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基于多转录组测序的胶质母细胞瘤间质样肿瘤亚群缺氧特征及预后研究

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[**摘要**] **目的** 通过多转录组测序数据分析,解析胶质母细胞瘤(glioblastoma, GBM)的肿瘤细胞异质性组成特点和生物学功能,以探究GBM间质样(mesenchymal-like, MES-like)肿瘤亚群的缺氧特点、空间定位特征以及对GBM恶性生物学行为的影响。**方法** 分析公共数据库18例GBM单细胞转录组测序(single-cell RNA sequencing, scRNA-seq)数据,以及GBM普通转录组测序(bulk RNA sequencing,bulk RNA-seq)和空间转录组测序(spatial transcriptomics,ST)数据,通过单细胞转录组定义GBM肿瘤亚群,基因本体论(Gene Ontology, GO)和基因集富集分析(gene set enrichment analysis, GSEA)进行功能富集。使用BayesPrism反卷积方法评估普通转录组的细胞亚群占比和定位,并收集2015年至2023年就诊于西南医院神经外科患者的12例符合胶质母细胞瘤病理学诊断标准的石蜡样本(男性10例,女性2例,平均年龄为53.50岁,中位年龄为54.50岁)进行免疫荧光验证。pySCENIC预测肿瘤细胞亚群特异转录因子。**结果** GBM肿瘤细胞具有高度异质性,主要分为4种亚群,即星形胶质细胞样(astrocyte-like,AC-like)、神经前体细胞样(neural progenitor-like,NPC-like)、少突前体细胞样(oligodendrocyte progenitor-like,OPC-like)和MES-like肿瘤亚群。差异基因分析发现MES-like肿瘤亚群高表达血管内皮生长因子A(vascular endothelial growth Factor A,VEGFA)、肾上腺髓质素(adrenomedullin,ADM)、N-myc下游调控基因1(N-myc downstream regulated 1,NDRG1)、胰岛素样生长因子结合蛋白5(insulin like growth factor binding protein 5,IGFBP5)、A-激酶锚定蛋白12(A-kinase anchoring protein 12,AKAP12)(P<0.001)。pySCENIC转录因子预测发现,MES-like肿瘤亚群的高活性转录因子为AT-rich交互域3A(AT-rich interaction domain 3A,ARID3A)、FOS like 2, AP-1转录因子亚基(FOS Like 2, AP-1 transcription factor subunit,FOSL2)、内皮PAS结构域蛋白1(endothelial PAS domain protein 1,EPAS1)、CCAAT增强子结合蛋白Delta(CCAAT enhancer binding protein delta,CEBPD)、CCAAT增强子结合蛋白β(CCAAT enhancer binding protein beta,CEBPB)(P<0.05)。GO和GSEA富集发现,MES-like肿瘤亚群富集于缺氧相关通路,如细胞对低氧水平的反应(NES=2.437,P<0.001)。BayesPrism反卷积显示,MES-like肿瘤亚群主要存在于假栅栏样坏死区和坏死周围。GBM石蜡样本免疫荧光证实$CD44^{+}$(CD44 antigen,CD44抗原)MES-like肿瘤亚群主要存在于$HIF1\alpha^{high}$(Hypoxia Inducible Factor 1 Subunit Alpha,缺氧诱导因子1α)的缺氧区域(P<0.01)。多因素Cox回归显示MES-like肿瘤亚群与患者不良预后显著相关(HR=1.71,95%CI:1.38~2.11,P<0.001)。**结论** GBM肿瘤细胞具有高度异质性,主要分为AC-like、NPC-like、OPC-like和MES-like肿瘤亚群,其MES-like肿瘤亚群具有缺氧特点,主要定位于假栅栏样坏死区和坏死周围,具有促进GBM恶性生物学行为的作用。

[**关键词**] 胶质母细胞瘤;缺氧;RNA测序分析

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Hypoxia characteristics and prognostic significance of glioblastoma MES-like subpopulation through multi-transcriptomics sequencing

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[**Abstract**] Objective To analyze the composition characteristics and biological functions of tumor cell subpopulations in glioblastoma (GBM) through multi-transcriptomics sequencing technology, and explore the hypoxia characteristics and spatial localization features of the mesenchymal-like (MES-like) tumor cell subpopulation in GBM and the influence on malignant biological behaviors. Methods Multi-transcriptomics sequencing data, including single-cell RNA sequencing (scRNA-seq) data (18 patients), bulk RNA sequencing (bulk RNA-seq) and spatial transcriptomics (ST) data of GBM, were employed to define cell subpopulations in GBM, and Gene Ontology (GO) and Gene Set Enrichment Analysis (GSEA) were utilized to analyze their functions. The proportions and locations of cell subpopulations in bulk RNA-seq data were evaluated with BayesPrism deconvolution. Immunofluorescence assay was conducted for verification on 12 paraffin samples of GBM from patients who visited the neurosurgical department of our hospital from 2015 to 2023 and met the pathological diagnostic criteria for GBM (10 males and 2 females, at an average age of 53.50 years and a median age of 54.50 years). pySCENIC was applied to predict specific transcription factors of tumor cell subpopulations. Results Tumor cells in GBM were highly heterogeneous, and could be mainly divided into 4 subpopulations: astrocyte-like (AC-like), neural progenitor-like (NPC-like), oligodendrocyte progenitor-like (OPC-like) and MES-like. Differential gene analysis found that the MES-like tumor cells highly expressed vascular endothelial growth factor A (VEGFA), adrenomedullin (ADM), N-myc downstream regulated 1 (NDRG1), insulin like growth factor binding protein 5 (IGFBP5), and A-kinase anchoring protein 12 (AKAP12) (P<0.001). pySCENIC transcription factor prediction found that the high-active transcription factors of the MES-like tumor cells were AT-rich interaction domain 3A (ARID3A), FOS like 2, AP-1 transcription factor subunit (FOSL2), endothelial PAS domain protein 1 (EPAS1), CCAAT enhancer binding protein delta (CEBPD), and CCAAT enhancer binding protein beta (CEBPB) (P<0.05). GO and GSEA enrichment analyses found that the MES-like tumor cells were enriched in hypoxia-related pathways, especially the pathway of cell responses to hypoxia levels (NES=2.437, P<0.001). BayesPrism deconvolution showed that the MES-like tumor cells mainly existed in PAN(Pseudopalisading cells around necrosis) and perinecrotic zone. Immunofluorescence assay confirmed $CD44^+$ (CD44 antigen) MES-like tumor cells were mainly located in hypoxia areas with highly expression of hypoxia inducible factor 1 subunit alpha (HIF1α) (P<0.01). Multivariate Cox regression analysis indicated that the MES-like tumor cells were significantly correlated with the adverse prognosis of GBM patients (HR=1.71, 95%CI: 1.38~2.11, P<0.001). Conclusion Tumor cells in GBM are of highly heterogeneity. They could be mainly divided into 4 subpopulations: AC-like, NPC-like, OPC-like and MES-like. MES-like tumor cells, mainly locating in PAN and perinecrotic zone, are characterized by hypoxia, which can promote the malignant progression of GBM.

[**Key words**] glioblastoma; hypoxia; sequence analysis, RNA

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胶质母细胞瘤(glioblastoma,GBM)作为最常见的原发恶性脑肿瘤,具有起病隐匿、进展快速、难以治愈的特点,患者中位生存期仅为14~16个月[1]。我国脑胶质瘤的年发病率为5/10万~8/10万,5年病死率仅次于胰腺癌和肺癌[2]。GBM异质性是导致其高侵袭性和放化疗抵抗的重要原因[3],其中间质样(mesenchymal-like,MES-like)肿瘤亚群可能具有高度恶性的特点[4-5],因此深入地解析其生物学特征能够为开发新型靶向药物和更高效的治疗方式提供重要参考。

普通转录组测序(bulk RNA sequencing,bulk RNA-seq)是最早开发出来的转录组测序技术,能衡量组织内所有细胞的平均转录水平[6],且具有成本低、易普及的优势,因此早期胶质瘤的临床样本测序数据大多是普通转录组测序数据。单细胞转录组测序(single-cell RNA sequencing,scRNA-seq)进一步在转录组层面全面刻画肿瘤内单个细胞状态[7],是解析肿瘤异质性和微环境特征的有力工具。GBM作为具有高度瘤内异质性的复杂生态系统,scRNA-seq能够识别GBM肿瘤细胞亚群,解析其转录特征和发育模式特点。空间转录组测序技术具有空间位置信息,能够直观展示GBM瘤内微环境特点和细胞间位置比邻关系[8]。通过整合上述3种转录组测序数据,能够获得GBM肿瘤亚群的临床预后信息和微环境特点,从而加深对GBM异质性的理解。

本研究以GBM单细胞转录组数据分析为主要方式,整合普通转录组和空间转录组数据,采用pySCENIC和BayesPrism反卷积等新型算法工具,根据转录状态对肿瘤细胞进行分群,并探究MES-like肿瘤亚群的微环境特点以及生物学功能特征,从多转录组学层面揭示GBM肿瘤细胞异质性和MES-like肿瘤亚群的缺氧状态,为靶向GBM的治疗方法开发提供新思路。

1 材料与方法

1.1 普通转录组测序数据获取与分析

胶质瘤普通转录组基因表达Counts数据以及相关临床预后信息下载自癌症基因组图谱(The Cancer Genome Atlas, TCGA)[9]和中国脑胶质瘤基因组图谱计划(Chinese Glioma Genome Atlas, CGGA)[10]数据库。GBM基于组织学的普通转录组基因表达Counts数据下载自Ivy胶质母细胞瘤图谱项目(Ivy Glioblastoma Atlas Project,Ivy-GAP)[11]。

1.2 单细胞转录组测序数据获取与分析

单细胞数据集下载自基因表达数据库(Gene Expression Omnibus, GEO)GSE182109,共包含18例GBM患者的23个样本。通过R语言Seurat包(v. 5.0.3)的Read 10X函数读入基因表达矩阵文件,过滤后共获得104 065个细胞用于聚类分析。利用Seurat中的AddModuleScore功能进行特定肿瘤细胞亚群基因集评分并进行注释。使用SCP包(v. 0.5.6)进行基因本体论(Gene Ontology, GO)和基因集富集分析(Gene Set Enrichment Analysis, GSEA)。

1.3 空间转录组测序数据获取与分析

GBM空间转录组数据集下载自GEO数据库GSE194329,通过R语言SPATA2包[12](v. 3.1.2)进行组织学区域划分,使用Seurat包(v. 5.0.3)中的AddModuleScore功能进行缺氧基因集评分。

1.4 pySCENIC转录因子预测

将单细胞转录组注释后的肿瘤细胞基因表达量Counts数据转置后作为输入文件,通过pySCENIC(v. 0.12.1)的grn命令、ctx命令进行顺式调控结构域富集,最后识别到123个调控子(Regulons),并且使用aucell命令计算转录因子活性。

1.5 BayesPrism反卷积

BayesPrism[13]是一种基于贝叶斯理论的反卷积工具,可以将单细胞转录组数据的信息映射到普通转录组数据中,从而推断肿瘤微环境的组成。将进行细胞类型注释后的单细胞转录组基因表达量Counts矩阵和普通转录组基因表达量Counts矩阵作为输入数据,选择"protein_coding"基因作为参照基因,设置肿瘤细胞作为键值以构建Prism对象,可视化采用ggplot2包完成。

1.6 免疫荧光染色

收集2015年至2023年就诊于西南医院神经外科患者的12例符合胶质母细胞瘤病理学诊断标准的石蜡样本(男性10例,女性2例,平均年龄为53.50岁,中位年龄为54.50岁)进行免疫荧光验证。GBM石蜡切片经脱蜡、梯度脱水、阻断和抗原修复后,加入一抗(鼠抗人CD44, Proteintech, 60224-1-IG-50UL, Mouse mAb, 1∶500;兔抗人HIF1α,Abcam, ab51608, Rabbit mAb,1∶300)4 ℃孵育过夜,次日孵育荧光偶连二抗,DAPI染核后Olympus VS200扫片。本研究经陆军军医大学第一附属医院伦理委员会审查批准(KY2020294)。

1.7 统计学分析

所有数据均采用R(4.3.1)、Python(3.12.6)和GraphPad Prism(9.0.0)软件进行统计学分析。采用多因素Cox回归分析探讨影响GBM患者预后的独立

危险因素,配对 t 检验分析免疫荧光染色样本中 $HIF1\alpha^{high}$ 和 $HIF1\alpha^{low}$ 区域中 CD44 平均荧光强度的差异。$P<0.05$ 认为有统计学意义。

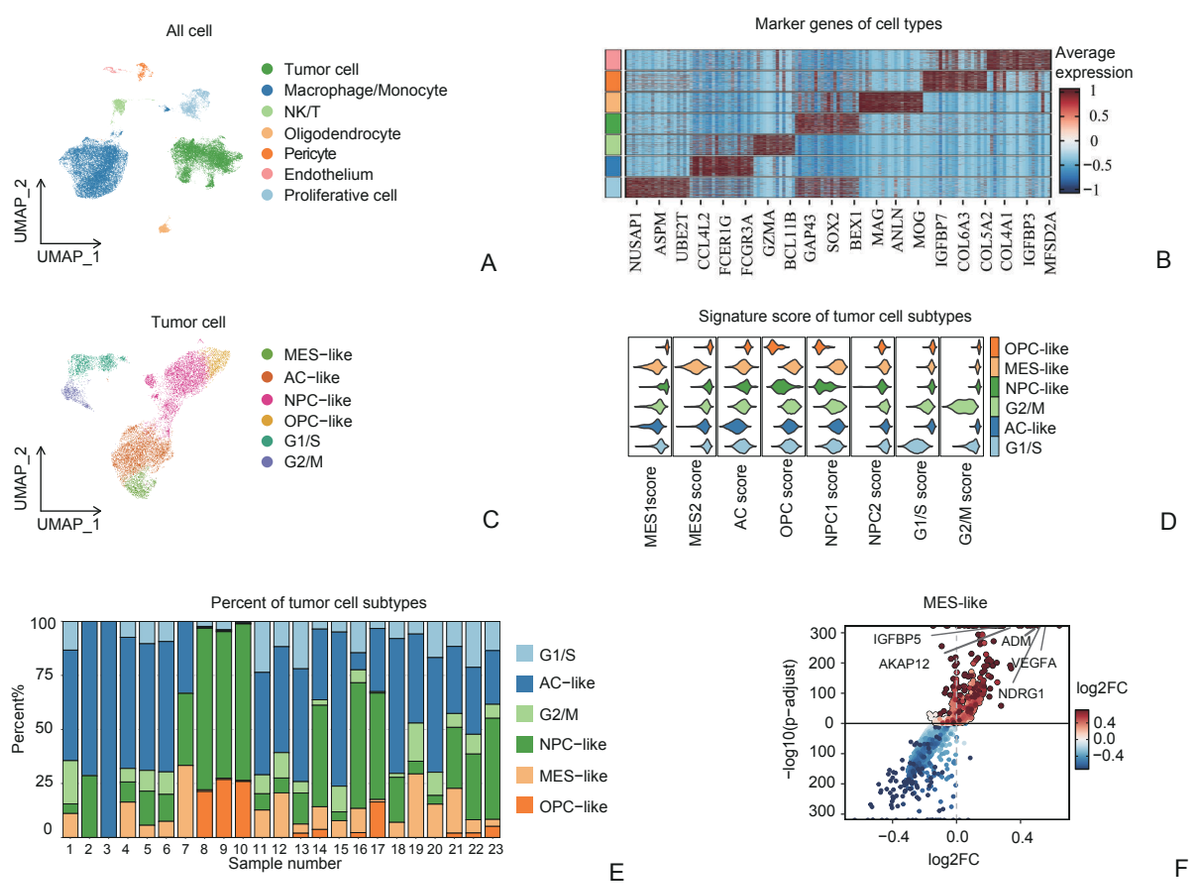
2　结果

2.1　GBM 肿瘤细胞中存在间质样肿瘤亚群

本研究通过对 18 例 GBM 患者的单细胞转录组数据进行分析,在去除双细胞并进行去批次效应等质控步骤后,一共获得 104 065 个细胞,降维后通过各细胞群标志基因[14]将其注释为 7 个主要细胞群,其中肿瘤细胞和巨噬/单核细胞最为丰富(图 1A、B)。进一步提取肿瘤细胞进行降维分群,按照 GBM 肿瘤亚群特征基因集评分[15]将肿瘤细胞注释为 6 个亚群(图 1C、D)。在分析的 GBM 样本中,星形胶质细胞样(astrocyte-like,AC-like)肿瘤亚群和神经前体细胞样(neural progenitor-like,NPC-like)肿瘤亚群占比较高,间质样肿瘤亚群(MES-like)和少突前体细胞样(oligodendrocyte progenitor-like,OPC-like)肿瘤亚群占比较低(图 1E),另外还存在少数处于细胞周期的 G1/S、G2/M 肿瘤细胞。差异基因分析发现,MES-like 肿瘤亚群表达量最高的前 5 个基因为 *VEGFA*、*ADM*、*NDRG1*、*IGFBP5*、*AKAP12*($P<0.001$,图 1F),提示 MES-like 肿瘤亚群具有通过高表达 VEGFA 促进血管新生的能力[16]。

2.2　GBM 细胞中的间质样肿瘤亚群具有缺氧和干性特征

对 GBM 间质样肿瘤亚群进行 GO 和 GSEA 功能富集发现,其主要富集了缺氧反应通路,如细胞对低氧水平的反应(NES=2.437,$P<0.001$)(图 2A、B)。利用 MsigDB 数据库[17]中的缺氧反应基因集对肿瘤细胞进行评分,也发现 MES-like 肿瘤亚群的缺氧评分最高,进一步证明了 MES-like 肿瘤亚群的缺氧特征。胶质瘤干细胞(glioma stem-like cell,GSC)标志基因 *CD133*



A: dimensional reduction of GBM scRNA-seq data; B: expression heatmap of marker genes among different cell types; C: dimensional reduction of GBM tumor cells; D: violin diagram of signature score among tumor cell subtypes; E: stacked column chart of tumor cell subtypes in 23 samples; F: the top 5 differential genes of MES-like tumor cells

图 1　单细胞转录组细胞分群结果及肿瘤细胞亚群鉴定

Figure 1　Cell types of GBM scRNA-seq data and identification of tumor cell subpopulations

(Prominin 1)主要集中在MES-like肿瘤亚群表达(图2C),通过对GEO数据库GSE85297中$CD133^+$ GSC与$CD133^-$ non-GSC差异基因分析,获得了86个在$CD133^+$ GSC中显著高表达(LogFC>2)的基因,并利用此基因集对GBM单细胞转录组和空间转录组数据进行富集评分,发现MES-like亚群显著富集GBM干性基因集,提示MES-like具有干性特征(图2C、D)。为了更加直观地展示MES-like肿瘤亚群的缺氧特征,本研究分析了公开数据库中GBM空间转录组数据,利用缺氧和干性基因集进行评分,发现MES-like肿瘤亚群同时具有缺氧高评分、CD44高表达量和干性高评分(图2D),进一步证实了$CD44^+$ MES-like肿瘤亚群的缺氧特征和干性特征。另外通过利用pySCENIC[18]对肿瘤细胞进行转录因子预测分析,发现MES-like肿瘤亚群的特征性转录因子为ARID3A、FOSL2、EPAS1、CEBPD、CEBPB($P<0.05$,图2E),提示上述转录因子对$CD44^+$ MES-like肿瘤亚群表型塑造可能具有重要作用。

2.3 GBM中的间质样肿瘤亚群主要定位于瘤内缺氧区域

通过将GBM单细胞转录组数据反卷积至TCGA胶质瘤普通转录组数据中,发现MES-like肿瘤亚群主要存在于GBM中,在低级别胶质瘤中所占比例极低(图3A)。为了进一步研究MES-like肿瘤亚群的空间定位,通过对Ivy-GAP数据库的GBM普通转录组数据进行反卷积,发现MES-like肿瘤亚群主要存在于GBM的假栅栏样坏死区(pseudopalisading cells around necrosis, PAN)和坏死周围(图3B),并在人GBM石蜡样本中的免疫荧光染色结果也证明$CD44^+$ MES-like肿瘤亚群主要存在于$HIF1\alpha^{high}$的缺氧区域($P<0.01$)(图3C、D)。对GBM空间转录组数据进行组织学区域划分后发现,假栅栏样坏死区存在大量异常拷贝数变异(图3E),提示此区域内的MES-like肿瘤细胞可能具有高度恶性生物学行为。

2.4 GBM中存在间质样肿瘤亚群与预后不良相关

为了进一步分析MES-like肿瘤亚群与临床预后的关系,本研究利用BayesPrism反卷积的方法整合GBM单细胞转录组数据和包含临床预后信息的TCGA、CGGA胶质瘤数据,发现MES-like肿瘤亚群主要存在于GBM中,并且存在MES-like肿瘤亚群富集的患者多为老年人群,其总生存期更短、异柠檬酸脱氢酶(isocitrate dehydrogenase,IDH)野生型和染色体1p/19q非共缺失占比更高(图4A)。通过多因素Cox回归分析发现,MES-like肿瘤亚群的存在是不良预后因素($HR=1.71$,95%CI: 1.38~2.11, $P<0.001$,图4B)。上述结果均表明,MES-like肿瘤亚群与GBM患者的不良预后相关。

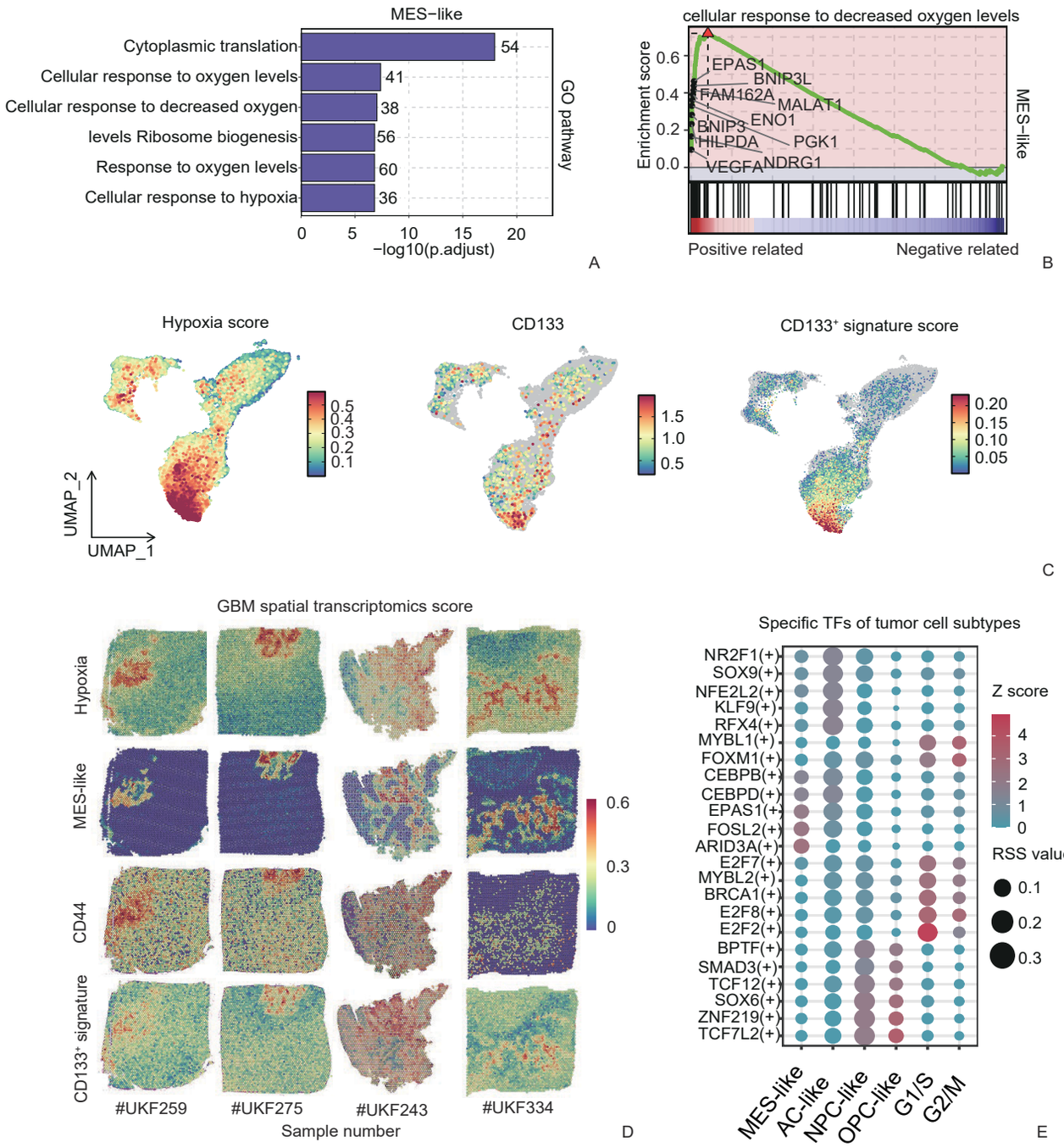
3 讨论

3.1 缺氧诱导的HIF1α/HIF2α塑造$CD44^+$ MES-like肿瘤亚群的恶性表型

作为缺氧响应的核心分子,HIF1α/HIF2α对$CD44^+$ MES-like肿瘤亚群的增殖、侵袭和自我更新等恶性生物学行为具有重要作用。最新研究发现,GSC中缺氧诱导的HIF1α促进Lucat1(Lung Cancer associated transcript 1,肺癌相关转录本1)新型异构体形成并与HIF1α、共激活因子CBP(CREB Binding Protein,CREB结合蛋白)形成复合体从而调控HIF1α靶基因表达,HIF1α-LUCAT1轴形成的正反馈环路强化HIF1α的功能,从而促进GSC增殖和自我更新[19]。肿瘤缺氧微环境还能增强GSC自噬能力从而促进其自我更新,缺氧区域GSC特异性表达的Gal-8(Galectin 8,半乳糖凝集素8)结合溶酶体膜的Ragulator-Rag复合体并灭活mTORC1(mechanistic target of rapamycin complex 1,雷帕霉素靶点复合体1),从而促进TFEB(Transcription Factor EB,EB转录因子)核转位和自噬性溶酶体的合成,最终维持GSC的增殖活性[20]。既往研究表明,骨桥蛋白作为CD44配体激活CD44,受γ分泌酶调控的CD44胞内段通过CBP/p300(E1A Binding Protein P300,E1A结合蛋白P300)增强HIF2α活性,从而促进GSC增殖和放疗抵抗[21]。另外缺氧通过上调GSC的HIF1α和HIF2α表达促进CDH5(Cadherin 5,钙黏蛋白5)转录,从而增强GSC的血管拟态形成能力[22]。可以发现,HIF1α/HIF2α对于维持$CD44^+$ MES-like肿瘤亚群的增殖、侵袭能力是十分重要的。

3.2 靶向$CD44^+$ MES-like肿瘤亚群具有临床治疗意义

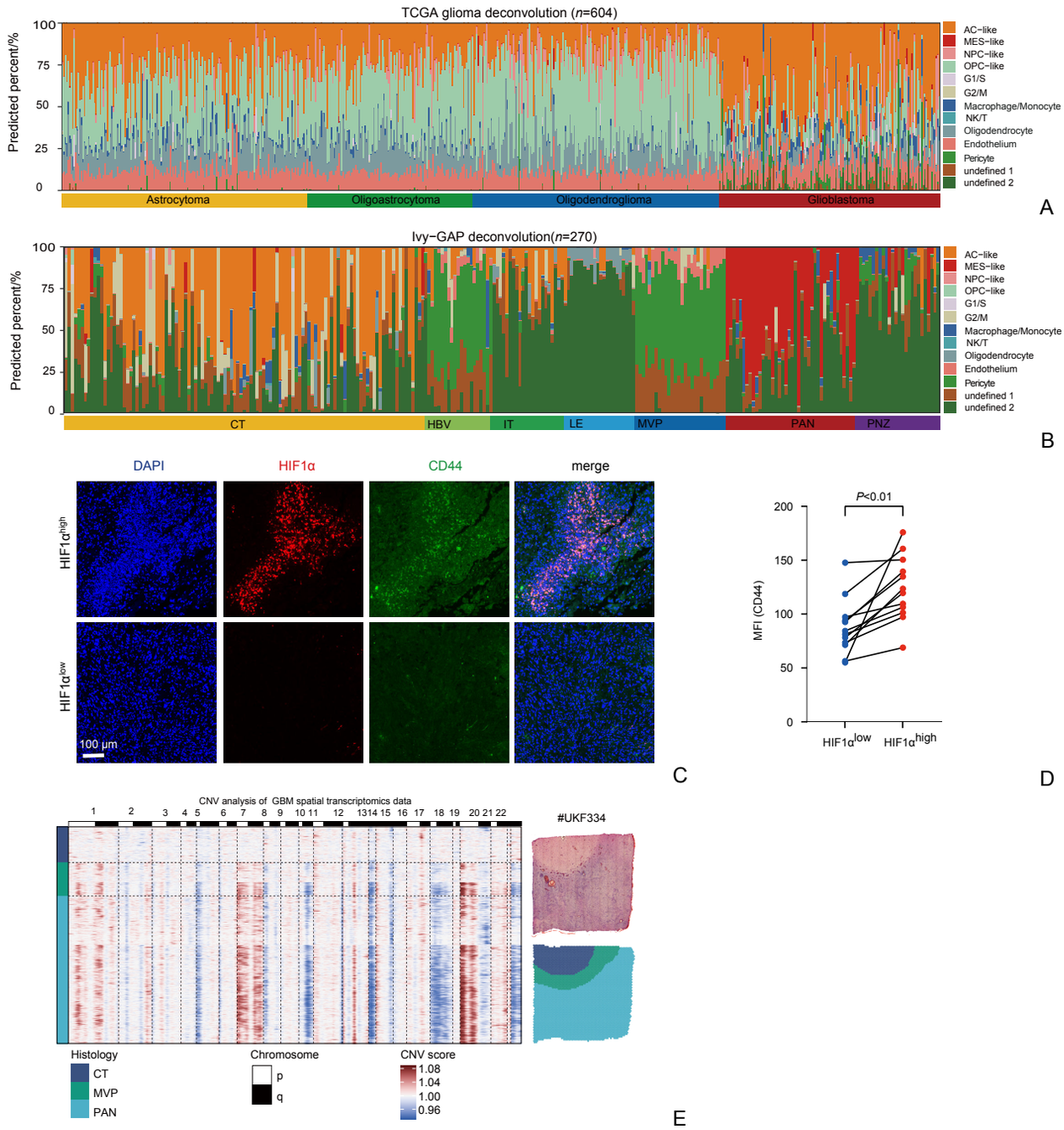
GBM作为具有高度异质性的中枢神经系统恶性肿瘤,其肿瘤细胞的异质性组成是导致目前常规放化疗疗效不佳的重要原因之一[23],缺氧状态的$CD44^+$ MES-like肿瘤亚群可能是最主要的恶性细胞群体。最新研究表明在小鼠GBM模型中诱导的血栓-缺氧-坏死级联反应将导致显著的肿瘤微环境重构和侵袭性进展,并且免疫抑制性肿瘤相关巨噬细胞和胶质瘤干细胞的数量在坏死周围显著增多,提示GBM缺氧微环境极有可能通过促进肿瘤细胞的间质性转化,从而促进其增殖、侵袭能力[24]。综上所述,GBM的$CD44^+$ MES-like肿瘤亚群具有显著的缺氧特征并且主要存在



A: GO enrichment analysis of MES-like tumor subpopulation; B: GSEA enrichment analysis of hypoxia response gene set of MES-like tumor subpopulation; C: visualization of tumor cell hypoxia score, CD133 expression and CD133$^+$ signature score of GBM; D: visualization of hypoxia score, MES-like score, CD44 expression and CD133$^+$ signature score of GBM on spatial transcriptomics data; E: bubble chart of predicted specific transcription factors among tumor cell subpopulations by pySCENIC. TFs: transcription factors

图2 间质样肿瘤亚群的功能富集

Figure 2 Functional enrichment of MES-like tumor subpopulation

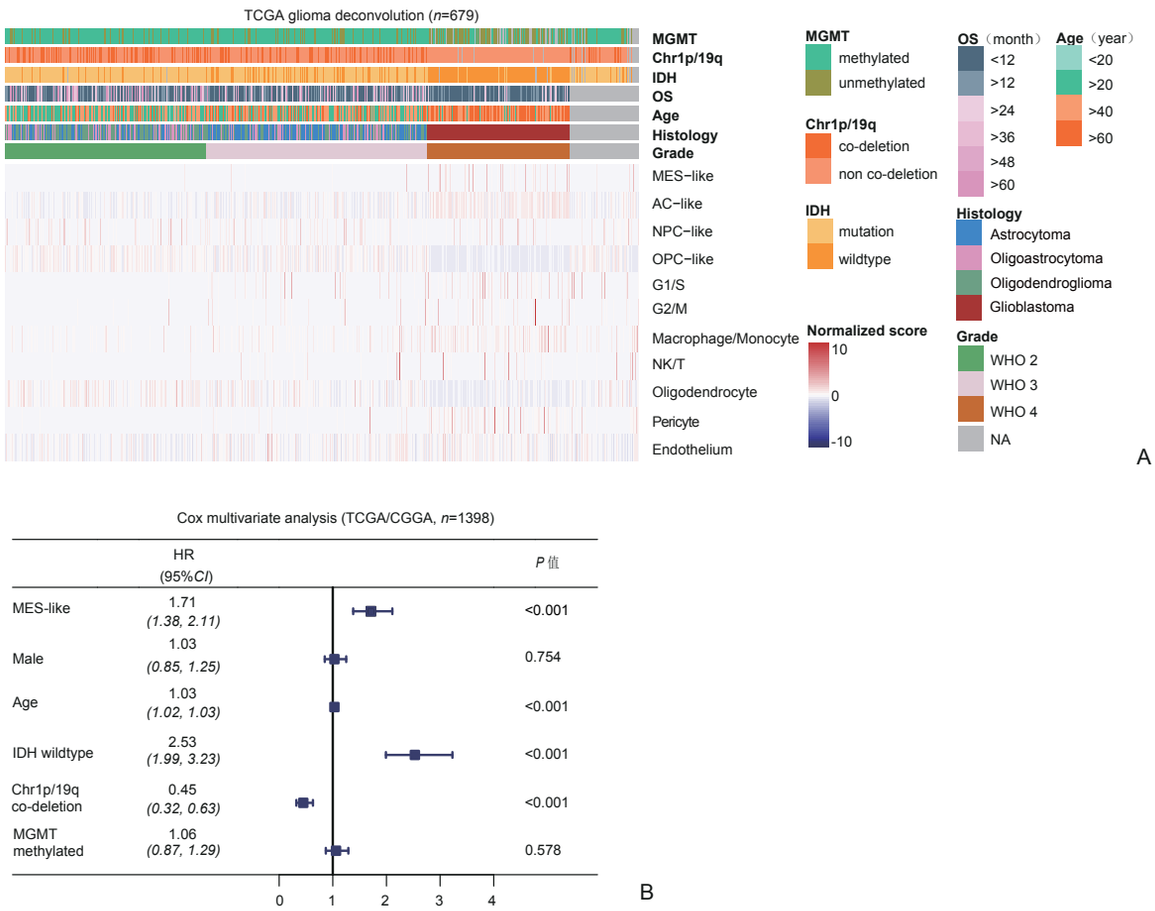


A: results of TCGA glioma BayesPrism deconvolution; B: results of Ivy-GAP BayesPrism deconvolution; C: immunofluorescent staining of GBM paraffin samples; D: statistical results of immunofluorescence staining; E: CNV analysis of GBM spatial transcriptomics data. CT: cellular tumor; HBV, hyperplastic blood vessels in cellular tumor; IT: infiltrating tumor; LE: leading edge; MVP: microvascular proliferation; PAN: pseudopalisading cells around necrosis; PNZ: perinecrotic zone; MFI: mean fluorescence intensity; CNV: copy number variation

图3 间质样肿瘤细胞的空间分布特点

Figure 3 Spatial distribution characteristics of MES-like tumor subpopulation

于GBM缺氧坏死区域,缺氧状态对$CD44^+$ MES-like肿瘤亚群的恶性生物学行为具有极大的塑造作用。靶向处于缺氧状态的$CD44^+$ MES-like肿瘤亚群可能具有减轻放化疗抵抗、抑制侵袭性转移和延长患者生存期的临床应用前景。



A: predicted proportion and clinical characteristics of each cell population in the TCGA glioma cohort; B: multivariate Cox analysis of overall survival in glioma cohorts combining TCGA and CGGA

图4 间质样肿瘤亚群的临床预后分析

Figure 4 Clinical prognosis analysis of MES-like tumor subpopulation

利益冲突 本文所有作者声明不存在利益冲突

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