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Original Article

Expression analysis of HIF-3α as a potent prognostic biomarker in various types of human cancers: a case of meta-analysis

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Abstract

Background and purpose: Hypoxia-inducible factors (HIFs) are transcription factors that get activated and stabilized in the heterodimerized form under hypoxic conditions. many studies have reported the importance of the HIF-1α and HIF-2α activity in biological pathways of hypoxic cancer cells. However, the importance of HIF-3α in a variety of cancers remains unknown.
Experimental approach: The expression profile of 13 different types of cancer samples from the Cancer Genome Atlas (TCGA) database were subjected to normalization, and differential gene expression analysis was performed using computational algorithms by R programming. Receiver operating characteristic tests and survival analyses were carried out for HIF-α subunits in different cancers.
Findings / Results: The expression status of HIF-3α was notably less in all cancer samples in contrast to their adjacent normal tissues. The expression degree of HIF-1α varied among distinct types of cancer and the expression degree of HIF-2α was lower in nearly all types of cancers. HIF-3α had very weak diagnostic potential, while HIF-2α had better diagnostic potential in most types of cancers compared to HIF-1α. Patients who had a higher level of HIF-3α had better survival, while the higher expression level of HIF-1α and HIF-2α were associated with worse survival in many types of cancers.
Conclusion and implications: Our findings showed that each HIF-α subunit had a unique heterogeneous expression pattern in different classes of cancers. The expression level of each HIF-α subunit correlated differently with the stages, tumor sizes, and survival rate of patients from different classes of TCGA cancers.

Keywords: Cancer; Expression analysis; HIF-3α; Hypoxia-inducible factors.

INTRODUCTION

Hypoxia refers to a state when the concentration of oxygen around the cell's microenvironment is less than 2% mmHg (1). A hypoxic environment can enhance the resisting behavior of solid tumor cells against drugs that are administrated for cancer treatments (2-4). An active form of hypoxia-inducible factor (HIF) is generated when alpha and beta subunits create a dimer whose activity and stability are tightly dependent on the status of oxygen tension in the cellular environment (5-7). Active HIF heterodimer is formed between HIF-α and HIF-β subunits (8,9). HIF subunits share high sequence similarities in their structure and domains. However, the HIF-β subunit lacks the oxygen-dependent degradation (ODD) domain and is not sensitive to oxygen levels, while the ODD domain is present in all three HIF-α subunits (HIF-1α, HIF-2α, and HIF-3α) and will lead to their degradation under normoxic conditions by hydroxylation and ubiquitination reactions mediated by prolyl hydroxylase domain and von Hippel-Lindau proteins, respectively (10-16).

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HIF-α heterodimers can perform transcriptional activity when they are stabilized under hypoxic conditions (13). HIF-1α and HIF-2α heterodimers produce the main transcription activation of genes that hold hypoxia response elements within their promoter sequence (17). Activation of HIF-α target genes in cancer cells can result in a 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 HIF-1α and HIF-2α activity in different types of cancer, little data exist to adequately explain the importance of HIF-3α expression level and molecular activity in different types of cancer (21). The HIF-3α contains an ODD domain and can get stabilized under hypoxic conditions and limit the activity of HIF-1α and HIF-2α by competing for dimerization with the HIF-β subunit (21-23).

Although HIF-3α has shown tissue-specific expression patterns, its exact expression pattern in many types of cancer remains unknown. This isoform encompasses multiple variants (23). The long variants of HIF-3α are able to heterodimerize with HIF-β subunits, bind to hypoxia response elements, and perform a weak transcriptional activity (24). While the short variant of HIF-3α, also known as the inhibitory Per-Arnt-Sim domain can prevent the transcriptional activity of HIF-1α through the direct formation of a dimer with HIF-1α, thereby hindering its binding on hypoxia response element elements (25).

In order to gain better insight into the expression pattern and importance of HIF-3α in cancers, we herein implemented a bioinformatics protocol containing differential gene expression (DGE) analysis along with receiver operating characteristic test and survival analysis on different types of the cancer genome atlas (TCGA) samples. The present study could help to clarify the expression pattern of HIF-α subunits in diverse kinds of cancer with different stages and sizes and also suggest the diagnostic and prognostic potential of these subunits in different types of cancer.

METHODS AND MATERIALS

Database

The TCGA database (https://docs.gdc.cancer.gov/) provides an expression matrix of different types of cancers. The Bioconductor tool (TCGAbiolinks package) was used to download the gene expression data of breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), head-neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), prostate adenocarcinoma (PRAD), stomach adenocarcinoma (STAD), and thyroid carcinoma (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 were normalized and the missing values of genes were removed to prepare the expression data for further analysis.

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 the Voom package in the R program and were converted into logarithmic form (log2 ratio). Limma and EdgeR packages were utilized for DGE analysis. Missing values from gene expression data were removed before DGE analysis. A cut-off of 0.01 was applied for the calculation of the *p*-value by t-test for measuring the differential expression level of HIF-1α, HIF-2α, and HIF-3α between tumor and normal paired tissue samples along different stages and tumor sizes of cancer samples.

Receiver operating characteristic test

The receiver operating characteristic (ROC) test is useful for measuring the performance of an interesting biomarker in the classification of tumor phenotype from the normal phenotype. To measure and compare the diagnostic potential of HIF-1α, HIF-2α, and HIF-3α in normalized gene expression data of different types of TCGA cancer, the ROC test was performed using GraphPad Prism software (version 8.4) and ROC curves were generated.

Survival analysis

In order to clarify the correlation between the expression level of HIF-α subunits with the

survival data in 13 different classes of cancer, the median of the gene expression values of each HIF-α subunit 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-α subunits was superior to the median value were considered 'higher than median' class and samples with gene expression status were less than the cut-off were considered lower than the median class. Survival analysis was operated employing the R tool (Survival package) and Kaplan-Meier (KM) plots were generated for HIF-α subunits in individual kinds of TCGA cancer.

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). The Voom package was used for the normalization of gene expression data in raw count format. Survival package Bioconductor tool was also employed for survival analysis.

RESULTS

HIF-α expression level in distinctive cancer tissues

DGE analysis was performed on normalized gene expression files of 13 types of TCGA cancer. The expression status of HIF-3α was notably little in all kinds of analyzed cancers in contrast to normal paired tissues (Fig.1A). The expression level of HIF-1α was high in uterine corpus endometrial carcinoma (UCEC), THCA, STAD, HNSC, BRCA, LUAD, and LUSC cancers but low in PRCA, LIHC, KIRC, and COAD cancers compared to normal paired tissues (Fig. 1B). The expression degree of HIF-2α was low in almost models of cancers, apart from KIRC and glioblastoma multiforme (GBM) cancers, whose expression scale was notably superior in cancer samples in contrast to normal paired tissues (Fig.1C).

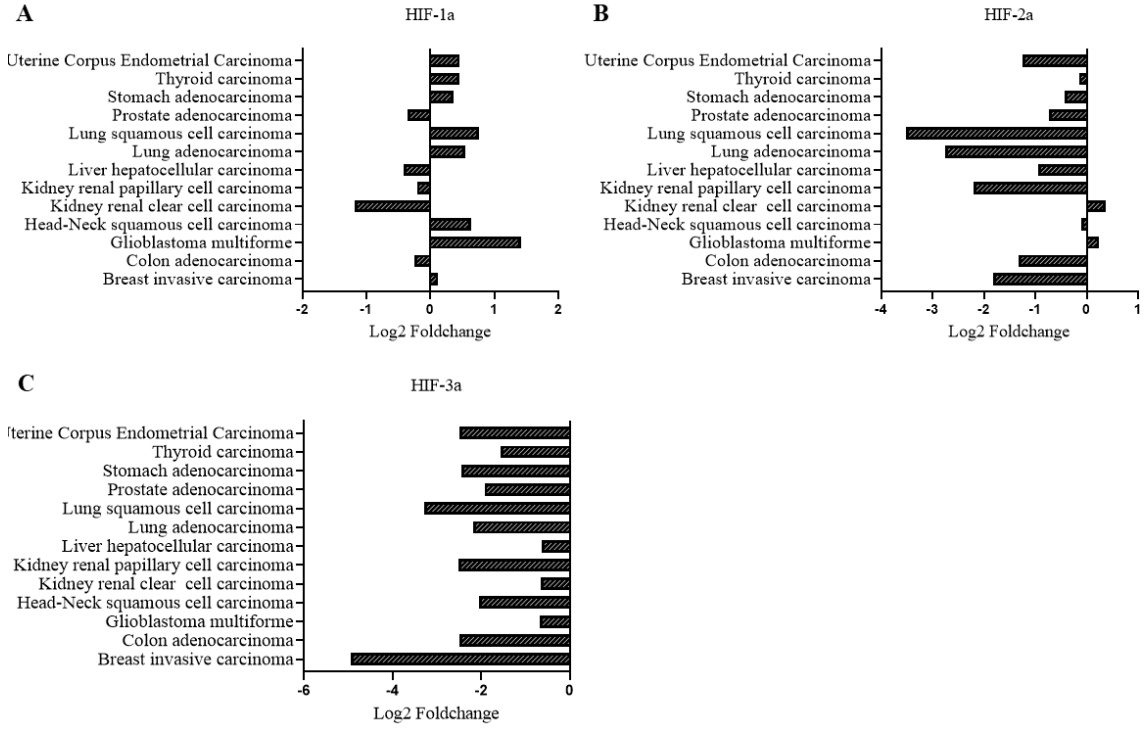

Fig. 1. Differential expression analysis of HIF-1α, HIF-2α, and HIF-3α in different types of cancers. (A) HIF-1α showed a heterogenous expression pattern in different types of cancers. Its expression level was lower in prostate adenocarcinoma, liver hepatocellular carcinoma, kidney renal papillary cell 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) HIF-2α expression level was lower in most types of cancer, except in kidney clear cell carcinoma and glioblastoma multiforme cancers, in which its expression level was higher in cancer tissues compared to adjacent normal tissues; (C) HIF-3α expression level was lower in all types of cancers compared to adjacent normal tissues, especially in breast invasive carcinoma tissues compared to normal adjacent tissues. HIF, Hypoxia-inducible factors.

HIF-3α expression level in different stages and sizes of tumor samples

The normalized expression level of HIF-1α, HIF-2α, and HIF-3α were analyzed based on the stage and size of cancer samples. The expression level of HIF-3α did not vary considerably in different sizes of cancer samples (Fig. 2), but its differential expression level in different stages of COAD, LUAD, and UCEC cancers was significant (Fig. 3). The differential expression level of HIF-1α was not significant in different sizes of cancer samples (Fig. S1), but its differential expression level was significant in different stages of COAD cancer samples (Fig. S2). The differential expression level of HIF-2α was significant only in different sizes of LUSC cancer samples (Fig. S3). Also, its differential expression level was only significant in different stages of BRCA cancer samples (Fig. S4).

HIF-3α as a potential cancer biomarker

ROC curve analysis was performed on HIF-1A, HIF-2α, and HIF-3α expression level in different types of TCGA cancers. The results revealed that HIF-3α has a very weak diagnostic potential in most types of analyzed cancers. However, it had better diagnostic potential in LUAD (Fig. S5). ROC curve analysis also showed that HIF-1α has a good diagnostic potential in GBM, KIRC, and LUSC cancers (Fig. S6). In addition, HIF-2α can be a useful diagnostic biomarker in BRCA, COAD, KIRP, LIHC, LUAD, LUSC, and UCEC cancers (Fig. S7).

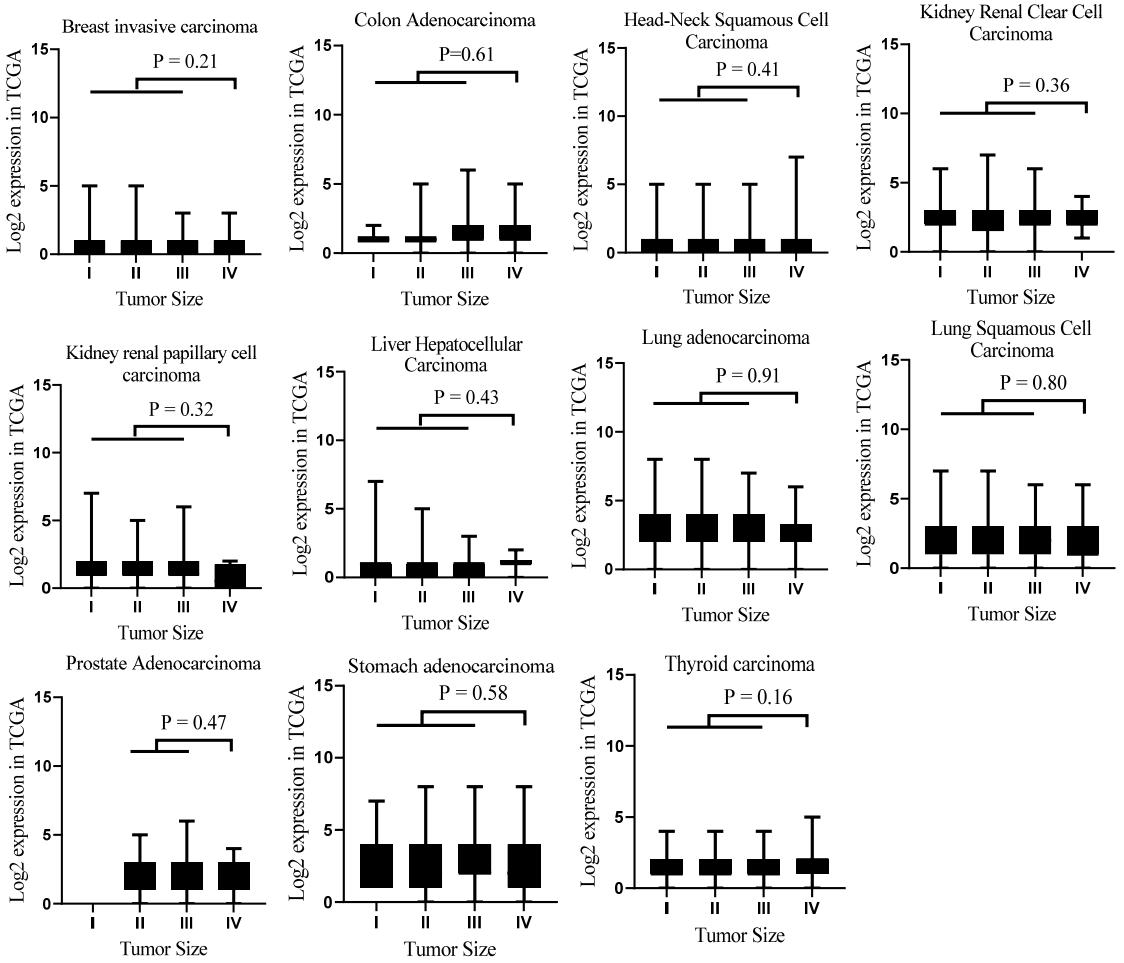

Fig. 2. HIF-3α expression level in different tumor sizes of various types of cancer. The expression level of HIF-3α did not significantly differ in different sizes of cancer tissues. Tumor samples were divided into four groups (I-IV) based on their phenotypic details. HIF, Hypoxia-inducible factors.

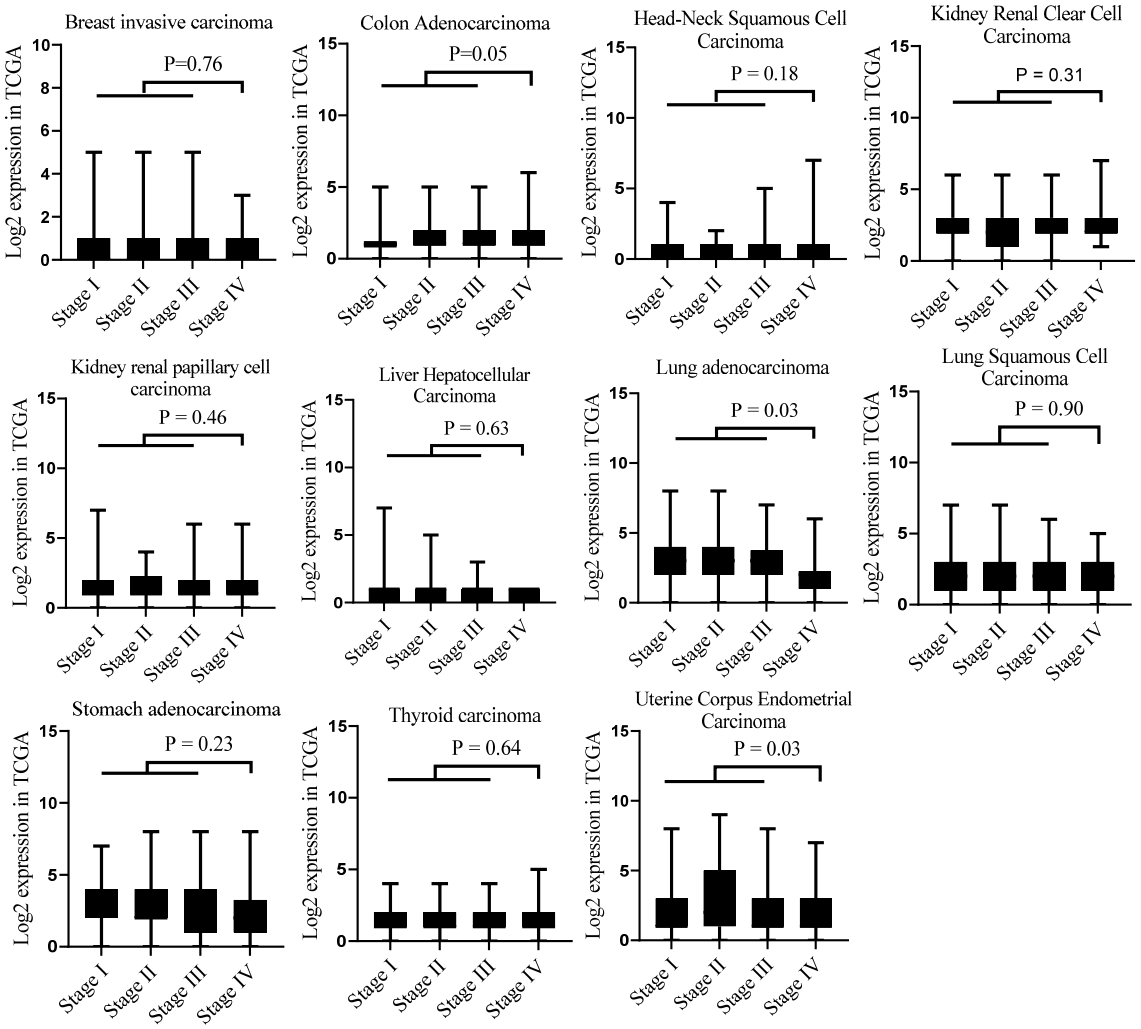

Fig. 3. Expression analysis of HIF-3α in different stages of cancer. HIF-3α expression level significantly correlated with different stages of colon adenocarcinoma, lung adenocarcinoma, and uterine endometrial carcinoma, but did not correlate with different stages of other types of cancer. HIF, Hypoxia-inducible factors.

Correlation of patient's survival chances with HIF-3α level

Survival analysis was performed on TCGA cancers to explore the importance of HIF-1α, HIF-2α, and HIF-3α expression levels on the survival of patients with varying kinds of cancer. A higher expression ratio of HIF-3α correlated with improved survival in various sorts of cancer. However, patients with GBM, KIRC, LIHC, and THCA cancers had a lower level of HIF-3α and better survival chances (Fig. S8). Survival analysis of HIF-1α showed that a greater expression degree of HIF-1α correlated with a lesser survival rate in most types of cancer, but patients with GBM, KIRC, LUSC, and STAD, who had higher expression levels of HIF-1α had better survival chances (Fig. S9). A high expression ratio of HIF-2α was linked with worse survival chances in most types of cancer; however, patients with KIRC and KIRP cancers, who had lower levels of HIF-2α had better chances of survival (Fig. S10). The differences between the survivals of patients who had high or low levels of HIF-α subunits were not significant in most types of cancer.

DISCUSSION

For long decades, many studies have described an association between the expression ratio of HIF-1α and the resisting

survival of hypoxic cancer cells against cancer treatment attempts with radiotherapy or different chemotherapy methods (26-30). Multiple studies have shown the role of HIF-1α and HIF-2α in the transcriptional activation of varying genes that participate in the 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 the first and second subunit of HIF-α in distinctive models of cancer cells have been previously shown by other's studies, little information exists to assess the importance of the expression pattern and function of the HIF-3α subunit in various kinds of cancer (21). In the present research, we applied DGE, receiver operating characteristics, and survival analyses on varying models of TCGA cancers to get a better perspective on the expression pattern and diagnostic potential of HIF-3α, 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-α is lesser in nearly many kinds of cancers compared to their paired normal tissues. Recently, Zhang *et al*. have shown that the expression status of HIF-3α was great in ovarian cancer tissues (36), a tissue that was not included in the present analysis. A study performed by Bjerre *et al.* has shown that the low expression level of HIF-3α in PRAD cells is highly correlated with a high methylation level in the promoter region of the HIF-3α gene (37) (Table 1).

Table 1. Overview of HIF-3α expression level in different types of cells or cancers.

Type of cells or cancer		References
Hep3B cells	Knockdown of HIF-3α influenced EPO expression level. Overexpression of the HIF3A2 variant correlated with higher mRNA levels of EPO, BMP6, PTX3 and lower mRNA levels of SPA17 and FZD6 genes under hypoxic conditions. HIF-3α2 variant showed weak transcriptional activity on EPO, glucose transporter 1, and angiopoietin-like 4 genes.	(24)
Prostate adeno carcinoma	The methylation degree in the HIF-3α promoter region was significantly higher in cancer cells compared to normal cells. Patients who had lower methylation in the HIF-3α promoter had a significantly better survival rate.	(37)
PC12 cells (rat)	The mRNA level of IPAS was shown to be regulated by tumour necrosis factor-alpha in an oxygen-independent manner.	(42)
Ovarian cancer (human)	The mRNA level of HIF-3α positively correlated with the mRNA level of LINC01342 and negatively correlated with the miR-30c-2-3P mRNA level. Higher mRNA level of HIF-3α correlated with proliferation and migration potential of ovarian cancer cells.	(36)
Colorectal cancer	HIF-3α1 variant expression correlated with the progression of colorectal cancer cells through the JAK-STAT3 pathway.	(40)
Caki-1 renal carcinoma	HIF-3α4 and HIF-3α2 mRNA levels were elevated under hypoxic conditions.	(43)
Lung epithelial cells (human)	The mRNA level of HIF-3α was shown to increase under hypoxic conditions.	(44)
Hela cells	Over-expressed level of mouse IPAS variant negatively correlated with the mRNA levels of vascular endothelial growth factor, and phosphoglycerate kinase 1 gene.	(38)
VHL-/CC- RCC cells	Over-expressed levels of IPAS negatively correlated with cellular growth and progression.	(45)
Pancreatic cells	Higher mRNA level of HIF-3α positively correlated with the metastatic potential of pancreatic cells, and poor survival rate, but showed no correlation with the stages and sizes of tumors. HIF-3α showed direct transcriptional activity on RhoC and ROCK1 genes under hypoxic conditions.	(39)
Hepatocellular carcinoma	HIF-3α expression level did not correlate with the overall survival rate of patients. A positive correlation was found between HIF-3α and HIF-2α protein levels.	(41)

HIF, Hypoxia-inducible factors; EPO, erythropoietin; IPAS, inhibitory per-Arnt-sim domain.

Tolonen *et al*. also showed that the induction of the long variant of HIF-3α expression level underneath a hypoxic environment in Hep3B cells and Kelly neuroblastoma cells positively correlated with the expression level of erythropoietin, bone morphogenetic protein 6, and pentraxin 3 genes (24). Zhang *et al.* also reported that HIF-3α expression levels have correlated positively with LINC01346 expression levels and induce metastatic potential in ovarian cancer cells (36). Makino *et al*. and Zhou *et al.* have shown respectively that the overexpression of the small variants of HIF-3α, negatively correlated with the expression level of vascular endothelial growth factor and phosphoglycerate kinase 1 gene 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 HIF-3α was not linked significantly with varying stages and sizes of various kinds of TCGA tumors 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 HIF-1α in different sizes of TCGA cancers. However, its expression level differed significantly in different stages of COAD cancer. At the same time, our analysis revealed that the expression level of the HIF-2α was also considerably altered in different tumor sizes of LUSC cancer and stages of BRCA cancer. As previously Zhou *et al*. also reported no correlation between the stages and tumor size of pancreatic cancer cells (39). Another study by Xue *et al*. reported that the expression level of the long variant of HIF-3α 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 revealed that a greater expression ratio of HIF-3α was associated with enhanced survival in patients affected by different types of cancer except for GBM, KIRC, LIHC, and THCA cancers. In previous studies, Zhou *et al*. and Liu *et al*. have shown respectively that a higher expression level of HIF-3α negatively correlated with the survival of pancreatic cells (39) and had no correlation with the overall survival rate of patients with hepatocellular carcinoma (41). Our knowledge of the molecular function of HIF-3α heterodimer is severely lacking. Further investigations are warranted to clarify the importance of expression levels of long and short variants of HIF-3α in different types of cancer as well as its exact molecular and transcriptional activity under hypoxic conditions and in oxygen-independent conditions such as inflammation.

CONCLUSION

In the present study, we introduced an overall new comparison of the expression patterns of all three members of the HIF-α factors, regarding the type of cancer, tumor size, and stage of the tumor samples. In our study, we presented the down-regulated expression levels of HIF-3α in varying models of cancers compared to their matched normal tissue samples. We also represented that the expression level of HIF-3α correlated with better survival in patients in certain classes of TCGA cancers. However, its diagnostic potential was weaker compared to HIF-1α and HIF-2α subunits. As earlier studies have established a positive association between the expression level of HIF-3α with the metastatic potential of ovarian cancer and pancreatic cancer cells and the progression of colorectal cancer cells, more extended investigations, including cell-culture experiments and knock-down models of HIF-3α under different ranges of oxygen tensions are needed to clarify the mechanism and function of different transcripts of HIF-3α subunit in distinctive classes of human cancers.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

The study design was performed by H. Sirous and B. Yazdani. Data analysis was done by B. Yazdani. Interpretations of the data and bioinformatics analysis were performed by B. Yazdani and H. Sirous. Manuscript writing was performed by B. Yazdani. The final version of the manuscript was approved by all authors.

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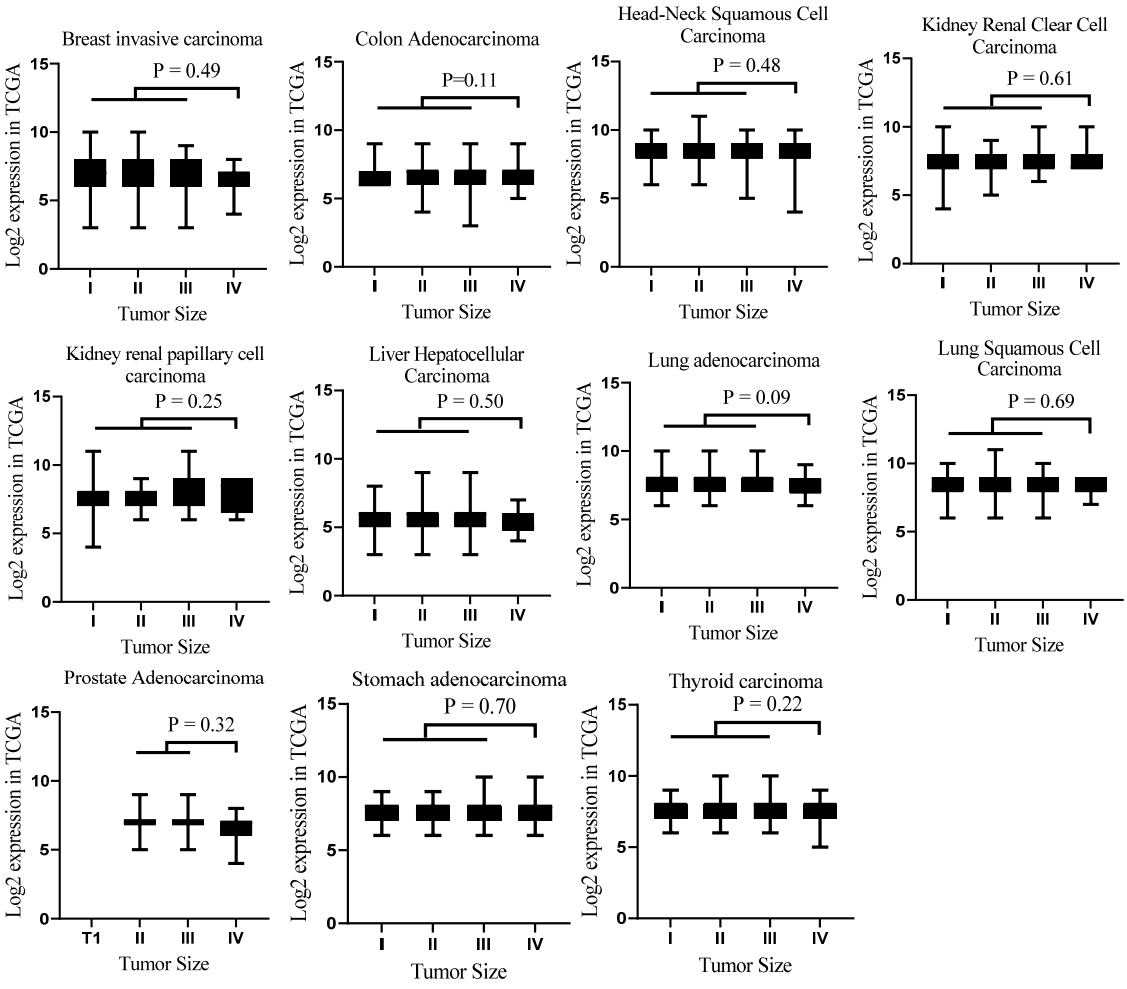

Fig. S1. Expression analysis of HIF-1α in different tumor sizes of various types of cancer. The expression level of HIF-1α did not significantly correlate with any types of selected cancer genome atlas (TCGA) cancer tissues. Tumor samples were divided into four groups (I-IV) based on their phenotypic details. HIF, Hypoxia-inducible factors.

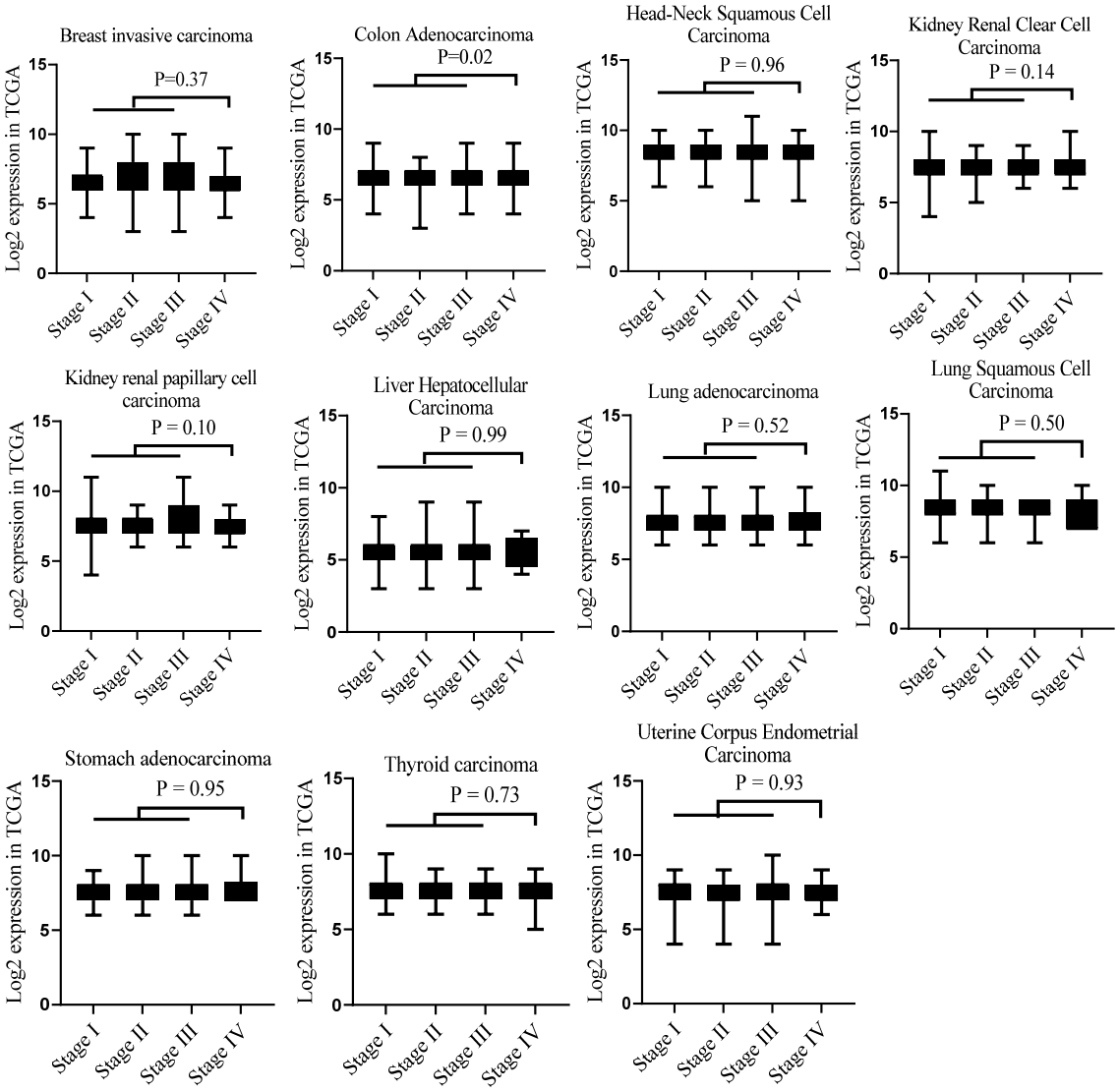

Fig. S2. Expression analysis of HIF-1α in different stages of cancer. HIF-1α expression level significantly correlated with different stages of colon adenocarcinoma, but did not correlate with different stages of other types of cancers. HIF, Hypoxia-inducible factors; TCGA, the cancer genome atlas.

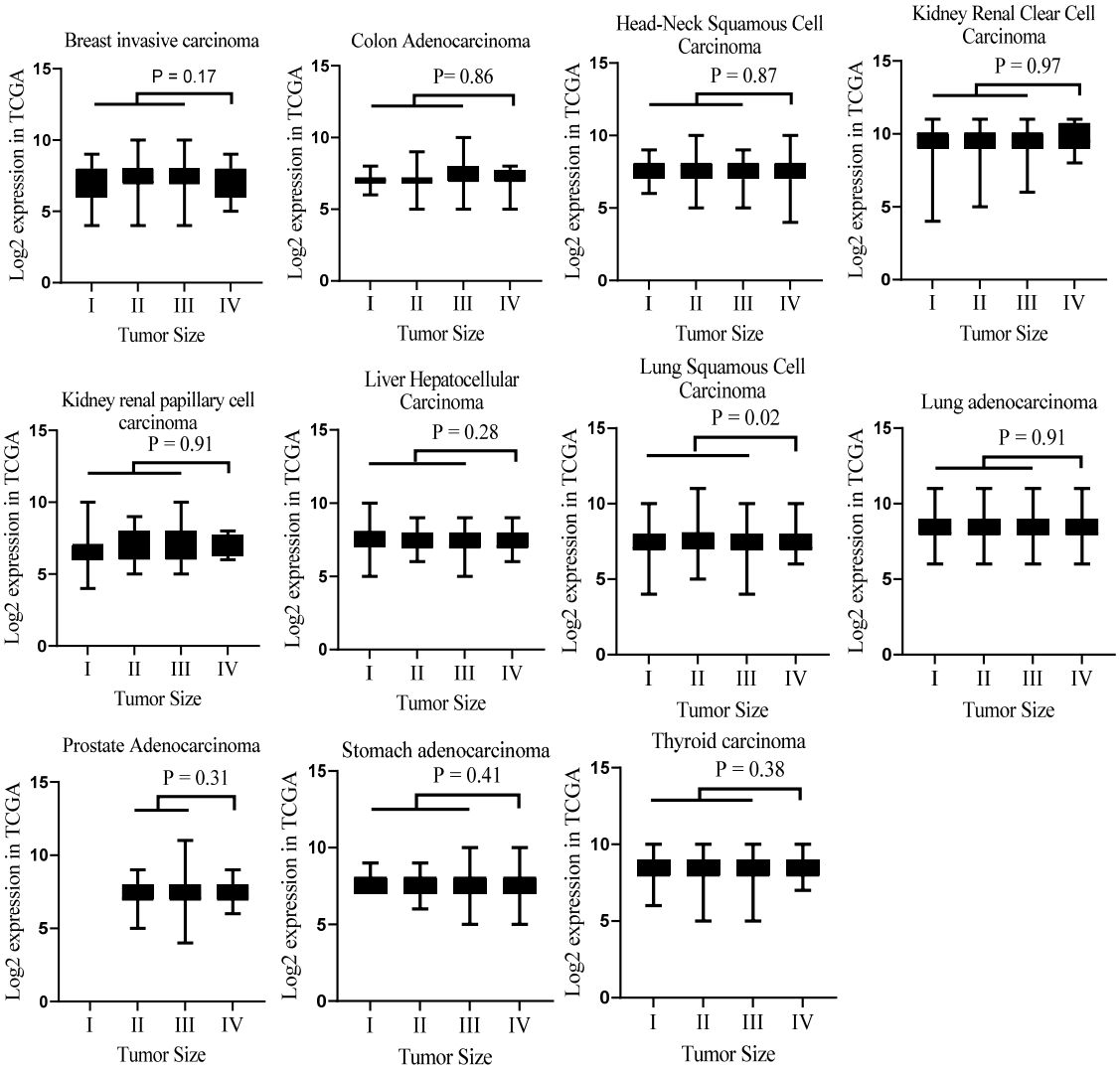

Fig. S3. Expression analysis of HIF-2α in different tumor sizes of various types of cancer. HIF-2α expression level only significantly correlated with different tumor sizes of lung squamous cell carcinoma but did not correlate with different sizes of other cancers. Tumor samples were divided into four groups (I-IV) based on their phenotypic details. HIF, Hypoxia-inducible factors; TCGA, the cancer genome atlas.

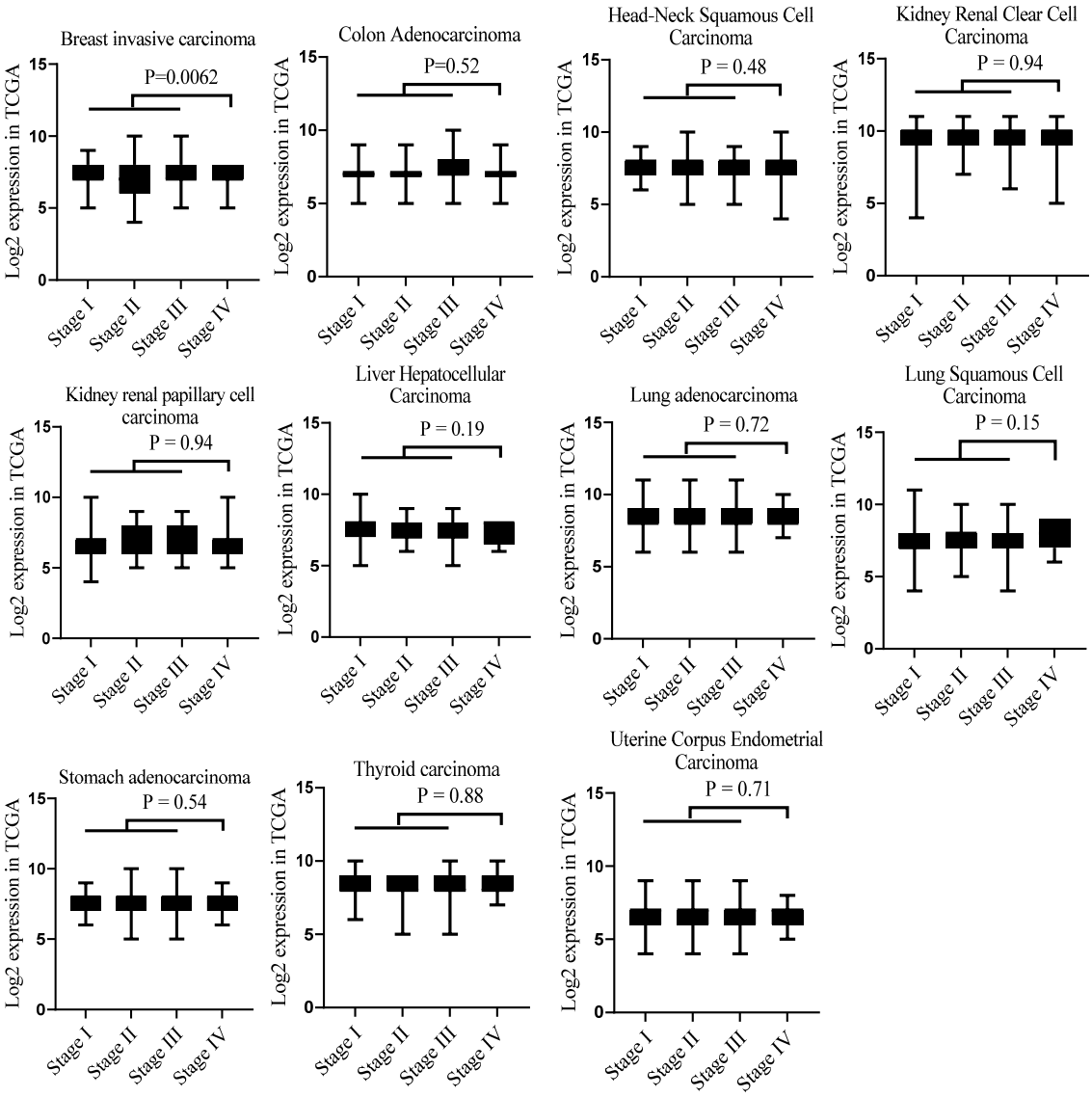

Fig. S4. Expression analysis of HIF-2α in different stages of cancer. HIF-2α expression level significantly correlated with different stages of breast invasive carcinoma, but did not correlate with different stages of other types of cancer. HIF, Hypoxia-inducible factors; TCGA, the cancer genome atlas.

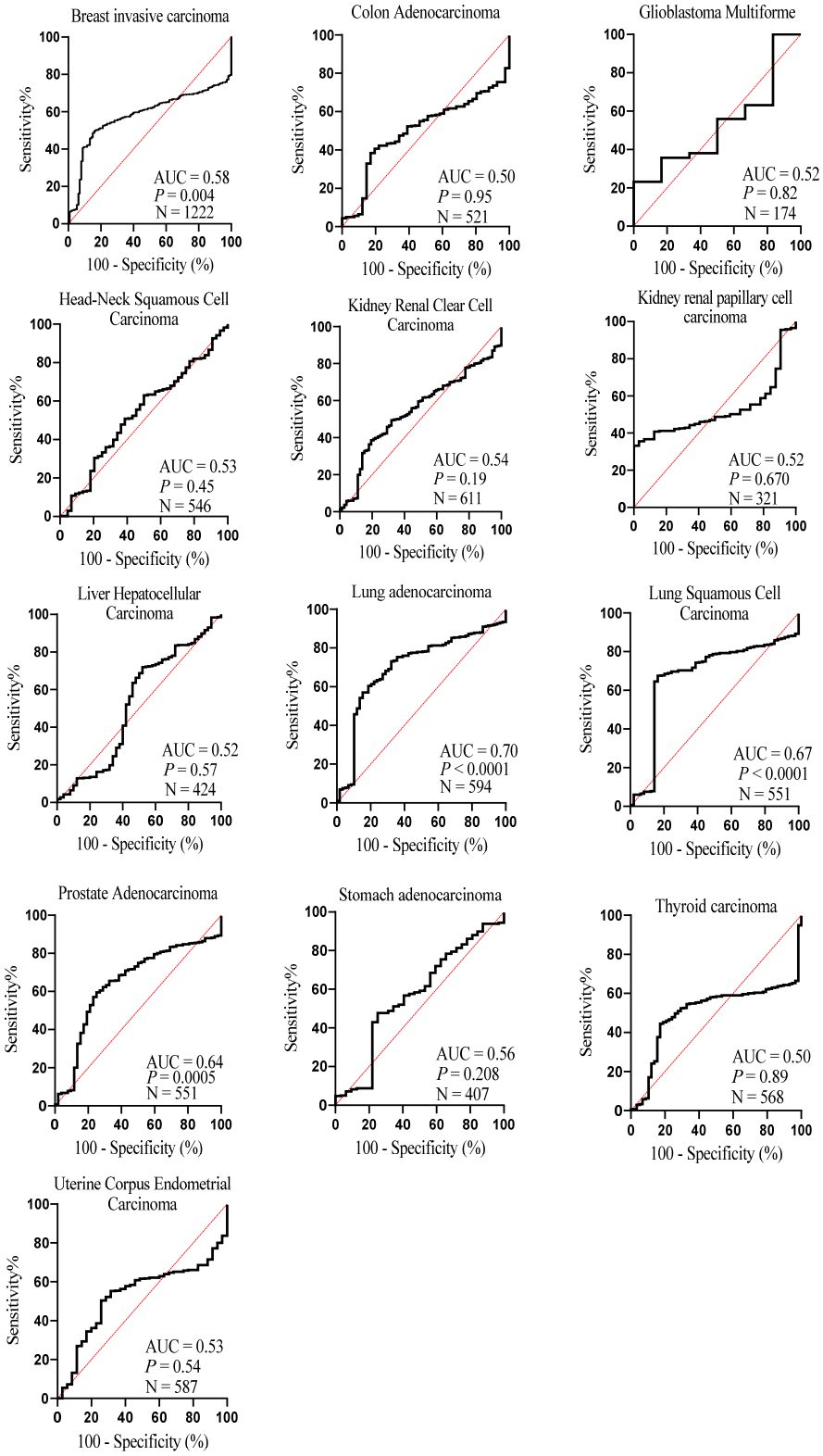

Fig. S5. Receiver operating characteristic test of HIF-3α in different types of cancer**. The** HIF-3α diagnostic potential was weak in most types of cancers, except in lung adenocarcinoma, which had better diagnostic potential and higher AUC value compared to other types of cancer. HIF, Hypoxia-inducible factors.

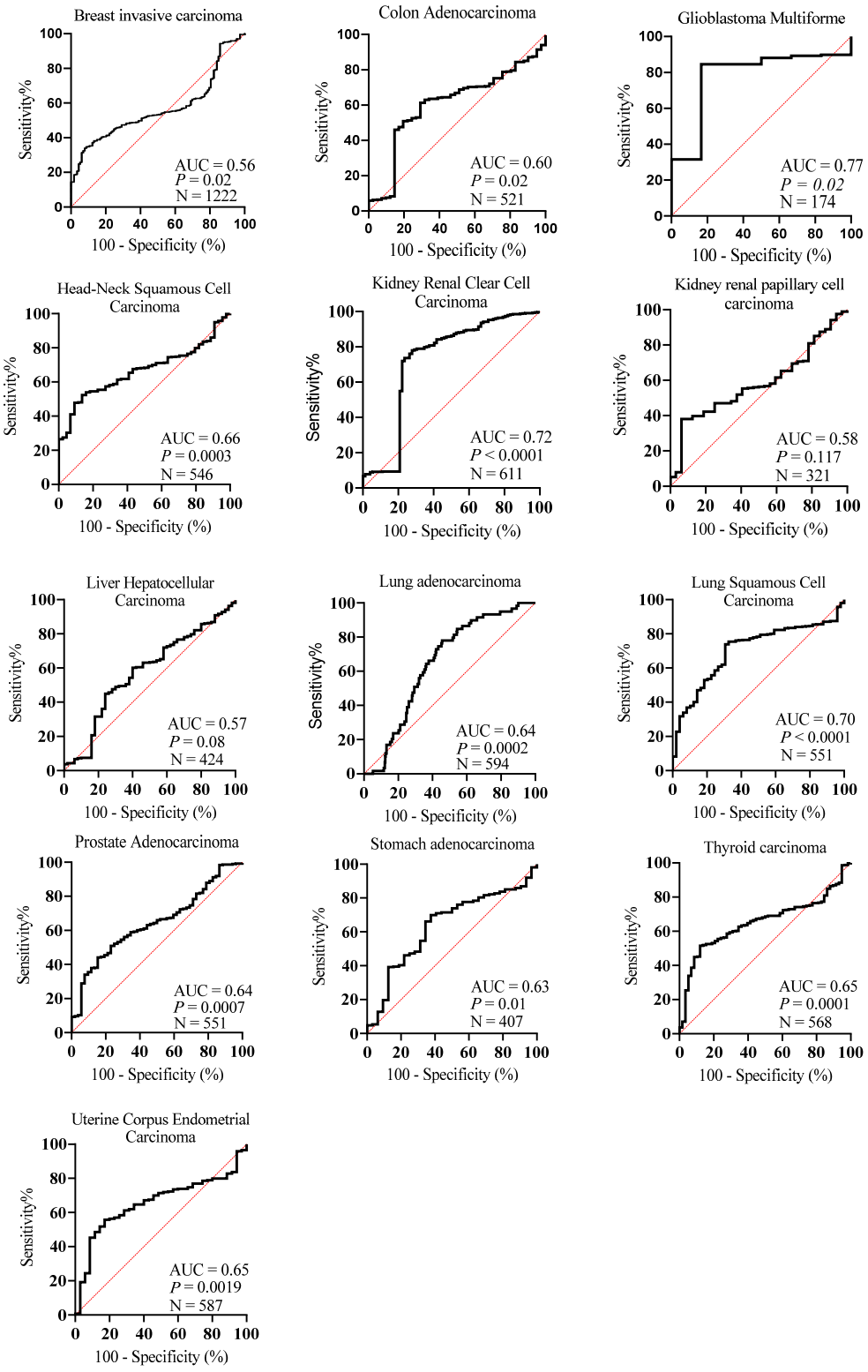

Fig. S6. Receiver operating characteristic test of HIF-1α in different types of cancer. HIF-1α had a weak diagnostic potential in most types of cancers, except in glioblastoma multiforme, kidney renal clear cell carcinoma, and lung squamous cell carcinoma cancers. HIF, Hypoxia-inducible factors.

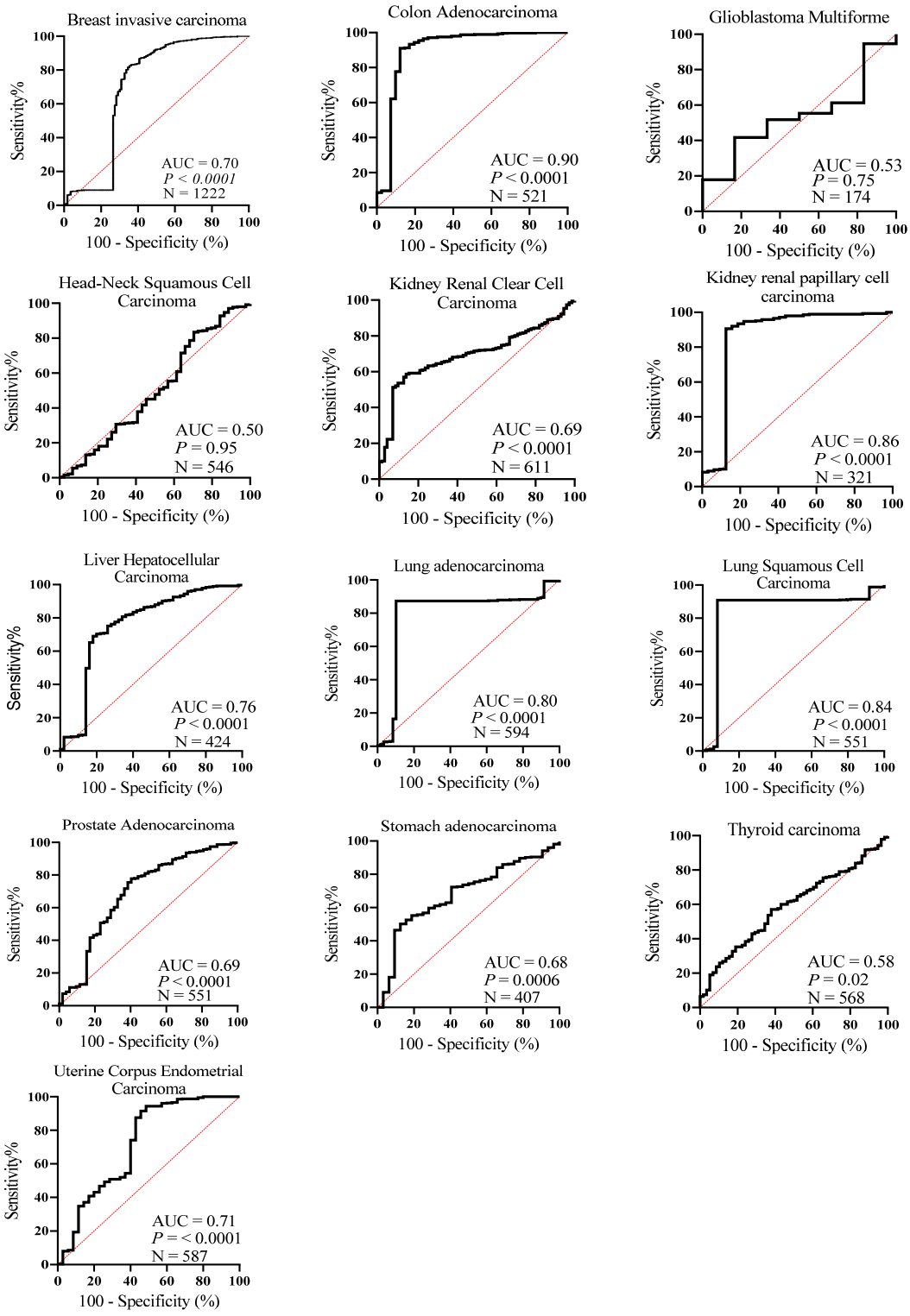

Fig. S7. Receiver operating characteristic test of HIF-2α in different types of cancer. The HIF-2α diagnostic potential was significant in most types of cancers, especially in breast invasive carcinoma, colon adenocarcinoma, kidney renal carcinoma, colon adenocarcinoma, liver hepatocellular carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, and uterine corpus endometrial carcinoma cancers. HIF, Hypoxia-inducible factors.

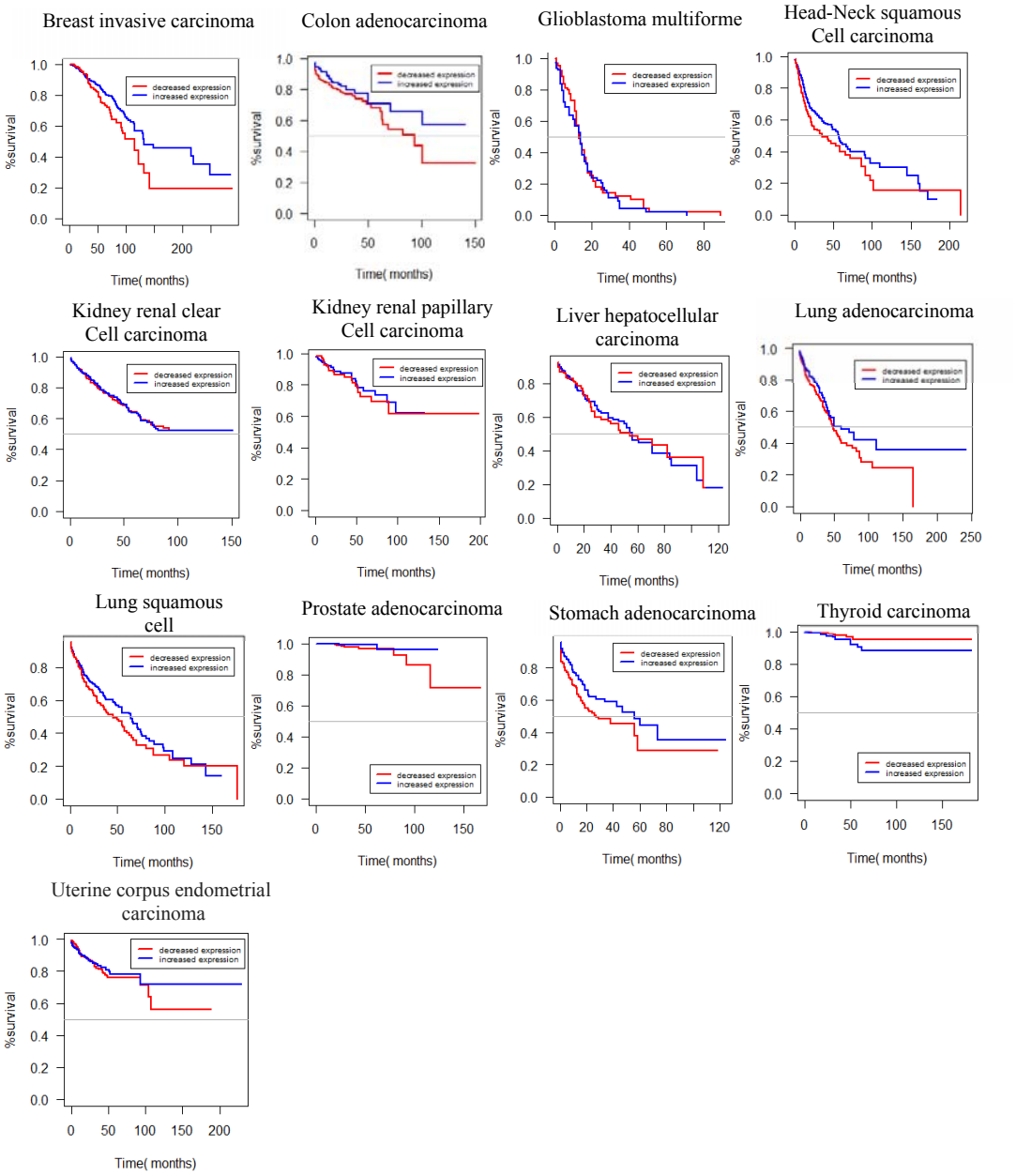

Fig. S8. Survival analysis of HIF-3α in different types of cancer. The higher expression level of HIF-3α correlated with better survival of patients with different types of cancers. Such as breast invasive carcinoma, colon adenocarcinoma, head-neck squamous cell carcinoma, kidney renal papillary cell carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, prostate adenocarcinoma, stomach adenocarcinoma, and uterine corpus endometrial carcinoma cancers. HIF, Hypoxia-inducible factors.

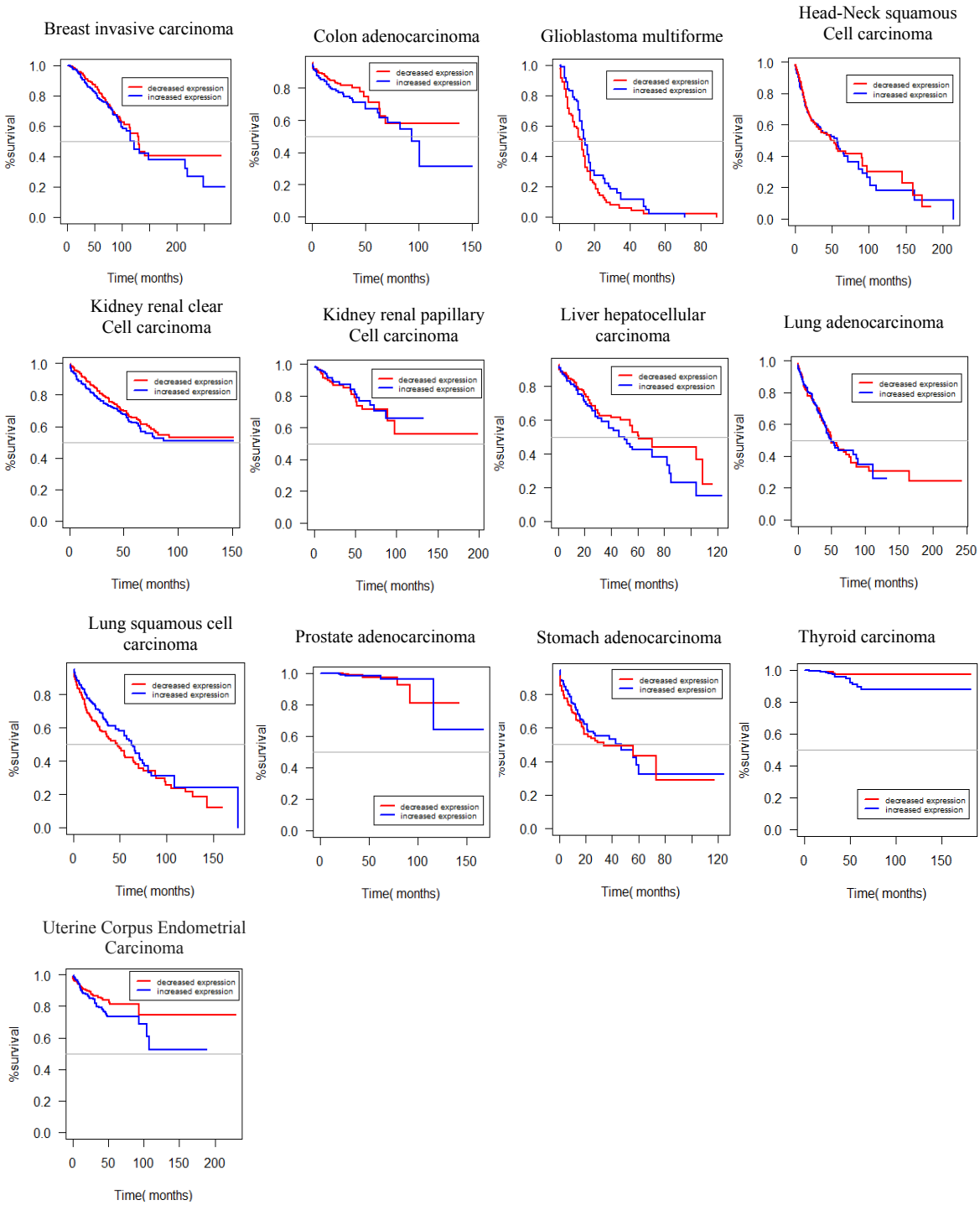

Fig. S9. Survival analysis of HIF-1α in different types of cancer. High expression levels of HIF-1α correlated with better survival in patients with glioblastoma multiforme, kidney papillary cell carcinoma, lung squamous cell carcinoma, and stomach adenocarcinoma cancers. In other types of cancer, the lower expression level of HIF-1α was associated with better survival. HIF, Hypoxia-inducible factors.

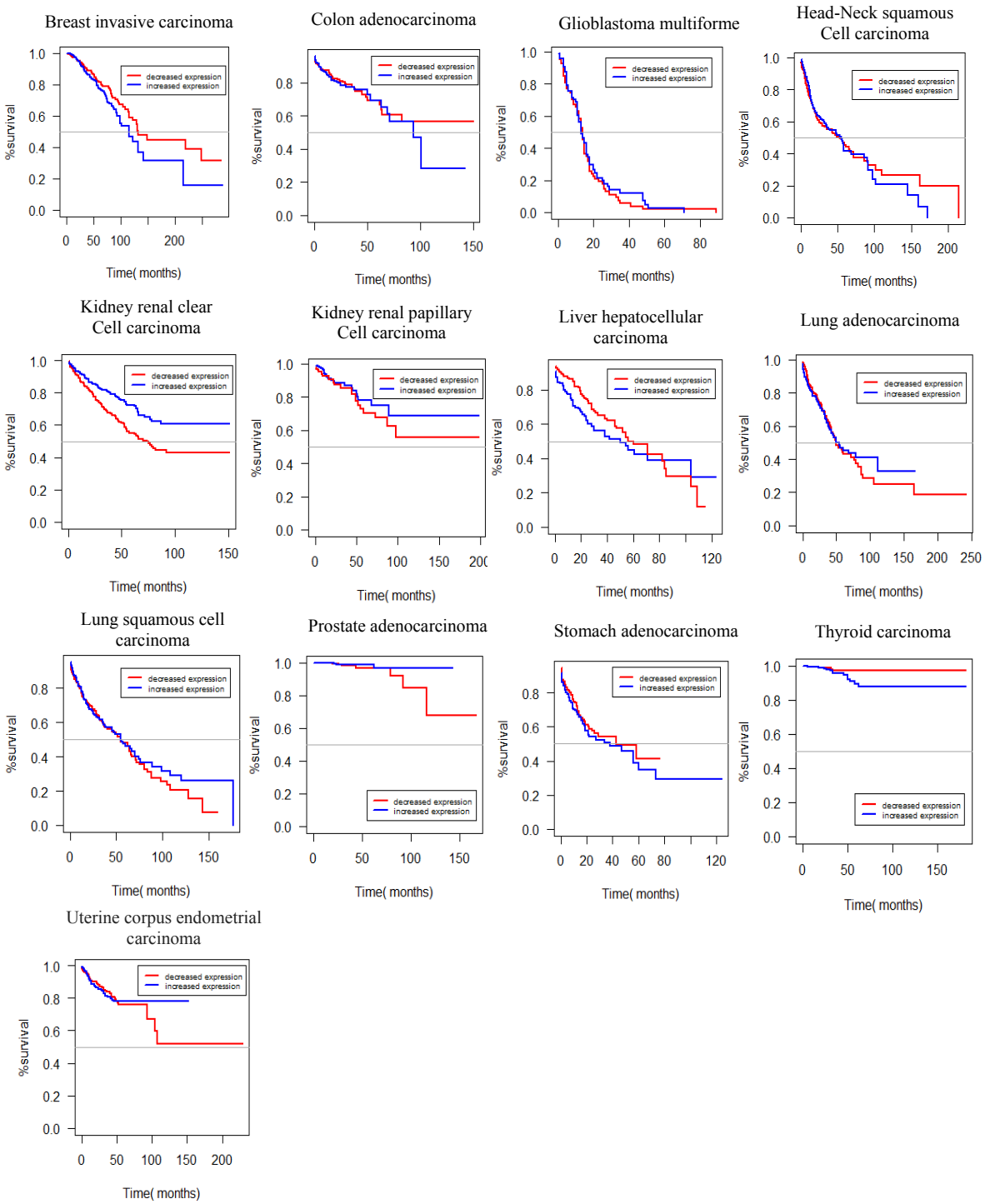

Fig. S10. Survival analysis of HIF-2α in different types of cancer. The higher expression level of HIF-2α correlated with better survival in patients with kidney renal cell clear carcinoma and kidney renal papillary cell carcinoma, but in most other types of cancer, a lower expression level of HIF-2α was associated with better survival. HIF, Hypoxia-inducible factors.