Activation of Alternate Prosurvival Pathways Accounts for Acquired Sunitinib Resistance in U87MG Glioma Xenografts^S

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ABSTRACT

Acquired drug resistance represents a major obstacle to using sunitinib for the treatment of solid tumors. Here, we examined the cellular and molecular alterations in tumors that are associated with acquired brain tumor resistance to sunitinib by using an *in vivo* model. U87MG tumors obtained from nude mice that received sunitinib (40 mg/kg/day) for 30 days were classified into sunitinib-sensitive and -resistant groups based on tumor volume and underwent targeted gene microarray and protein array analyses. The expression of several angiogenesis-associated genes was significantly modulated in sunitinib-treated tumors compared with those in control tumors ($p < 0.05$), whereas no significant differences were observed between sunitinib-sensitive and -resistant tumors ($p > 0.05$). Tumor vasculature based on microvessel density, neurogenin 2 chondroitin sulfate proteoglycan density, and α -smooth muscle actin density was also similar in sunitinib-treatment groups ($p >$

0.05). The moderate increase in unbound sunitinib tumor-to-plasma area-under-the-curve ratio in sunitinib-resistant mice was accompanied by up-regulated ATP-binding cassette G2 expression in tumor. The most profound difference between the sunitinib-sensitive and -resistant groups was found in the expression of several phosphorylated proteins involved in intracellular signaling. In particular, phospholipase C- γ 1 phosphorylation in sunitinib-resistant tumors was up-regulated by 2.6-fold compared with that in sunitinib-sensitive tumors ($p < 0.05$). In conclusion, acquired sunitinib resistance in U87MG tumors is not associated with revascularization in tumors, but rather with the activation of alternate prosurvival pathways involved in an escape mechanism facilitating tumor growth and possibly insufficient drug uptake in tumor cells caused by an up-regulated membrane efflux transporter.

Introduction

The concept that sustained angiogenesis is an essential feature of many human cancers has rendered the inhibition of tumor angiogenesis as a promising strategy for cancer treatment (Hanahan and Weinberg, 2000). The finding of vascular endothelial growth factor (VEGF) as a key mediator

of angiogenesis in cancer was a milestone that spurred appreciable research efforts to developing therapeutic agents selectively targeting VEGF ligands and their receptors (Leung et al., 1989; Ferrara et al., 2004). In spite of impressive preclinical results and initial positive responses of patients, anti-VEGF therapy has yet to show an overall survival benefit, and most patients eventually relapse because of drug resistance (Shojaei and Ferrara, 2007). The postulated resistance mechanisms are diverse and as such attest to the challenge of devising treatment strategies that might prevent drug resistance (Bergers and Hanahan, 2008; Azam et al., 2010).

Acquired tumor resistance to antiangiogenic therapy occurs after treatment with selective inhibitors targeting

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ABBREVIATIONS: VEGF, vascular endothelial growth factor; ABCB1, ATP-binding cassette B1; ABCG2, ATP-binding cassette G2; ANOVA, analysis of variance; AUC, area under the curve; c-Jun, Jun proto-oncogene; CREB, cAMP response element-binding protein; C_T, cycle threshold; CT, control tumor; ERK1/2, extracellular signal-regulated kinase 1/2; p-ERK1/2, phosphorylated ERK1/2; GSK3 α/β , glycogen synthase kinase 3 α/β ; p-GSK3 α/β , phosphorylated GSK3 α/β ; HSP27, heat shock protein 27; IF, interstitial fluid; IL-8, interleukin-8; JNK, c-Jun NH₂-terminal kinase; p-c-Jun, phosphorylated c-Jun; MMP, matrix metalloproteinase; NG2, neurogenin 2 chondroitin sulfate proteoglycan; NIH, National Institutes of Health; PCA, principal component analysis; PCR, polymerase chain reaction; PK, pharmacokinetic; PLC- γ 1, phospholipase C- γ 1; p-PLC- γ 1, phosphorylated PLC- γ 1; RSK1/2/3, ribosomal S6 kinase 1/2/3; RT, resistant tumor; α -SMA, α -smooth muscle actin; ST, sensitive tumor; STAT5 α/β , signal transducers and activators of transcription 5 α/β ; U73122, 1-(6-((3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione.

VEGF receptors (Casanovas et al., 2005; Lucio-Eterovic et al., 2009) and other multitargeted antiangiogenic agents, such as sunitinib. Sunitinib is a small-molecule multikinase inhibitor that has antiangiogenic and antitumor activities achieved through the inhibition of several related receptor tyrosine kinases, including VEGF receptors 1 to 3, platelet-derived growth factor receptor α/β , stem cell factor receptor, and FMS-like tyrosine kinase 3 (Mendel et al., 2003; Sun et al., 2003). Despite initial reports suggesting clinical efficacy in various types of solid tumors (Motzer et al., 2006; George, 2007; Liljegren et al., 2009), acquired resistance to sunitinib has emerged as a major obstacle for improving overall response rate and survival of patients with cancer. Even though there is an urgent need to understanding the mechanisms underlying acquired sunitinib resistance, only a handful of experimental studies have been performed to date. A diversity of mechanisms underlying the sunitinib-resistance phenotype has been elucidated under different experimental conditions, including various *in vitro* and/or *in vivo* approaches using different tumor cell lines (Huang et al., 2010; Gotink et al., 2011; Kutikov et al., 2011; Bender and Ullrich, 2012; Yang et al., 2012). Each individual study has focused merely on one aspect of resistance, either a distinct proangiogenic factor, a prosurvival signaling pathway (Huang et al., 2010; Kutikov et al., 2011; Bender and Ullrich, 2012; Yang et al., 2012), or lysosomal sequestration (Gotink et al., 2011); thus, in-depth knowledge about the mechanisms involved in acquired sunitinib resistance is still lacking.

Brain tumor chemotherapy also suffers from the development of acquired drug resistance, and in addition, because of the presence of the blood-brain barrier, it can negatively affect drug penetration and might be considered an intrinsic resistance factor. Nonetheless, one study demonstrated the potent antiangiogenic and anti-invasive effects of sunitinib on experimental human glioblastomas by using mouse brain slices implanted with GL15 glioblastoma cells or fresh human glioma biopsy specimens (de Boüard et al., 2007), providing a rationale for using sunitinib for patients with malignant gliomas. In fact, the National Institutes of Health (NIH) has recently completed accrual of a phase II trial investigating sunitinib in patients with recurrent malignant gliomas, with final results pending (NCT00923117). (<http://clinicaltrials.gov/ct2/show/NCT00923117>). In light of the potential use of sunitinib for the treatment of brain cancer and possible occurrence of sunitinib resistance over time, additional knowledge about the molecular alterations and features of brain tumors in relation to tumor resistance to sunitinib would be essential for the design of effective drug combinations that may maximize patient responses. The aim of this study was to identify pharmacokinetic (PK), cellular, and molecular alterations in gliomas that are associated with phenotypic resistance to sunitinib. To accomplish this, an *in vivo* drug resistance tumor model was established based on the differential growth of subcutaneous tumors in mice to determine the role of both cellular and vascular components in the acquisition of sunitinib resistance. The expression levels of angiogenesis markers and angiogenesis-associated genes in tumors were measured as well as the phosphorylation levels of various proteins that regulate various prosurvival signaling pathways in the sensitive and resistant phenotypes. This approach enabled us to provide a broader understanding of sunitinib resistance.

Materials and Methods

Materials. Sunitinib malate [Sutent, Pfizer, New York, NY; *N*-[2-(diethylamino)ethyl]-5-[(*Z*)-(5-fluoro-1,2-dihydro-2-oxo-3*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxamide(2*S*)-2-hydroxybutanedioic acid (1:1) salt] was purchased from LC Laboratories (Woburn, MA) and dissolved in deionized water at a stock concentration of 5.35 mg/ml (4 mg of sunitinib base per milliliter). All other chemicals, solvents and reagents were obtained from commercial sources.

Male NIH Swiss nude mice (*nu/nu*; 6–7 weeks old) were purchased from Taconic Farms (Germantown, NY). All animal experiments were approved by the Institutional Animal Care and Use Committee and performed according to NIH guidelines (Institute of Laboratory Animal Resources, 1996).

U87MG human glioma cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin and maintained in a humidified atmosphere of 5% CO₂ in air at 37°C.

Sunitinib-Sensitive and -Resistant Xenograft Tumor Model. U87MG cells (5×10^6) suspended in 0.2 ml of Matrigel (BD Biosciences, San Jose, CA) were inoculated subcutaneously in the dorsal neck region of the nude mice. Tumor growth was monitored once a week with the volume calculated as $0.5 \times \text{length} \times \text{width}^2$. Tumor engraftment and growth were observed in all animals 14 days after tumor implantation. Tumor-bearing mice were randomized into two groups to initiate treatment, vehicle control ($n = 5$) and 40 mg/kg sunitinib group ($n = 21$), based on tumor volume. Each mouse received oral once-daily administration of either vehicle or sunitinib with a 6-day-on and 1-day-off dosing schedule for 30 days. Phenotypic sensitivity of individual mice to sunitinib treatment was defined based on the degree of suppression of tumor growth. Sunitinib-treated mice were classified into sunitinib-sensitive and -resistant groups according to the median value of fold change of tumor volume after the 30-day treatment period, which was calculated as the ratio of tumor volume on day 30 to that on day 0.

Pharmacokinetic Blood Sampling and Tumor Microdialysis. On day 30, a subgroup of sunitinib-treated tumor-bearing mice that consisted of six sunitinib-sensitive and four sunitinib-resistant animals underwent PK measurements. The day before the last dose of sunitinib the carotid artery of each mouse was catheterized for blood sampling (Zhou et al., 2008). Blood samples were taken before, 5, 15, and 30 min after, and 1, 2, 3, 4, 6, 8, and 24 h after sunitinib administration on day 30. Plasma was separated by centrifugation and stored at -80°C until being analyzed for sunitinib. Tumor microdialysis was performed to determine unbound sunitinib concentrations in tumor interstitial fluid (IF). In brief, on the day of PK study, a CMA/20 Elite microdialysis probe (Harvard Apparatus Inc., Holliston, MA) with a membrane length of 4 mm and molecular mass cutoff of 20 kDa was inserted into the central region of the tumor and perfused with Ringer's solution containing 5% (w/v) 2-hydroxypropyl- β -cyclodextrin at a flow rate of 0.6 μ l/min. After the first 5 h of sample collection after drug administration, dialysate samples were collected at different flow rates (0.6, 1.2, and 2 μ l/min) at a time interval of 30 min, which is consistent with the zero flow rate calibration method as described under Supplemental Methods (Elmehy et al., 2011).

Sunitinib concentrations in plasma and tumor IF were determined by using a validated liquid chromatography-tandem mass spectrometry method as described previously (Zhou and Gallo, 2010).

Immunofluorescence Double Staining. Frozen subcutaneous tumor samples collected from the PK study were cryosectioned at 10 μ m, fixed in 4% paraformaldehyde, and then blocked with 1.5% goat serum in phosphate-buffered saline. Sections were incubated overnight at 4°C in either a mixture of 1:400 rat anti-mouse CD31 (BD Pharmingen, San Diego, CA) and 1:200 rabbit anti-mouse α -SMA (Abcam Inc., Cambridge, MA) or a mixture of 1:400 rat anti-mouse CD31 and 1:200 rabbit anti-mouse NG2 (Millipore Corporation, Billerica, MA). After being washed with phosphate-buffered saline,

tumor sections were incubated for 1 h at room temperature in the dark with a mixture of 1:200 Alexa Fluor 488-conjugated goat anti-rat IgG and 1:200 Alexa Fluor 568-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). Fluorescent immunostained sections were examined under a Zeiss Axioplan2IE fluorescence microscope with Zeiss HC PL Fluotar 10 and 20×/0.5 NA dry objective (Carl Zeiss Inc., Thornwood, NY). Images were processed with MetaMorph 4.6-5 (Molecular Devices, Sunnyvale, CA).

Angiogenesis Polymerase Chain Reaction Array. Tumor samples obtained from another subgroup of sunitinib-treated animals, which were not used in the PK study and consisted of four sunitinib-sensitive and seven sunitinib-resistant mice, were used in the angiogenesis real-time PCR arrays designed to determine the expression levels of genes involved in modulating the biological processes of angiogenesis in either humans or mice (SA Biosciences, Valencia, CA). Total RNA extracted from tumor tissues with TRIzol reagent (Invitrogen) was converted to cDNA by using random hexamer primers and avian myeloblastosis virus reverse transcription reagents according to the manufacturer's protocol (Promega, Madison, WI). The cDNA samples were then subjected to the real-time PCR arrays. To visualize the results, we subtracted the C_T values of angiogenesis genes from the glyceraldehyde-3-phosphate dehydrogenase expression and then subtracted the mean and divided by the S.D. across all samples (Z-score normalization). To identify differentially expressed genes across all groups the analysis of variance (ANOVA) was applied with Benjamini-Hochberg correction ($p < 0.05$). To identify differentially expressed genes between sensitive and resistant groups a t test was used with the Benjamini-Hochberg correction ($p < 0.05$). Principal component analysis (PCA) was applied to the expression vectors across all samples and to an average vector for each of the three groups: control, sunitinib-sensitive, and sunitinib-resistant.

Human Phospho-Kinase Antibody Array Study. Representative subcutaneous tumor samples from the individual study groups ($n = 4$ from each group) were processed by using the human phospho-kinase array kit (ARY003; R&D Systems, Minneapolis, MN), which included 46 intracellular serine/threonine/tyrosine kinases according to the manufacturer's protocol. A qualitative assessment of the modulation effect of sunitinib on the expression of phosphorylated kinases was performed with the following two criteria: 1) a protein expression ratio of the sunitinib-treated tumor to the control tumor ≥ 1.5 was considered up-regulation; and 2) a protein expression ratio of the control tumor to the sunitinib-treated tumor ≥ 1.5 was considered down-regulation.

Western Blot Analysis. Tumor lysate samples were prepared as described previously (Zhou et al., 2008) and subjected to immunoblotting with the following antibodies purchased from Cell Signaling Technology (Danvers, MA): Jun proto-oncogene (c-Jun; 1:1000), phosphorylated c-Jun (p-c-Jun; 1:1000), extracellular signal-regulated kinase 1/2 (ERK1/2; 1:1000), phosphorylated ERK1/2 (p-ERK1/2; 1:1000), glycogen synthase kinase 3 α/β (GSK3 α/β ; 1:1000), phosphorylated GSK 3 α/β (p-GSK3 α/β ; 1:1000), phospholipase C- γ 1 (PLC- γ 1; 1:1000), phosphorylated PLC- γ 1 (1:1000), signal transducers and activators of transcription 5 α/β (STAT5 α/β ; 1:1000), and phosphorylated STAT5 α/β (1:1000). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:15,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and immunoreactive protein bands were visualized by the enhanced chemiluminescence system (PerkinElmer Life and Analytical Sciences, Waltham, MA). Band areas were quantified by using NIH's ImageJ software (<http://rsb.info.nih.gov/ij/>). Normalization for loading differences was achieved by dividing the densitometry values for individual bands by the densitometry values for β -actin in the same lane. The expression levels of individual proteins in sunitinib-treated tumors are presented as the percentage change compared with those in the control tumors.

To measure the expression levels of ABCB1 and ABCG2, the crude membrane was extracted from individual tumor tissues as described

under Supplemental Methods and subjected to Western blotting by using the ABCB1 (1:100; Calbiochem, San Diego, CA) and ABCG2 antibodies (1:500; Santa Cruz Biotechnology, Inc.) following the abovementioned protocol.

Statistical Analyses. Except for the real-time PCR array data, data analyses were performed by using NCSS 2007 (NCSS Statistical Software, Kaysville, UT). Data are presented as the mean $\pm$ S.D. Comparison of means between two independent groups was made by using the Mann-Whitney U test. Comparison of means among three study groups was made by using one-way ANOVA followed by the post hoc Tukey-Kramer multiple comparison test. Spearman's rank correlation was used to describe relations between two variables. A two-sided p value < 0.05 was considered statistically significant.

Results

Inhibitory Effect of Sunitinib on Tumor Growth. Significant suppression of tumor growth in sunitinib treatment groups was observed on days 9 and 16 ($p < 0.01$ for both compared with the vehicle control). Vehicle-treated mice were sacrificed 16 days after the initiation of the treatment because their tumors reached the allowed maximum size (2000 mm³). Sunitinib-treated mice exhibited different tumor growth rates during the 30-day treatment period. The fold change of tumor volume after the 30-day treatment period ranged from 0.7 to 11.5 with the median value of 4.7. This median value was then used as the cutoff point to classify sunitinib-treated mice into sunitinib-sensitive and -resistant groups (Fig. 1A; Supplemental Table S1). Significant differences in mean tumor volume (Supplemental Fig. S1) and tumor volume ratio (the ratio of tumor volume measured at an indicated time to tumor volume at the start of vehicle or sunitinib treatment) (Fig. 1B) between the sunitinib-sensitive and -resistant groups were observed on days 23 and 30 ($p < 0.01$ for both), indicating that sunitinib exhibits greater antitumor efficacy in sunitinib-sensitive mice than in sunitinib-resistant mice.

Sunitinib Pharmacokinetic Study in Sunitinib-Sensitive and -Resistant Mice. The PK study was performed to determine whether the reduced effectiveness of sunitinib was attributable to insufficient drug penetration into tumor cells. The unbound sunitinib plasma AUC was calculated based on the reported sunitinib unbound fraction value of 9% in the mouse plasma (Haznedar et al., 2009). Although there were trends of altered PK characteristics (decreased unbound plasma AUC and increased clearance) in the sunitinib-resistant group (Table 1), the differences were insignificant compared with the sunitinib-sensitive group ($p > 0.05$). Nonetheless, a 19% increase in the mean unbound sunitinib tumor IF-to-plasma AUC ratio in the sunitinib-resistant group compared with that in the sunitinib-sensitive group raised speculation that activated efflux pumps in the plasma membrane of tumor cells efflux drug from the intracellular space to the interstitial space.

Evaluation of Tumor Angiogenesis in Sunitinib-Sensitive and -Resistant Tumors. Pericytes on tumor blood vessels express distinct markers at different stages of differentiation (Morikawa et al., 2002). NG2 proteoglycan is a cell surface molecule and a prominent component of pericytes in tumor microvessels. It has the potential to affect tumor progression by contributing to pericyte recruitment and pericyte-endothelial cell interactions (Ozerdem et al., 2002; Brekke et al., 2006). In contrast, α -SMA has been reported as

Fig. 1. Differential tumor growth rate in sunitinib-treated tumor-bearing animals. U87MG human glioblastoma xenografts were grown subcutaneously in male NIH Swiss nude mice (*nu/nu*). Once-daily oral administration of vehicle or 40 mg/kg of sunitinib was initiated 14 days after tumor implantation and continued for 30 days. A, the distribution and median value of the day 30-to-day 0 tumor volume ratio in sunitinib-treated tumor-bearing mice ($n = 21$). Sunitinib-treated mice with the day 30-to-day 0 tumor volume ratio value being no less than the median value of 4.7 were classified as sunitinib-resistant animals ($n = 11$), whereas the rest were defined as the sunitinib-sensitive animals ($n = 10$). Subgroup 1: Tumor samples collected from this group were used for the angiogenesis PCR array, human phosphor-kinase antibody array and immunofluorescence double staining. Subgroup 2: Animals from this group were used in the PK study, and tumor samples collected were used for the immunofluorescence double staining and Western blotting. B, comparison of tumor growth ratio among control, sunitinib-sensitive, and sunitinib-resistant groups. Control animals ($n = 5$) were sacrificed 16 days after the initiation of the treatment because their tumors reached the allowed maximum size (2000 mm³). ++, $p < 0.01$ compared with the control group by using one-way ANOVA followed by the post hoc Tukey-Kramer multiple comparison test. **, $p < 0.01$ compared with the sunitinib-sensitive group by using the Mann-Whitney U test.

a marker of mature mural cells including both pericytes and smooth muscle cells (Yonenaga et al., 2005). In this study, sunitinib treatment significantly reduced the tumor microvessel density and pericyte recruitment as indicated by the CD31 and NG2 immunostaining ($p < 0.01$ for both; Fig. 2, A and C; Table 2). Sunitinib had no effect on the

α -SMA density, but significantly increased the number of α -SMA-positive cells lining CD31-positive endothelial cells relative to the total number of CD31-positive endothelial cells, which is reflected by the percentage of α -SMA/CD31 double-positive structures ($p < 0.01$ for both sunitinib-resistant and -sensitive tumors; Fig. 2, B and D; Table 2) and is considered an index of vessel maturation (Djokovic et al., 2010). The mean percentage of CD31-positive structures covered by NG2-positive area was significantly reduced in both sunitinib-sensitive and -resistant tumors compared with that in the control tumors ($p < 0.05$ and 0.01 for sunitinib-sensitive and -resistant tumors, respectively; Fig. 2, A and C), suggesting markedly impaired pericyte recruitment and reduced new blood vessel formation in tumors. Overall, the results suggest that sunitinib targets mainly newly formed immature tumor vessels, thereby increasing the proportion of functional vessels in tumors. No significant differences were found between sunitinib-sensitive and -resistant groups in tumor microvessel density, NG2 density, α -SMA density, and the percentage of either α -SMA/CD31 or NG2/CD31 double-positive structures. This implies that even though tumor angiogenesis is inhibited to the same extent in both sunitinib-sensitive and -resistant tumors alternative prosurvival pathways that allow tumor growth to depend less on tumor neovascularization may be activated to a greater degree in the resistant tumors compared with those in the sensitive tumors.

Angiogenesis Polymerase Chain Reaction Array. Species-specific detection of the expression levels of human and mouse angiogenesis-associated genes was carried out by using real-time PCR array. The C_T values for individual genes are presented in Supplemental Tables S2 and S3. The unsupervised hierarchical biclustering approach was used to organize and explore the data and highlight groups of samples with similar gene expression patterns. Hierarchical clustering using the normalized C_T values generated two distinct clusters for both mouse and human angiogenesis-related genes. Cluster 1 contained all vehicle control animals ($n = 5$), and cluster 2 contained all sunitinib-treated animals, including four sunitinib-sensitive and seven resistant animals (Fig. 3, A and B). Likewise, PCA demonstrated that sunitinib-sensitive and -resistant groups displayed overall similar expression patterns for both human and mouse angiogenesis-associated genes, whereas the control group showed differential gene expression pattern compared with the two sunitinib treatment groups (Fig. 3, C-F). Moreover, 21 of the 84 mouse genes and 35 of the 84 human genes were differentially expressed between the control tumors and either sunitinib-sensitive or -resistant tumors ($p < 0.05$; Fig. 3, G and H). Although most resistant and sensitive groups seem to be segregated into two distinct groups by visually inspecting the hierarchical clustering and PCA plots, no significantly differentially expressed genes were found. Hence, the

TABLE 1
Unbound sunitinib systemic and tumor exposure in sunitinib-sensitive and -resistant tumors
 p values are 0.594, 0.166, and 0.594 for unbound tumor IF AUC_{0–24}, unbound plasma AUC_{0–24}, and unbound tumor IF/plasma AUC ratio, respectively.

Group	Unbound Tumor IF AUC _{0–24}	Unbound Plasma AUC _{0–24}	Unbound Tumor IF/Plasma AUC Ratio
	ng · h/ml		
Sensitive tumor, $n = 6$	881.0 ± 682.1	548.6 ± 178.4	1.93 ± 1.70
Resistant tumor, $n = 4$	871.8 ± 268.7	405.2 ± 158.9	2.30 ± 0.79

Fig. 2. Quantitation of CD31, α -SMA, and NG2 expression in control tumors ($n = 5$; open columns), sunitinib-sensitive tumors ($n = 8$; striped columns), and sunitinib-resistant tumors ($n = 10$; solid columns). A, results of double fluorescent staining for CD31 and α -SMA. B, results of double fluorescent staining for CD31 and NG2. Columns, mean; bars, S.D. *, $p < 0.05$ and **, $p < 0.01$ compared with the control group by using one-way ANOVA followed by the post hoc Tukey-Kramer multiple comparison test. C and D, representative images for the double fluorescent staining of CD31/NG2 (C) and of CD31/ α -SMA (D) in tumor sections from the control, sunitinib-sensitive and sunitinib-resistant groups.

TABLE 2
Tumor angiogenesis in control, sunitinib-sensitive and -resistant tumors

Group	CD31/ α -SMA Double Staining			CD31/NG2 Double Staining		
	MVD	α -SMA Density	Vessel Maturity	MVD	NG2 Density	Pericyte Recruitment
		%			%	
Control	7.49 $\pm$ 2.06	2.05 $\pm$ 0.75	8.49 $\pm$ 3.09	8.91 $\pm$ 1.39	3.08 $\pm$ 1.14	18.39 $\pm$ 3.08
Sensitive	1.88 $\pm$ 0.55**	1.28 $\pm$ 0.47	27.95 $\pm$ 10.37**	1.86 $\pm$ 0.51**	0.64 $\pm$ 0.16**	7.85 $\pm$ 5.93*
Resistant	1.81 $\pm$ 0.47**	1.18 $\pm$ 0.64	25.17 $\pm$ 9.56*	1.52 $\pm$ 0.26**	0.43 $\pm$ 0.12**	9.88 $\pm$ 5.72*

* $p < 0.05$; ** $p < 0.01$ compared with control using one-way ANOVA and Tukey-Kramer multiple comparison test.

difference in the expression levels of either mouse or human genes was not significant between sunitinib-sensitive and -resistant groups, suggesting similar patterns of angiogenesis-related gene expression in the sunitinib-sensitive and -resistant tumors.

Human Phospho-Kinase Antibody Array Study. Even though the results of both immunostaining and angiogenesis PCR array showed that tumor angiogenesis was inhibited to a similar degree in both sunitinib-sensitive and -resistant tumors, the relatively rapid growth rate of sunitinib-resistant tumors suggests that there are potentially cell signaling pathways that differentially regulate tumor cell proliferation and survival in the resistant tumors. Because prosurvival signaling pathways are known to play an important role in drug resistance, a human phosphor-kinase antibody array was used to screen for potential markers for the acquisition of sunitinib resistance. Figure 4, A and B shows the fold changes in protein expression of phosphorylated kinases and their substrates that were either up-regulated (≥ 1.5 , sunitinib-treated to control) or down-regulated (≥ 1.5 , control to sunitinib-treated), respectively. A network diagram depicting the relationships between these proteins and their substrates in the context of cell signaling pathways is shown in Fig. 4, C and D; however, it should be viewed as the definitive network because there are many other regulators involved that are not shown. In addition, the references for the links between components are from different cell types, either human or mouse. However, it can be seen that the up-regulated proteins in the resistant tumors and the down-regulated proteins in the sensitive tumors form a pathway that can mediate the phosphorylation of ERK1/2, which subsequently up-regulates c-Jun expression, and another set of transcription factors, STAT5 α/β . Moreover, the up-regulation of GSK3 α/β is also likely to play a role in the activation of c-Jun, suggesting a coherent feed-forward network motif among effectors of c-Jun in sunitinib-resistant tumors.

Western Blot Analysis. Based on the phosphor-kinase antibody array results, semiquantitative Western blot analyses were conducted to confirm that the following proteins were up-regulated in the sunitinib-resistant tumors: ERK1/2 (Thr202/Tyr204), GSK3 α/β (Ser21/Ser9), PLC γ -1 (Tyr783), c-Jun (Ser63), and STAT5 α/β (Tyr699). As shown in Fig. 5, B and C, the expression levels of p-PLC γ -1 ($p < 0.01$), total PLC γ -1 ($p < 0.05$), and total c-Jun ($p < 0.05$) in the sunitinib-resistant tumor were significantly up-regulated compared with those in the control tumors. The expression level of p-PLC γ -1 and total GSK3 β in sunitinib-resistant tumors was significantly higher than that in sunitinib-sensitive tumors ($p < 0.05$ and 0.01 for total GSK3 β and p-PLC γ -1, respectively). Expression of p-c-Jun seemed to be up-regulated in the sunitinib-resistant tumors but down-regulated in the

sensitive tumors, and the difference between those two groups was significant ($p < 0.05$). Differences in the expression levels of p-ERK1/2, total ERK, p-GSK3 α/β , and total GSK3 α among the three study groups were not statistically significant (Fig. 5C). The expression levels of phosphorylated and total STAT5 α/β were not detectable by Western blotting analysis. To examine whether the expression of the tested proteins would be associated with tumor growth after sunitinib treatment, the expression levels of individual proteins were compared with tumor volume on day 30 or fold changes of tumor volume after the 30-day treatment period by using Spearman correlation coefficients. Because the relationship between any two variables might be linear or log-linear, rank-based coefficients, such as Spearman's coefficients, would yield more robust estimates of correlation than linear coefficients, such as Pearson's coefficients, which could be strongly biased by extreme values. Using Spearman correlation analyses, the fold change of tumor volume was found to be significantly correlated with p-PLC γ -1 ($r = 0.636$; $p = 0.048$; Supplemental Fig. S2A), total ERK1/2 ($r = 0.661$; $p = 0.038$; Supplemental Fig. S2B), and total GSK3 β ($r = 0.636$; $p = 0.048$; Supplemental Fig. S2C).

Sunitinib is known to be a substrate (Tang et al., 2012) and inhibitor (Shukla et al., 2009) of ABCG2, a membrane transporter that serves as a drug efflux pump. In this study, the ABCG2 expression levels in tumors seemed to be up-regulated in both sunitinib-sensitive (by 36%) and sunitinib-resistant (by 72%) tumors compared with that in the control tumors, although only the difference between the sunitinib-resistant and control groups was statistically significant ($p < 0.05$; Fig. 5C). This result suggests that the up-regulation of ABCG2 in tumor cells may contribute to the acquired tumor resistance to sunitinib treatment. However, significant correlations were not found between tumor ABCG2 expression levels and either unbound sunitinib tumor or plasma AUC values or the tumor-to-plasma AUC ratios. The expression level of ABCB1 was not detected by the Western blot in all tumor samples.

Discussion

Efforts to identify mechanisms of sunitinib resistance in various cancer types have begun but none have focused on gliomas (Huang et al., 2010; Gotink et al., 2011; Kutikov et al., 2011; Bender and Ullrich, 2012; Yang et al., 2012). In this study, a U87MG xenograft model was used to explore the potential mechanism involved in acquired sunitinib resistance. In contrast to in vitro models, the subcutaneous glioma model used here has the advantage of flexibility of treatment duration and direct assessment of a resistant phenotype based on tumor size, yet suffers a drawback of not

Fig. 3. A and B, unsupervised hierarchical biclustering of the expression patterns of 84 mouse (A) and 84 human (B) angiogenesis associated genes in five control tumors (CT), four sunitinib-sensitive tumors (ST), and seven sunitinib-resistant tumors (RT). The colors represent relative expression levels. Orange represents expression levels greater than the mean for a given gene across all samples; blue represents expression levels less than the mean. Each colored cell in the heat map represents the gene expression value for a probe in a sample. C to F, PCA of the mouse (C, mean values, and E, individual animals) and human (D, mean values, and F, individual animals) angiogenesis-associated gene expression was performed to visualize the overall difference in expression levels across samples and groups. The percentage near each axis of the PCA plots denotes the contribution of the principle component to capturing the overall variability within the data. G and H, expression levels of differentially expressed mouse (G) and human (H) angiogenesis-associated genes exhibiting significant difference between the control, sunitinib-sensitive, and sunitinib-resistant groups ($p < 0.05$ compared with the control) are visualized as a bi-clustering heat map using the same color scheme and normalization procedure as in A and B.

capturing the native environment of brain tumors. Nonetheless, this study provides a framework for understanding the sequence of biologically programmed events in brain tumors leading to acquired sunitinib resistance.

In this study, sunitinib-treated animals were classified into sunitinib-sensitive and -resistant groups based on the median value of fold change of tumor volume after the 30-day treatment period (Fig. 1). Although the designation of sensitive and resistant groups is partially arbitrary, having two phenotypically distinct groups aids in characterizing differences in sunitinib-induced cellular and molecular alterations in tumor and sunitinib's PK behavior. A similar approach was reported by Huang et al. (2010), who defined the sunitinib-resistant renal cell carcinoma xenografts as those with more than 25% increase in the initial tumor volume, whereas those with the initial tumor volume increase being less than 25% were considered sunitinib-sensitive.

Consistent with the early study showing that sunitinib significantly reduced blood vessel formation but had little effect on existing blood vessels (Osusky et al., 2004), the double immunofluorescent staining results of this study suggest that the immature tumor vessels are more susceptible to sunitinib, whereas mature vessels are relatively resistant to it (Fig. 2). The remaining mature vessels in the tumors are able to maintain blood flow and provide oxygen and nutrients, thereby supporting tumor growth (Jain 2001), which explains the observed gradually increased tumor volume in all sunitinib-treated animals in this study. Because there was no difference in tumor angiogenesis, measured in terms of microvessel density, pericyte density, and percentage of microvessel with pericyte coverage, between sunitinib-sensitive and -resistant groups, it is unlikely that the relatively rapid growth observed in sunitinib-resistant tumors is caused by the restoration of sprouting tumor angiogenesis.

Fig. 4. A and B, phosphorylated protein kinases and other kinase substrates protein expression levels in sunitinib-sensitive (A) and sunitinib-resistant tumors (B) compared with control tumors. Up-regulation: a protein expression ratio of the sunitinib-treated tumor to the control tumor being ≥ 1.5 . Down-regulation: a protein expression ratio of the control tumor to the sunitinib-treated tumor being ≥ 1.5 . C and D, network created from up-regulated (C) and down-regulated (D) kinases and other phospho-proteins. Links denote activation (arrowheads) and inhibition (ball-head); dashed arrows represent indirect effects; nodes are color coded by up-regulated (red), down-regulated (blue), and unchanged (light gray); additional nodes that were not measured are color-coded in dark gray. Links are marked based on their database sources, and numbers represent PubMed IDs. eNOS, endothelial nitric-oxide synthase.

Fig. 5. Modulated expression levels of ABCG2 and selected phosphorylated and total kinases in sunitinib-sensitive and -resistant tumors. A, Western blot analysis of ABCG2 levels in untreated ($n = 4$), sunitinib-sensitive ($n = 6$), and sunitinib-resistant ($n = 4$) tumors. Anti- β -actin immunoblot is shown as an independent loading control. B, Western blot analysis of phosphorylated and total c-Jun, ERK1/2, GSK3 α/β , and PLC γ 1 in untreated, sunitinib-sensitive and -resistant tumors. ST^s indicate sample was not included in the study because of the missing dose on day 30. C, quantitation of Western blotting results using densitometric analysis. The results are plotted as percentage of control. Columns, mean; bars, S.D. Open columns, control group; striped columns, sunitinib-sensitive group; solid columns, sunitinib-resistant group. *, $p < 0.05$; **, $p < 0.01$ compared with the control group by using one-way ANOVA followed by the post hoc Tukey-Kramer multiple comparison test. +, $p < 0.05$; ++, $p < 0.01$ compared with the sunitinib-sensitive group by using one-way ANOVA followed by the post hoc Tukey-Kramer multiple comparison test.

Resistance to antiangiogenic therapy in cancer involves both tumor cells and stromal components (Casanovas, 2011). Although a human tumor xenograft in a mouse background is a mixture of human and mouse tissues, most studies on antiangiogenic drug resistance that included gene expression analyses of tumor xenografts have determined only human genes (Casanovas et al., 2005; Huang et al., 2010; Zhang et al., 2011). In this study, the real-time PCR array assay was performed to obtain species-specific gene expression profiles. This enabled us to distinguish between angiogenesis-associated factors derived from human tumor cells and those from the mouse stroma, thus gaining insight into tumor-stromal interactions related to the acquisition of sunitinib resistance. In line with the findings of the immunohistochemistry study, results of the real-time PCR array demonstrated that the expression patterns of both human and mouse angiogenesis-related genes were different between control and sunitinib treatment groups, but not between sunitinib-sensitive and -resistant groups. However, the directed angiogenesis arrays would not have captured genes related to other resistant mechanisms. For example, treatment with antiangiogenic agents has been demonstrated to increase local invasion and accelerate metastases (Ebos et al., 2009; Páez-Ribes et al., 2009). In this regard, whole genomewide expression analysis would be necessary to locate likely differences not captured in the scope of this study, which focused on tumor neovascularization. Nonetheless, several differentially expressed genes observed in this study were also reported by other research groups. For example, Finke et al. (2011) reported that sunitinib-persistent myeloid-derived suppressor cells in metastatic renal cell carcinoma were associated with the up-regulated expression of intratumoral matrix metalloproteinase (MMP) 9, MMP8, and interleukin 8 (IL-8). In a preclinical study by Huang et al. (2010), plasma IL-8 levels were higher in sunitinib-resistant mice compared with those in sunitinib-sensitive mice. In the present study, the expression levels of *Mmp9* and IL-8 were significantly up-regulated in sunitinib-treated tumors com-

pared with control tumors. However, no difference was found between sunitinib-sensitive and -resistant tumors (Fig. 3, G and H). The discrepancy between findings of this study and those of Finke et al. (2011) and Huang et al. (2010) may be caused by differences in study designs, including various tumor model used, different tissue samples examined, and different treatment regimens applied.

The angiogenic array results revealed that the phenotypic resistance to sunitinib treatment was not attributed to the up-regulation of alternative proangiogenic factors, which raised the question of whether the modulation of other pro-survival pathways might contribute to the acquisition of sunitinib resistance. Using an antibody array followed by Western blotting verification, we identified a few differentially expressed kinase proteins in sunitinib-sensitive and -resistant tumors. There was some discrepancy between phosphor-kinase antibody arrays and Western blotting in terms of the magnitude of the fold changes, which is possibly attributable to the different efficacy of applied antibodies.

Results of the Western blot analysis showed that the expression levels of phosphorylated and total PLC γ 1 were elevated in sunitinib-sensitive and -resistant tumors compared with control tumors, but to various degrees. Moreover, the p-PLC γ 1 expression level in sunitinib-treated tumors was significantly correlated with the fold change of tumor volume ($p < 0.05$; Supplemental Fig. S2A). The intracellular signaling molecule PLC γ 1 can be activated by various growth factors and hormones through their corresponding receptor tyrosine kinases (Burgess et al., 1990; Kundra et al., 1994). Activation of PLC γ 1 results in the formation of second messengers inositol 1,4,5-trisphosphate and diacylglycerol, which subsequently mobilize the release of calcium and activate protein kinase C isoforms, respectively, leading to diverse cellular responses. Phosphorylation on tyrosine residue 783 of PLC γ 1 is critical to its activation (Yu et al., 1998; Poulin et al., 2005). A growing body of evidence has shown that PLC γ 1 promotes tumor invasion. For example, the PLC γ 1 expression in a metastatic tumor-derived head and

neck squamous cell carcinoma cell line was significantly up-regulated compared with the paired primary tumor-derived cell line. Treatment with the PLC inhibitor 1-(6-((3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U73122) attenuated epidermal growth factor-stimulated head and neck squamous cell carcinoma invasion in vitro (Nozawa et al., 2008). A mechanistic study using the MDA-MB-231 breast cancer cell line revealed that the phosphoinositide 3-kinase-mediated activation of PLC- γ 1 provided a link between integrin- and growth factor-mediated signaling pathways to regulate cell motility (Piccolo et al., 2002). In this study, the up-regulation of PLC- γ 1 expression in sunitinib-treated tumors suggests that tumors change part of their molecular characteristics in response to sunitinib treatment to not only sustain tumor growth but also promote tumor invasion.

The expression of p-c-Jun was up-regulated in the sunitinib-resistant tumors but down-regulated in the sensitive tumors (Fig. 5C). c-Jun and its upstream regulator c-Jun NH₂-terminal kinase (JNK) belong to one subgroup of mitogen-activated protein kinases that can be stimulated by environmental stresses, cytokines, and DNA-damaging agents (Weston and Davis, 2007). The overexpression or activation of c-Jun seems to be antiapoptotic in various cancer cell lines, and targeting c-Jun increases the sensitivity of resistant cancer cells to DNA-damaging or microtubule-interacting agents (Pan et al., 2002; Obey et al., 2005; Duan et al., 2007). In our case, the up-regulation of phosphorylated and total c-Jun in addition to PLC- γ 1 in sunitinib-resistant tumors indicates that more than one prosurvival pathway is activated in tumors resistant to sunitinib.

Tumor resistance to pharmaceutical intervention may be caused by PK resistance that would culminate in lower intracellular drug exposure. In this study, the up-regulated ABCG2 expression level in sunitinib-treated tumors was associated with increased unbound tumor IF-to-plasma AUC ratios. Sunitinib being a substrate of ABCG2 could be subjected to enhanced tumor cell efflux, causing lower intracellular concentrations but higher concentrations in tumor IF. This interesting phenomenon that was detected in vivo by using tumor microdialysis has not been reported previously and offers an experimental approach to ascertain how intracellular drug concentrations may be altered by drug transport modulation. Even though no correlation was found between tumor ABCG2 expression levels and unbound sunitinib tumor IF-to-plasma AUC ratios, possibly because of a limited sample size, there is a sufficient basis to further study the role of ABCG2 in acquired sunitinib resistance.

In summary, based on the findings of this study, acquired tumor resistance to sunitinib is not associated with revascularization in tumor, but is associated with the activation of alternate prosurvival pathways, notably those mediated by PLC- γ 1 and c-Jun proteins. The reduced drug uptake in tumor cells attributable to up-regulated ABCG2 also seems to play a role in acquired sunitinib resistance. Further studies are needed to clarify the role of these potential resistance factors in sunitinib resistance; however, without the use of an in vivo model of drug resistance that provides a foundation for further exploration including novel combination therapies these leads are not likely to be identified.

Authorship Contributions

Participated in research design: Zhou and Gallo.

Conducted experiments: Zhou and Lv.

Contributed new reagents or analytic tools: Lv.

Performed data analysis: Zhou, Mazloom, Xu, and Ma'ayan.

Wrote or contributed to the writing of the manuscript: Zhou, Xu, Ma'ayan, and Gallo.

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