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Research Paper

The Prognostic Significance of Combining VEGFA, FLT1 and KDR mRNA Expressions in Brain Tumors

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Abstract

Tumor cells require angiogenesis to deliver nutrients and oxygen to support their fast growth and metabolism. The vascular endothelial growth factor (VEGF) pathway plays an important role in promoting angiogenesis, including tumor-induced angiogenesis. Recent clinical trials have demonstrated the benefit of targeting VEGF in the treatment of glioblastoma. However, the prognostic significance of the expression of *VEGFA* and its receptors VEGFR1 (*FLT1*) and VEGFR2 (*KDR*) are still largely elusive. In the present study, we aimed to investigate the prognostic significance of these three factors, alone or in combination, in glioma patients. Gene mRNA expression was extracted from three independent brain tumor cohorts totaling 242 patients and the association between gene expression and survival was tested. We found that when *VEGFA*, *FLT1* and *KDR* expressions were considered alone, only *VEGFA* demonstrated a significant association with patient survival. Patients with high expression of both *VEGFA* and either receptor had significantly worse survival than patients expressing both factors at a low level. Importantly, we found that those patients whose tumors overexpressed all three genes had a significantly shorter survival compared to those patients with a low level expression of these genes. Our results suggest that a high level expression of *VEGFA* and its receptors, both *FLT1* and *KDR*, may be required for brain tumor progression, and that these three factors should be considered together as a prognostic indicator for brain tumor patients.

Key words: Brain tumor, *VEGFA*, *FLT1*, *KDR*, Angiogenesis, Survival

Introduction

Gliomas are the most common type of primary brain tumors, of which grade 3 astrocytoma and grade IV glioblastoma are highly malignant and are associated with a very poor prognosis [1]. As with other malignancies, the growth and development of gliomas is reliant on the angiogenic process. VEGF is an important factor promoting tumor angiogenesis, and the activation of VEGF pathway requires the binding of the ligand, such as VEGFA, to one of its receptors, such as *FLT1* or *KDR*, thus generating downstream signals to stimulate the proliferation and structural reorganization of endothelial cells [2]. The VEGF family is divided into four members, including *VEGFA*, *VEGFB*, *VEGFC* and *VEGFD*, and VEGF receptors consist of three subtypes, including VEGFR1 (*FLT1*), VEGFR2 (*KDR*), and VEGFR3 [3]. *VEGFA* is a key regulator of developmental vasculogenesis and angiogenesis and it mainly interacts with *FLT1* and *KDR* to generate downstream signaling [3]. *FLT1* is a tyrosine kinase receptor that binds VEGFA with about 10-fold higher affinity than *KDR*, and although it is poorly activated by VEGF ligands, it has been shown

to promote tumor growth and metastasis [3]. On the other hand, VEGF ligand binding to *KDR*, also a tyrosine kinase receptor, generates potent downstream signals that leads to endothelial cell growth and migration [3]. The development of targeted therapy for malignant glioma has had limited success mainly due to the parallel and converging complex interactions between several important pathways, including pathways that regulate tumor angiogenesis [4].

VEGF, *FLT1* and *KDR*, were all shown to be up-regulated in brain tumor vasculature [5], signifying the importance of angiogenesis in glioma. Suppressing expression of VEGF in tumoral cells by anti-sense RNA technology was shown to inhibit the growth of glioma cells in vivo in nude mice [6]. In addition, VEGF also plays an important role in angiogenesis induced by stem cell-like glioma cells [7]. Inhibition of angiogenesis acts synergistically with chemotherapy in reducing the tumor sphere forming ability of brain tumor stem-like cells in glioma xenograft model [8]. Together, these results suggest that the VEGF pathway plays a central role in initiation and progression of glioma, especially in the glioma stem cells.

Bevacizumab is a monoclonal antibody that binds to VEGFA and neutralizes VEGFA from binding to *FLT1* and *KDR*, thereby inhibiting the downstream growth signal that could induce angiongenesis [9]. Indeed, bevacizumab has been shown to be effective in combination with irinotecan for recurrent high grade glioma with a radiographic response noted in around 60% of patients, and a 6-month overall survival of 77% in patients treated with this regimen [10, 11]. Other phase II trials have shown and confirmed that bevacizumab as a single agent or in combination with irinotecan are active and tolerable in patients with recurrent glioblastoma [12, 13]. The Food and Drug Administration in United States approved bevacizumab as a single agent in patients with glioblastoma multiforme (GBM) whose disease had progressed following prior therapy[14]. Recently, the results from a phase II study reported that bevacizumab in combination with lomustine has clinical activity to recurrent GBM [15]. Two studies have assessed the potential of bevacizumab for the treatment of newly diagnosed glioblastoma. Combination of bevacizumab with temozolomide and radiation therapy increased progression-free survival compared to a retrospective control cohort [16], while combination of bevacizumab with other chemotherapeutic agents may improve efficacy in newly diagnosed glioblastoma [17]. The results from two Phase III studies have confirmed that addition of bevaicumab to radiotherapy and chemotherapy led to improvement in progression-free survival but not overall survival [18, 19].

VEGFA, *FLT1* and *KDR* mRNAs are expressed at an elevated level in malignant tumor endothelial compared to normal brain vasculature [20]. Overexpression of *VEGFA*, but not *FLT1*, was seen in high-grade astrocytoma compared to low-grade astrocytoma [21], while increased expression of *FLT1* was observed in grade 4 diffusely infiltrating astrocytomas [22]. Very recently, a SNP located in the 5′ UTR of the *VEGFA* gene, was demonstrated to be associated with higher plasma *VEGFA* concentration and a longer progression-free survival in GBM patients treated with bevacizumab [23].

Despite the unquestionable role of angiogenesis in progression of malignancies of the brain, and despite recognition of VEGF pathway-targeting treatment strategies, expression profiling of the VEGF pathway role-players for brain tumor prognostication has not been done. Recently, we demonstrated the prognostic value of combined expression profiling of *VEGFA*, *FLT1* and *KDR* in lung and colon cancers, in which anti-VEGF therapy has been shown to be effective. In the present study, we aimed to investigate the prognostic significance of these three factors in combination in glioma, whose most malignant subtype, glioblastoma, is susceptible to bevacizumab.

Materials and Methods

Extraction of clinical and microarray gene expression data from glioma patient datasets

Three glioma patient datasets, GSE4271 [24], GSE4412 [25] and GSE7696 [26] were identified in the Gene Expression Omnibus (GEO) Database; datasets using the HG-U133 microarray platform, and comprising >70 patients for whom survival data were available were included in this study. Microarray gene expression data were retrieved from the data matrixes deposited to the GEO database by the original authors. R scripting was used to extract the expression values from probesets of VEGFA, *FLT1* and *KDR*, and the clinical data from the data matrixes downloaded from GEO as previously described [27].

Patient stratification

Expression levels were divided into four groups using quartile as the cut-off points for association analysis. The median expression level was used as the cut-off point for survival analysis. Patients were further divided into three groups based on the expression levels of *VEGFA* and *FLT1*; the *VEGFA*-*FLT1*-low group consisted of patients who expressed both of these two genes at low levels; the *VEGFA*-*FLT1*-high group consisted of patients who expressed both of these two genes at high levels; the

VEGFA-*FLT1*-intermediate group consisted of the rest of patients; patients were similarly stratified into *VEGFA*-*KDR* subgroups. Patients were also divided into four groups based on the number of genes, among *VEGFA*, *FLT1* and *KDR*, that were expressed at a high level.

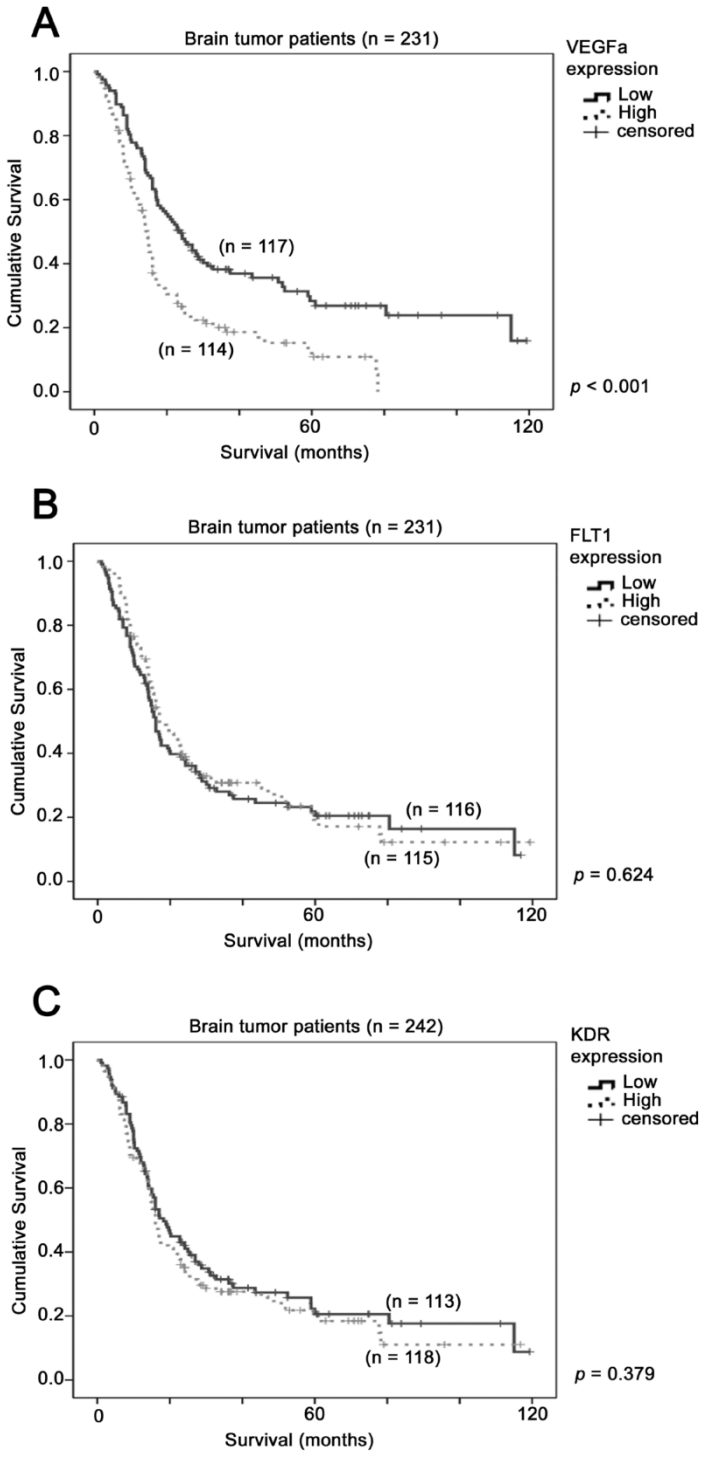

Figure 1. The association between VEGFA mRNA expression and brain tumor patient survival. Kaplan-Meier analyses for mRNA expression of (A) *VEGFA* (B) *FLT1* and (C) *KDR* in the brain tumor patient cohort consisting of 231 patients. P-value for log-rank test is presented alongside each Kaplan-Meier plot.

Correlations of gene expression levels and clinical data

Since the sample size was small, the three patient cohorts were combined and analyzed together. The combined brain tumor dataset comprised 258 patients, 231 of whose survival data were available. Of these 231 patients, 175 were deceased at data collection. Of the 231 samples, 186 were glioblastoma, and the remaining 45 were of other subtypes. All statistical analyses were performed using SPSS19.0. The associations between expression levels of genes were tested by Chi-Square test. For Kaplan-Meier survival analysis, results were compared by log-rank test. Cox regression analysis was used to correlate the gene expression levels and patient survival.

Results

The prognostic significance of VEGFA, FLT1 and KDR in glioma patients

The prognostic significance of *VEGFA*, *FLT1* and *KDR* was tested in a combined dataset comprising a total of 231 patients from GSE4271, GSE4412 and GSE7696. Using median expression level as a cut-off point, we found that patients whose tumors expressed a high level of *VEGFA* had a mean survival of 23 months (95% CI = 18 – 28 months), while those whose tumors expressed a low level of *VEGFA* had a mean survival of 46 months (95% CI = 37 – 54 months), with a Hazard ratio of 1.86 (95% CI = 1.38 – 2.52, $p < 0.001$; Figure 1A). This result suggests that a high level of *VEGFA* in glioma specimens predicted a shorter survival time of glioma patients. On the other hand, the survival time was not significantly different between patients whose tumors expressed different levels of *FLT1* ($p = 0.624$; Figure 1B) or *KDR* ($p = 0.379$; Figure 1C). Together, our results suggest that *VEGFA* is a better prognostic factor for glioma patients.

Association between VEGFA, FLT1 and KDR expression

FLT1 expression level was significantly different among patients expressing various levels of *VEGFA* (Chi Square test, $p < 0.001$; Figure 2A). Around 11% (25 out of 231) of patients expressed both *VEGFA* and *FLT1* at a very high level and around 10% (23 out of 231) of patients expressed both *VEGFA* and *FLT1* at a low level, while only 3% (6 out of 231) of patients who expressed *VEGFA* at a low level expressed *FLT1* at a very high level and only 6% (13 out of 231) of patients who expressed *VEGFA*

at a very high level expressed *FLT1* at a low level. Similar results were observed when the association between *VEGFA* and *KDR* expression levels was tested (Chi Sqaure test, $p = 0.037$; Figure 2B). Merely 3% (6 out of 231) of patients who expressed *VEGFA* at a low level expressed *KDR* at a very high level, but around 18% patients expressed these two genes both at a very high level or both at a low level. The expression levels between the two receptors, *FLT1* and *KDR*, were significantly associated (Chi Square test, $p < 0.001$; Figure 2C). As shown in Figure 2C, only 4% patients expressed *FLT1* at a low level and *KDR* at a very high level, and vice versa, but 29% of patients expressed *FLT1* and *KDR* at a very high level, and at a low level, respectively. Our results suggest that these three factors were co-expressed at a high level in glioma specimens leading us to further investigate the prognostic significance of the co-expression of these factors.

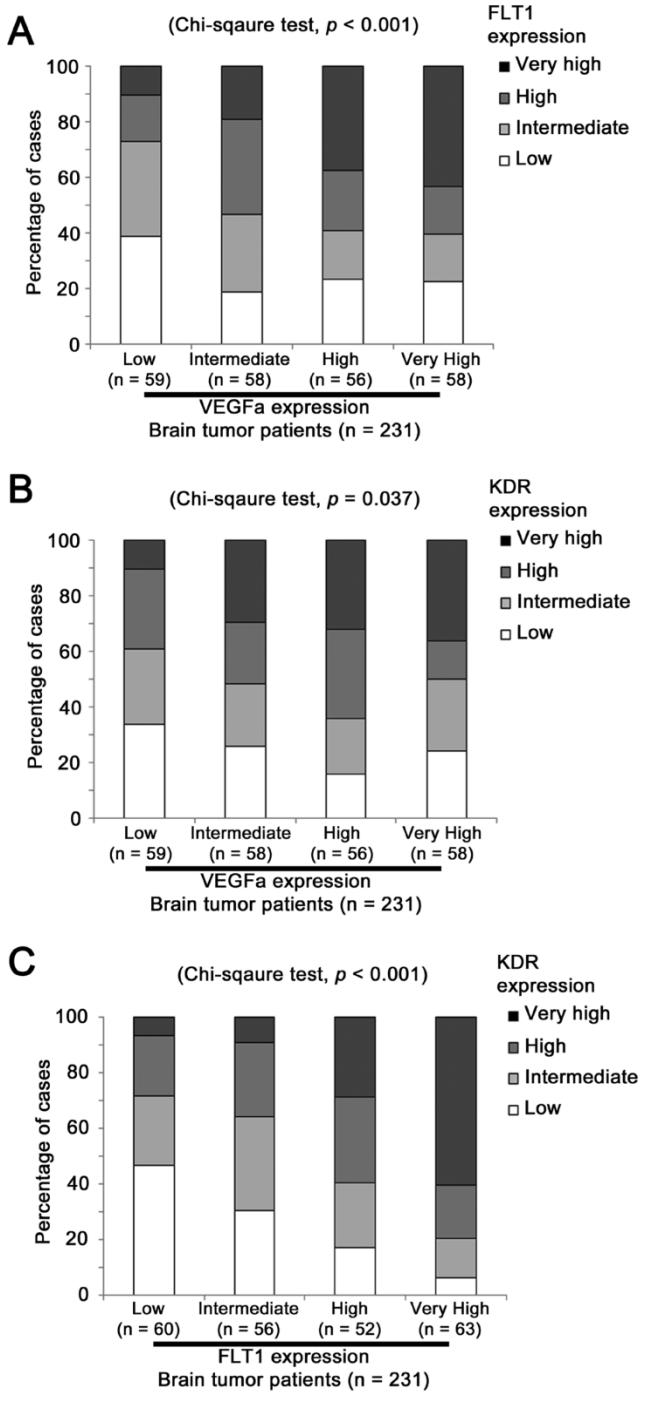

Figure 2. The association between VEGFA, FLT1 and KDR mRNA expression in brain tumor patients. Histograms showing the percentage of patients whose tumor expressed different levels of (A) *VEGFA* and *FLT1*, (B) *VEGFA* and *KDR* and (C) *FLT1* and *KDR*.

High level expression of both VEGFA and FLT1 mRNA in the same patients predicts shorter survival outcome

Although *FLT1* alone did not predict patient survival in our combined glioma dataset, the molecular mechanism for *VEGFA-FLT1* signaling led us to hypothesize that glioma patients whose tumors expressed high levels of both *VEGFA* and *FLT1* may have the worst prognosis. To investigate this hypothesis, we stratified the patients into three groups based on the levels of expression of *VEGFA* and *FLT1*. As shown in Figure 3, patients whose tumors expressed both *VEGFA* and *FLT1* at low levels had a mean survival of 43 months (32 – 53 months), patients whose tumors expressed one of these two genes at high level had a mean survival of 37 months (28 – 46 months), and patients whose tumors expressed both genes at high level had a mean survival of 24 months (18 – 31 months), which was significantly shorter than that of the *VEGFA-FLT1*-low group of patients ($p = 0.017$). Patients whose tumors expressed both genes at high level had a significantly higher risk of death than those whose tumors expressed both genes at low level with a hazard ratio of 1.57 (95% CI = 1.06 – 2.31, $p = 0.023$). Our results suggest that glioma patients whose tumors co-expressed *VEGFA* and *FLT1* at a high level had a poorer prognosis.

High level expression of both VEGFA and KDR mRNA in the same patients predicts shorter survival outcome

Similarly, although *KDR* alone did not predict patient survival in our combined glioma dataset, the co-overexpression of *VEGFA* and *KDR* may be important for activation of such signals. We stratified the patients into three groups based on the levels of expression of *VEGFA* and *KDR*. As shown in Figure 4, patients whose tumors expressed both *VEGFA* and *KDR* at low levels had a mean survival of 48 months (36 – 59 months), patients whose tumors expressed one of these two genes at high level had a mean survival of 35 months (27 – 44 months), and patients whose tumors expressed both genes at high level had

a mean survival of 24 months (17 – 30 months), which was significantly shorter than the *VEGFA-KDR*-low group of patients (p = 0.001). Patients whose tumors expressed both genes at high level had a significantly higher risk of death than those whose tumors expressed both genes at low level with a hazard ratio of 1.94 (95% CI = 1.30 – 2.92, p = 0.001). Our results suggest that glioma patients whose tumor co-expressed *VEGFA* and *KDR* at a high level had a poorer prognosis.

High level expression of VEGFA, FLT1 and KDR mRNA in the same patients predicts shorter survival outcome

Since both *FLT1* and *KDR* could be activated upon binding by its ligand, *VEGFA*, we then investigated whether the co-expression of these three factors have value in patient prognosis. Interestingly, we found that patient survival time shortened with increasing number of VEGF pathway genes overexpressed. As shown in Figure 5, patients whose tumors expressed none of these three genes at a high level had a mean survival time of 43 months (95% CI = 30 – 57 months), patients whose tumors expressed one of these three genes at a high level had a mean survival time of 39 months (95% CI = 29 – 50 months), patients whose tumors expressed two of these three genes at a high level had a mean survival time of 30 months (95% CI = 22 – 38 months), and patients whose tumors expressed all three genes at a high level had a mean survival time of 26 months (95% CI = 19 – 34 months), which was significantly shorter than group of patients who expressed all three genes to a low degree. Importantly, patients whose tumors expressed all three genes at a high level had a higher risk of death compared to those whose tumors expressed none of these three genes at a high level (Hazard Ratio = 1.56, 95% CI = 0.98 – 2.47, p = 0.06). Together, our results suggest that co-overexpression of *VEGFA* and its two receptors, *FLT1* and *KDR*, in glioma biopsies is a prognostic indicator for glioma patients.

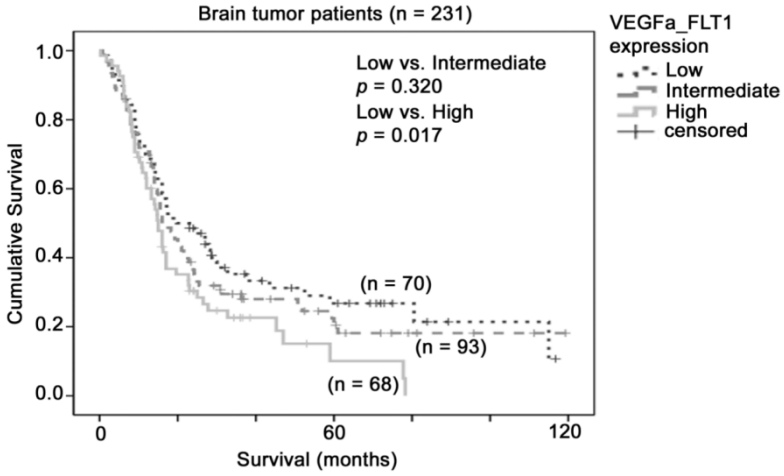

Figure 3. The association between combination of VEGFA-FLT1 mRNA expression and brain tumor patient survival. Kaplan-Meier analyses for VEGFA and FLT1 mRNA expression in combination in the brain tumor patient cohort

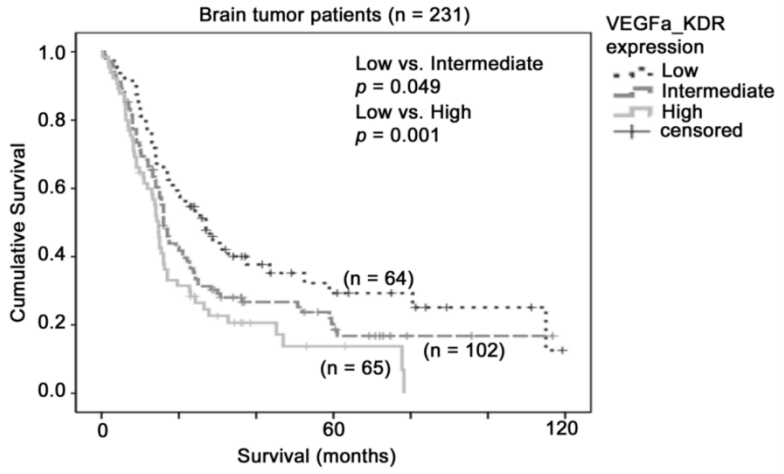

Figure 4. The association between combination of VEGFA-KDR mRNA expression and brain tumor patient survival. Kaplan-Meier analyses for VEGFA and KDR mRNA expression in combination in the brain tumor patient cohort

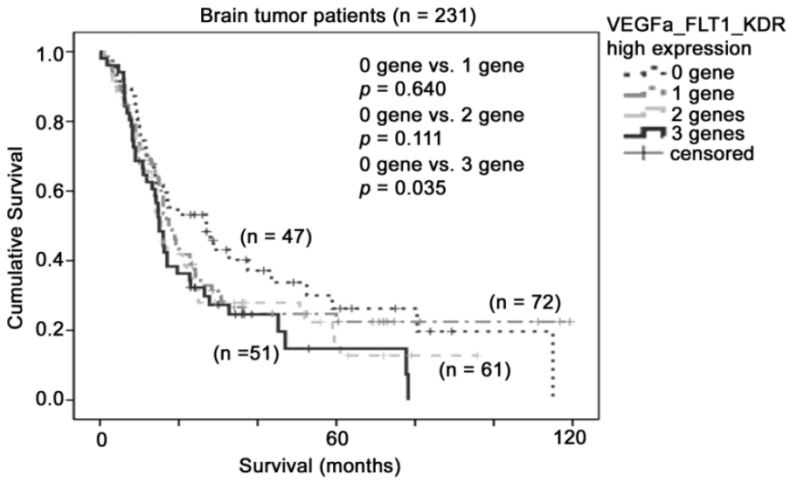

Figure 5. The association between combination of VEGFA-FLT1-KDR mRNA expression and brain tumor patient survival. Kaplan-Meier analyses for VEGFA, FLT1 and KDR mRNA expression in combination in the brain tumor patient cohort.

Discussion

Anti-angiogenesis has been shown to be an effective strategy in treatment of patients with glioblastoma, the most aggressive type of glioma. Previously, we have shown that the co-overexpression of *VEGFA* and its receptors, *FLT1* and *KDR*, is associated with poor prognosis in both lung and colon cancers [28, 29], malignancies that benefit from anti-angiogenesis therapy. Interestingly, in the present study, we have shown that co-expression of these genes also confers prognostic significance in glioma, suggesting that *VEGFA-FLT1-KDR* three-gene signature may be a potential prognostic indicator for glioma patients. We previously reported that expression profiling of this three-gene signature could also predict response to bevacizumab in colon cancer patients [29]. Bevacizumab is an anti-angiogenic agent that has also been shown to be effective in glioblastoma patients; as no dataset comprising glioblastoma patients who have received bevacizumab exist in the public domain, and as there is also a lack of success in identification of biomarker for anti-angiogenic agents [30], the value of this three-gene signature for prediction of response to bevacizumab in glioblastoma patients warrants further investigation.

Tumor angiogenesis is a highly dynamic process that is controlled by the interaction between tumor and endothelial cells as well as the tumor microenvironment and is required for tumor growth, invasion, and metastasis [31]. Activation of a cell surface receptor requires the presence of its ligand, however, overexpression of the ligand alone may not be sufficient to drive sufficient signaling to promote cancer progression. Overexpression of a cell surface receptor, such as HER2, has been shown to be sufficient to promote breast cancer progression and predict tumor response to anti-HER2 therapy [32]. In addition, mutation that led to constitutive activation of a cell surface receptor, EGFR, was found to be sufficient to promote lung cancer progression and predict tumor response to EGFR tyrosine kinase inhibitors [33]. In the present report, we have demonstrated the importance of considering the co-overexpression of both the ligand, *VEGFA*, and its receptors, *FLT1* and *KDR*, in prognosis of glioma patients.

Glioblastoma is a very aggressive subtype of glioma with very short life expectancy and limited treatment options. Although anti-angiogenesis has been shown to be effective in delaying tumor progression in glioblastoma patients, mixed results on other clinical endpoints in phase III trials studying bevacizumab in newly diagnosed glioblastoma patients suggest that not all patients will be benefit from anti-angiogenic therapy [18, 19]. Similarly, mixed results have also been observed in phase III trials in lung [34, 35] and colon cancers [36, 37]. Together with our previous findings in lung and colon cancer patient cohorts [28, 29], our results suggest that this three-gene signature may have clinical application in terms of predicting response towards anti-angiogenic agents in various types of cancer, and that this warrants further clinical evaluation, especially in a prospective trial setting.

Since glioblastoma patients have only limited treatment options, bevacizumab, which has been shown to relieve symptoms related to brain tumor, could be a valuable option to them. However, the clinical use with bevacizumab is still hindered by the lack of a suitable biomarker for identifying a sub-population of patients that could definitely benefit from treatment of bevacizumab. Although we have been unable to determine the anti-angiogenesis response-prediction capacity of this three-gene signa-

ture in glioblastoma patients, we have validated its use for predicting patient morbidity, and encourage others who may have access to data on glioblastoma patient response to bevacizumab to test the value of the three-gene signature for predicting response to the therapy.

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Competing Interests

The authors have declared that no competing interest exists.

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