POLISH ACADEMY OF SCIENCES
NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY
Pasteur 3, 02-093 Warsaw, Poland
Phone: (48-22) 58 92 09; Fax: (48-22) 822 53 42; http://www.nencki.gov.pl

Prof. Bozena Kaminska
Laboratory of Molecular Neurobiology
Neurobiology Center

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Evaluation of the PhD thesis
"Analysis of gene expression pattern in renal cell carcinoma stem cells derived from primary and metastatic cancer site" by Mr Mohammad Imran Khan

The PhD candidate has addressed a very timely and interesting topic, namely a comprehensive biological and molecular characterization of cancer stem cells from renal cell carcinoma (RCC) and understanding the role of those cells in RCC progression. The recent concepts regarding cancer origin are evolving into the idea of cancer origin from stem/progenitor/dedifferentiated cells accumulating genomic alterations leading to acquisition of oncogenic transformation. This is based on discovery of cellular heterogeneity of cancer and identification of subpopulation of cancer cells with stem like or dedifferentiated features in many hematopoietic and solid tumors. Cancer stem cells (CSCs) are rare tumor cells that exhibit stem cell properties such as self-renewal capacity and pluripotency. In recent years, CSCs have been recognized as key tumor-initiating cells which are therapy-resistant and highly tumorigenic and may play a pivotal role in cancer recurrence following chemotherapy. It is becoming clear that due to their specific features, CSC survive standard therapies and re-initiate tumors. There is growing number of evidence of their increase after standard therapies. Therefore, to better fight cancer it is obligatory to characterize those cells in different types of cancer, find their markers and deregulated pathways, search for new inhibitors with therapeutic potential. The current methods are not fully standardized and there is a need to find good CSC markers for specific tumor types.

Mr Khan's work addressed this important issue employing state of the art cell biology techniques and modern molecular biology methods to isolate CSC from primary and metastatic renal cell carcinoma and characterize underlying mechanisms and signaling pathways using transcriptomic approach. There are many aims of this study as the author addressed several molecular aspects related to CSC biology that are based on prior studies in the supervisor laboratory. It should be mentioned that there is no consensus regarding markers of CSC, which seem to be specific for each tumor type, except a few embryonic stemness markers (NANOG, OCT4, SOX2 etc…) which are universal. Therefore, it is necessary to define culture conditions and markers for each cell type. The scope of studies was very broad and ambitious. It encompasses:

1) developing a cell culture system for cancer stem cells from RCC;
2) characterizing such population from primary and metastatic RCC;
3) determining gene expression profiles of CSCs from primary and metastatic RCC;
4) detecting altered signaling pathways based on transcriptomic data using computational analyses.

To achieve his goals the applicant employed a broad set of methods. It is important to underlie that the appropriate and state of the art methods have been used in this study. In particular, it is worth to mention that the applicant used two methods of enrichments in RCC CSCs, namely specific culture conditions and flow cytometry, and used a battery functional assays and markers to characterize their stemness features. The methods description section is complete and very precise, all information required to control those experiments is provided. The part of method description is even too detailed and sounds as a technical protocol from a company page.

The thesis is well constructed, according to general rules and with good proportions and a reasonable list of well selected references. Rationale for this study is well explained. Introduction is a concise, brief summary of information regarding biology of RCC, cancer stem cells in RCC and some markers which could be used to their detection. The presented goals of the study have been achieved and the applicant has collected a very impressive set of original results and reported several important findings.

A section of Methods presents the applied procedures clearly and in most cases in sufficient details. It is noteworthy that a wide scope of different techniques has been used in this study, such as cellular and wound healing assays, flow cytometry evaluation of protein expression and immunohistochemistry, molecular biology techniques of gene expression analysis and computational analyses. The methods fit to the objectives very well. Experiments are logic and well designed. Many techniques have been employed to verify author's queries and hypotheses. The author took in general a good care for quantitative analysis of data. The quality of figures is relatively good, layout of the text is suitable. The sample size fits the objective and in some experiments was rather large. Information concerning number of experiments for cytometry, cell culture assays, q-PCR studies was provided.

The findings are original, very interesting and have potential clinical importance. The results are highly innovative because this is one of the first comprehensive characterization of CSC in established renal clear cell carcinoma cell lines, their properties and gene expression networks. The achievements and findings may provide a rationale for development of novel drugs targeting specific transcriptional regulators and signaling pathways in this cancer.

In the Discussion part, the Author summarizes briefly his findings and presents their biological meaning that allows him to make reasonable and original conclusions. The conclusions are based on interpretation of results and are well supported. The discussion is too much of a summary of results, and in particular the description of transcriptomic data analyses, could be more elaborated and critical.

Minor comments:

1. INTRODUCTION. This section, which contains 6 pages, is laconic and a bit too terse. It is limited strongly to the topic of cancer stem cells and methods used by the applicant in his work. One would expect broader description of CSCs in general and in RCC based on available literature. For instance, the features responsible for CSCs therapy resistance should be discussed along with discrepancies and controversies in the field. CSC biology and selective markers are still poorly defined and there is an ongoing debate whether these cells represent a "real" subpopulation amongst cancer cells, or they are just cell culture artifacts or a case of cellular adaptative plasticity. Other studies on RCC gene expression profiling

should be mentioned. The presented objectives suggests that the idea of this study appeared "out of thin air" while in fact there is quantity of data on this subject.

2. METHODS. In qPCR primers description of stemness gene names should be in italics (as these are human genes). Some protocols sounds like taken directly from the internet or product description. Some descriptions such as Crystal violet+methanol are too laconic (what was a concentration?).
3. RESULTS section. A few technical details should be presented to verify quality of data.

- For example fig.4 immunohistochemistry for CD10 and CK7 in RCC cell lines, but negative controls for antibody specificity and scale bars are missing. There is no figure legend for fig.4. The last raw seems to have no staining, and this is not discussed. The last panel on p.34 looks like phase contrast microscopy images not immunohistochemistry.
- In the fig.5 there is a reference to a PLOS paper instead of figure legend, there is no negative controls with an antibody omitted. Higher magnifications images should be provided as insets to make it more convincing.
- Gating strategy for CD105 stained cells is not well explained. In the discussion it mentioned that Wharton yelly cells expressing CD105 were used to establish gating strategy in flow cytometry and test specificity of antibody, but this is not shown. Isotype controls should be provided. Why there is so different background in the fig.8?
- In the fig. 9 with qPCR, data are presented in strange way; it should be delta CT. What means ratio in this case? In the fig. 10 there are additional bands in some cells lines, this is not discussed in the thesis.
- Wound healing assay is quantitative and there are methods to quantify cell fee areas or cell covered areas. Again there are different backgrounds (different shades of yellow) in the figure.
- The right panel at the fig. 15 how the same data as the other one and is unnecessary.

4. A list of abbreviations should be alphabetical.
5. DISCUSSION. This part is written well, summarizing briefly results and discussing their significance. There are a few comments not very clear i.e. p.86 there is a statement regarding *"differentially expressed genes in CD105+ cells isolated form the primary RCCCaki-2 cell line and compared with a healthy kidney ASE cell line"* , but this is not described how the expression in normal cells was compared to the cancer one. Overrepresentation of gene GO categories should be presented with p values.
6. SUMMARY. The first sentence is imprecise: *The biology of RCC is heterogenous*", I think it was meant the RCC is heterogeneous.
7. Gene names should in italics and because all work in done on human material these should be in capital letters.

The thesis is (generally) well written (it could benefit from some editing for English syntax and there are a few unfinished and badly worded sentences in the Discussion) and clearly delivers the important message.

Altogether, findings presented in the thesis are very important, significantly contribute to our understanding of renal clear cell carcinoma pathogenesis and cancer stem cell biology, and have a potential value for the field of oncology.

This reviewer judges that the thesis entitled **"Analysis of gene expression pattern in renal cell carcinoma stem cells derived from primary and metastatic cancer site " by Mr Mohammad Imran Khan** fulfills all requirements for PhD thesis and this work is sufficient to get Doctor of Philosophy (Ph.D.) degree at the Military Institute of Medicine (Wojskowy Instytut Medyczny). The candidate is well prepared for independent work and seems to be a good candidate for scientific carrier.

Uważam, że przedstawiona mi do recenzji praca rozprawy doktorskiej mgr **Mohammada Imran Khana pod tytułem; "Analysis of gene expression pattern in renal cell carcinoma stem cells derived from primary and metastatic cancer site**" spełnia wymagania stawiane rozprawom doktorskim i wnioskuję do Rady Naukowej Wojskowego Instytutu Medycznego o dopuszczenie mgr **Mohammada Imran Khana** do dalszych etapów przewodu doktorskiego.

Sincerely yours

Prof. dr hab. Bożena Kamińska-Kaczmarek