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Gdansk, 28 January 2024

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Chairman of the Biotechnology
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Review of the doctoral thesis of Kenneth Weke, MSc. entitled "Expanding the proteome: Advancing proteomics methodologies to uncover new insights into cancers"

Doctoral thesis of Kenneth Weke is a collection of three papers of which two were published and one is an unpublished manuscript. All three papers are original studies. PhD candidate is first (or co-first) author in all of them. Two papers are published in a very good journals: *J Proteome Res* and *Anal Chim Acta* that that both are in first quartile.

Thesis contains summaries in English and Polish, a list of abbreviations, list of papers included into the thesis, list of figures, list of tables, table of contents, introduction ending with aims of the doctoral thesis, three chapters dedicated to papers included, and conclusions and future directions, appendices and references and authors contribution statement. Thesis has 126 pages, 37 figures and 5 tables. It is prepared with the highest standards with regard to content and quality of presentation.

Search for early detection and optimal cure for cancer is the one of the most important ongoing medical tasks. New methodologies can provide solutions for this very complex challenge. Mass spectrometry is rapidly evolving technology with spectacular progress in the recent years resulting in orders of magnitude improvements in sensitivity, resolution and robustness. This tool can provide chemical insight that is needed to identify sometimes very subtle differences between normal cells those that become cancerous. Before this could be achieved adequate procedures have to be developed to maximize information that could be obtained. This include widest possible spectrum of chemical molecules that could be detected in a smallest possible amount of sample.

The thesis is an important step into this direction. Its overall aim is the development and implementation of methodologies that are based on mass spectrometry to detect proteins and immune system related peptides for studies of cancer, with a special focus on glioblastoma (GBM) with overall aim to facilitate diagnosis and identify new treatment possibilities.

Thesis introduction focuses on two areas. First part is related to cancer, providing description of pathological, clinical, diagnostic and therapeutic aspects of glioblastoma.

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Second part describes mass spectrometry technology from its basic principles to current status with special focus on its applications in proteins and peptides analysis. Such introduction is appropriate considering content of the thesis. Both aspects are very broad but its description gives right balance between general aspects, new trends and problems specific to the thesis.

Specific aims of the thesis included:

1. Development of a microscale MS-based proteomics method capable for analysis of limited numbers of cells.

2. Development of procedure for analysis of protein pattern in formalin-fixed paraffin-embedded glioblastoma tissues

3. Investigation how hypoxia affect antigen presentation in glioblastoma using analysis of proteins and immune system related peptides.

Thesis results section starts with Chapter 2 that describes results included in the paper: "MicroPOTS analysis of Barrett's oesophageal cell line models identifies proteomic changes after physiologic and radiation stress". This chapter/paper describes move from macroscale preparative systems in proteomics to micro- and nanotechnology. Microdroplet processing in one pot for trace samples (microPOTS) was adopted to identify proteomic changes in just a few hundred Barrett's esophageal cells following physiologic and radiation stress exposure. This procedure identified >1500 protein groups and achieved a high reproducibility and in a Barrett's cell line treated with either lithocholic acid (LCA) or X-ray identified 21 overexpressed proteins. This study not only proved feasibility of the method in a small samples but also provided interesting data relevant to oesophageal cancer.

Chapter 3 describes study/paper: "DIA-MS proteome analysis of formalin-fixed paraffin-embedded glioblastoma tissues". Suboptimal sample fixation for proteomic analysis is common problem that is especially important in retrospective analyses. The study aims to implement procedure for data independent quantification of proteins by mass spectrometry (DIA-MS) in formalin-fixed paraffin-embedded (FFPE) tissues. A simple and precise DIA-MS workflow was established that facilitated detection and quantification of immune- and glioblastoma (GBM) - relevant proteins from FFPE patient materials, More than 1700 proteins were detected and over 1400 proteins were quantified from GBM FFPE tissue microdissections. Several GBM-relevant proteins (e.g., GFAP, FN1, VIM, and MBP) and immune system-related proteins (e.g., ILF2, MIF, and CD38) were detected and quantified proving that this strategy holds potential for routine protein quantification in FFPE tissues.

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Chapter 4 describes unpublished manuscript: "The interplay of hypoxia and antigen presentation in glioblastoma tumor: Insights from proteomics and immunopeptidomics".

Glioblastoma (GBM) is a highly aggressive and deadly brain cancer that is characterized by hypoxic tumor microenvironment (TME) suppressing antitumor immune responses. Antigen presentation via the human leukocyte antigen class I (HLA-I) molecules is a critical process for T cells to recognize and eliminate tumor cells, but this could be affected by hypoxia. This study used mass spectrometry (MS)-based proteomics and immunopeptidomics to investigate the effect of hypoxia on antigen presentation. Severe hypoxic stress led to downregulation of key enzymes in the antigen processing (ERAP1 and ERAP2) and presentation machinery (APM) as well as to reduction in HLA-I-associated antigen peptide repertoire. This study suggested that carboxypeptidase A4 (CPA4) has a potential role in shaping the tumor immunopeptidome landscape under a hypoxic microenvironment.

Research methodology repertoire of this Thesis involved use of complex and technically demanding methodologies such as high resolution mass spectrometry linked to liquid nanochromatography. Diverse sample preparation procedures were applied including design of new approaches. Furthermore, cell culture, Western blotting and flow cytometry were used. Number of standard biochemical procedures were also used.

The research described had been conducted according to the highest standards. I can add only a few minor comments:

While journals policy is not uniform on this I feel that it is better to avoid abbreviations in the title. Expanding DIA-MS in the second paper would not complicate title too much, making it easier for readers.

In the third unpublished study: why cell proteins were not extracted directly from culture plates? Additional step such as scratching cells and centrifugation may induce losses and errors.

P41, last line: ammonium bicarbonate is not an amine.

p.63, l.21 ..impact of hypoxic on antigen… should be: ..impact of hypoxic environment (or hypoxia) on antigen….

p.67, l.1: Samples were frozen overnight at -80^{o}C.. could be: Samples were frozen and maintained at -80^{o}C overnight.

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p.70,l.2. Experimental design and study workflow belongs to Methods section, not Results and Discussion. Further down, repeating methods description in Results and Discussion section is not necessary.

p.71,l.1 Have you tried to get relative HIF1α contents from MS data to compare it with Western blot analysis?

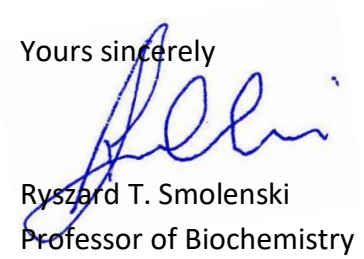
p.72.l.9 and onward. Is the term “quantified”, “quantitative values” correctly used? Typically this means weight or molar values per volume or weight of tissue.

p.76.l.13 Would it be possible to speculate on the reasons and the consequences of metabolic reprograming induced by hypoxia in addition to listing affected proteins and pathways in a similar way it was nicely proposed for CPA4? The same point is applicable to changes in peptide repertoire (p.80.l1).

My last point is that overall discussion (Chapter 5) is perhaps too modest. This would be the place to highlight broader implications and long term goals of this research. How this could translate to better diagnostics? How this could help to develop cancer vaccines? It would be good to include potential specific scenarios.

The above comments on the work I am assessing do not in any way diminish its high value. Kenneth Weke doctoral thesis constitutes a significant contribution to the development of methodology for detection and therapy in cancer diseases, setting further directions and standards for such research. The assessed work meets all the criteria for a doctoral dissertation specified in the currently applicable legal regulations in Poland. I submit to the Scientific Council of the Discipline Biotechnology of the University of Gdansk my recommendation to admit Kenneth Weke to further stages of the doctoral procedure. Furthermore, considering very important nature of this work, its technical difficulty and publications of the results in two very good journals with perspective of third even better publication I recommend awarding distinction to this PhD.

Yours sincerely



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