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Running title: HIF3A: A potent prognostic biomarker in different kinds of cancer

Manuscript Title: The importance of HIF3A expression level and prognostic biomarker potential in different types of cancer

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Abstract

Background: Hypoxia-inducible factors (HIFs) are transcription factors that get activated and stabilized in heterodimerized form under hypoxic conditions. The three members of the HIF alpha factors share high structural similarity but have tissue- specific expression patterns. A majority of studies have reported the importance of the HIF1A and HIF2A activity in survival, proliferation, metastatic potential and metabolic regulation of hypoxic cancer cells. However, the importance of the expression pattern and activity of HIF3A in a variety of cancers remains unknown.

Method and materials: The expression profile of 13 different types of The Cancer Genome Atlas (TCGA) cancer samples were downloaded, normalized and differential gene expression analysis (DGE) was performed to compare the expression pattern of HIF alpha family members in cancer and adjacent normal tissues, as well as at different stages and tumor-sizes. Receiver operating characteristic (ROC) test and survival analysis were carried out to estimate the diagnostic potential of HIF alpha isomers in different cancers, as well as the survival rate of patients with varying expression level of HIF alpha factors.

Results: The expression status of HIF3A was notably less in all cancer samples in contrast to their adjacent normal tissues. The expression degree of HIF1A varied among distinct types of cancer and expression degree of HIF2A was lower in nearly all types of cancers. The expression level of HIF alpha isomers did not significantly correlate with different sizes of tumor samples and stages of different tumor tissue samples. HIF3A had very weak diagnostic potential, while the HIF2A had better diagnostic potential in most types of cancers compared to HIF1A. Patients who had higher level of HIF3A had better survival, while higher expression level of HIF1A and HIF2A were associated with worse survival in many types of cancers.

Conclusion: Our study shows the heterogenous expression pattern of HIF alpha subunits in distinctive kinds of cancers and the influence of HIF3A expression level in the survival of patients with varying types of cancers.

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Keywords: cancer**,** hypoxia-inducible factors, HIF3A, expression analysis

Abbreviations

AUC, Area under curve; BRCA, Breast invasive carcinoma; COAD, Colon adenocarcinoma; DGE, Differential gene expression; EPO, Erythropoietin; HIF, Hypoxia-inducible factor; HNSC, Head-neck squamous cell carcinoma; HRE, Hypoxia response element; IPAS, Inhibitory PAS domain; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; OC, Ovarian cancer; ODD, Oxygen-dependent degradation domain; PAS, Per-Arnt-Sim; PHD, Prolyl hydroxylase domain; PRAD, Prostate adenocarcinoma; PGK1, Phosphoglycerate kinase 1 ; ROC, Receiver operating characteristic; STAD, Stomach adenocarcinoma; THCA, Thyroid carcinoma; VEGF, Vascular endothelial growth factor; VHL,Von Hippel-Lindau.

1. Introduction

Hypoxia refers to a state when the concentration of oxygen around the cell's microenvironment is less than 2% mmHg (1). Hypoxic environment can enhance the resisting behavior of solid tumor cells against drugs that are administrated for cancer treatments (2-4). Active dimer of HIF factors is generated when alpha and beta subunits create a dimer whose activity and stability is tightly dependent on the status of oxygen tension in cellular environment (5-7). Active HIF heterodimer is formed between HIF alpha and HIF beta subunits (8, 9). HIF subunits share high sequence similarity in their structure and domains. However, HIF beta subunit lacks the ODD domain and is not sensitive to oxygen level, while ODD domain is present in all three of HIF alpha subunits (HIF1A, HIF2A, and HIF3A) and will lead to their degradation under normoxic condition by hydroxylation and ubiquination reactions mediated by PHD and VHL proteins respectively (10-16).

HIF alpha heterodimers can perform transcriptional activity when they are stabilized under hypoxic conditions (13). HIF1A and HIF2A heterodimers produce the main transcription activation of genes that hold HRE within their promoter sequence (17). Activation of HIF alpha target genes in cancer cells can result in metabolism shift from oxidative phosphorylation to glycolysis, activation of survival, angiogenesis, metastasis and proliferation pathways (18-20).

While most of the past studies had focused on the importance of HIF1A and HIF2A activity in different types of cancer, little data exist to adequately explain the importance of HIF3a expression level and molecular activity in different types of cancer (21). HIF3A contains ODD domain and can get stabilized under hypoxic conditions and limit the activity of HIF1A and HIF2A by competing for dimerization with the HIF beta subunit (21-23).

HIF3A has shown tissue-specific expression patterns, but its exact expression pattern in many types of cancer remains unknown. HIF3A has multiple variants(23). The long variants of HIF3A have been shown to be able to heterodimerize with HIF beta subunits, bind to HRE element and perform weak transcriptional activity (24). While the short variant of HIF3A, also known as IPAS (Inhibitory PAS domain) can prevent

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the transcriptional activity of HIF1A by forming a dimer with HIF1A directly and prevents its binding on HRE elements (25).

In order to gain better insight on the expression pattern and importance of HIF3A in cancers, we have taken a bioinformatic approach and performed differential gene expression (DGE) analysis along receiver operating characteristic test and survival analysis on different types of TCGA cancer samples. Our study will help clarifying the expression pattern, diagnostic, and prognostic potential of HIF alpha subunits in diverse kinds of cancer with different stages and sizes.

2. Methods and Materials

2.1. Database

The TCGA database (https://docs.gdc.cancer.gov/) provides expression matrix of different types of cancers. The Bioconductor tool (TCGAbiolinks package) was used to download the gene expression data of BRCA, COAD, HNSC, KIRC, KIRP, LIHC, LUAD, LUSC, PRAD, STAD, and THCA of the TCGA tissue samples, as well as the clinical data of patients, such as vital-status, tumor-stage and tumor-size. Expression data was normalized and the missing values of genes were removed to prepare the expression data for further analysis.

2.2. Differential gene expression analysis

Downloaded gene expression data of TCGA cancer samples are in single raw count form. Therefore, the count data was normalized using Voom package in R program and were converted into logarithmic form (log2 ratio). Limma and EdgeR packages were utilized for differential gene expression analysis (DGE). Missing values from gene expression data were removed before DGE analysis. Cut-off of 0.01 was applied for calculation of p-value by t-test for measuring differential expression level of HIF1A, HIF2A, and HIF3A between tumor and normal paired tissue samples along different stages and tumor. sizes of cancer samples.

2.3. Receiver operating characteristic (ROC) test

ROC test is useful for measuring the performance of an interest biomarker in the classification of tumor phenotype from the normal phenotype. To measure and compare the diagnostic potential of HIF1A, HIF2A,

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and HIF3A in normalized gene expression data of different types of TCGA cancer, the receiver operating characteristic test was performed using GraphPad Prism software (version 8.4) was utilized and ROC curves were generated.

2.4. Survival analysis

In order to reveal the influence of HIF alpha members expression status on the survival rate of patients diagnosed with various kinds of cancer, the median of the gene expression values of each HIF alpha isomer was selected as a cut-off value to group the samples of patients based on their gene expression level. Patients whose gene expression level of different HIF alpha subunits was superior than the median value were considered as 'Higher than median ' class and samples with gene expression status was less than the cut off were considered as 'Lower than the median' class. Survival analysis was operated employing the R tool (Survival package) and Kaplan-Meier (KM) plots were generated for HIF alpha subunits in individual kinds of TCGA cancer.

2.5. Data analysis

DGE and survival analysis were performed employing the RStudio program (version 4.1.0). ROC curves were created using GraphPad Prism software (version 8.4). Voom package was used for normalization of gene expression data in raw count format. Survival package Bioconductor tool was used for survival analysis.

3. Results

3.1. HIF3A expression level is significantly less in distinctive cancer tissues.

DGE analysis was performed on normalized gene expression files of 13 types of TCGA cancer and the expression status of HIF3A was notably little in all kinds of analyzed cancers in contrast to normal paired tissues (Figure.1A). The expression level of HIF1A was high in UCEC, THCA, STAD, HNSC, BRCA, LUAD, and LUSC cancers but low in PRCA, LIHC, KIRC and COAD cancers compared to normal paired tissues (Figure.1B). The expression degree of HIF2A was low in almost models of cancers, apart from KIRC and GBM cancers, whose expression scale was notably superior in cancer samples in contrast to normal paired tissues (Figure.1C).

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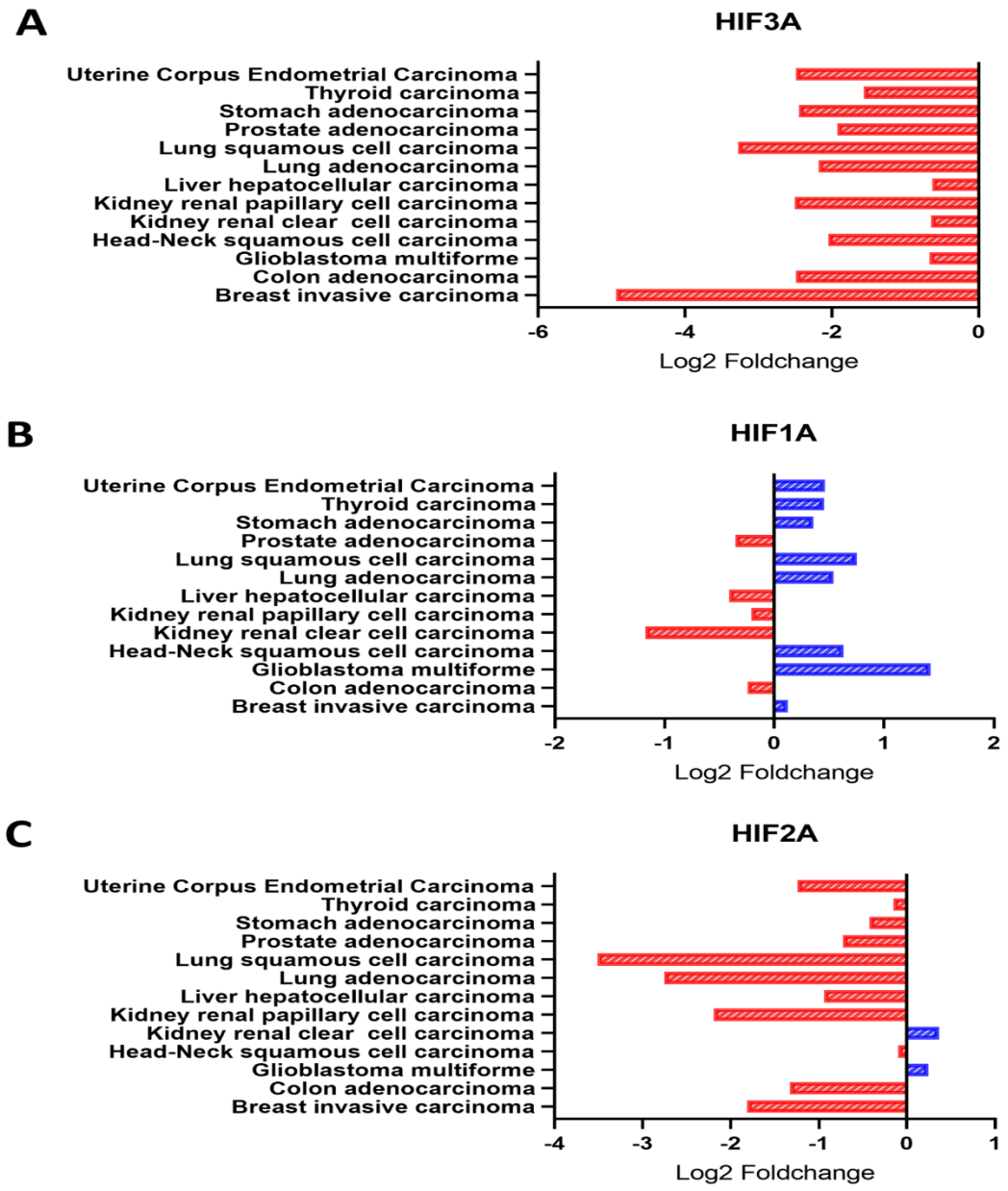

Figure 1. Differential expression analysis of HIF1A, HIF2A, and HIF3A in different types of cancers. **A**. HIF1A showed heterogenous expression pattern in different types of cancers. Its expression level was lower in prostate adenocarcinoma, liver hepatocellular carcinoma, kidney renal papillary cell carcinoma, kidney clear cell carcinoma, and colon adenocarcinoma. But its expression level was higher in other types of cancers compared to adjacent normal tissues. **B**. HIF2A expression level was lower in most types of cancer, except in kidney clear cell carcinoma and glioblastoma multiforme cancers, which its expression level was higher in cancer tissues compared to adjacent normal tissues. **C**. HIF3A expression level was lower in all

154 types of cancers compared to adjacent normal tissues, especially in breast invasive carcinoma tissues
155 compared to normal adjacent tissues.
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157 **3.2. HIF3A expression level in different stages and sizes of tumor samples**

158 The normalized expression level of HIF1A, HIF2A, and HIF3A were analyzed based on the stage and
159 size of cancer samples. The expression level of HIF3A did not vary considerably in different sizes of cancer
160 samples (Supplementary Figure.1), but its differential expression level in different stages of COAD (p-value
161 =0.05), LUAD (p-value =0.03), and UCEC (p-value = 0.03) cancers was significant (Supplementary
162 Figure.2). The differential expression level of HIF1A was not significant in different sizes of cancer samples
163 (Supplementary Figure.3), but its differential expression level was significant (p-value = 0.02) in different
164 stages of COAD cancer samples (Supplementary Figure.4). The differential expression level of HIF2A was
165 significant (p-value= 0.02) only in different sizes of LUSC cancer samples (Supplementary Figure.5). Also,
166 its differential expression level was only significant (p-value =0.006) in different stages of BRCA cancer
167 samples (Supplementary Figure.6).

168 **3.3. Potential of HIF3A as cancer biomarker.**

169 ROC curve analysis was performed on HIF1A, HIF2A, and HIF3A expression level in different types of
170 TCGA cancers. The results revealed that HIF3A has a very weak diagnostic potential in most types of
171 analyzed cancers. However, it had better diagnostic potential in LUAD (AUC= 0.70, p-value <0.0001)
172 (Supplementary Figure.7). ROC curve analysis also showed that HIF1A has a good diagnostic potential in
173 GBM (AUC= 0.77, p-value= 0.02), KIRC (AUC=0.72, p-value <0.0001), and LUSC (AUC= 0.70, p-value
174 <0.0001) cancers (Supplementary Figure.8). In addition, HIF2A can be a useful diagnostic biomarker in
175 BRCA (AUC=0.70, p-value<0.0001), COAD (AUC= 0.90, p-value <0.0001), KIRP (AUC =0.86, p-value
176 <0.0001), LIHC (AUC =0.76, p-value <0.0001), LUAD (AUC=0.80, p-value<0.0001), LUSC (AUC=0.84,
177 p-value<0.0001), and UCEC (AUC = 0.71, p-value<0.0001) cancers (Supplementary Figure.9).
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3.4. Correlation of patient's survival chances with HIF3A level

Survival analysis was performed on TCGA cancers to explore the importance of HIF1A, HIF2A, and HIF3A expression level on the survival of patients with varying kinds of cancer. Higher expression ratio of HIF3A correlated with improved survival in various sorts of cancer. However, patients with GBM, KIRC, LIHC, and THCA cancers had lower level of HIF3A and better survival chances (Supplementary Figure.10). Survival analysis of HIF1A showed that greater expression degree of HIF1A correlated with lesser survival rate in most types of cancer, but patients with GBM, KIRC, LUSC, and STAD, who had higher expression level of HIF1A had better survival chances (Supplementary Figure.11). High expression ratio of HIF2A was linked with worse survival chances in most types of cancer; However, patients with KIRC and KIRP cancers, had lower level of HIF2A had better chances of survival (Supplementary Figure.12). The differences between the survival of patients who had high or low levels of HIF alpha subunits was not significant in most types of cancer.

4. Discussion

For long decades, many studies have described an association between the expression ratio of HIF1A and the resisting behavior of cancer cells against cancer treatment attempts under hypoxic conditions (26-30). HIF1A and HIF2A have been shown to induce the expression scale of varying genes that are participating in adaption of cancer cells to hypoxic conditions, such as activation of angiogenic, survival, metastatic, proliferative, and glycolytic pathways (26, 31-35).

While the role and importance of first and second subunit of HIF alpha in distinctive models of cancer cells has been shown, little information exist to assess the importance of the expression ration and function of HIF3A subunit in various kinds of cancer (21). In the present research, we applied differential gene expression, receiver operating characteristic and survival analyses on varying models of TCGA cancers to get a better perspective on the expression pattern and diagnostic potential of HIF3A, as well as its correlation with the survival ratio of patients with diverse types of cancer.

By DGE analysis, we have shown that the mRNA ratio of the third subunit of HIF alpha is lesser in nearly many kinds of cancers compared to their paired normal tissues. Only one published study has shown that the expression status of HIF3A was great in ovarian cancer tissues (36), a tissue that was not included in the present analysis. Low expression level of HIF3A in prostate adenocarcinoma cells highly correlated with high methylation level in the promoter region of HIF3A gene (37) (Table 1). Induction of the long variant of HIF3A expression level underneath hypoxic environment in Hep3B cells and Kelly neuroblastoma cells positively correlated with the expression level of Erythropoietin (EPO), Bone morphogenetic protein 6 (BMP6), and Pentraxin 3 (PTX3) genes (24). HIF3A expression level have been shown to positively correlate with LINC01346 expression level and induce metastatic potential in ovarian cancer (OC) cells (36).

Over expression of the small variants of HIF3A, negatively correlated with the expression level of VEGF and PGK1 genes in Hela cells (38), while positively correlated with higher metastatic potential and lower survival rate in pancreatic cells (39).

By differential expression analysis we revealed that the expression scale of HIF3A was not linked significantly with varying stages and sizes of various kinds of TCGA tumor that were analyzed in this study. However, its expression level significantly correlated with different stages of COAD, LUAD, and UCEC cancers. In addition, we found no correlation between the expression level of HIF1A in different sizes of TCGA cancers. However, its expression level differed significantly in different stages of COAD cancer. At the same time, the expression degree of HIF2A was also considerably different in different tumor-sizes of LUSC cancer and different stages of BRCA cancer. Another study had also reported no correlation between the stages and tumor-size of pancreatic cancer cells (39). The expression level of the long variant of HIF3A was previously indicated to influence the progression and growth of colorectal cancer cells through participating in the Jak-Stat3 signaling pathway (40).

By survival analysis we have shown that a greater expression ratio of HIF3A was linked with enhanced survival in patients affected by different types of cancer except for GBM, KIRC, LIHC, and THCA cancers. Previous studies had shown that higher expression level of HIF3A negatively correlated with survival of pancreatic cells (39) and had no correlation with the overall survival rate of patients with hepatocellular carcinoma (41). Our knowledge on the molecular function of HIF3A heterodimer is severely lacking. Further

investigations are needed to clarify the importance of altered expression level of long and short variants of HIF3A in different types of cancer and reveal its exact molecular and transcriptional activity under hypoxic conditions and in oxygen-independent conditions such as inflammation.

5. Conclusion

In our study, we made an overall new comparison of the expression patterns of all three members of the HIF alpha factors. The expression ratio of HIF3A was little in varying models of cancers and its expression level significantly correlated with better survival of affected patients. However, its diagnostic potential was weaker compared to HIF1A and HIF2A heterodimers. As earlier studies have established a positive association between the expression level of HIF3A with metastatic potential of ovarian cancer and pancreatic cancer cells and the progression of colorectal cancer cells, more extended investigations are desired to define these differences and, more in general, the importance of HIF3A expression and function in distinctive groups of cancer.

Ethical Approval and Consent form

As the TCGA data base shares its data in public form, approval from the ethics committee was not necessary for this study.

Consent for publication

All authors agreed on the submission of the present research to this journal.

Availability of supporting data

Supporting and raw data are available upon a reasonable request to the corresponding author.

Competing Interest

All the authors affirm that they have no competing financial desire that could influence the work announced in this paper.

Funding

Not available.

Authors' Contributions

Study design was performed by B.Y. Data analysis was done by B.Y. Interpretations of data were performed with B.Y, H.S. Bioinformatics analysis was performed with B.Y. Manuscript writing was performed by B.Y. Final approval of the manuscript was performed with H.S.

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