Author’s response to reviews

Title: HIF-1 transcription activity: HIF1A driven response in normoxia and in hypoxia.

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Author’s response to reviews:

Dear Editor

Submitted with this covering letter is our revised manuscript entitled “HIF-1 transcription activity: HIF1A driven response in normoxia and in hypoxia” that we kindly ask you to consider for publication in BMC Medical Genetics.

We appreciate the reviewers’ suggestions to improve the manuscript. All the comments have been addressed carefully. All the authors have reviewed the final version of the manuscript and declare no conflict of interest. All changes to the manuscript are indicated in the text by highlighting.

We look forward to hearing from you regarding to our resubmission.

Sincerely

Flora Cimmino
Response to reviewers’ comments:

We appreciate the reviewers for their careful work and thoughtful suggestions. All the comments are helpful to improve the manuscript. According to all suggestions, the manuscript has been carefully revised and point-by-point response is listed below.

Reviewer#1

The study is a characterization of the HIF1A-dependent gene expression profiles and methylation status of the genome in neuroblastoma cells, by knockdown of the HIF1A gene using shRNAs under normoxic and hypoxic conditions. In addition, the authors have examined methylation profiles under hypoxia using methylation chips. Thus, they identify sets of genes regulated exclusively in hypoxia, exclusively in normoxia and those regulated by HIF1A. To address the concern of the effect of NMYC amplification in the NB cells primarily analyzed, the gene expression profiles were confirmed in a different cell line without NMYC amplification.

1) Though the methods and analyses of the gene expression and methylation overall are appropriate, one concern with such studies is the use of established cell lines such as neuroblastoma, regardless of their N-MYC status. These cells tend to show a deregulated gene expression that may not respond to changes in the environment, and are not comparable with normal cells.

Answer. We agree with the Reviewer that the use of established cell lines, as other in vitro models, reflects limited aspects of in vivo tumor microenvironments. It lacks geometrical complexity, cellular components including immune cells and organ-specific stromal cells, and extracellular matrix components. The aim of this report has been to establish a HIF1A-based method useful in the investigation of undiscovered mechanisms of neuroblastoma tumorigenesis under hypoxic microenvironments. The validity of our methods is also supported from the capacity of the hypoxia gene expression profiles to predict the prognosis and to stratify neuroblastoma tumors in low and high risk sub-groups (Figure 4C and 4D and Figure S6). We believe that information extracted from these cancer cells is relevant to identify novel mechanisms associated with variation of oxygen in tumor microenvironment or in cells with HIF1A depleted expression. Indeed, in “Discussion” section, we have emphasized the need to confirm our results by functional validation and mechanistic studies to further improve in vitro cell line models predictive validity.

2) It is not clear how the authors evaluated for hypoxic status of the cells after their exposure to low oxygen conditions. There should be an independent test (not expression of HIF) to check that hypoxia is established in these cells. This aspect is missing from the study. Is it conclusive that just 2 hrs of incubation in low oxygen made the cells hypoxic?

Answer. We thank the Reviewer for this observation. As suggested, we performed an independent test to check that hypoxia is established in these cells. By RT-PCR, we tested the gene expression of well-known targets of HIF1A and hypoxia. As shown in the new Figure S1, hypoxia targets levels increase in SKNBE2 and SHSY5Y cells (Figure S5) exposed to hypoxia, and suggested that 2 hrs of incubation in low oxygen made the cells hypoxic.
Another concern I have is that as seen from Figures 1A and S3, in both cell lines used, the knockdown of HIF1A does not appear to be complete in the presence of hypoxia (lanes 4-6 in both figures). If there is "leaky" expression of HIF1A despite knockdown, the gene expression data obtained may not be specific for HIF1A-inducible genes.

Answer. In the presented work we aim to evaluate the effects of HIF1A depletion on HIF-1 activity and not direct/specific HIF1A effects on NB cells. We performed HIF1A inactivation by short hairpin RNA silencing HIF1A, in normoxia and in hypoxia (2h). Several studies reported in literature used HIF1A/HIF2A silencing with short hairpin system to evaluate the effects down-stream its depletion. We also applied the same gene knock-down strategy in the published paper Cimmino et al (Sci Rep. 2015 Jun 9;5:11158. doi: 10.1038/srep11158.) and showed HIF1A involvement in neuronal differentiation and response to retinoic acid treatment. Furthermore, because HIF-1α protein amount is rapidly stabilized within 2hours of hypoxia, we can not obtain a total knockdown of HIF-1α (short hairpin silencing) in these hypoxia conditions (2 hours) as also reported by Krutilina et al. (Breast Cancer Research 2014, 16:R78). Based on our previous manuscript and in line with the bibliographic reports, we believe that it is not possible to achieve a complete reduction of HIF1A protein in hypoxia (2h) and that small amount of stabilized HIF1A protein does not interfere in the assessment of downstream effects on HIF1A transcription activity because HIF1A is a very essential protein for cellular functions.

Reviewer #2: Editor

In this paper the authors underscore the important role of hypoxia in cancer. Cell response to hypoxia is primarily regulated by the transcription factor HIF- that triggers protective and adaptive mechanisms, promoting cell survival establishing resistance to therapeutic approaches as radiotherapy. Therefore, the identification of factors able to influence the expression levels of HIF1A could allow greater therapeutic success.

1. Recent papers have emphasized the pathophysiological effects of methylglyoxal (MG) and the glyoxalase system in acute hypoxic injury since the methylglyoxal led to a rapid proteasome-dependent degradation of HIF-1 (The Role of Glyoxalase System in Renal Hypoxia. Advances in Experimental Medicine and Biology 662:49-55)
(The Chaperone-Dependent Ubiquitin Ligase CHIP Targets HIF-1α for Degradation in the Presence of Methylglyoxal. PLoS ONE 5(11): e15062)
These aspects should be taken into consideration by the authors and described in introduction.

Answer. We have now described the aspects pointed out from the Editor in “Introduction” section.