition in restoring cellular processes. These findings represent a significant contribution to the literature and hold great potential for advancing understanding of responses on various oxygen level in cancer cell. Particularly noteworthy is the efficiency with which, despite a number of planes, the PhD Student analyzes the results of large-scale metabolite analysis, contributing to a more accurate

understanding of the biology and biochemistry of processes related to cancer. This proves a very good knowledge of literature and scientific maturity of the PhD Student.

The last part of the assessed dissertation is a list of all publications of the doctoral student and participation in scientific conferences. Based on these data, it can be concluded that the PhD Student is an active researcher, working in various scientific area. However, the lack of publication of the results of the experimental work constituting this dissertation causes a certain degree of scarcity.

In conclusion, I state that the PhD thesis of M.Sc. Badr Saif Mohsen Qasem is an original scientific achievement, the research is innovative and important from the scientific and social point of view. The PhD Student showed theoretical knowledge and analytical skills as well as the ability to solve scientific problems. Thus, the PhD thesis of M.Sc. Badr Saif Mohsen Qasem entitled "Metabolomics analysis of time and oxygen effect on Fibrosarcoma cell line (HT1080) – model studies" I evaluate positively.

I hereby declare that the doctoral thesis of M.Sc. Badr Saif Mohsen Qasem entitled "Metabolomics analysis of time and oxygen effect on Fibrosarcoma cell line (HT1080) – model studies" meets all requirements for doctoral theses ("określone w art. 13 ustawy z dnia 14 marca 2003 r. o stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki (t.j. Dz.U. z 2017 r. poz. 1789 z późn. zm.)") and hereby submit that its Author can be admitted to the next stages of the procedure for obtaining a doctoral degree.