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 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 miRNA profiles were associated with patients' Overall Survival (OS) and Progression Free Survival (PFS).

Results. At 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. As second step, a minisignature of two gene transcripts (*HIF1A, CDKN1A*) and three 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 months).

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

Keywords: glioblastoma, regorafenib, miR-93-5p, *HIF1A*, *CDKN1A*

Keypoints:

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 glioblastoma (GBM), regorafenib has gained interest after the REGOMA clinical trial reported an overall survival advantage of the patients of the regorafenib arm compared to 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 identifying 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.

Introduction

The standard of care for glioblastoma (GBM), the most common and severe brain malignancy in adults, is based on maximal surgical resection followed by radio-chemotherapy. 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 post-surgical 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 VEGFR 1-3, PDGF Receptor (PDGFR), FGF Receptor (FGFR). An inhibitory effect has also been reported against kinases of Mitogen-Activated Protein Kinases (MAPKs) family such as Extracellular signal Regulated Kinases (ERKs) 1/2, MAP kinase kinase (MEK) 1-2, which are involved in tumor angiogenesis and in controlling tumor micro-environment and tumor immunity.[3-5] After extensive pre-clinical investigation, regorafenib has been approved for treatment of patients with metastatic colorectal cancer, gastro-intestinal tumors and hepatocellular carcinoma.[7-8] Concerning brain tumors, regorafenib has suggested to inhibit proliferation and angiogenesis in tumor cell models *in vitro* and *ex vivo*, thus providing a pre-clinical rationale for use in these malignancies.[9-10] 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).[11] The OS was improved in the regorafenib group compared with the lomustine group (i.e., 7.4 versus 5.6 months, respectively),[11] 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 into NCCN 2020 guidelines v 1.2020 for Central Nervous System Cancers as 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.[11]

MicroRNAs (miRNAs) are a family of small (19 to 25 nucleotides in length) non-coding RNAs playing an important role in post-transcriptional control of gene expression. The miRNA-dependent recognition of 3'-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.[12] Dysregulation of miRNAs 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 insights that differential expression of miRNAs underlies on 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.[13]

With the aim of identifying a signature potentially predictive of response to therapy with regorafenib, we have hence 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 two 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 have been published elsewhere.[11] Clinical features of the patients included in this study are reported in Supplementary sTable 1. Genome-wide miRNA and mRNA biomarker analysis 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 regorafenib arm and 36 in lomustine arm, respectively.

Ethics statement

All participating centres 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 Istituto Oncologico Veneto.

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 MiRNeasy FFPE minikit (Qiagen, Venlo, Netherlands). RNA was quantified with Qubit 3.0 and Qubit RNA HS Assay kits (Applied Biosystems).

mRNA and miRNA profiling by RNAseq analysis

RNAseq libraries were prepared from 100 ng of total RNA using the QuantSeq 3'mRNA-Seq Library Prep Kit-FWD (Lexogen, Vienna, Austria) 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 1,400 miRNAs and were detected as expressed in each sample (Supplementary sFigure1). The detailed RNAseq protocols and data analysis pipelines are described in the Supplementary material and method section. RNA-sequencing data presented in this study have been deposited in NCBI's 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 Tissue 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 was associated with OS data of patients undergoing first-line therapy with post-surgery radio-chemotherapy. To explore the prognostic power of the miRNAs, subjects were divided in two groups as a function of expression of each miRNA as above (HIGH) or below (LOW) median levels. Median OS was calculated from Kaplan-Meier (K-M) curves and log-rank test was applied for statistical significance.

Statistical analysis

The statistical analysis 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 cut-off has been defined in the literature for mRNA and miRNAs expression in GBM tissue, mRNA and miRNA median expression levels were arbitrarily chosen for separating samples in two groups, thus subjects were assigned to "HIGH" or "LOW" subgroups in reference to the median values. For each of the two 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 flow-chart of Figure 1. A list of biomarkers from K-M 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 K-M curves for OS only within the regorafenib arm, resulting in a more restricted mRNA and miRNA minisignature (Fig. 1).

Results

Identication 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).[11] The clinical characteristics were homogeneous between the group of patients enrolled in the regorafenib and lomustine arms as well as with the clinical set of the REGOMA study (Supplementary sTable 1). Sequencing data and the number of detected genes were consistent between regorafenib and lomustine groups (Supplementary sFigure 1). In order to select

those gene transcripts with potential predictivity on patients' survival, we associated the expression levels of each mRNA with patients' Overall Survival (OS) and Progression Free Survival (PFS), as depicted in Figure 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, CD53* mRNAs and low expression of *CBR4, NIFK-AS1, RAB30-DT* mRNAs (Figure 2) (Supplementary sTable 2). The median OS in the regorafenib arm was ranging from 10.6 to 20.8 months compared to 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 sTable 2). Focusing on OS, these results strengthened the findings previously reported in the REGOMA trial where a more favourable survival was observed in the whole group of patients treated with regorafenib (median OS 7.4 months) compared to the group treated with lomustine (median OS 5.6 months).[11] Thus, we verified whether the expression analysis of the 11 selected mRNAs could further help indicating subgroups of patients with a selective advantage. At 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 HIF1A and CDKN1A mRNA (log-rank test p 0.0011 and 0.00083, respectively) (Fig. 4A and B). Interestingly, the median OS in high *HIF1A* and *CDKN1A* expression subgroups was prolonged by several months (median OS 20.8 months) as compared to that of patients with low gene expression (median OS 5.9 and 6.0 months, respectively). Parallel Kaplan-Meier analysis of PFS did not reach statistical significance (data not shown). Collectively, transcriptome analysis of tumour tissue at first surgery identified HIF1A and CDKN1A mRNAs expression as a molecular minisignature 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,[14] 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 (Figure 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, 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 to 5.5-7.3 months in lomustine arm, with median OS in regorafenib arm enhanced from 3.3 to 7.9 months (Supplementary sTable 3). Similar results were observed for PFS (Supplementary sTable 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 sTable 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, that are associated with prolonged OS in regorafenib-treated patients, could be correlated with a better prognosis regardless of regorafenib treatment. We interrogated the dataset of the Tissue Cancer Genome Atlas (TCGA) for GBM by cBioPortal, in which expression levels of several miRNAs has been subdivided in two groups according to their median values. The expression of miRNAs was associated with OS of 592 GBM patients treated with the post-surgery first-line protocol including radio- and chemo-therapy. Seven of the ten miRNAs under investigation have already been included in the TCGA dataset for GBM, but only two of them have been significantly associated with prolonged OS, namely miR-17-5p and miR-222-3p (Supplementary sTable 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

TCGA dataset. Overall, miR-93-5p, miR-203a-3p, miR-101-3p, miR-301a-3p, 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 sTable 6). Interestingly, the median OS was prolonged by several months (median OS range of 3 miRNAs: 12.2-14.6 months) compared to patients with high expression of the 3 miRNAs (median OS range of the 3 miRNAs: 7.1-7.6 months). 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 Figure 4 C, D and 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 sTable 7). In summary, these results suggest a minisignature 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 Figure 1, allowed to identify 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 if the 11 selected gene transcripts are target of the 10 miRNAs utilizing the MiRTARBase algorithm

(http://miRTarBase.mbc.nctu.edu.tw).[15] *HIF1A* was identified as 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 (Figure 5B), indicating a potential interesting interplay between miRNAs and mRNAs associated with the response to regorafenib.

Considering the broad molecular targets of regorafenib as 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. At this aim we selected a set of significant genes using less stringent filtering criteria than those previously described, i.e. 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 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 other related to more general aspects of tumor biology, such as metabolism of carbohydrates, innate immune system, antigen processing (Figure 5A). With the aim to analyze 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 sTable 9). Of these, 15 genes were potential targets of the 10 selected miRNAs according to MiRTARBase predictions (Figure 5C and Supplementary sTable 8). Many of them were found as potential targets of miR-17-5p. MiR-93-5p was a predicted regulator of *VEGFA*, *CXCL8* and *CXCL2* transcripts, in addition to *HIF1A*, whereas miR-301a-3p of TIMP2 (Figure 5C). Considering the relevance of miR-93-5p and miR-301-3p in the minimiRNA-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 (Figure 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 to identify a subgroup of patients treated with regorafenib with favorable benefit. Although descriptive in nature due to the small sample size, our analyses suggest to include 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* (Hypoxia Inducible Factor) 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 Hypoxia Inducible Factor (HIF), which has been widely recognized as a master regulator of tumor angiogenesis, proliferation and metabolism in several malignancies, including GBM.[17] Expression and activation of *HIF1A* is mainly regulated by hypoxia but also upon phosphorylation by several kinases, such as phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), Mitogen Activated Protein Kinases (MAPK) Extracellular Regulated Kinase (ERK),[18-21] that can be potentially targeted by the inhibitory effect of regorafenib.[3-5] *CDKN1A* (Cyclin Dependent Kinase Inhibitor 1A, *alias* p21/Cip1/Waf1) is a cyclin-dependent kinase inhibitor, transcriptionally regulated by p53-dependent and several p-53-independent pathways, playing 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.[22-23]

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.[24] *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.[25] Interestingly its expression can be regulated by Transient Receptor Potential Channel (TRPC) 6 and HIF1A-related induction mechanisms aimed to increase glucose transport in hypoxic conditions.[26] 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,[27-28] a potential role could be devised in GBM where overexpression of collagen has been found associated with worse prognosis, the remodelling of collagen architecture being strictly involved in GBM angiogenesis.[29-30] *CBR4* (Carbonyl Reductase 4 *alias SDR45C1*) intervenes in activation or inactivation of endogenous signaling molecules (e.g. steroids, prostaglandins, biogenic amines) and inactivation of xenobiotics and drugs.[31] Little is known on *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.[32-33] *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.[34] *CA12* has been found overexpressed in GBM and preliminary investigation on drug inhibitors was found to delay GBM growth.[35-36] *WDR1* (WD Repeat Domain 1), a gene encoding a protein with 9 WD aminoacid repeats, induces disassembly of acting filaments intervening in cytokinesis and potentially in tumor cell invasion.[37] 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 on this gene as a risk target.[38-39] *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.[40] *NIFK-AS1* and *RAB30-DT* transcribe two long non-coding RNA (lncRNA), a family of molecules gaining increasing interest

in cancer.[41] Although these two lncRNAs have not been reported in GBM so far, NIFK-AS1 lncRNA has been involved in cancer by inhibiting M2 macrophage polarization.[42]

Recalling the anti-angiogenic effect of regorafenib, the involvement of angiogenesis-related genes is conceivable. The approach utilized here allowed to identify different genes involved in angiogenesis (e.g. *HIF1A*, *CTSK*, *KLHL12*) or at least modulated in the hypoxic milieu (e.g. *SLC2A1*, *CA12*). Interestingly, exploring further the issue of angiogenesis-related in a wider list of angiogenesis-related genes (Supplementary sTable 9) we found that four genes of the list are targeted by the 3 miRNA-signature (Fig. 5C), namely *VEGFA*, *CXCL8*, *CXCL2*, *TIMP2*, that all presented a survival advantage as a function of their expression (Fig. 6). Among these four genes, *CXCL8* deserves a particular interest in our viewpoint. The expression of IL-8 in GBM tumor tissue has been found close to the areas of hypoxic necrosis, similarly to VEGFA.[43] 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.[44] This would make CXCL8 an important player in the development or in the 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 cells proliferation, chemotaxis, survival and production of metalloproteases.[44] 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 MAPKs ERK 1/2.[45] Their inhibition, mediated by regorafenib, may provide an hypothetical advantage in case if 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 the regorafenib, being the advantage 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 GBM with low expression of miR-93.[46-47] Interestingly, we previously found that miR-93-5p is an epigenetic down-regulator not only of CXCL8 but also of VEGFA.[48] 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 (e.g. *HIF1A*, *CDKN1A*, *WDR1*, *CXCL8*, *CXCL2*, *VEGFA*) associated with prolonged survival in the 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 (e.g. angiogenesis, proliferation, invasion) and their expression/action can be mediated by different kinases that are known to be inhibited by regorafenib, gaining insights on the mechanisms providing a survival advantage in the subgroups of patients treated with this drug will require future experimental pre-clinical 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, that we utilized here to avoid reducing the power of the statistical analysis, to support the stratification of patient into subgroups with different survival. For example, the mutational status of key cancer genes can provide additional insights on 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 radio-therapy. 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.

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Figure legends

Fig. 1

Outline of study design and biomarker filtering strategy.

Fig. 2

Kaplan-Meier plots for OS probability for levels of expression of gene transcripts. Significant survival curves of patients in 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.

Fig. 3

Kaplan-Meier plots for OS probability as a function of expression levels of miRNAs.

Significant survival curves of patients in 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, **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.

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.

Fig. 6

Kaplan-Meier plots for OS probability of VEGFA, CXCL8, CXCL2 and TIMP2 mRNAs.

Significant survival curves of patients in 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.

Figure 1

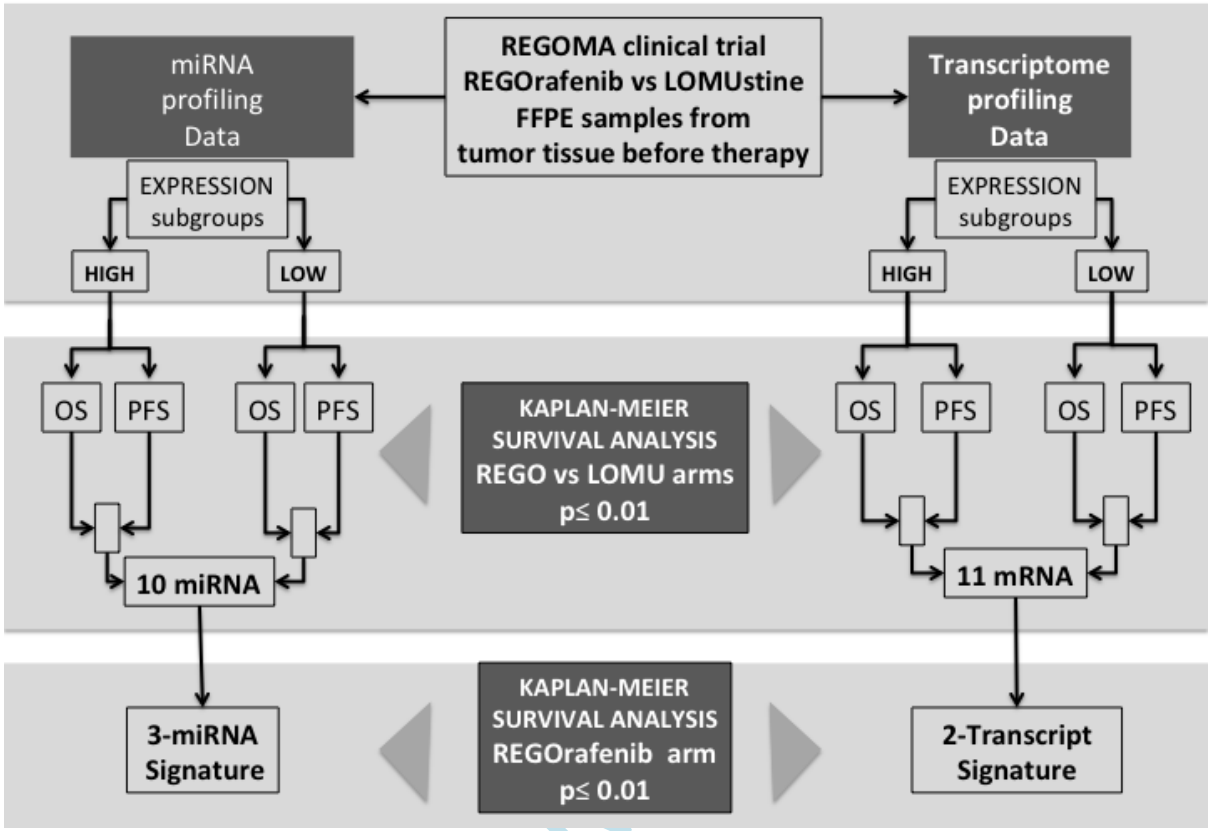


Figure 2

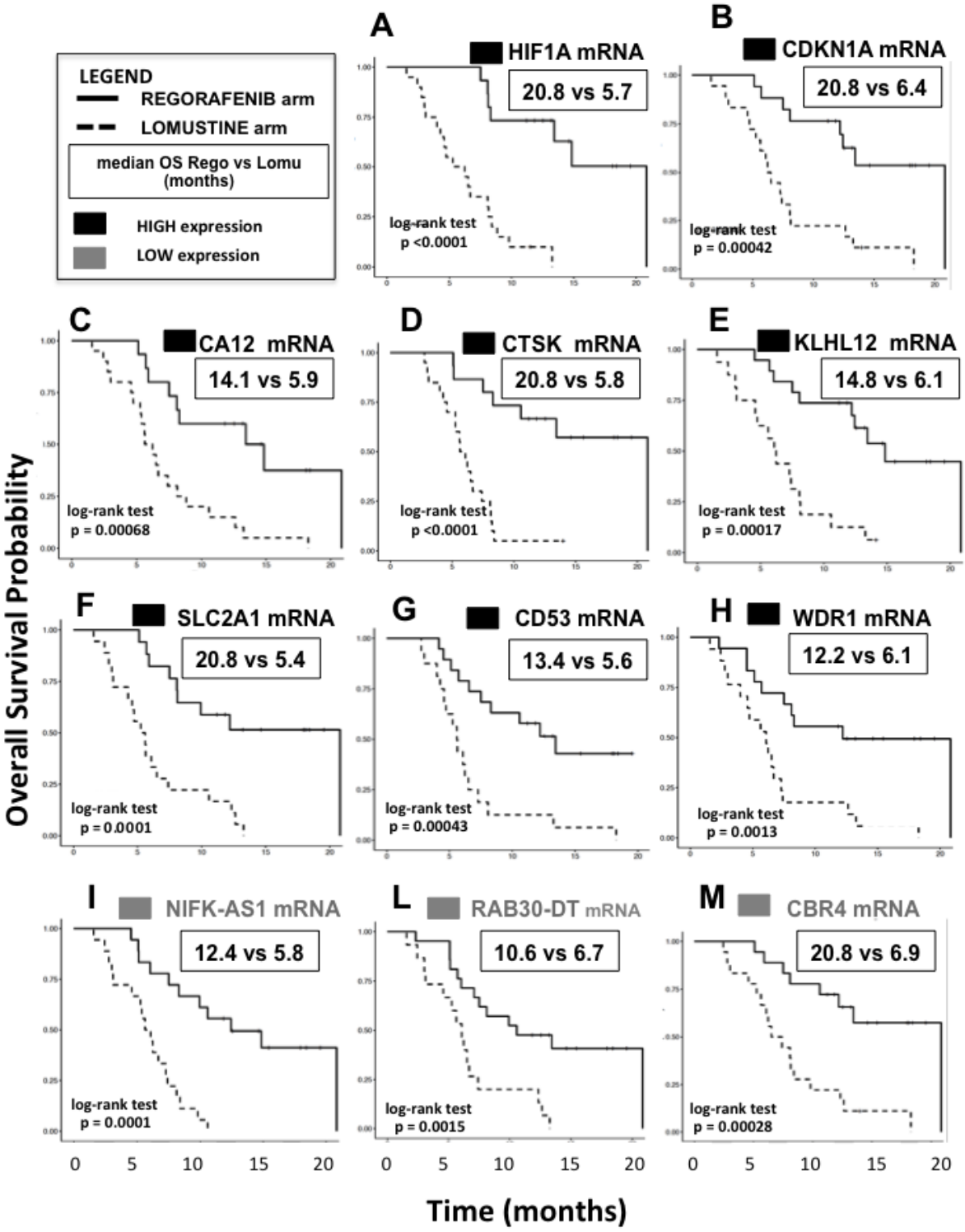


Figure 3

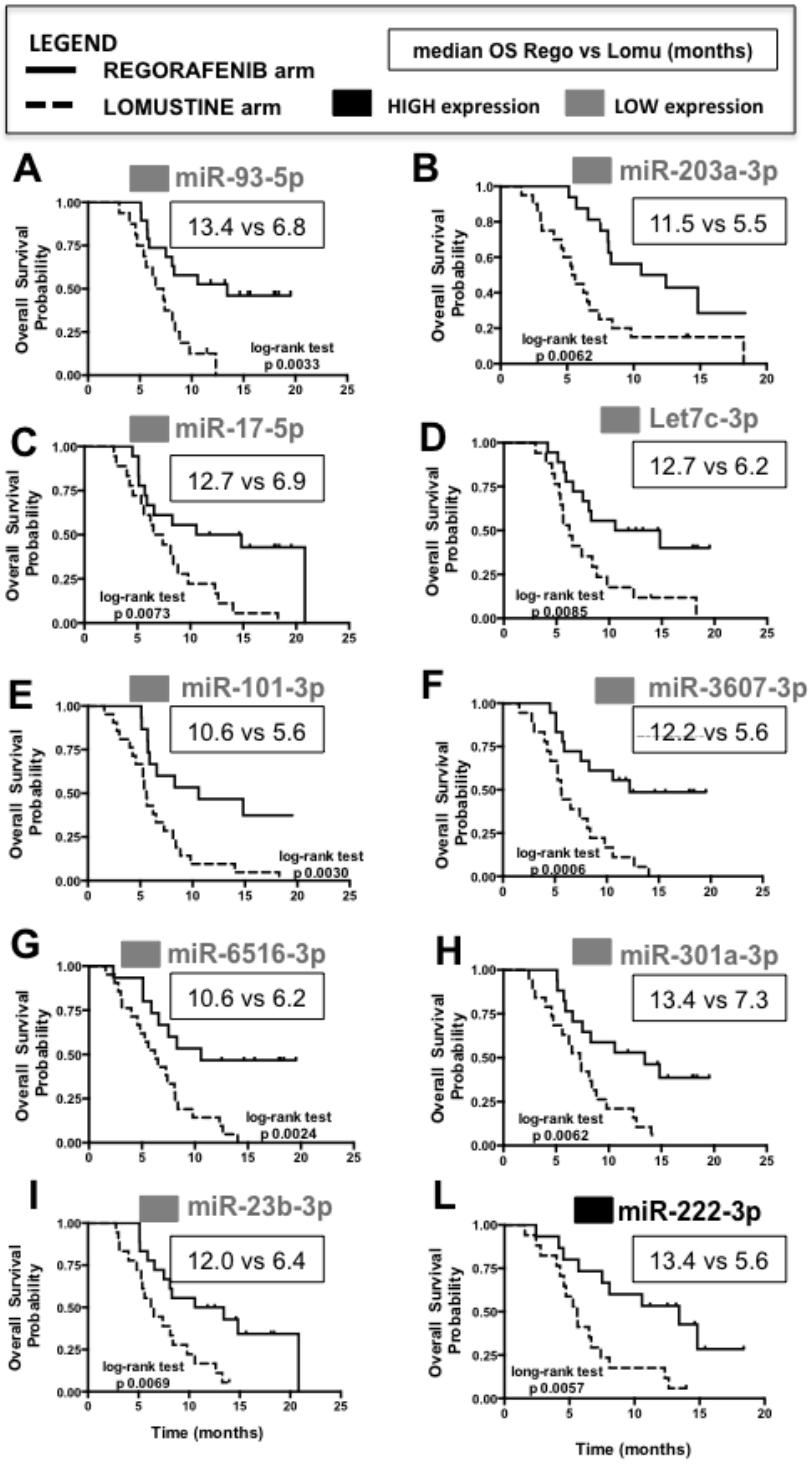


Figure 4

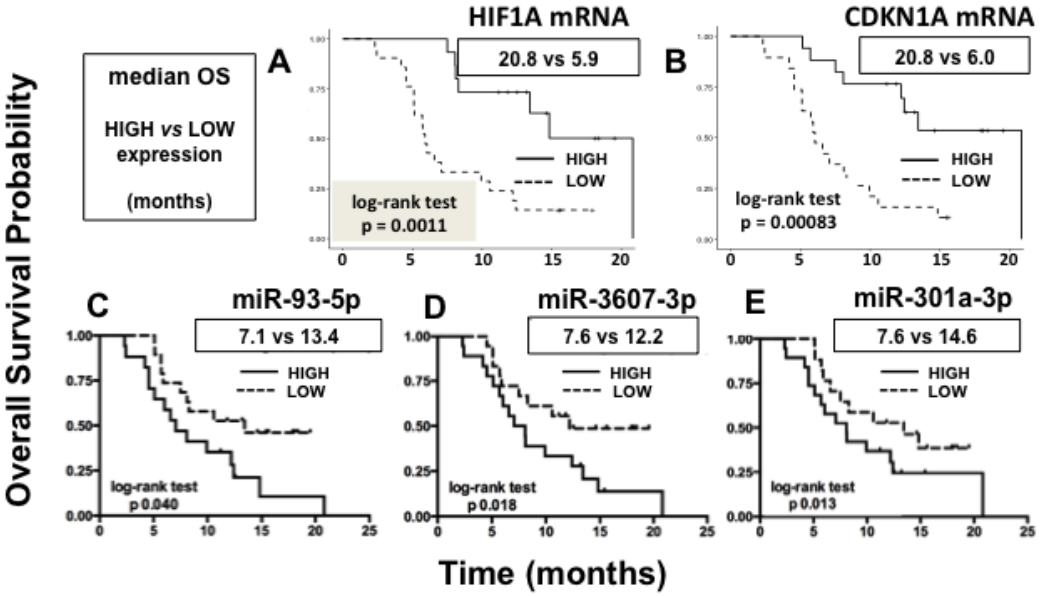
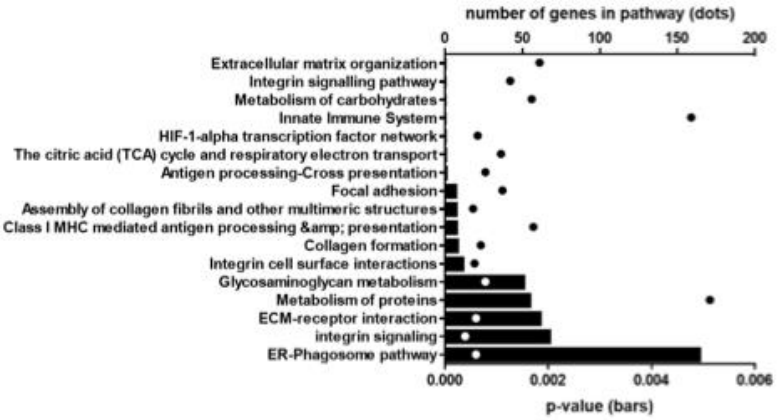


Figure 5

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			-	-						-

Figure 6