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Characterization of Hypoxia-associated Molecular Features to Aid Hypoxia-Targeted Therapy

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Abstract

Tumor hypoxia is a major contributor to resistance to anti-cancer therapies. Given that the results of hypoxia-targeted therapy trials have been disappointing, a more personalized approach may be needed. Here we characterize multi-OMIC molecular features associated with tumor hypoxia and identify molecular alterations that correlate with both drug-resistant and drug-sensitive responses to anti-cancer drugs. Based on a well-established hypoxia gene expression signature, we classify about 10,000 tumor samples into hypoxia score-high and score-low groups across different cancer types from The Cancer Genome Atlas and demonstrate their prognostic associations. We then identify various types of molecular features associated with hypoxia status that correlate with drug resistance but, in some cases, also with drug sensitivity, contrasting the conventional view that hypoxia confers drug resistance. We further show that 110 out of 121 (90.9%) clinically actionable genes can be affected by hypoxia status and experimentally validate the predicted effects of hypoxia on the response to several drugs in cultured cells. Our study provides a comprehensive molecular-level understanding of tumor hypoxia and may have practical implications for clinical cancer therapy.

Introduction

Hypoxia is a condition characterized by limited oxygen supply, which is a feature of most tumors and has been correlated with advanced tumor progression, treatment resistance and poor clinical outcome^{1,2}. Tumor hypoxia is linked to many cancer “hallmarks”, including impaired immune responses, metabolic reprogramming, increased cancer stem cells, stimulation of tumor vascularization, promotion of tumor invasion and metastasis, increased genomic instability, facilitation of apoptosis, and reduced cell proliferation³. Nevertheless, it is still difficult to define hypoxia status in tumors due to variations in oxygen levels among different tissues. Researchers have employed different methods to diagnose tumor hypoxia, including direct methods (e.g., oxygen electrode and phosphorescence quenching), physiologic methods (e.g., photoacoustic tomography and near-infrared spectroscopy/tomography), and/or endogenous markers of hypoxia (e.g., hypoxia-inducible factor (HIF)-1 α and glucose transporter 1)², but none of these methods can be easily applied to large numbers of patient samples. Therefore, several recent studies have identified gene expression signatures that reflect hypoxia status⁴⁻⁶. Among them, a 15-gene signature appears to perform the best^{5,7}.

The tumor hypoxia microenvironment is associated with multiple layers of molecular alterations, from genomics and epigenomics to transcriptomics and proteomics. Hypoxia drives transient site-specific copy alterations⁸ and increases the mutation frequency of key cancer genes⁹. Hypoxia induces the hypermethylation of promoter regions for several tumor suppressor genes, such as PTEN and APC, and thus leads to low expression of these tumor suppressors⁷. Furthermore, hypoxia dysregulates genes in cancer-related pathways such as the glycolytic pathway and PI3K/AKT/mTOR pathway¹⁰, as well as pro-angiogenic factors¹¹ and oncogenic growth factors¹². In addition, in response to hypoxia, miRNAs linked to multiple key signaling pathways are altered, such as miR-210¹³. Hypoxia also profoundly impacts protein synthesis and phosphorylation, such as the activation of PERK and phosphorylation of eIF2 α ¹⁴. Taken together, previous studies have established that hypoxia can lead to multiple layers of molecular changes and thus plays a pivotal role in cancer development.

Hypoxia is a major contributor to resistance to anti-cancer therapies, including chemotherapy, radiation therapy, targeted therapy and immunotherapy, thereby making hypoxia-targeted therapy attractive¹⁵. Severe hypoxia can induce resistance to chemotherapy in cervical tumors¹⁶, and blocking HIF activity in breast cancer can increase the chemotherapy treatment effect¹⁷. Hypoxic tumors have not responded well to radiation therapy in head and neck cancer¹⁸. Hypoxia can also cause resistance to gefitinib in both EGFR mutant and wild-type non-small-cell lung cancer (NSCLC)¹⁹. Metformin-induced reduction of tumor hypoxia can potentiate the efficacy of immunotherapy with the PD-1 checkpoint inhibitor²⁰. Thus, combination treatment of hypoxia-targeted therapy with other anti-cancer therapies would improve treatment effects. Indeed, combination of the HIF-1 α inhibitor with a molecular-targeted agent (e.g., a p-Stat3 inhibitor: T40214)²¹ or with a chemotherapeutic agent (e.g., cisplatin)²² has demonstrated greater clinical efficacy than either therapy alone. Strikingly, changes in hypoxic cells can also result in sensitivity to specific therapies¹⁵. For example, some tumors appear to be more sensitive to PARP inhibitors, veliparib and olaparib, under hypoxic conditions^{23,24}. Patients with kidney cancers that had high levels of HIF1 α or HIF2 α responded better to sunitinib²⁵. These studies suggest that the contribution of hypoxia status in cancer treatment is complex. Unfortunately, the results of hypoxia-targeted therapy trials, which have included evofosfamide and tarloxotinib bromide in lung cancer²⁶, PX-12 in pancreatic cancer²⁷, and tirapazamine and nitroglycerin in lung cancer^{28,29}, have been disappointing. There is still a lack of predictive therapeutic biomarkers to make hypoxia-targeted therapy part of standard treatments²⁶. The availability of genomic, epigenomic, transcriptomic and proteomic profiles across a broad range of cancer types from The Cancer Genome Atlas (TCGA) project^{30,31} provides an unprecedented opportunity to explore hypoxia-associated molecular signatures in great depth.

Results

Rigorous classification of hypoxia status by an established gene expression signature

To classify the hypoxia status of tumor samples, we focused on a 15-gene expression signature^{6,7,32} that was shown to perform the best in a recent comprehensive study assessing

the robustness of different hypoxia signatures⁵. We performed multiple analyses to validate the performance and assess the robustness of this hypoxia signature. First, we collected 10 independent gene expression datasets of cancer cell lines and tumor fragments of multiple cancer types under hypoxic and normoxic conditions (Supplementary Table 1). We calculated a hypoxia score for each sample based on this 15-gene signature. Indeed, in all cases, cells under the hypoxic conditions show significantly higher hypoxia scores than those under the normoxic conditions (Fig. 1a–k). Second, the score for this signature highly correlates with the hypoxia scores based on the other two hypoxia signatures (Winter signature³³ and Hu signature³⁴) (Fig. 1l). The observed consistency suggests that the 15-gene signature is robust and that different classifiers would lead to similar groups. These results demonstrate the robustness of the 15-gene signature to define hypoxia status in different cancer types.

To classify the hypoxia status of tumor samples, we analyzed 24 TCGA cancer types with a sample size ≥ 100 ³⁰. We further excluded kidney renal clear cell carcinoma (KIRC) and colon adenocarcinoma (COAD) samples with relatively high mutation frequency in VHL (5%), which directly regulates HIF1A to induce pseudohypoxia³⁵. In each cancer type, we classified samples into hypoxia score-high, hypoxia score-low, and hypoxia score-intermediate groups based on the unsupervised clustering pattern of the 15 genes (Fig. 2a; Supplementary Fig. 1; see Methods). In the 21 cancer types surveyed, both hypoxia score-high and hypoxia score-low groups contained ≥ 30 samples, and we focused on these cancer types for the subsequent analysis. Indeed, the two sample groups showed distinct hypoxia score distributions (Supplementary Fig. 2a). To validate our mRNA-based sample classification, we further performed the analysis using independent proteomic data over the same TCGA sample sets. Using mass spectrum data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC)^{36,37}, we assessed the same hypoxia signature at the protein levels and found that the signature was indeed enriched in hypoxia score-high groups for both BRCA (normalized enrichment score [NES] = 1.92, false discovery rate [FDR] < 0.001) and ovarian serous cystadenocarcinoma (OV; NES = 2.15, FDR < 0.001) (Supplementary Fig. 2b). This is likely due to high correlations between mRNA expression and protein expression levels. Taken together, our analyses based on mRNA expression and protein expression data support the validation of our classification of hypoxia status across different cancer types.

The proportions of different hypoxia groups greatly vary among different cancer types (Fig. 2a). For example, in kidney renal papillary cell carcinoma (KIRP), 10.3% (30/290) are hypoxia score-high samples versus 50.7% (147/290) hypoxia score-low samples, while in thymoma (THYM), 48.3% (58/120) are hypoxia score-high samples versus 27.5% (33/120) hypoxia score-low samples (Fig. 2a; Supplementary Table 2). These results suggest that patients with different cancer types may have distinct response rates to hypoxia-targeted therapy. For example, for certain cancer types such as THYM, head and neck squamous cell carcinoma (HNSC), and lung squamous cell carcinoma (LUSC), larger proportions of patients are more likely to benefit from hypoxia-targeted therapy. In contrast, only a small proportion of patients with KIRP and prostate adenocarcinoma (PRAD) may benefit from hypoxia-targeted therapy as the majority of those tumors are classified as hypoxia score-low.

To assess the relevance of our sample classification in a clinical context, we examined the correlations of our hypoxia status classification with patients' overall survival times. We observed that the hypoxia score-high tumors were consistently associated with worse prognosis across cancer types in univariate or multivariate survival analysis by Cox proportional hazards model (Fig. 2b), such as HNSC (log-rank test, $p = 2.9 \times 10^{-4}$), and lung adenocarcinoma (LUAD; log-rank test, $p = 5.1 \times 10^{-4}$; Fig. 2c). These results suggest the potential prognostic power of our hypoxia status classification.

Global patterns of multidimensional hypoxia-associated molecular signatures across cancer types

To identify the molecular signatures associated with hypoxia status in cancer, we employed a propensity score algorithm³⁸ to reduce potential confounding effects (Supplementary Fig. 3; Supplementary Table 3) (e.g., gender, age at diagnosis, tumor purity, race and tumor stage^{39,40}; see Methods). As outlined in Fig. 3a, we compared molecular features between the hypoxia score-high and hypoxia score-low groups with balanced confounding factors. Molecular features of consideration included mRNA expression (~20,000 genes), protein expression (~200 proteins), miRNA expression (~2,000 miRNAs), DNA methylation (~16,000 protein-coding genes), highly mutated genes (genes with >5% mutation frequency in each cancer type) and significant somatic copy number alterations (SCNAs) identified by GISTIC²⁴¹. We identified significantly differential features of these six types between the two hypoxia status groups (Fig. 3b; Supplementary Data 1; FDR < 0.05; see Methods).

The effects of hypoxia status on the molecular data in different cancer types varied significantly. For example, alterations of mRNA expressions ranged from 399 genes in OV to 4,795 genes in testicular germ cell tumors (TGCT; Fig. 3b). Alterations of miRNA expression ranged from two in skin cutaneous melanoma (SKCM) to 213 in THYM. Stomach adenocarcinoma (STAD) showed the largest number of alterations at the protein level, while THYM showed the largest number of alterations in DNA methylation, and BRCA showed the largest number of SCNAs. The total number of hypoxia-associated features across multiple layers also varied. For example, STAD had many hypoxia-associated features in six molecular layers, including 4,169 mRNAs, 186 miRNAs, 91 proteins, 294 methylation probes, one gene mutation and 10 SCNAs, while glioblastoma multiforme (GBM) had hypoxia-associated features in 629 mRNAs and five proteins. Furthermore, previous studies demonstrated the effects of hypoxia status on metabolomics⁴². Based on 399 metabolites from 23 TCGA BRCA samples⁴³, we observed that 86 metabolites positively correlated with hypoxia score ($R_s > 0.3$, $p < 0.05$; Supplementary Fig. 4a). We further identified 45 metabolites that were upregulated in 7 hypoxia score-high samples compared to 6 hypoxia score-low samples (two-sided t-test $p < 0.05$; Supplementary Fig. 4b). These results provide an overview of molecular differences associated with hypoxia status across tumor lineages.

To assess the potential effects of hypoxia-associated features on drug response, we focused on 1060 genes with at least one type of hypoxia-associated molecular signature in at least nine cancer types. We calculated Spearman's correlations between the expression of these genes and drug sensitivity for 252 anti-cancer drugs from the Genomics of Drug Sensitivity

in Cancer (GDSC)⁴⁴ across 1,074 cancer cell lines. These anti-cancer drugs target multiple biological processes, including the chromatin signature, cell cycle, metabolism, EGFR signaling and RTK signaling pathways. We identified 143 hypoxia-associated genes that significantly correlated to sensitivity of at least three anti-cancer drugs ($|Rs| \geq 0.3$, FDR < 0.05; Fig. 3c; Supplementary Fig. 5; Supplementary Data 2). For example, the protein level of YAP1 is upregulated in hypoxia score-high samples in nine cancer types; and its mRNA expression is linked to drug resistance to 49 anti-cancer drugs (e.g., navitoclax, $Rs = 0.52$, FDR < 1.0×10^{-55}) and linked to drug sensitivity to five anti-cancer drugs in (e.g., docetaxel, $Rs = -0.42$, FDR = 1.7×10^{-35}). Dysregulation of the RTK signaling pathway is an established feature in multiple cancer types, and RTK signaling can be stimulated by hypoxia⁴⁵. In our analysis, 19 drugs that targeted genes involved in the RTK signaling pathway highly correlated to hypoxia-associated genes (Supplementary Fig. 5a). Taken together, our results across cancer cell lines show extensive interactions between hypoxia-associated molecular features and drug response, highlighting the potential of combining anti-hypoxia drugs with other cancer therapies.

Hypoxia effects on mRNA, microRNA, protein expression, and DNA methylation

We observed significant alterations of mRNA expression across different cancer types. For example, several genes involved in invasion and metastasis were significantly biased in hypoxia score-high samples in TGCT, including L1CAM (fold change [FC] = 4.5, FDR = 8.4×10^{-28}), LOXL4 (FC = 2.2, FDR = 1.6×10^{-5}), LOX (FC = 2.3, FDR = 1.5×10^{-7}), and MET (FC = 2.6, FDR = 1.6×10^{-9}). Genes involved in glycolysis, the p53 pathway, apoptosis, epithelial mesenchymal transition (EMT) and angiogenesis were more likely to be upregulated in hypoxia score-high samples across cancer types (Supplementary Fig. 6a). These hypoxia-associated mRNA expression patterns are partially due to alterations of DNA methylation (Supplementary Fig. 6b). Indeed, the hypoxia-biased mRNA expression level of a gene tended to be the opposite of its DNA methylation level (Supplementary Fig. 6c), which is consistent with the view that hypermethylation generally leads to gene silencing, while hypomethylation results in overexpression⁴⁶.

To further investigate the effects of hypoxia on miRNA expression, we identified miRNAs that were differentially expressed between hypoxia score-high and hypoxia score-low samples. The hypoxia-induced miRNA, miR-210-3p¹³, was upregulated in hypoxia score-high tumors in 16 cancer types (Fig. 4a; Supplementary Fig. 6d). In contrast, hypoxia-inhibited miR-139-3p⁴⁷ was downregulated in hypoxia score-high samples in four cancer types. Several target genes of miR-139-3p were upregulated, including ANGPT1, which is associated with promotion of vascularization; CTSC, MET and PLUAR, which are associated with invasion and metastasis; PKM2, which is related to metabolic reprogramming; and SOX2 and WWTR1, which maintain cancer cell stemness. We further examined the targeted genes of 87 miRNAs that were also altered in at least three cancer types. We identified 3,793 miRNA targeted genes with significantly opposing alterations. Genes targeted by these miRNAs are significantly enriched in cancer-related pathways, including PI3K/AKT signaling, Hippo signaling, Ras signaling, p53 signaling, EGFR signaling, and HIF-1 signaling pathways (Fig. 4a). For example, miR-455-3p, miR-205-5p and 11 other miRNAs were significantly upregulated. Their target tumor suppressor gene,

TP53INP1, was significantly downregulated in hypoxia score-high samples in esophageal carcinoma (ESCA), pancreatic adenocarcinoma (PAAD), STAD and TGCT. In contrast, miRNA-30a-5p/miRNA-30a-3p was significantly downregulated, and the target gene MET, a marker for invasion and metastasis and the therapeutic target of crizotinib in lung cancer⁴⁸, was upregulated in hypoxia score-high samples in GBM, low-grade glioma (LGG) and TGCT. Furthermore, hypoxia-associated miRNAs were highly associated with drug response in patients represented in TCGA data (Supplementary Fig. 6d, upper panel). For example, the expression of hypoxia score-high biased miR-210-3p highly correlated to the response to at least one drug in 13 cancer types (Supplementary Fig. 6e). In liver hepatocellular carcinoma (LIHC), this miR-210-3p negatively correlated (drug-sensitive) to the response to KU-55933 ($R_s = -0.44$, $FDR = 4.2 \times 10^{-5}$; Supplementary Fig. 6f), an inhibitor of ATM in the genome integrity pathway, while the expression of miR-210-3p positively correlated (drug-resistant) to vinorelbine ($R_s = 0.48$, $FDR = 1.9 \times 10^{-6}$; Supplementary Fig. 6f), a microtubule destabilizer.

Using functional proteomic data of reverse-phase protein arrays that cover key cancer-related total and phosphorylated proteins, we identified hypoxia-associated protein alterations in cancer signaling pathways (Fig. 4b). For example, fibronectin, which positively regulates EMT⁴⁹, was significantly upregulated in hypoxia score-high samples in seven cancer types. PTEN, which negatively regulates the PI3K/AKT signaling pathway⁵⁰, was significantly downregulated in hypoxia score-high samples in five cancer types. We observed that the anti-apoptosis protein, BCL-2, was significantly downregulated in hypoxia score-high samples in seven cancer types, while the pro-apoptosis protein, caspase-7, was significantly upregulated in four cancer types. Furthermore, hypoxia-associated proteins correlated to the response to anti-cancer drugs (Fig. 4b). PTEN negatively correlated (drug-sensitive) to the response to dasatinib (LGG, $R_s = -0.47$, $FDR = 5.8 \times 10^{-5}$), which targets Src and many other kinases, but positively correlated (drug-resistant) to pictilisib (LGG, $R_s = 0.42$, $FDR = 1.6 \times 10^{-3}$), a PI3K inhibitor (Supplementary Fig. 6g).

Integrative analysis of hypoxia-associated molecular features on drug response

To further understand the effects of the hypoxia microenvironment on drug response, we performed an integrative analysis to assess the associations between multidimensional hypoxia-associated molecular features and response to anti-cancer drugs in TCGA patients (see Methods). Taking ESCA as an example, we identified 61 genes that were overexpressed in hypoxia score-high samples. These genes are hypomethylated and regulated by nine miRNAs. The expression levels of these genes negatively correlated (drug-sensitive) to the response to anti-cancer drugs, while their DNA methylation level and targeting miRNAs positively correlated (drug-resistant) to the same anti-cancer drugs. These drugs target important pathways, including PI3K and ERK/MAPK signaling pathways (Fig. 5a). For example, EGFR was upregulated in hypoxia score-high tumors ($FC = 2.65$, $FDR = 3.2 \times 10^{-5}$; Fig. 5b) in ESCA, and EGFR expression negatively correlated (drug-sensitive) to the response to the RTK signaling pathway inhibitor crizotinib ($R_s = -0.28$, $FDR = 0.02$), FNTA inhibitor FTI-277 ($R_s = -0.29$, $FDR = 0.025$), AKT inhibitor A-443654 ($R_s = -0.26$, $FDR = 0.015$), BRAF inhibitor PLX4720 ($R_s = -0.31$, $FDR = 2.0 \times 10^{-3}$) and microtubule destabilizer (vinorelbine: $R_s = -0.30$, $FDR = 7.2 \times 10^{-3}$; vinblastine: $R_s = -0.32$, $FDR = 1.4$

$\times 10^{-3}$). EGFR showed hypomethylation in the promoter region (difference = -0.24 , FDR = 4.5×10^{-8}), and the methylation level of EGFR positively correlated (drug-resistant) to the response to A-443654 ($R_s = 0.34$, FDR = 5.5×10^{-4}), PLX4720 ($R_s = 0.47$, FDR = 5.3×10^{-7}), vinblastine ($R_s = 0.38$, FDR = 5.2×10^{-5}) and vinorelbine ($R_s = 0.32$, FDR = 2.3×10^{-3}). Meanwhile, four miRNAs that target EGFR showed significant downregulation in hypoxia score-high tumors (miR-375: FC = -13.5 , FDR = 4.4×10^{-9} ; miR-153-5p: FC = -2.03 , FDR = 5.2×10^{-4} ; miR-200a-3p: FC = -2.4 , FDR = 4.6×10^{-3} ; miR-146a-5p: FC = -2.5 , FDR = 9.8×10^{-7}). These miRNAs positively correlated (drug-resistant) to the corresponding drugs that have negative correlations with EGFR expression. For example, miR-375 positively correlated (drug-resistant) to A-443654 ($R_s = 0.37$, FDR = 9.0×10^{-4}), PLX4720 ($R_s = 0.39$, FDR = 9.4×10^{-5}) and vinblastine ($R_s = 0.35$, FDR = 8.5×10^{-4}), and miR-200a-3p positively correlated (drug-resistant) to A-443654 ($R_s = 0.35$, FDR = 1.3×10^{-4}), PLX4720 ($R_s = 0.29$, FDR = 6.1×10^{-3}) and vinblastine ($R_s = 0.44$, FDR = 1.2×10^{-5}). For 29 genes overexpressed in hypoxia score-low samples, we also observed a similar regulatory network in that the downregulation of gene expression was consistent with hypermethylation of promoter regions and upregulation of miRNAs, and highly correlated to response to anti-cancer drugs (Supplementary Fig. 7a). Furthermore, we observed significant correlations between protein expression and drug response, which is consistent with the correlations between mRNA expression and drug response (Supplementary Fig. 7b). Taken together, our results suggest that regulatory networks are affected in complicated ways by the hypoxia microenvironment at multiple layers, and these regulatory networks may largely affect drug response.

Hypoxia-associated somatic mutations and copy number alterations

To reveal the global pattern of hypoxia-associated mutations, we examined mutation frequency across multiple cancer types and identified hypoxia-associated mutated genes ranging from one in THYM to 14 in LUAD (Fig. 6a; Supplementary Fig. 8a). For example, TP53 had significantly higher mutation frequency in hypoxia score-high tumors across multiple cancer types: 62.3% (137/220) and 73.5% (83/113) of samples had TP53 mutations in hypoxia score-high BCRA and LUAD, while only 8.0% (19/236) and 34.6% (44/127) of samples had TP53 mutations in hypoxia score-low BCRA and LUAD, respectively (Fig. 6b). This is consistent with previous reports that mutations of p53 contribute to diminished oxygen consumption⁵¹. We further observed that TP53 mutations were associated with reduced drug response to the BRAF inhibitor, AZ-628 (FDR = 5.0×10^{-4} ; Fig. 6c), and increased response to the HIF-PH inhibitor dimethyloxalylglycine (FDR = 8.9×10^{-3} ; Fig. 6c). In contrast, IDH1 has 95.5% (63/66) mutation frequency in hypoxia score-low samples, while it only has 38.1% (32/84) in hypoxia score-high samples in LGG. Mutation of IDH1 promotes the degradation of HIF1 α ⁵², thus reducing the hypoxic effects in tumors and leading to better patient survival times (log-rank test, $p = 2.6 \times 10^{-13}$, Supplementary Fig. 8b).

Hypoxia can induce transient site-specific copy number gains in tumor cells⁸. We comprehensively analyzed hypoxia-associated SCNAs across different cancer types and identified significantly hypoxia-associated SCNAs in 13 cancer types, ranging from two in THYM to 29 in BCRA (Fig. 3; Fig. 6d; Supplementary Data 1). Notably, these hypoxia-

associated SCNAs harbor a number of clinically actionable genes (Fig. 6d). In LGG, the 7q32.3 amplicon, which harbors BRAF and MET and leads to gefitinib resistance⁵³, occurred more frequently in hypoxia score-high samples (FDR = 0.045, Fig. 6d). The 7p11.2 amplicon, which harbors EGFR, occurred more frequently in hypoxia score-high LGG samples (FDR = 6.5×10^{-5} , Fig. 6e). In particular, we observed that 7p11.2-amplified LGG tumors were more sensitive to erlotinib (two-sided t-test, FDR = 3.1×10^{-4}), an anti-EGFR drug (Fig. 6e), which is consistent with a previous study⁵⁴. Deletion of 2q37.3 (FDR = 4.2×10^{-3}) occurred more frequently in hypoxia score-high samples. This region harbors PDCD1, which is the target of pembrolizumab and nivolumab for cancer immunotherapy⁵⁵. In addition, some other SCNAs occur more frequently in hypoxia score-low samples. For example, 7q31.2 amplification, which harbors MET, occurred more frequently in hypoxia score-low KIRP (FDR = 5.3×10^{-3}). Deletion of 9p23, which harbors CDKN2A and CDKN2B, occurred more frequently in hypoxia score-low BRCA (FDR = 0.014). These results suggest that the hypoxia microenvironment could affect tumor response to drugs, including immunotherapy drugs, by altering the somatic copy numbers.

Hypoxia-associated molecular signatures in clinically actionable genes and their therapeutic liability

To characterize clinically applicable therapeutic implications of hypoxia-associated molecular signatures, we examined the molecular alterations between hypoxia score-high and hypoxia score-low samples across five molecular dimensions of 121 clinically actionable genes targeted by 89 FDA-approved drugs⁵⁶ (Fig. 7a; Supplementary Fig. 9a). We identified hypoxia-associated features, ranging from six features in sarcoma (SARC) to 93 in TGCT (Supplementary Fig. 9b). For example, EGFR was biased in hypoxia score-high samples with overexpression of total proteins or phosphorylated proteins in eight cancer types (e.g., ESCA, diff = 0.54, FDR = 5.5×10^{-5} ; BLCA, diff = 0.42, FDR = 7.1×10^{-3}), amplification in two cancer types (STAD, FDR = 0.022; LGG, FDR = 6.5×10^{-5}) and hypomethylation in two cancer types (cervical squamous cell carcinoma and endocervical adenocarcinoma [CESC], diff = -0.29, FDR = 1.8×10^{-8} ; ESCA, diff = -0.24, FDR = 4.5×10^{-8}). Strikingly, 90.9% (110/121) of clinically actionable genes were associated with at least one type of hypoxia-associated molecular signature in at least one cancer type (Fig. 7a). Interestingly, several immunotherapeutic targets were also affected by hypoxia. PDCD1 (PD-1) was highly expressed in hypoxia score-low samples in LUSC, suggesting that PDCD1 inhibitors, such as nivolumab and pembrolizumab^{57,58}, could have better efficacy in hypoxia score-low tumors. We observed many more such incidences in hypoxia score-low samples (453) than in hypoxia score-high samples (237), which may partially explain why drugs are generally more effective in hypoxia score-low tumors. Patients with tumors having greater incidence of hypoxia score-high groups may benefit from combination treatment with hypoxia-targeted therapy.

We further evaluated the hypoxia effects on drug response in patient samples from imputed drug data⁵⁹ (Supplementary Data 3). Our comprehensive analysis of hypoxia effects on clinically actionable genes could link to hypoxia effects on drug response directly⁶⁰ (Fig. 7a). For 21 FDA-approved anti-cancer drugs available in the GDSC database, we showed alterations of drug responses across multiple cancer types. Furthermore, we observed that

the response to paclitaxel positively correlated (drug-resistant) to hypoxia status in CESC ($R_s = 0.40$, $FDR = 6.2 \times 10^{-4}$; Supplementary Fig. 10a,b), which is consistent with the resistance reported in a cervical cancer cell line⁶¹. We also observed that the response to AKT-inhibitor-VIII negatively correlated (drug-sensitive) to hypoxia status in LUAD ($R_s = -0.25$, $FDR = 0.02$; Supplementary Fig. 10a,b), which is consistent with the sensitivity reported for AKT-inhibitor-VIII in lung cancer cell lines⁶². These observations suggest that our analysis is reliable and provides meaningful clinical insights. Tumors under hypoxic conditions are resistant to many drugs, including erlotinib in LIHC ($R_s = 0.42$, $FDR = 1.5 \times 10^{-4}$) and lapatinib in KIRP ($R_s = 0.49$, $FDR = 7.1 \times 10^{-6}$), suggesting a potential clinical benefit of combining the cancer treatment with hypoxia-targeted therapy for patients with LIHC or KIRP. Strikingly, some tumors may become sensitive to several drugs under hypoxic conditions, such as thapsigargin in PAAD ($R_s = -0.66$, $FDR < 1.0 \times 10^{-55}$) and imatinib in HNSC ($R_s = -0.31$, $FDR = 4.3 \times 10^{-4}$), which suggests that patients with these cancers may not benefit from hypoxia-targeted therapy.

To directly validate our findings on drug response, we performed drug sensitivity experiments on selected drugs under a hypoxic condition (1% O_2) and a normoxic condition (21% O_2) in two lung cancer cell lines (A549 and H1299). Consistent with our computational prediction, our experimental results using drugs camptothecin and bexarotene showed greater drug resistance under the hypoxic condition, while using drugs AKT-inhibitor-VIII and PHA-665752 showed greater sensitivity under the hypoxic condition for both A549 and H1299 cell lines (Fig. 7b; Supplementary Fig. 10c). Furthermore, we observed that patients with advanced NSCLC with high hypoxia scores were associated with worse prognosis after sorafenib treatment (log-rank test, $P = 8.6 \times 10^{-3}$; Supplementary Fig. 10d) in a clinical trial^{63,64} (sorafenib in patients with NSCLC). Taken together, these results show that the hypoxia microenvironment largely affects tumor response to anti-cancer drugs, and that the tumor hypoxia microenvironment should be considered to improve the efficacy of cancer therapy.

Discussion

Hypoxia induces a series of biological changes that contribute to tumorigenesis and are associated with resistance to chemotherapy, radiation therapy, drug therapy and immunotherapy. Therefore, understanding the effect of hypoxia on molecular signatures is crucial to improving outcomes to cancer therapy, and the degree of tumor hypoxia varies across cancer types. Here, we first demonstrated the robustness of a 15-gene signature to define the hypoxia status across multiple cancer types. Considering that the hypoxia gene signature is a relative signature, we then classified tumor samples into hypoxia score-high, hypoxia score-low and hypoxia score-intermediate groups based on this signature in each cancer type. Focusing on the comparison between hypoxia score-high and hypoxia score-low groups in each cancer type, we utilized a well-controlled statistical approach, the propensity score algorithm, to control potential confounders, including gender, race, age at diagnosis, smoking status, tumor stage, histological type and tumor purity. In this way, we identified hypoxia-biased molecular signatures that are largely independent from the potential confounders across 21 cancer types. Our study provides a comprehensive view of hypoxia-associated molecular signatures, including mRNA, miRNA and protein expression,

DNA methylation, somatic mutations, and SCNAs. These molecular alterations that are driven by the hypoxia microenvironment will likely impact a broad range of biological processes, including metabolic reprogramming, angiogenesis, apoptosis, and multiple signaling pathways. Our integrative analysis further suggests that the hypoxia microenvironment can impact tumor molecular signatures at multi-omic levels through gene regulatory networks.

One striking observation is that 110 out of 121 (90.9%) clinically actionable genes are biased in at least one layer of molecular signatures across multiple cancer types. These clinically actionable genes are targets of FDA-approved cancer drugs, including drugs for immunotherapy, chemotherapy, hormone therapy and targeted therapy. Our comprehensive analysis demonstrates that many clinically actionable genes are biased toward hypoxia score-high samples and confirms that hypoxia-targeted therapy is an attractive cancer therapy, likely as a component of combination therapy targeting clinically actionable genes. Unfortunately, the results from several trials of hypoxia-targeted therapy have been disappointing^{17,27–29}. This is likely because of our limited understanding of how molecular signatures are affected by the hypoxia microenvironment and our lack of rational combination therapies. Most clinical trials focus on overcoming the drug-resistant effects of hypoxia, while hypoxia may also result in increasing drug sensitivity in some patients¹⁵. These patients may not receive clinical benefit from hypoxia-targeted therapy and/or combination treatments. Our systematic classification of hypoxia status and identification of hypoxia-biased signatures thus have crucial clinical implications; this analysis can help to evaluate the clinical benefit of hypoxia-targeted therapy.

There are several limitations in our study. First, large-scale tumor samples with multiple omic datasets (e.g., TCGA) generally do not provide direct values for hypoxia status, e.g., O₂ levels. Therefore, we have to indirectly infer the relative hypoxia status through the hypoxia gene signature in each cancer type, as described in previous studies^{6,7,32}. We have validated the performance of this hypoxia gene signature using independent datasets in which the hypoxia status is known. Second, large-scale datasets often provide the bulk of information across different cell types within a sample. With advancements in single-cell profiling technology, future efforts should take tumor heterogeneity into consideration. Third, our analyses provide a comprehensive catalog of molecular alterations associated with hypoxia. Despite the causal effects demonstrated by many previous studies and a few cases within our experiments, further efforts are necessary to identify which alterations are directly affected by hypoxia. Finally, most clinical trials do not have the information of the hypoxia status of patients' tumors, so we have limited data to further validate our observations in more rigorous clinical settings. Our study calls attention to the need to include tumor hypoxia status in future clinical studies.

Methods

Multi-omic data and clinical data for TCGA samples

Molecular data, including mRNA expression, miRNA expression, protein expression, DNA methylation, somatic mutations, somatic copy number alterations, and clinical data, including tumor stage, histology subtype, gender and overall survival times, across 33 cancer

types were downloaded from the TCGA data portal (<https://tcga-data.nci.nih.gov/tcga/>). One gene may have multiple methylation probes, and we selected the probe that most negatively correlated with the expression of the corresponding gene³⁹. We downloaded tumor purity data from Tumor IMmune Estimation Resource⁶⁵ (<http://cistrome.org/TIMER/download.html>), and if not available, we obtained tumor purity data from a previous study⁶⁶ (<http://doi.org/10.5281/zenodo.253193>) to complement our data. Normalized metabolite levels of the 23 TCGA breast cancer samples were downloaded from a previous study⁴³. We identified different metabolites using a two-sided t-test p-value < 0.05.

Classification of hypoxia status across different cancer types

We selected a 15-gene expression signature (ACOT7, ADM, ALDOA, CDKN3, ENO1, LDHA, MIF, MRPS17, NDRG1, P4HA1, PGAM1, SLC2A1, TPI1, TUBB6 and VEGFA)⁶ that has been shown to perform the best when classifying hypoxia status. This gene signature was defined based on gene function and analysis of *in vivo* co-expression patterns, and was highly enriched for hypoxia-regulated pathways⁶. The hypoxia score for each tumor sample or cancer cell line was calculated by using gene set variation analysis (GSVA)⁶⁷ based on 15 mRNA-based hypoxia signatures. The student t-test was used to assess the statistical difference between hypoxic and normoxic conditions in different cancer cell lines. Spearman's correlation was used to assess the correlation among hypoxia scores based on different gene signatures. We kept 24 cancer types with sample size > 100, and filtered KIRC and COAD samples with relatively high mutation frequency in VHL (> 5%) to avoid effects of pseudohypoxia in the tumors. To classify the hypoxia status, we employed unsupervised hierarchical clustering⁷ to cluster samples in each cancer type based on the 15 mRNA-based hypoxia signatures. The top three sub-clusters were assigned as hypoxia score-high, -intermediate and -low groups in each cancer type. We included 21 cancer types with > 30 samples in both hypoxia score-high and hypoxia score-low groups for further analysis (Supplementary Fig. 2; Supplementary Table 2). To avoid the confounding factors from the potential mixture, we excluded samples from the hypoxia score-intermediate group for further analysis.

Identification of alterations between hypoxia score-high and hypoxia score-low tumors

To balance the potentially confounding factors (such as tumor stage, histology subtype, and the other factors listed previously) between hypoxia score-high and hypoxia score-low groups, we performed the propensity score algorithm^{39,40}. In brief, we first calculated the propensity score using logistic regression with the hypoxia status as a variable, and performed matching weights³⁸ to reweight samples based on the propensity scores. We considered the clinical confounding factors to be balanced between weighted hypoxia score-high and hypoxia score-low samples if the standardized difference between their weighted propensity scores was less than 10%. We then compared the molecular data between these two balanced groups and calculated the *p*-values and FDRs. To eliminate random noise in signal detection, we employed permutation tests by randomly selecting hypoxia score-high or hypoxia score-low labels of patient samples and repeated the above steps 100 times. We calculated the ratio of the appearance of a significant feature set in these permuted datasets and retained the feature sets with permutation test *p*-value < 0.05 for further analysis. The statistical significance for each molecular data type in each cancer type is as follows. For

mRNA and miRNA expression: FC > 2, FDR < 0.05; total protein or phosphorylated protein: difference > 0.2, FDR < 0.05; DNA methylation: difference > 0.2, FDR < 0.05; somatic mutation and SCNA: FDR < 0.05.

Analysis of clinically actionable genes and drug response

We downloaded clinically actionable genes identified as targets of FDA-approved therapeutic drugs or biomarkers from a previous study⁵⁶ (<http://archive.broadinstitute.org/cancer/cga/target>). We collected the therapeutic drugs and their prescription information from <https://www.fda.gov/Drugs/InformationOnDrugs/60>. We retained clinically actionable genes with the corresponding drugs as therapeutic targets for further analysis. To assess drug response in cancer cell lines, we downloaded the drug sensitivity area under the dose-response curve (AUC) and gene expression profiles for cancer cell lines from Genomics of Drug Sensitivity in Cancer (<http://www.cancerrxgene.org/downloads>, GDSC)⁴⁴. We calculated Spearman's correlation between gene expression and the AUCs from GDSC⁶⁸ and used Spearman's correlation coefficient $|R_s| > 0.3$ and FDR < 0.05 as statistical significance.

To assess the drug response in TCGA patient samples, we downloaded the imputed tumor response to 138 anti-cancer drugs in cancer patients from a previous study⁵⁹. We calculated the correlation between imputed drug response and hypoxia-associated mRNA expression, miRNA expression, protein expression and DNA methylation using Spearman's correlation, considering $|R_s| > 0.2$ and FDR < 0.05 as statistical significance. To compare the imputed drug response between groups with or without mutations and SCNAs, we used the student t-test and considered FDR < 0.05 as statistical significance.

Cell culture and reagents

A549 and H1299 were purchased from the American Type Culture Collection (ATCC) and Characterized Cell Line Core Facility (MD Anderson Cancer Center) and were cultured in DMEM supplemented with 10% FBS (Gibco) at 37 °C in 5% CO₂ (v/v). AKT-inhibitor-VIII (612847-09-3) was purchased from Cayman Chemical; PHA-665752 (S1070), camptothecin (S1288) and bexarotene (S2098) were purchased from Selleckchem.

Cell proliferation assay

The effect of the drug on cell proliferation was determined using a CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Cells were plated in 96-well plates (4 replicates per condition). The following day, cells were treated with a range of drug concentrations prepared by serial dilution. Plates were incubated in normoxic conditions (37°C, 5% CO₂, 21% O₂) or in hypoxic conditions (37°C, 5% CO₂, 1% O₂). After 3 days of treatment, assays were performed by adding 20µl of the CellTiter 96® AQueous One Solution Reagent directly to the culture wells, incubating for 1 hour and then recording absorbance at 490nm with an Envision plate reader (Perkin Elmer). The relative growth was normalized to the untreated samples in each group. Drug response data analysis was performed using GraphPad Prism version 7.00 (GraphPad software).

Analysis of clinical trial data

We examined clinical trials with the data of related drugs and cancer types in our study from [ClinicalTrials.gov](https://clinicaltrials.gov/) (<https://clinicaltrials.gov/>). We were able to identify only one clinical trial (, NSCLC patients treated with sorafenib) that had both detailed clinical outcomes and mRNA expression data (NCBI GEO GSE33072). Patients who received sorafenib treatment were classified into two groups based on the hypoxia scores of their tumor samples. We used a log-rank test to assess the difference in the overall survival times between the two groups.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code Availability

Codes were implemented in R and deposited in GitHub: <https://github.com/youqiongye/HAMFA>.

Data Availability

All data supporting the findings of the current study are listed in Supplementary Tables 1–3 and Supplementary Data files 1–3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing interests

G.B.M. has sponsored research support from AstraZeneca, Critical Outcomes Technology, Karus, Illumina, Immunomet, Nanostring, Tarveda and Immunomet and is on the Scientific Advisory Board for AstraZeneca, Critical Outcomes Technology, ImmunoMet, Ionis, Nuevolution, Symphogen, and Tarveda. H.L. is a shareholder and scientific advisor of Precision Scientific and Eagle Nebula. J.G. serves as a consultant for ARMO Biosciences, AstraZeneca, Jounce, Nektar, and Pfizer.

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Fig. 1 |. Validation of a 15-gene expression signature for hypoxia status.

(a-j) Hypoxia score of cancer cell lines and tumor fragments under hypoxic and normoxic conditions in 10 datasets. The boxes show the median ± 1 quartile, with whiskers extending from the hinge to the smallest or largest value within 1.5 interquartile range from the box boundaries. One-sided student t-test was used to assess the difference. *: $p < 0.05$. Sample size for hypoxic (a: $n = 3$; b: $n = 4$; c: $n = 5$; d: $n = 5$; e: $n = 6$; f: $n = 3$; g: $n = 2$; h: $n = 3$; i: $n = 3$; j: $n = 10$) and normoxic (a: $n = 3$; b: $n = 4$; c: $n = 5$; d: $n = 2$; e: $n = 6$; f: $n = 3$; g: $n = 2$; h: $n = 3$; i: $n = 3$; j: $n = 10$) conditions. (k) Summarized differences of hypoxia scores under hypoxic and normoxic conditions across cancer cell line datasets from (a-j). (l) Spearman's

correlation of hypoxia scores between this 15-gene signature and the other two gene signatures across cancer types ($n = 9686$). The color intensity indicates Spearman's correlation coefficient R_s , and the point size indicates p value for Spearman's correlation.

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Fig. 2 |. Classification of hypoxia status across different cancer types.

(a) Percentage of samples with hypoxia score-high, -low and -intermediate classifications across multiple cancer types based on this 15-gene signature. (b) Association of hypoxia status with patients' overall survival times based on both univariate and multivariate Cox proportional hazards model in different cancer types (n = 3495). Size denotes statistical significance at a given false discovery rate (FDR); color denotes hazard ratio (HR). (c) Kaplan-Meier curves show hypoxia score-high status is associated with worse survival time in multiple cancer types. Two-sided log-rank test $p < 0.05$ is considered as a statistically significant difference.

Fig. 3 |. Overview of the propensity score algorithm and the hypoxia-associated molecular patterns across cancer types.

(a) Overview of the propensity score algorithm to balance clinical features. (b) Relative abundance and numbers of multidimensional hypoxia-associated molecular signatures identified by the propensity score algorithm. The percentage of significant features over total features for each molecular signature in each cancer type is displayed as a solid circle; the number of significant features for the corresponding molecular signature is displayed as a bar plot. (c) Association between mRNA expression levels of hypoxia-associated genes and drug sensitivity across 1,074 cancer cell lines by Spearman's correlation. Dark green dots

along the x-axis indicate hypoxia-related genes; orange dots denote drugs that are clustered by different signaling pathways. The size of the orange dot indicates the number of genes correlated to drug sensitivity ($|Rs| \geq 0.3$, FDR < 0.05); the bar plot shows the number of drugs correlated to genes. The pink and cyan lines indicate positive and negative correlation, respectively.

(a) Hypoxia-associated miRNA-regulated mRNA expression in multiple key signaling pathways. Number of cancer types with miRNA alterations in hypoxia score-high tumors (outer loop). “miR” is omitted from the name of miRNA. Genes are marked as magenta dots and organized in pathways (middle loop). Gold bar denotes enrichment of miRNA targeted genes in signaling pathways (inner loop), Fisher’s exact test $p < 0.05$ is considered as statistical significance. Gray lines link the miRNAs and their targeted genes. (b) Alterations of protein or protein phosphorylation in hypoxia score-high samples in at least three cancer types. Upper panel bars display the accumulated number of drugs positively (dark magenta) or negatively (dark green) correlated to hypoxia-associated proteins in different cancer types.

The correlation was performed by Spearman's correlation. Statistical analysis was performed using propensity score algorithm to identify hypoxia-associated miRNAs and proteins; details are in Methods.

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Fig. 5 |. Effects of multidimensional hypoxia-associated signatures on drug response.
(a) Interaction of hypoxia-associated mRNAs, DNA methylation, miRNAs and drug response. Upregulated genes (red bars in upper panel) in hypoxia score-high ESCA negatively correlate (green line, drug-sensitive) to response to 17 drugs. Downregulated miRNAs (blue bars in left panel), hypomethylation (blue bars in bottom panel) for corresponding upregulated genes positively correlate (magenta line, drug-resistant) to drug response. The target genes and targeted pathways of drugs are listed in the panel to the right. Gold line indicates miRNA-targeted mRNA, red line links the drug to its target and targeted pathway. (b) EGFR is upregulated in hypoxia score-high ESCA and negatively correlates to six drugs (green line, drug-sensitive). The hypomethylation of EGFR positively correlates to

four of six drugs (magenta line, drug-resistant). Downregulation of miR-375, miR-153-5p, miR-200a-3p and miR-146a-5p may upregulate EGFR, and positively correlates to four of the six drugs (magenta line, drug-resistant); $n = 21$ for hypoxia score-high ESCA samples, $n = 80$ for hypoxia score-low ESCA samples. Associations among hypoxia-associated mRNAs, DNA methylation, miRNAs and drug response were calculated by Spearman's correlation. The boxes in b show the median ± 1 quartile, with whiskers extending from the hinge to the smallest or largest value within 1.5 interquartile range from the box boundaries.

Fig. 6 | Hypoxia-associated somatic mutation and somatic copy number alteration (SCNA) signatures.

(a) Hypoxia-associated somatic mutations across seven cancer types. Results show significantly higher mutation frequency in hypoxia score-high samples (red), and lower mutation frequency in hypoxia score-low samples (blue). (b) TP53 mutation signature in patients with hypoxia score-high (upper) and hypoxia score-low (bottom) tumors in BRCA and LUAD in the lollipop plots. Percentages of samples with TP53 mutation in hypoxia score-high and hypoxia score-low samples are respectively summarized in parentheses. (c) Two-sided student test to compare imputed drug response to AZ-628 and dimethylxalylglycine in patients with HNSC with ($n = 46$) or without ($n = 138$) TP53 mutation. (d) Chromosome plot displays the distributions of hypoxia-associated SCNAs with significant alterations in the tumor. Red indicates hypoxia score-high associated SCNAs; blue indicates hypoxia score-low associated SCNAs. Different shapes of points indicate different cancer types. Position of point above or below the chromosome respectively indicates copy number gains or losses in different cancer types. (e) Two-sided student test to compare percentage of 7p11.2 amplification in hypoxia score-high and -low LGG samples (left). Imputed drug response to erlotinib in patients with LGG with ($n = 29$) or without ($n = 79$) 7p11.2 amplification (right). Note that lower imputed drug responses on the y-axis in

images c and e imply greater drug sensitivity. The boxes in c and e indicate the median $\pm$ 1 quartile, with whiskers extending from the hinge to the smallest or largest value within 1.5 interquartile range from the box boundaries.

Fig. 7 | Hypoxia-associated molecular signatures in clinically actionable genes and effects on the response to individual drugs.

(a) Association between FDA-approved drugs and their linked clinically actionable genes (right) and alterations of these genes at mRNA, protein, DNA methylation, mutation and SCNA levels based on hypoxia score-high (red) or hypoxia score-low (blue) samples across 21 cancer types (left). Different symbol shapes represent different types of molecular signatures. Filled cells indicate that the gene is a therapeutic target of clinical practice in the corresponding cancer type. Bar plots in right panel indicate the number of cancer types with positive correlation (drug-sensitive, magenta) and negative correlation (drug-resistant, green)

between hypoxia score and drug response (Spearman's correlation). (b) Dose-response curves for mean value of cell viability of camptothecin, bexarotene, AKT-inhibitor-VIII, and PHA-665752 in hypoxic conditions (red, $n = 4$) and normoxic conditions (blue, $n = 4$) in the lung cancer cell line A549. Cell viability was normalized to that treated with DMSO. Error bars indicate mean $\pm$ SD. Sigmoidal dose-response curves were fitted to data.

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