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Review report of the doctoral dissertation by Ms. Effi Haque

Thesis tile: "*Evaluation of the effect of the NRF2 and HNF family genes mutations found in human liver cancer on transcriptional activity using mammalian cells*"

The doctoral dissertation submitted for evaluation comprises of two original publications and a review article published in indexed journals along with a descriptive part. The descriptive part summarizes the results of the published experimental work carried out by the doctoral student under the supervision of Prof. dr hab. Mariusz Pierzchała and Dr Hiroaki Taniguchi at the Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences in Jastrzębiec. In all three attached publications, the candidate is the first author with significant contributions ranging between 50% - 57%.

Primary liver cancer is the sixth most commonly diagnosed cancer and the fourth leading cause of cancer mortality worldwide. Approximately, 75% of all liver cancer cases are of the type hepatocellular carcinoma (HCC), and whereas, the incidence of HCC in the US has tripled over the last four decades, it is also increasing in the Europe. Therefore, identifying different mechanisms that may be involved in HCC progression is important to find therapeutic strategies. Towards this end, the work presented in the thesis, evaluates the functional significance of the mutations found in the functional domain of the nuclear factor erythroid2-related factor 2 (NRF2) and hepatocyte nuclear factor 1A (HNF1A) transcription factors responsible for HCC development. Ms. Haque started her doctoral work with writing a review article detailing role of aberrant NRF2-signaling in controlling the fate of hepatocarcinogenesis, which in my opinion is an excellent approach to start working on a research problem.

The review by Haque et. al. (2020), *Molecular mechanisms underlying hepatocellular carcinoma induction by aberrant NRF2 activation-mediated transcription networks: interaction of NRF2-KEAP1 controls the fate of hepatocarcinogenesis,* presents an up to date literature survey and discussion of the dysregulation of oxidation-stress response induced NRF2-KEAP1 signaling in developing HCC. Ms. Haque begins by discussing molecular

control of oxidative stress-dependent HCC pathogenesis followed by a detailed description of role of various NRF2 domains in the control or repression of its transcriptional activity. Ms. Haque then describes the role of NRF2 mutations in its constitutive activity leading to upregulation of its target genes. The review lists a number of HCC somatic mutations found in DLG and ETGE motifs (essential for NRF2-KEAP1 interaction) of NRF2 and their clinical significance in HCC. Role of somatic mutation in DLG and ETGE motifs in interfering with NRF2-KEAP1 interaction, and importance of the loss of this interaction in promoting HCC through expression of various NRF2 target genes is elegantly presented. Finally, the review outlines the role of aberrant NRF2 activation on regulation of lipid and cholesterol metabolism in liver diseases leading to HCC. The final part of the review describes the emerging roles of transcription factors closely related to NRF2 in liver cancer. The authors conclude that in NRF2 over-activation induced through mutations plays a cytoprotective role a diseased microenvironment. In my opinion, the Ph. D student has put tremendous effort to put together this review, read as many as 154 publications, compiled all the relevant information, and presented and discussed the results that are easy for a reader to follow. Most importantly, writing review prepared her for planning doctoral work to fill the gaps that were pointed out in the review.

In the first original research: "*NRF2 DLG domain mutations identified in Japanese liver cancer patients affect the transcriptional activity in HCC cell lines*". The aim of the work was to elucidate the role of NRF2 somatic mutations in oncogenic transformation of liver cells. Ms. Haque used mouse hepatoma cells, Hepa1-6 cells and human hepatocyte-derived carcinoma cells, Huh7 cells for her research work. It was demonstrated that NRF2 DLG motif mutations (NRF2 D29A and L30F), found in Japanese liver cancer patients, upregulate the transcriptional activity of NRF2 in HCC cell lines. These mutations interfered with NRF2-KEAP1 binding resulting in blocking of KEAP1-mediated degradation of NRF2. To achieve the goals following was done:

- First, molecular graphics was used to show that whereas, D29A mutation reduces binding affinity between NRF2 and KEAP1, L30F mutation causes steric hindrance that interferes with binding between amino acid residues of NRF2 and KEAP1.
- Using Luciferase reporter assay, Ms. Haque evaluated transcriptional activity of wild type (WT) and mutant (MT) NRF2 by transfecting cell lines with WT and MT NRF2

in the presence of 3xARE reporter. The mutations induced a gain-of-function phenotype in the cells.

- Exogenous KEAP1 expression in cells transfected with WT and MT NRF2 was shown to decreases transcription activity of WT NRF2 only and there was no effect of KEAP1 on MT NRF2 activity proving a role for loss of interaction between MT NRF2 and KEAP1. Moreover, the student showed that MT NRF2 transcriptional activity on MMP9 promoter is significantly higher than WT NRF2.
- Finally, Ms. Haque showed the transcriptional activity of NRF2 on MMP9 and 3xARE promoter was further increased by its co-expression with pro-oncogenic BRAF or its V600E mutant gene in Hepa1-6 and Huh7 cells. The highest transcriptional activity was achieved in cells co-transfected with BRAF V600E and NRF2 L30F mutations.

In the second paper: "*HNF1A POU domain mutations found in Japanese liver cancer patients cause downregulation of HNF4A promoter activity with possible disruption in transcription networks*" Ms. Haque aimed to evaluate role of HNF1A POU domain mutations in its loss of function leading to HCC development and demonstrated that decreased transcriptional activity of HNF1A mutants are due to impaired DNA binding that leads to downregulation of HNF4A gene expression in HEK293 and Huh7 cells. Important findings of the study are:

- Evolutionary conserved mutations in POU domain of HNF1A were identified and overexpression of WT and MT HNF1A in mouse and human cells was used for stimulation of HNF1A-responsive elements containing HNF4A promoter. Mutations resulted in decreased transactivation of HNF1A.
- As both WT and MT proteins were shown to be localized in nucleus, the loss of function due to HNF1A POU domain mutations was as a result of the decrease in the DNA-binding ability of mutant proteins towards HNF4A promoter regions.
- V259 mutation effects stability of HNF1A and reduces its binding affinity.
- To gain a better understanding of HNF1A loss of function, the student silenced *HNF4A* in Huh7 cells and performed RNA-Seq analysis to determine significantly dysregulated gene networks. A total of 748 genes were differentially regulated and

found to be associated with processes such as lipid and cholesterol metabolism, extracellular matrix organization and binding activity.

General Comments and Concluding Remarks:

The work presented is already published and undergone refinement through the review process, which makes it easier to review the thesis, I have just couple of general questions that came to my mind:

1. Are these HNF1A (Y122C, R229Q and V259F) and NRF2 mutations (NRF2 D29A and L30F) exclusively found only in Japanese populations of liver cancer patients and do NRF2 and BRAF mutations occur simultaneously in HCC or other cancer types.
2. In second article, though I understand that HNF1A mutations lead to HNF4 downregulation, why didn't author carry out a transcriptomic analysis of cells with HNF1A POU domain mutations? Wouldn't it lead to broader understanding (more direct results) and yield changes not only because of its effect on HNF4A downregulation?

Summing up, research work of Ms. Haque has generated significant new data and knowledge in the field of HCC progression. The methods employed are advanced, scientifically sound and described in detailed manner. In my opinion, PhD student has demonstrated excellent knowledge in the area of research, use of good and varied research techniques and shown the ability to analyze and interpret the results. The work carried out has scope for further investigation in the field of HCC research. Therefore, I can confidently say that doctoral dissertation presented for evaluation by Ms. Effi Haque fulfils all criteria and meets the requirements of Art. 187 of The Act of July 20, 2018 Law on Higher Education and Science (Journal of Laws of 2021, item 478) for the award of Ph.D degree. Considering the high scientific level of the analytical methods employed for the research and scope of the work, I recommend and request the award of Ph.D degree with distinction.

Sincerely,

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Uchwała Nr 15/2022

Rady Naukowej Instytutu Genetyki i Biotechnologii Zwierząt Pan w Jastrzębcu

z dnia 23 września 2022 r.
w sprawie zmiany promotora w przewodzie doktorskim

Na podstawie art. 14 ust. 2 pkt 5 ustawy z dnia 14 marca 2003 r. o stopniach naukowych i tytule naukowym oraz stopniach i tytule w zakresie sztuki (Dz. U. Nr 65, poz. 595, z póź. zm.) w związku z art. 33 ust. 2 ustawy z dnia 18 marca 2011 r. o zmianie ustawy – Prawo o szkolnictwie wyższym, ustawy o stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki oraz o zmianie niektórych innych ustaw (Dz. U. Nr 84, poz. 455) Rada Naukowa IGiBZ PAN uchwala co następuje:

§ 1.

Zmienia się promotora w przewodzie doktorskim **mgr Effi Haque** z **prof. dr hab. Mariusza Pierzchały** na **dr hab. Hiroaki Taniguchi**.

§ 2.

Uchwała wchodzi w życie z dniem podjęcia.

Przewodniczący Rady Naukowej IGiBZ
PAN w Jastrzębcu

Prof. dr hab. Ryszard Słomski