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A molecular signature associated with prolonged survival in glioblastoma patients treated with regorafenib

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Abstract

Background. Patients with glioblastoma (GBM) have a dramatically poor prognosis. The recent REGOMA trial suggested an overall survival (OS) benefit of regorafenib in recurrent GBM patients. Considering the extreme genetic heterogeneity of GBMs, we aimed to identify molecular biomarkers predictive of differential response to the drug.

Methods. Total RNA was extracted from tumor samples of patients enrolled in the REGOMA trial. Genome-wide transcriptome and micro (mi)RNA profiles were associated with patients' OS and progression-free survival.

Results. In the first step, a set of 11 gene transcripts (*HIF1A, CTSK, SLC2A1, KLHL12, CDKN1A, CA12, WDR1, CD53, CBR4, NIFK-AS1, RAB30-DT*) and 10 miRNAs (miR-93-5p, miR-203a-3p, miR-17-5p, let-7c-3p, miR-101-3p, miR-3607-3p, miR-6516-3p, miR-301a-3p, miR-23b-3p, miR-222-3p) was filtered by comparing survival between regorafenib and lomustine arms. In the second step, a mini-signature of 2 gene transcripts (*HIF1A*, *CDKN1A*) and 3 miRNAs (miR-3607-3p, miR-301a-3p, miR-93-5p) identified a subgroup of patients showing prolonged survival after regorafenib administration (median OS range, 10.6–20.8 mo).

Conclusions. The study provides evidence that a signature based on the expression of 5 biomarkers could help identify a subgroup of GBM patients exhibiting a striking survival advantage when treated with regorafenib. Although the presented results must be confirmed in larger replication cohorts, the study highlights potential biomarker options to help guide the clinical decision among regorafenib and other treatments in patients with relapsing GBM.

Key Points

1. Predictive biomarkers for second-line therapy of glioblastoma are lacking.
2. A transcriptional signature identifies patients with significant benefit with regorafenib.
3. These biomarkers can guide clinical decision in second-line treatment in glioblastoma.

Importance of the Study

Among anti-angiogenic drugs for second-line therapy of patients with GBM, regorafenib has gained interest after the REGOMA clinical trial reported an OS advantage of the patients of the regorafenib arm compared with those enrolled in the lomustine arm. Considering the huge molecular variability among GBM tumors, we investigated by genome-wide analyses whether expression levels of transcripts and miRNAs could help identify patients with specific advantage or disadvantage in the choice of regorafenib as second-line therapy. Our findings propose to assess expression levels of a specific signature of transcripts and miRNAs in tumor tissue in support of a precision medicine–oriented therapeutic choice in these patients.

The standard of care for glioblastoma (GBM), the most common and severe brain malignancy in adults, is based on maximal surgical resection followed by radiochemotherapy. GBMs are characterized by intense angiogenesis driven by the modulation of expression of a family of genes promoting the formation of new vessels.[1] As intense angiogenesis is associated with biological aggressiveness and postsurgical recurrence in patients with GBM, several direct and indirect anti-angiogenic drugs have been under scrutiny.[2]The limited improvement of overall survival (OS) in patients enrolled in clinical trials with this class of molecules prompted development of novel anti-angiogenic drugs.[2]

Regorafenib, a recently designed drug, inhibits the activation of several kinases, including some of the class of receptor tyrosine kinases, such as vascular endothelial growth factor receptor (VEGFR) 1–3, platelet derived growth factor receptor, and fibroblast growth factor receptor. An inhibitory effect has also been reported against kinases of the mitogen-activated protein kinase (MAPK) family such as extracellular signal-regulated kinase 1 and 2 (ERK1/2), and mitogen-activated protein kinase kinase 1 and 2 (MEK1/2), which are involved in tumor angiogenesis and in controlling the tumor microenvironment and tumor immunity.[3–5] After extensive preclinical investigation, regorafenib has been approved for treatment of patients with metastatic colorectal cancer, gastrointestinal tumors, and hepatocellular carcinoma.[6,7] Concerning brain tumors, regorafenib has suggested inhibition of proliferation and angiogenesis in tumor cell models in vitro and ex vivo, thus providing a preclinical rationale for use in these malignancies.[8,9] We recently concluded a multicenter, open-label, randomized, controlled phase 2 trial (REGOMA) for investigating the effect of regorafenib in patients with recurrent GBM (ClinicalTrials.gov NCT02926222).[10] OS was improved in the regorafenib group compared with the lomustine group (ie, 7.4 vs 5.6 mo, respectively),[10] thus indicating that this drug could represent an advancement in patient management. Based on these results, regorafenib has recently been approved by the Italian Medicines Agency (AIFA) and included in the National Comprehensive Cancer Network 2020 guidelines v1.2020 for central nervous system cancers as a new treatment option for recurrent GBM. A translational research program was associated with the REGOMA trial, including the genome-wide evaluation of expression of transcripts and microRNAs, which were analyzed in tumor tissue samples obtained at the time of first surgery.[10]

MicroRNAs (miRNAs) are a family of small (19 to 25 nucleotides in length) noncoding RNAs playing an important role in posttranscriptional control of gene expression. The miRNA-dependent recognition of 3′ untranslated region (UTR), coding sequence, and 5′-UTR mRNA sequences controls mRNA translation processes, ultimately leading to decreased protein synthesis. The molecular interaction of miRNAs with regulated mRNAs is associated with repression of protein translation or mRNA degradation or both.[11] Dysregulation of miRNA expression in several cancers has been consistently observed, so that it has been postulated that miRNAs may exert either pro-oncogenic or tumor-suppressive functions. Besides the relevant insight that differential expression of miRNAs underlies the mechanisms of tumor growth and progression, miRNA signatures in cancer have been proposed as biomarkers for diagnosis, prognosis, and prediction of therapeutic responses in different cancers.[12]

With the aim of identifying a signature potentially predictive of response to therapy with regorafenib, we analyzed the genome-wide transcriptome and miRNA profiles

in tumor samples of patients enrolled in the REGOMA trial and we then correlated the expression levels of detected mRNAs/miRNAs with the OS and progression-free survival (PFS) from tumor relapse in the 2 arms of treatment with regorafenib or lomustine. These results collectively provide preliminary information on specific mRNAs and miRNAs for developing a signature useful to guide personalized treatment in patients with GBM.

Materials and Methods

Patients and Samples

The clinical information of the 119 patients enrolled in the REGOMA trial has been published elsewhere.[10] Clinical features of the patients included in this study are reported in Supplementary Table 1. Genome-wide miRNA and mRNA biomarker analyses were performed in formalin-fixed paraffin-embedded (FFPE) slices obtained from tumor tissue at first surgery in 72 of such patients (60.5%), 36 in the regorafenib arm and 36 in the lomustine arm.

Ethics Statement

All participating centers obtained written approval for the study from their local authorities and ethics committees. All patients signed an informed consent approval form approved by the ethics committee of the enrolling institution according to national regulations. The study was done in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines, reviewed and approved by the Veneto Institute of Oncology.

RNA Isolation from Tumor Tissues

Total RNA for analysis of both mRNA and miRNAs was extracted from formalin-free alcoholic-based fixative FineFIX and paraffin-embedded samples by the MiRNeasy FFPE minikit (Qiagen). RNA was quantified with Qubit 3.0 and Qubit RNA HS Assay kits (Applied Biosystems).

Profiling of mRNA and miRNA by RNA-Sequencing Analysis

RNA-seq libraries were prepared from 100 ng of total RNA using the QuantSeq 3′ mRNA-Seq Library Prep Kit-FWD (Lexogen) for mRNA analysis or from 350 ng using the QiAseq miRNA kit (Qiagen) for miRNA profiling, according to manufacturer's instructions including the recommendation for FFPE samples. On average 15 314 genes and 1400 miRNAs were detected as expressed in each sample (Supplementary Figure 1). The detailed RNA-seq protocols and data analysis pipelines are described in the Supplementary Material. RNA-sequencing data presented in this study have been deposited Gene Expression Omnibus (GEO) database: Project accession number GSE154043, RNaseq accession number GSE154041, and miRNA-seq accession number GSE154042.

Analysis of Prognostic Value of miRNA from TCGA

Survival data of patients with GBM (n = 592) were extracted from The Cancer Genome Atlas (TCGA) for GBM (TCGA, PanCancer Atlas) dataset by using cBioPortal for cancer genomics software (http://www.cbioportal.org). The miRNA expression levels detected in tumor tissues at surgery were associated with OS data of patients undergoing first-line therapy with postsurgery radiochemotherapy. To explore the prognostic power of the miRNAs, subjects were divided in 2 groups as a function of expression of each miRNA as above (high) or below (low) median levels. Median OS was calculated from Kaplan–Meier curves, and the log-rank test was applied for statistical significance.

Statistical Analysis

Statistical analysis was aimed at assessing the efficiency of different mRNAs and miRNAs in stratifying subgroups of patients of regorafenib and lomustine arms in terms of OS and PFS. As no reference interval or cutoff has been defined in the literature for mRNA and miRNA expression in GBM tissue, mRNA and miRNA median expression levels were arbitrarily chosen for separating samples in 2 groups, thus subjects were assigned to "high" or "low" subgroups in reference to the median values. For each of the 2 subgroups (high and low expression) OS and PFS were calculated with Kaplan–Meier survival analysis. Heterogeneity in survival of the two treatments in the REGOMA trial (regorafenib vs lomustine) was assessed by two-sided long-rank test. After Kaplan–Meier survival analyses and assessment of log-rank test probability for each of the mRNA and miRNAs subdivided in high and low groups comparing regorafenib versus lomustine arms, log-rank test probability at $P \leq 0.01$ for both OS and PFS was the criterion for selecting miRNAs and mRNA for further analysis, as depicted in the filtering diagram represented in the flowchart of Fig. 1. A list of biomarkers from Kaplan–Meier curves comparing regorafenib and lomustine arms with statistical significance at $P \leq 0.01$ levels for both OS and PFS ended to select a first set of candidate gene transcripts and miRNAs. These biomarkers were further filtered in a second step with Kaplan–Meier curves for OS only within the regorafenib arm, resulting in a more restricted mRNA and miRNA mini-signature (Fig. 1).

Results

Identification of Transcripts Predicting Regorafenib-Associated Survival

Genome-wide mRNA profiling was assessed on RNA extracted from FFPE slices of tumor tissues available from 72 patients with GBM enrolled in the REGOMA clinical trial (regorafenib n = 36 and lomustine, n = 36).[10] The clinical characteristics were homogeneous between the groups of patients enrolled in the regorafenib and lomustine arms as well as with the clinical set of the REGOMA study (Supplementary Table 1). Sequencing data and the number

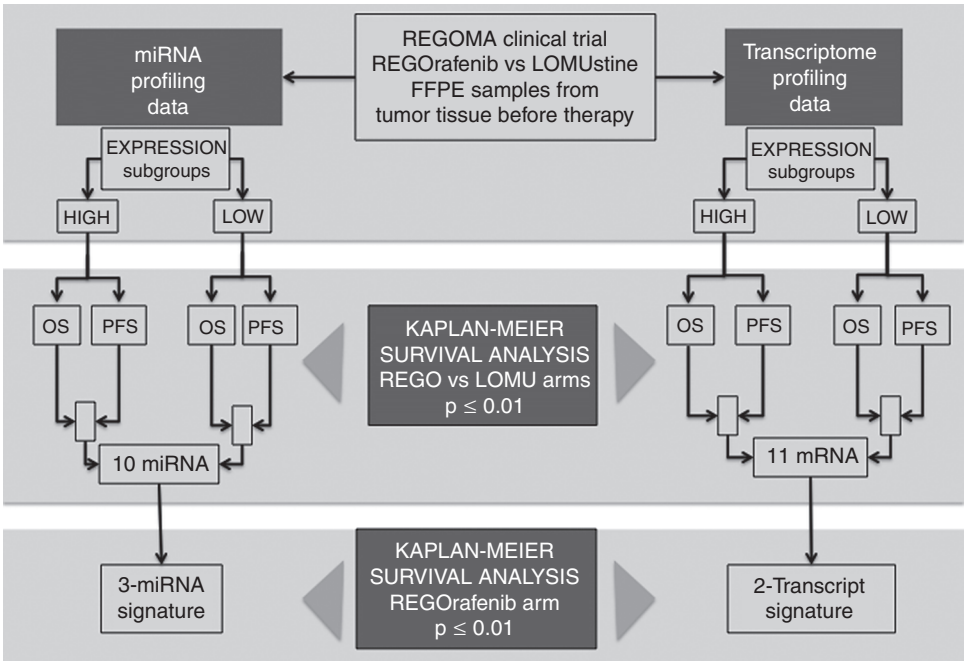


Fig. 1 Outline of study design and biomarker filtering strategy.

of detected genes were consistent between regorafenib and lomustine groups (Supplementary Figure 1). In order to select those gene transcripts with potential predictivity of patients' survival, we associated the expression levels of each mRNA with patients' OS and PFS, as depicted in Fig. 1. A significant difference in both OS and PFS was found between regorafenib and lomustine groups for 11 mRNAs. In particular, OS was prolonged in patients treated with regorafenib in respect to the treatment with lomustine in the subgroup of patients with high expression of *HIF1A, CTSK, SLC2A1, KLHL12, CDKN1A, CA12, WDR1*, and *CD53* mRNAs and low expression of *CBR4, NIFK-AS1*, and *RAB30-DT* mRNAs (Fig. 2) (Supplementary Table 2). The median OS in the regorafenib arm ranged 10.6–20.8 months compared with 5.4–8.4 months in the lomustine arm, with a difference of median OS (delta OS) enhanced from a minimum of 5.1 to a maximum of 12.4 months in the regorafenib arm. Similar results were observed for PFS, though smaller differences in time were evidenced between median PFS (Supplementary Table 2). Focusing on OS, these results strengthened the findings previously reported in the REGOMA trial where a more favorable survival was observed in the whole group of patients treated with regorafenib (median OS, 7.4 mo) compared with the group treated with lomustine (median OS, 5.6 mo).[10] Thus, we verified whether the expression analysis of the 11 selected mRNAs could further help to indicate subgroups of patients with a selective advantage. For this aim, we compared the OS survival of patients enrolled in the regorafenib arm after stratification according to expression levels of the 11 mRNAs. Significant differences in median OS were observed in the regorafenib arm only in the patients presenting high expression of hypoxia-inducible factor 1A (HIF1A) and cyclin-dependent kinase inhibitor 1A (CDKN1A) mRNA (log-rank test $P = 0.0011$ and 0.00083, respectively) (Fig. 4A, B). Interestingly, the median OS in high *HIF1A* and *CDKN1A* expression subgroups was prolonged by several months (median OS, 20.8 mo) compared with that of patients with low gene expression (median OS, 5.9 and 6.0 mo, respectively). Parallel Kaplan–Meier analysis of PFS did not reach statistical significance (data not shown). Collectively, transcriptome analysis of tumor tissue at first surgery identified HIF1A and CDKN1A mRNA expression as a molecular mini-signature capable of identifying specific OS advantage in subgroups of patients affected by recurrent GBM and undergoing treatment with regorafenib.

MiRNA Profiling and Regorafenib-Associated Survival

Since miRNAs are key regulators of gene expression and dysregulated expression of several miRNAs has been proposed as prognostic index in patients with GBM,[13] whole-genome miRNA profiling was assessed in parallel on the same RNA samples extracted from FFPE slices of tumor tissues. A significant difference in both OS and PFS was found between regorafenib and lomustine groups for 10 miRNAs (Fig. 3). OS was prolonged in patients treated with regorafenib in the group with low expression of miR-93-5p, miR-203a-3p, miR-17-5p, let-7c-3p, miR-101-3p, miR-3607-3p, miR-6516-3p, miR-301a-3p, and miR-23b-3p and high expression of miR-222-3p. The range of median OS in the regorafenib arm was 10.6–13.4 months compared with 5.5–7.3 months in the lomustine arm, with median OS in the regorafenib arm enhanced from 3.3 to 7.9 months (Supplementary Table 3). Similar results were observed for

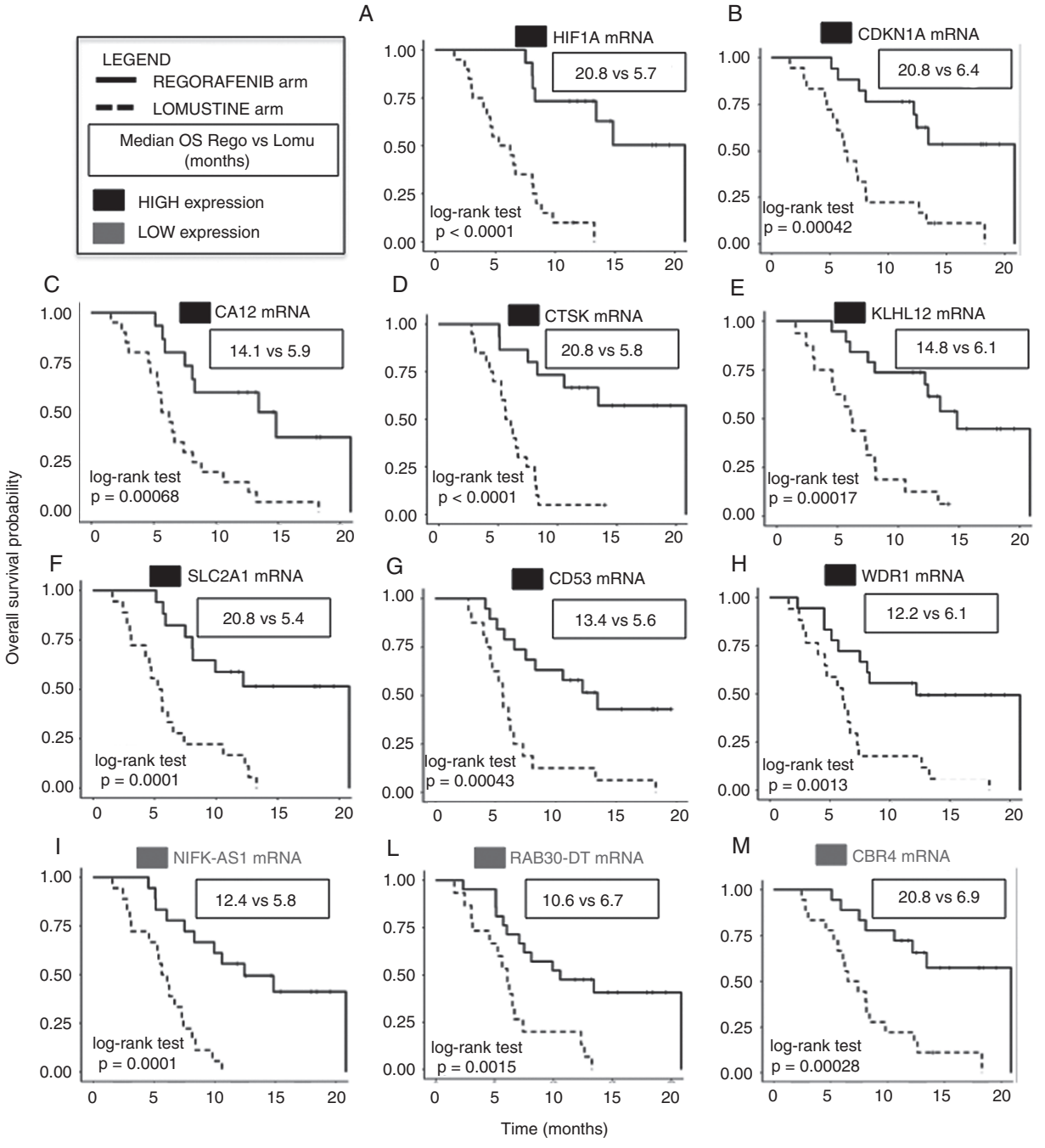


Fig. 2 Kaplan–Meier plots for OS probability for levels of expression of gene transcripts. Significant survival curves of patients in the regorafenib (solid line) versus lomustine (dashed line) arms of gene transcripts with high expression, (A) *HIF1A*, (B) *CDKN1A*, (C) *CA12*, (D) *CTSK*, (E) *KLHL 12*, (F) *SLC2A1*, (G) *CD53*, (H) *WDR1* or low expression, (I) *NIFK-AS1*, (L) *RAB30-DT*, (M) *CBR4*. Median OS in months of regorafenib arm versus lomustine arm is reported in the boxes.

PFS (SupplementaryTable 4), though differences in median PFS were relatively small. In order to verify whether high or low expression levels of the 10 miRNAs could predict OS or PFS independently of treatment, median OS and PFS in high versus low groups were compared. No significant differences could be observed for median OS or PFS from the analysis in both arms (Supplementary Tables 3 and 4). Then, we investigated whether reduced expression of miR-93-5p, miR-203a-3p, miR-17-5p, let-7c-3p, miR-101-3p, miR-3607-3p, miR-6516-3p, miR-301a-3p, miR-23b-3p, and high expression of miR-222-3p, which are associated with prolonged OS in regorafenib-treated patients,

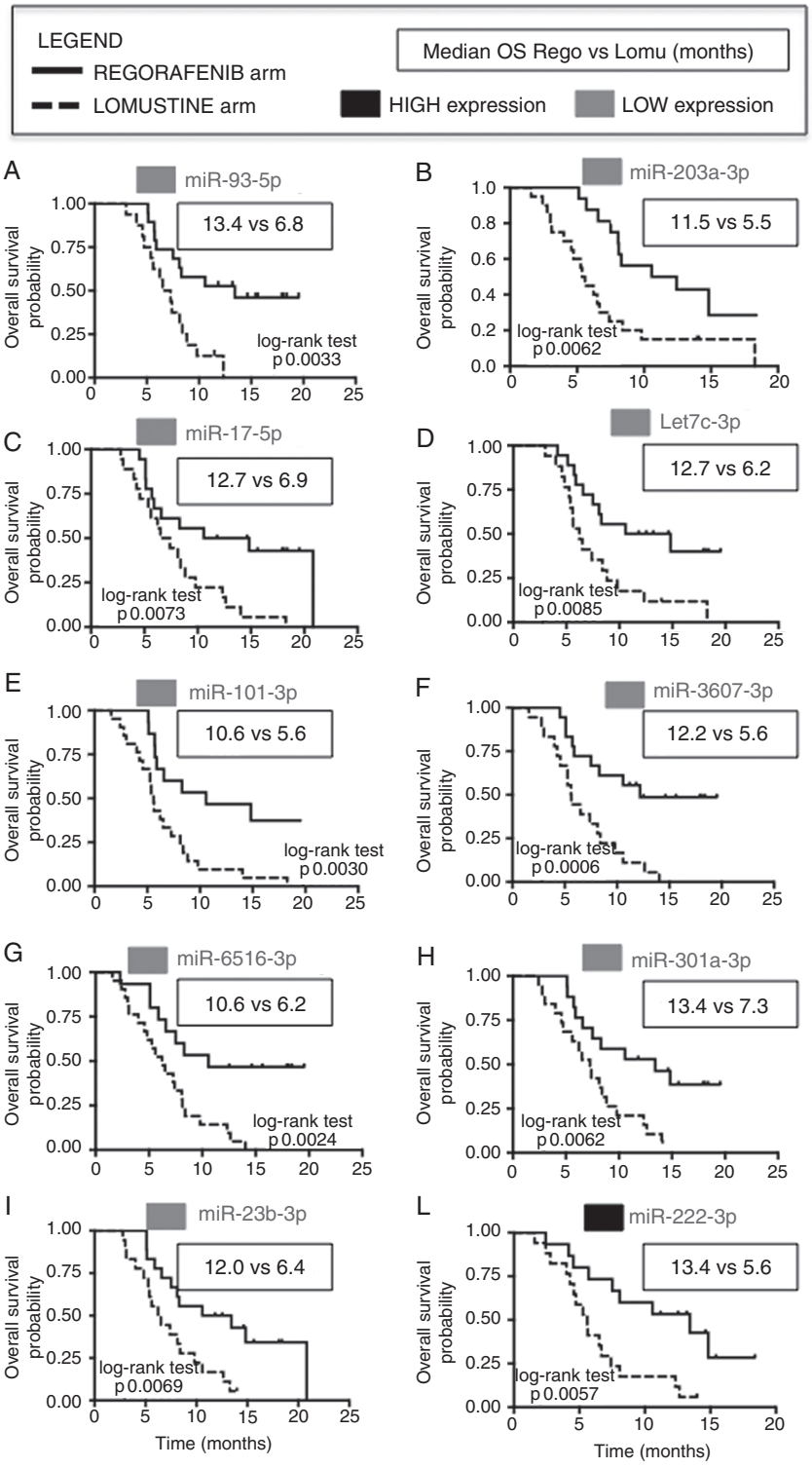


Fig. 3 Kaplan–Meier plots for OS probability as a function of expression levels of miRNAs. Significant survival curves of patients in the regorafenib (solid line) versus lomustine (dashed line) arms of gene transcripts with low expression, (A) miR-93-5p, (B) miR-203a-3p, (C) miR-17-5p, (D) let-7c-3p, (E) miR-101-3p, (F) miR-3607-3p, (G) miR-6516-3p*53*, (H) miR-301a-3p, (I) miR-23b-3p, or high expression, (L) miR-222-3p. Median OS in months of regorafenib arm versus lomustine arm is reported in the boxes.

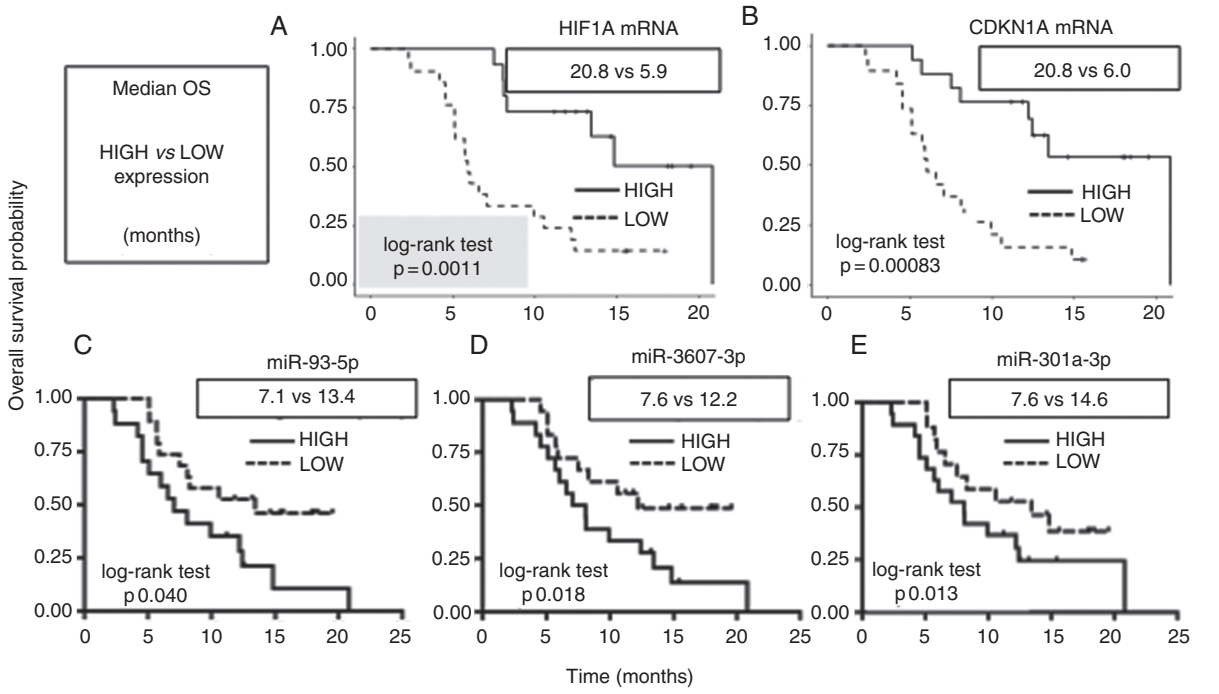


Fig. 4 Kaplan–Meier plots for OS probability in the regorafenib arm. Significant survival curves of patients in the regorafenib arm comparing high and low expression levels of the biomarkers (A) HIF1A mRNA, (B) CDKN1A mRNA, (C) miR-93-5p, (D) miR-3607-3p, (E) miR-301a-3p. Median OS in months of regorafenib arm versus lomustine arm is reported in the boxes.

could be correlated with a better prognosis regardless of regorafenib treatment. We interrogated the dataset of The Cancer Genome Atlas (TCGA) for GBM by cBioPortal, in which expression levels of several miRNAs have been subdivided into 2 groups according to their median values. The expression of miRNAs was associated with OS of 592 GBM patients treated with the postsurgery first-line protocol including radio- and chemotherapy. Seven of the 10 miRNAs under investigation have already been included in the GBM dataset of TCGA, but only 2 of them have been significantly associated with prolonged OS, namely miR-17-5p and miR-222-3p (Supplementary Table 5). Nevertheless, the expression level associated with favorable prognosis was the opposite to that observed in our patients treated with regorafenib, whereby prolonged OS can be observed at high levels of miR-17-5p and low levels of miR-222-3p in the dataset of TCGA. Overall, miR-93-5p, miR-203a-3p, miR-101-3p, miR-301a-3p, and miR-23b-3p do not seem to be associated with OS of GBM patients undergoing first-line therapy.

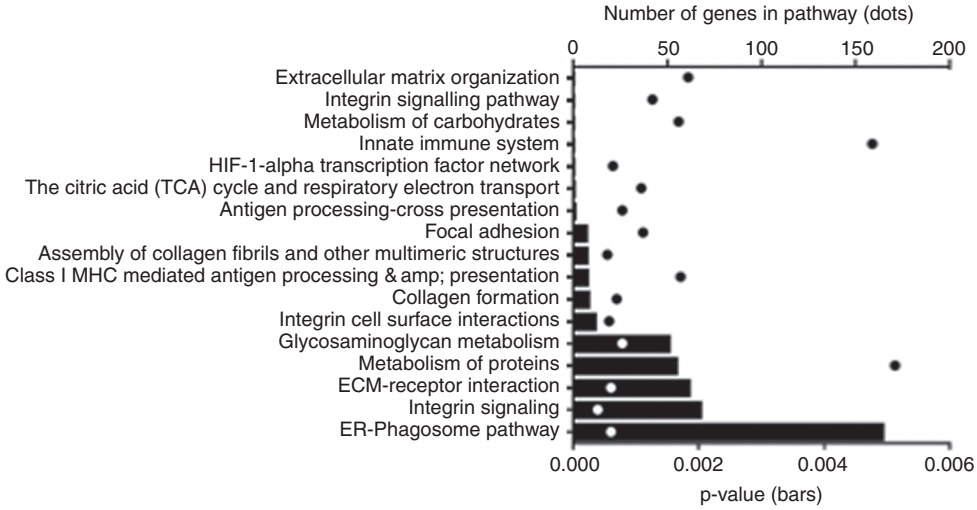
To understand whether the 10 selected miRNAs could further indicate patients with selective advantage, we compared the OS survival of patients enrolled in the regorafenib arm after stratification according to high and low expression levels of the 10 miRNAs. Significant differences were observed in median OS for patients treated with regorafenib and low expression of miR-93-5p, miR-3607-3p, and miR-301a-3p (log-rank test P = 0.040, 0.018, and 0.013, respectively) (Supplementary Table 6). Interestingly, the median OS was prolonged by several months (median OS range of 3 miRNAs: 12.2–14.6 months) compared with patients with high expression of the 3 miRNAs (median OS range of the 3 miRNAs: 7.1–7.6 mo). The graphical representation of Kaplan–Meier curves for OS according to expression levels of miR-93-5p, miR-3607-3p, and miR-301a-3p is shown in Fig. 4C–E. Parallel Kaplan–Meier analysis of PFS did not reach statistical significance (data not shown). In order to verify whether the association between expression of the 3 miRNAs and OS was specifically linked to regorafenib treatment, we analyzed Kaplan–Meier curves for OS in patients treated with lomustine. The expression levels of the 3 miRNAs in lomustine arm did not discriminate patients with significantly different OS (Supplementary Table 7). In summary, these results suggest a mini-signature of miR-93-5p, miR-3607-3p, and miR-301a-3p that could identify patients with clear OS advantage when treated with regorafenib.

Pro-Angiogenic Gene Pathway and Survival in Regorafenib Treatment

Survival analyses presented so far, based on the selection criteria described in Fig. 1, allowed identification of 11 gene transcripts and 10 miRNA associated with significantly prolonged OS in patients affected by GBM when treated with the anti-angiogenic drug regorafenib. To assess whether the transcriptome profile associated with the prolonged survival upon regorafenib treatment could be related to a miRNA-mediated epigenetic regulation, we verified

whether the 11 selected gene transcripts were targets of the 10 miRNAs utilizing the MiRTARBase algorithm (http://miRTarBase.mbc.nctu.edu.tw).[14] *HIF1A* was identified as a target of miR-101-3p and miR-93-5p, *CDKN1A* of miR-101-3p, miR-17-5p, miR-203-3p, and miR-93-5p, *WDR1* of miR-17-5p and miR-93-5p (Fig. 5B), indicating a potential

A



B

	let-7c-3p	miR-101-3p	miR-17-5p	miR-203a-3p	miR-222-3p	miR-23b-3p	miR-301a-3p	miR-3607-3p	miR-6516-3p	miR-93-5p
HIF1A		–								–
CTSK										
SLC2A1										
KLHL12										
CBR4										
CDKN1A		–	–	–						–
CA12										
NIFK-AS1										
WDR1			–							–
RAB30-DT										
CD53										

C

	let-7c-3p	miR-101-3p	miR-17-5p	miR-203a-3p	miR-222-3p	miR-23b-3p	miR-301a-3p	miR-3607-3p	miR-6516-3p	miR-93-5p
HIF1A		–								–
TIMP2		–	–				–			
CDH5		–								
CXCL12			–							
MDK			–							
S1PR1			–							
MMP14			–	–						
COL4A2			–							
CXCL8			–	–		–				–
FLT1			–							
THBS1			–							
CXCL2			–	–						–
AMOT			–							
VEGFA			–	–						–

Fig. 5 Pathway enrichment analysis and miRNA target genes. (A) Pathway enrichment analysis of genes significant in patients with significantly prolonged survival. Genes targeted by the 10 identified miRNAs predictive of response (B) from genome-wide transcriptome profiling and (C) from angiogenesis-related genes listed.

interesting interplay between miRNAs and mRNAs associated with the response to regorafenib.

Considering the broad molecular targets of regorafenib as a multikinase inhibitor, we investigated which molecular pathway could be related to the specific survival advantage provided by this drug, by performing a pathway enrichment analysis. With this aim we selected a set of significant genes using less stringent filtering criteria than those previously described, that is, including the gene transcripts showing both OS and PFS with statistical significance at $P \leq 0.05$ (instead of $P \leq 0.01$) in the Kaplan–Meier analyses between the regorafenib and lomustine arms. Pathway analysis ranked on *P*-values showed the enrichment of several processes closely related to angiogenesis, such as *HIF1A* transcription factor network, extracellular matrix organization, integrin signaling pathway, and others related to more general aspects of tumor biology, such as metabolism of carbohydrates, innate immune system, antigen processing (Fig. 5A). With the aim of analyzing more in-depth the angiogenesis-related genes (besides the already mentioned *HIF1A*), we investigated further 39 genes with log-rank significance of $P \leq 0.05$ and classified as angiogenesis-related from the literature (Supplementary Table 9). Of these, 15 genes were potential targets of the 10 selected miRNAs according to MiRTARBase predictions (Fig. 5C and Supplementary Table 8). Many of them were found as potential targets of miR-17-5p. MiR-93-5p was a predicted regulator of *VEGFA*, C-X-C motif chemokine ligand 8 (*CXCL8*), and *CXCL2* transcripts, in addition to *HIF1A*, whereas miR-301a-3p was predicted regulator of metalloproteinase 2 (TIMP2) (Fig. 5C). Considering the relevance of miR-93-5p and miR-301-3p in the mini-RNA-signature identified by this study, we plotted the Kaplan–Meier plots of VEGFA, CXCL8, CXCL2, and TIMP2 mRNAs and observed that high levels of expression of these 4 transcripts were associated with a statistically significant prolonged OS in the patients treated with regorafenib (Fig. 6).

Discussion

Here we report that elevated expression levels of *HIF1A* mRNA and *CDKN1A* mRNA as well as reduced expression levels of miR-93-5p, miR-3607-3p, and miR-301a-3p in tumor tissue at first surgery are capable of identifying a subgroup of patients treated with regorafenib with favorable benefit. Although descriptive in nature due to the small sample size, our analyses suggest inclusion of this biomarker signature in future replication studies with large cohorts of GBM patients for confirming the efficiency of these biomarkers in supporting the clinical decision on regorafenib use.

That high expression of the pro-angiogenic *HIF1A* is associated with a better OS in patients treated with regorafenib could be explained considering the major anti-angiogenic action of this drug.[3,4] HIF1A is the alpha subunit of the heteromeric HIF, which has been widely recognized as a master regulator of tumor angiogenesis, proliferation, and metabolism in several malignancies, including GBM.[15] Expression and activation of *HIF1A* is mainly regulated by hypoxia but also upon phosphorylation by several kinases, such as phosphatidylinositol-3 kinase, protein kinase B (AKT), MAPK, and ERK,[16–19] which can be potentially targeted by the inhibitory effect of regorafenib.[3–5] *CDKN1A* (alias p21/Cip1/Waf1), a cyclin-dependent kinase inhibitor transcriptionally regulated by p53-dependent and several p53-independent pathways, plays different roles besides cell cycle arrest, such as cell migration, invasion, cytoskeletal dynamics, apoptosis, reprogramming of induced pluripotent stem cells, and autophagy, and it is believed to act either as tumor suppressor or oncogene depending on the cellular context.[20,21]

Considering also the other 9 gene transcripts filtered in the first step, *CTSK* (cathepsin K) is a cysteine protease overexpressed in GBM involved in tissue invasion and angiogenesis.[22] *SLC2A1* (solute carrier family 2 member 1 alias glucose transporter-1/GLUT1), involved in many malignancies, is expressed on perivascular and pseudopalisaded cell membranes in GBM tissues.[23] Interestingly its expression can be regulated by TRPC 6 (transient receptor potential channel 6) and HIF1A-related induction mechanisms aimed to increase glucose transport in hypoxic conditions.[24] The role of high levels of *KLHL12* (Kelch like family member 12 alias *C3IP1*) in GBM has not been described previously. Recalling that *KLHL12* intervenes in pro-collagen secretion,[25,26] a potential role could be devised in GBM where overexpression of collagen has been found associated with worse prognosis, the remodeling of collagen architecture being strictly involved in GBM angiogenesis.[27,28] *CBR4* (carbonyl reductase 4 alias *SDR45C1*) intervenes in activation or inactivation of endogenous signaling molecules (eg, steroids, prostaglandins, biogenic amines) and inactivation of xenobiotics and drugs.[29] Little is known of *CBR4* in malignancies, although reduced expression of carbonyl reductases has been associated with worse prognosis and metastasis in lung and ovarian cancers, whereas its potential implication in GBM is novel.[30,31] *CA12* (carbonic anhydrase 12/XII, carbonic dehydratase) catalyzes the reversible hydration of carbon dioxide into bicarbonate and protons. In tumor biology, *CA12* is overexpressed in the hypoxic milieu counteracting acidosis.[32] *CA12* has been found overexpressed in GBM and preliminary investigation on drug inhibitors was found to delay GBM growth.[33,34] *WDR1* (WD repeat domain 1), a gene encoding a protein with 9 WD amino acid repeats, induces disassembly of acting filaments intervening in cytokinesis and potentially in tumor cell invasion.[35] A strong prognostic role of high *WDR1* expression has been already reported upon TCGA and genome-wide analyses in GBM, and the results presented here further stress the interest in this gene as a risk target.[36,37] *CD53* (alias *TSPAN25*) is a member of the tetraspanin family mediating signal transduction events that play a role in the regulation of cell development, activation, growth, and motility. Although never reported in GBM, *CD53* has been indicated as a tumor-initiating marker in cancer stem cells.[38] *NIFK-AS1* and *RAB30-DT* transcribe 2 long noncoding RNA (lncRNA), a family of molecules gaining increasing interest in cancer.[39] Although these 2 lncRNAs have not been reported in GBM so far, NIFK-AS1 lncRNA has been involved in cancer by inhibiting M2 macrophage polarization.[40]

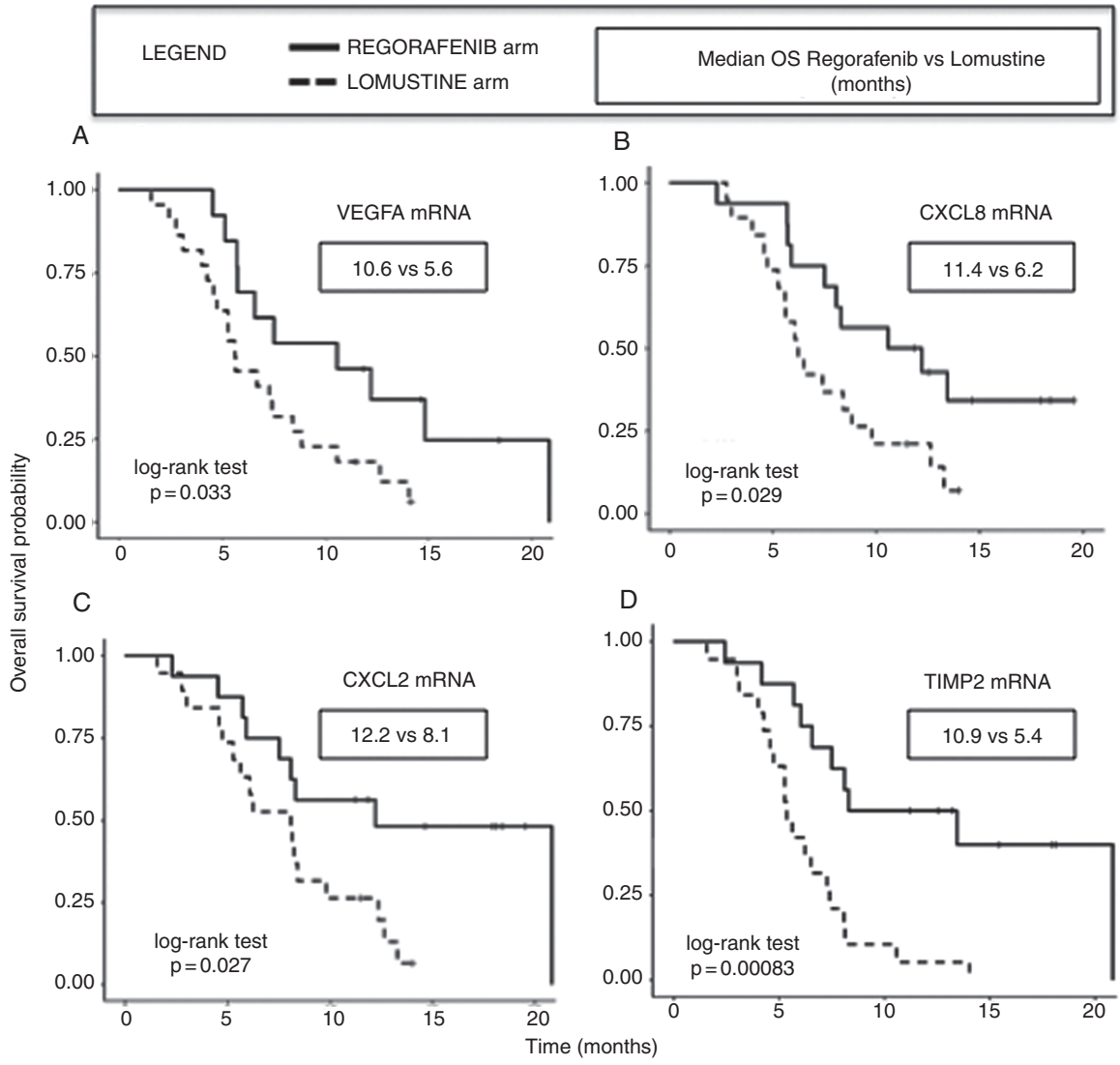


Fig. 6 Kaplan–Meier plots for OS probability of VEGFA, CXCL8, CXCL2, and TIMP2 mRNAs. Significant survival curves of patients in the regorafenib (solid line) versus lomustine (dashed line) arms of gene transcripts with high expression, (A) *VEGFA*, (B) *CXCL8*, (C) *CXCL2*, (D) *TIMP2*. Median OS in months of regorafenib arm versus lomustine arm is reported in the boxes.

Recalling the anti-angiogenic effect of regorafenib, the involvement of angiogenesis-related genes is conceivable. The approach utilized here allowed identification of different genes involved in angiogenesis (eg, *HIF1A, CTSK, KLHL12*) or at least modulated in the hypoxic milieu (eg, *SLC2A1, CA12*). Interestingly, exploring further the issue of angiogenesis relation in a wider list of angiogenesis-related genes (Supplementary Table 9), we found that 4 genes of the list are targeted by the 3 miRNA-signature (Fig. 5C), namely *VEGFA, CXCL8, CXCL2*, and *TIMP2*, which all presented a survival advantage as a function of their expression (Fig. 6). Among these 4 genes, *CXCL8* deserves a particular interest in our viewpoint. The expression of interleukin (IL)-8 in GBM tumor tissue has been found close to the areas of hypoxic necrosis, similarly to VEGFA.[41] Besides glial cells, other components of the complex GBM microenvironment can contribute to IL-8 release in the tumor milieu, including macrophages, microglia, neutrophils, and lymphocytes.[42] This would make CXCL8 an important player in the development or progression of GBM. Many of the effects of CXCL8 in GBM tissue are mediated by its binding to CXCR1/2 receptors expressed on the endothelial cells. In this setting CXCL8 can promote different angiogenic properties, such as endothelial cell proliferation, chemotaxis, survival, and production of metalloproteases.[42] Focusing on the molecular action of regorafenib, it could be recalled that the pro-angiogenic effects induced by the binding of IL-8 to CXCR1 and CXCR2 are mediated through a sharp activation of MAPK ERK1/2.[43] Their inhibition, mediated by regorafenib, may provide a hypothetical advantage in case of CXCL8 overexpression.

We found an OS advantage of similar magnitude in patients with lower expression levels of miR-93-5p, miR-3607-3p, and miR-301a-3p. This survival advantage was clearly associated with regorafenib, but the advantage was virtually insignificant in patients in the lomustine arm. Different molecular targets have been identified for miR-3607-3p and miR-301a-3p, although to the best of our knowledge, they have not been reported in the GBM literature so far. Unlike miR-3607-3p and miR-301a-3p, it should be stressed that miR-93-5p has been already extensively investigated in gliomas. Elevated expression of miR-93-5p in GBM promotes cell proliferation and angiogenesis, these properties explaining in principle a survival advantage in those GBMs with low expression of miR-93.[44,45] Interestingly, we previously found that miR-93-5p is an epigenetic downregulator not only of CXCL8 but also of VEGFA.[46] Moreover, both genome-wide trascriptome profiling and the further analysis of a subset of angiogenesis genes highlight that downregulation of miR-93-5p is mirrored by upregulation of several target gene transcripts (eg, *HIF1A*, *CDKN1A*, *WDR1*, *CXCL8*, *CXCL2*, *VEGFA*) associated with prolonged survival in patients treated with regorafenib, which supports the interest in verifying miR-93-5p in future replication studies to select GBM patients who may benefit more from treatment with regorafenib.

In conclusion, even though the 11 genes selected from the transcriptome profiling are potentially relevant to different aspects of GBM tumor biology (eg, angiogenesis, proliferation, invasion) and their expression/action can be mediated by different kinases that are known to be inhibited by regorafenib, gaining insights into the mechanisms providing a survival advantage in the subgroups of patients treated with this drug will require future experimental preclinical and clinical investigation. Thus, these results must be validated in future clinical trials, possibly testing different cutoff strategies such as quartiles and continuous variables instead of medians, which we utilized here to avoid reducing the power of the statistical analysis, to support the stratification of patients into subgroups with different survivals. For example, the mutational status of key cancer genes can provide additional insights into the pathways involved. In addition, MRI and fluoroethyltyrosine PET data could be correlated with the gene transcripts proposed in this paper to allow the identification of patients more responsive to regorafenib. Moreover, it could be of interest to investigate, in patients undergoing tissue biopsy at progression, whether the proposed molecular signature is maintained over time or will change due to the effects of first-line chemo- and radiotherapy. Further validation of these biomarkers is needed to confirm their usefulness in the clinical decision between regorafenib and other target therapies in patients with relapsing GBM.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Keywords

CDKN1A | *HIF1A* | glioblastoma | miR-93-5p | regorafenib

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