Clinical Cancer Research

Molecular and Clinical Effects of Notch Inhibition in Glioma Patients: A Phase 0/I Trial

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Abstract

Purpose: High-grade gliomas are associated with a dismal prognosis. Notch inhibition via the gamma-secretase inhibitor RO4929097 has emerged as a potential therapeutic option based on modulation of the cancer-initiating cell (CIS) population and a presumed antiangiogenic role.

Experimental Design: In this phase 0/I trial, 21 patients with newly diagnosed glioblastoma or anaplastic astrocytoma received RO4929097 combined with temozolomide and radiotherapy. In addition to establishing the MTD, the study design enabled exploratory studies evaluating tumor and brain drug penetration and neuroimaging parameters. We also determined functional effects on the Notch pathway and targeting of CISs through analysis of tumor tissue sampled from areas with and without blood–brain barrier disruption. Finally, recurrent tumors were also sampled and assessed for Notch pathway responses while on treatment.

Results: Treatment was well tolerated and no dose-limiting toxicities were observed. IHC of treated tumors showed a significant decrease in proliferation and in the expression of the Notch intracellular domain (NICD) by tumor cells and blood vessels. Patient-specific organotypic tumor explants cultures revealed a specific decrease in the $CD133^+$ CIS population upon treatment. Perfusion MRI demonstrated a significant decrease in relative plasma volume after drug exposure. Gene expression data in recurrent tumors suggested low Notch signaling activity, the upregulation of key mesenchymal genes, and an increase in VEGF-dependent angiogenic factors.

Conclusions: The addition of RO4929097 to temozolomide and radiotherapy was well tolerated; the drug has a variable blood–brain barrier penetration. Evidence of target modulation was observed, but recurrence occurred, associated with alterations in angiogenesis signaling pathways. *Clin Cancer Res; 22(19); 4786–96. ©2016 AACR.*

Introduction

High-grade gliomas, including glioblastoma and WHO grade III anaplastic astrocytomas (AA), are the most common and aggressive primary brain tumor in adults. Despite multidisciplinary approaches that combine maximal surgical resection and radiotherapy with concomitant and adjuvant temozolomide, glioblastomas remain associated with a dismal prognosis, with clinical trials in newly diagnosed disease reporting median overall survival (OS) of 15 to 18 months (1–3).

Recent literature supports a role for glioma stem cells (GSC), which represent a subfraction of the tumor cell pool and are thought to be crucial to tumor growth (4). The Notch signaling pathway plays a key role during the development of the central nervous system, including maintenance of self-renewing neural progenitors and regulation of their fate decisions by promoting glial differentiation (5). Notch signaling has also been implicated

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Translational Relevance

In this phase 0/I trial, gamma-secretase/Notch inhibitor RO4929097 was added to chemoradiotherapy to treat glioblastomas and anaplastic astrocytomas. A comprehensive integrated translational approach demonstrated tumor penetration and effective target modulation of the drug, as measured by effects on neuroimaging, gene expression, modulation of glioma stem cells, and promising efficacy.

in cancer with putative roles in tumor stem cell biology, angiogenesis, and tumor progression (6–8). The four transmembrane Notch receptors (*NOTCH* 1–4) interact closely with membrane-bound ligands that belong to the Jagged (Jagged ligands 1 and 2) or Delta-like family (DLL 1, 3, and 4; ref. 9). Upon ligand–receptor binding, the Notch receptor undergoes proteolytic cleavage by metalloprotease and gamma-secretase, which releases the Notch intracellular domain (NICD) that translocates to the nucleus and activates target gene transcription (10).

Our laboratory and others have investigated the role of Notch inhibition in the GSC population (11–14). Using three-dimensional (3D) organotypic tumor explants, we demonstrated Notch inhibition via a gamma-secretase inhibitor (GSI) is associated with a decrease in GSCs, as well as a decrease in endothelial cells (11). We also demonstrated that combination treatment of tumor explants with Notch inhibition and radiation was more effective than either treatment alone, suggesting a synergistic effect (11, 15). These findings formed the scientific rationale for the design of the current trial.

Given its significant role in tumor biology, *in vivo* Notch pathway modulation has been studied with a range of inhibitors, including GSIs, other small-molecule inhibitors as well as targeted mAbs (16, 17). RO4929097 is an orally bioavailable small-molecule GSI, capable of a potent, inhibitory effect on Notch signaling (17, 18). It has been evaluated in early-phase trials in solid tumors, alone or in combination with other agents (19–24), with responses observed in a range of tumor types. This early experience also raised some concerns about drug–drug interactions and autoinduction, and identified GSI-associated toxicities. Of note is a phase I trial of 103 patients with advanced solid tumors conducted with GSI MK-0742, which reported a complete response in one AA and stable disease in 10 patients with glioblastoma (25).

Treatment of brain tumors is often limited by the ability of small molecules to cross the blood–brain barrier (BBB) and achieve therapeutic concentrations in tumor tissue. There are currently no data pertaining to BBB permeability of RO4929097 or its effectiveness in achieving inhibition of Notch signaling in brain tumors upon systemic administration.

Here, we report a phase 0/I study of RO4929097 in combination with temozolomide and radiotherapy in patients with newly diagnosed glioblastoma or WHO grade III AA. The primary goals were to determine the MTD, toxicities, and pharmacokinetic effects. Secondary goals of this proof-of-concept study included exploratory analyses of tumor drug penetration, evaluation of patients' GSC populations, and *in vivo* Notch target modulation, as well as the evaluation of drug effects on advanced neuroimaging, including dynamic contrast-enhanced (DCE) perfusion MRI. The trial also included analyses of tissue samples obtained intraoperatively and grown in 3D organotypic cultures as a means of evaluating the drug effects on tumor tissue *ex vivo*, following a clinical laboratory codevelopment paradigm. We also had the opportunity of sampling recurrent tumors while on treatment, thus establishing a comparative profile of gene expression.

Materials and Methods

Study design

Patients with newly diagnosed glioblastoma or AA who had undergone biopsy or partial resections before enrollment, and that upon further neurosurgical evaluation had an indication for additional debulking surgery were eligible for this study; anaplastic oligodendroglioma or 1p/19q codeleted tumors were excluded. Other standard inclusion and exclusion criteria are detailed in Supplementary Methods.

The study design is summarized in Fig. 1 and Supplementary Fig. S1. Upon establishment of date of surgery, patients received RO4929097 daily for 7 days, followed by surgical resection on day 7. The last dose was administered 2 to 3 hours before resection, at which time tissue and blood samples were obtained for correlative studies. After surgery, RO4929097 was discontinued to allow for recovery and wound healing.

A phase I 3+3 design was then applied to evaluate the combination of radiotherapy, temozolomide, and RO4929097. Patients with adequate performance status after surgery were restarted on daily RO4929097 and received standard radiotherapy concomitant with temozolomide 75 mg/m^2 daily, followed by adjuvant temozolomide given at a dose of 150 to 200 mg/m^2 days 1 to 5 of 28-day cycles, for a minimum of 6 and a maximum of 12 cycles. Standard 3+3 dose escalation rules were utilized, with dose-liming toxicities based on adverse events occurring in the first 30 days of radiotherapy.

Three prespecified dose levels of RO4929097 were examined (10 mg, 15 mg, and 20 mg once a day), selected on the basis of available pharmacokinetic studies of RO4929097 in solid tumors that indicated doses above 20 mg do not increase drug exposure due to autoinduction.

To avoid delays in establishing the MTD, patients who were not candidates for postoperative treatment due to poor performance status or comorbidities were replaced by patients already resected off-study ($n = 11$). Enrollment of additional patients who were surgical candidates was permitted while a given cohort was being examined, for a maximum of 10 additional patients.

Tumor status (MacDonald criteria) was assessed every 2 months. Patients with suspected pseudoprogression were allowed to stay on treatment if deemed appropriate by the treating physician, or offered surgical resection. If progression was confirmed on next scan or histologically, patients were removed from study and the date of first MRI showing radiographic progression was considered the date of progression. If further tumor resections were indicated during the study, that is, in patients with suspicion of tumor progression or pseudoprogression, patients were asked to remain on RO4929097 until day of surgery, during which tumor tissue samples were also collected for further comparative analysis ($n = 7$).

Exploratory neuroimaging evaluation

To explore early pharmacodynamic effects via changes in advanced neuroimaging parameters, notably perfusion and vascular permeability, patients undergoing surgical resection were evaluated with DCE-MRI at baseline and then again after 7 days of

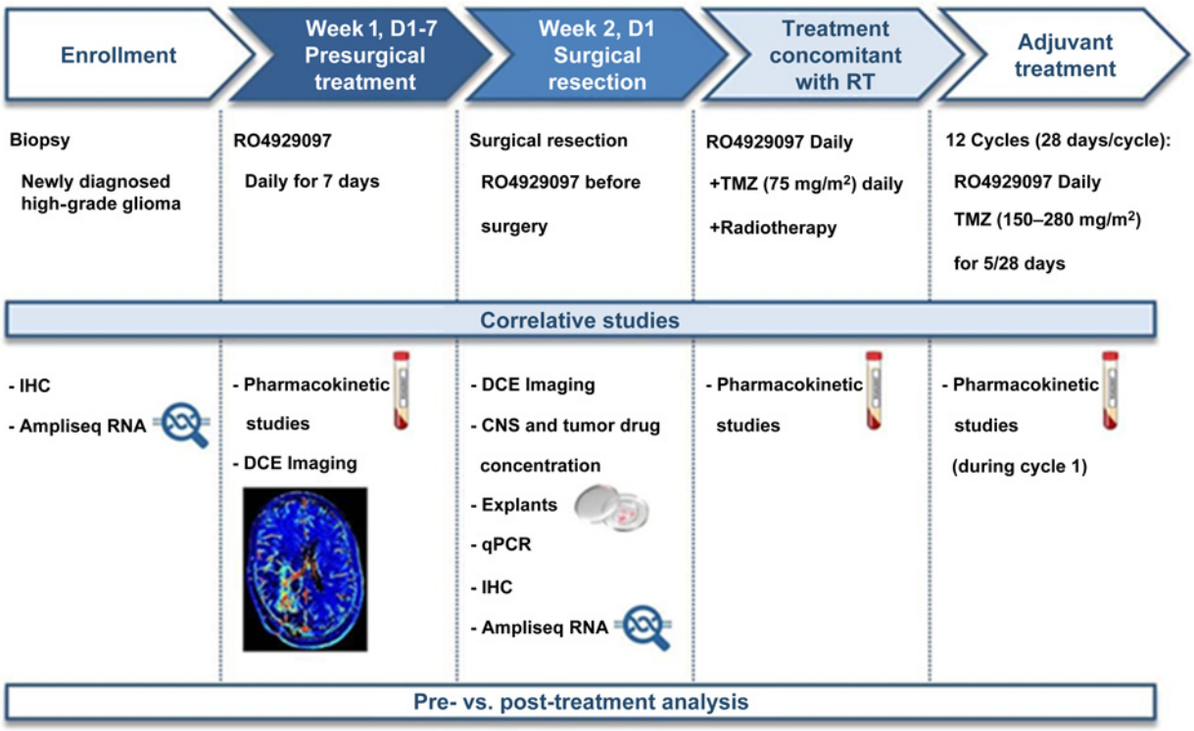

Figure 1.
Study design and treatment plan for enrolled patients. Patients with newly diagnosed glioblastoma or anaplastic astrocytoma were treated with RO4929097 (in escalating doses/3+3 phase I design) for 7 days, followed by surgical resection, radiotherapy (RT) and temozolomide (TMZ) administration. Pharmacokinetic studies were performed on plasma and tumor tissue; surgical samples were also used for histochemical and molecular studies.

RO4929097 (i.e., immediately before surgery; Supplementary Methods). Analyses focused on blood perfusion as measured by relative fractional plasma volume (Vp) and vascular permeability as measured by volume transfer coefficient between plasma and extravascular extracellular space (K^{trans}).

Pharmacokinetic studies

High-performance liquid chromatography with tandem mass spectrometry was utilized to determine levels of RO4929097 in blood and tumor tissue. Tumor areas with and without contrast enhancement, as defined by intraoperative MRI, were differentially sampled. In addition, standard blood pharmacokinetic parameters of RO4929097 were evaluated in combination with temozolomide 75 mg/m^2 and 150 mg/m^2 (More detail in Supplementary Methods).

qRT-PCR, IHC, morphometry, and RNA sequencing

Methods utilized for the evaluation of effects of RO4929097 on gene expression, Notch pathway, angiogenesis, and GSC are detailed in Supplementary methods. Archival and on-study surgery formalin-fixed paraffin-embedded tumor tissue was utilized for qRT-PCR and RNA sequencing. IHC was performed utilizing anti-NICD and anti-CD31 antibodies. Morphometry was performed using ImageJ.

Viability and flow cytometric analysis of organotypic explants

In addition to the analyses above, fresh tumor tissue from patients operated on study were grown as 3D organotypic explants for further characterization of *in vitro* effects of Notch inhibition (ref. 20; Supplement Methods). Established explants were treated with radiotherapy with temozolomide, or combined radiotherapy, temozolomide, and RO4929097, or single-agent RO4929097, then evaluated for effects on cell proliferation (Ki-67 immunofluorescent staining), tumor cell viability (CellTiter Glo Luminescent Assay), and proportion of GSC-like CD133$^+$ cells (FACS analysis).

Statistical analysis

All values are expressed as mean ± SEM unless otherwise stated. Statistical significance was determined by unpaired Student *t* tests. Survival was calculated from date of initial histologic diagnosis to either death (OS), or progression (PFS) utilizing Kaplan–Meier methodology (Stata 9.3). Patients were censored at last follow-up.

Study approval

This study was an investigator-initiated, prospective phase 0/I clinical trial (ClinicalTrials.gov identifier: NCT01119599), primarily sponsored by the NCI/Cancer Treatment and Evaluation Program (CTEP); NCI# 8556. The study was approved by the MSKCC IRB. Written informed consent was obtained from all participants or guardians before enrollment.

Results

Study population

Twenty-one patients with newly diagnosed glioblastoma ($n = 17$) or WHO grade III AA ($n = 4$) were enrolled between

Table 1. Baseline characteristics of patients enrolled in the study

Characteristic	All patients	GBM	AA
Number of patients enrolled	21	17	4
Median age	57	58	38
Range	28–68	36–65	28–68
Gender			
Women	7	6	1
Men	14	11	3
Median KPS	90	90	95
Range	80–100	80–100	90–100
RO4929097 dosage			
10 mg	4	3	1
15 mg	7	6	1
20 mg	10	8	2
Surgical candidates[a]	10	10	0
Replacement patients[a]	11	7	4
MGMT promoter			
Methylated	4	4	0
Unmethylated	12	11	1
Undetermined	5	2	3
IDH-1 R132H mutation			
Present	2	*0*	*1*
Absent	5	*5*	*1*[b]
Not tested	16	12	*2*

Abbreviations: AA, anaplastic astrocytoma; GBM, glioblastoma.

[a]Patients who were candidates for surgical resection were primarily enrolled. Patients who were not candidates for a clinical trial after surgery were replaced by patients with tumor already resected off trial.

[b]This patient had an IDH-1 R132C mutation on next generation sequencing. MGMT promoter status determined by methylation-specific PCR.

5/2010-6/2012. Median age was 57 years (range: 28–68) and median KPS was 90. Patient characteristics are summarized in Table 1.

All three prespecified RO4929097 dose levels were examined: 10 mg ($N = 4$), 15 mg ($N = 7$), and 20 mg ($N = 10$). Seven patients required repeat surgical resection for tumor progression while on RO4929097 and temozolomide, and had tissue analyzed for comparative analyses.

Treatment, dose escalation, and toxicity

Treatment was well tolerated, and dose escalation proceeded as planned. No dose-limiting toxicities were observed in any of the prespecified dose levels. The 20-mg daily dose was deemed the recommended phase II dose (RP2D). A summary of all reported grades 3 and 4 adverse events is shown in Supplementary Table S1. Toxicities were manageable or reversible; no patient discontinued treatment due to toxicities, and there were no treatment-related deaths. The toxicity profile is comparable with previously reported profiles for temozolomide and RO4929097, consisting mainly of hematologic toxicities and elevated transaminases. The relationship between electrolyte disturbances and treatment is uncertain, but such events were conservatively designated as possibly related to treatment. Other unrelated or unlikely related adverse events, inherent to brain tumors (e.g., intracranial hemorrhage, complications from surgery, neurologic symptoms, thromboembolic events, and hyperglycemia in the setting of corticosteroids), were observed as expected. One patient developed retroperitoneal bleeding in the setting of a mechanical fall and use of aspirin. There was no significant QTc prolongation.

Pharmacokinetic evaluation

To evaluate effects of temozolomide on the plasma pharmacokinetic parameters of RO4929097, standard 24-hour pharmacokinetic evaluations were performed during temozolomide 75 mg/m^2 (first day of treatment and after 5 weeks) and during first day of adjuvant temozolomide 150 mg/m^2/day. Mean (±SD) values of maximum plasma concentration (C_{max}) and the area under the plasma concentration–time curve for the 24-hour dosing interval (AUC_{24}) across the different RO4929097 dose levels are presented in Supplementary Table S2. The mean C_{max} and AUC_{24} for the initial dose of RO4929097 increased as the dose was escalated and values were consistent with previously reported clinical studies of single-agent RO4929097 (19, 23). In addition, the mean C_{max} and AUC_{24} values determined after 10 weeks of continuous daily dosing on day 1 of adjuvant therapy were lower than the corresponding values for the initial dose at each dose level, consistent with the previously reported autoinduction of RO4929097 elimination.

Effect of RO4929097 on MRI perfusion parameters

Eight patients underwent the exploratory pharmacodynamic evaluation with DCE-perfusion MRI obtained before and after 7 days of exposure to RO4929097 (Supplementary Fig. S2A). All patients demonstrated a decrease in perfusion, with mean Vp ratio decreasing from 9.34 ± 2.4 at baseline to 3.92 ± 0.9 after drug exposure ($P < 0.05$; Supplementary Fig. S2B). Likewise, there was a trend toward a decrease in vascular permeability, with a mean K^{trans} of 0.05 ± 0.01/minute at baseline and 0.03 ± 0.01/minute after treatment (Supplementary Fig. S2C). These findings suggest an early attenuating effect on tumor blood perfusion after exposure to RO4929097.

Brain and tumor penetration of RO4929097

Intraoperative biopsy of contrast enhancing and nonenhancing tumor tissue and concomitant acquisition of plasma samples were obtained in 11 patients. Areas of enhancement on T1 post-gadolinium MRI represent areas of BBB breakdown and increased permeability, whereas areas without contrast enhancement have a relatively intact BBB. It has been posited that tumor cells in areas of intact BBB (no enhancement) are often not exposed to adequate drug levels, contributing to the lack of efficacy of treatments for glioma. Our data show drug levels upon initial administration of RO4929097 (i.e., 7 days culminating in surgery) were significantly higher in enhancing (0.73 ± 0.13 μmol/L) versus nonenhancing (0.33 ± 0.04 μmol/L) tissue (Fig. 2A). Available literature demonstrate a reduction in target genes (e.g., *Hes1*) with RO4929097 in a dose-dependent manner starting at 0.1 μmol/L (24), thus suggesting the achieved drug levels were within therapeutic levels regardless of BBB integrity. Interestingly, in patients who presented with tumor progression that required a second surgery after a prolonged course of RO4929097 (mean, 181 days; range, 104–399 days), tumor drug concentrations were similar in enhancing and nonenhancing areas, reaching an average of 0.77 ± 0.19 μmol/L and 0.71 ± 0.24 μmol/L, respectively (Fig. 2A).

To assess the extent to which drug concentrations in the blood influenced intratumoral drug concentrations, the corresponding ratios of tumor-to-plasma molar drug concentrations were determined at the time of surgery (after 7 days of RO4929097 administration), and were found to be approximately 1 in the nonenhancing areas, but twice as high in the areas of BBB breakdown and enhancement (Fig. 2; Supplementary Table S3). Upon repeat surgery for recurrence in the setting of prolonged RO4929097 use, drug accumulated in areas of presumed normal

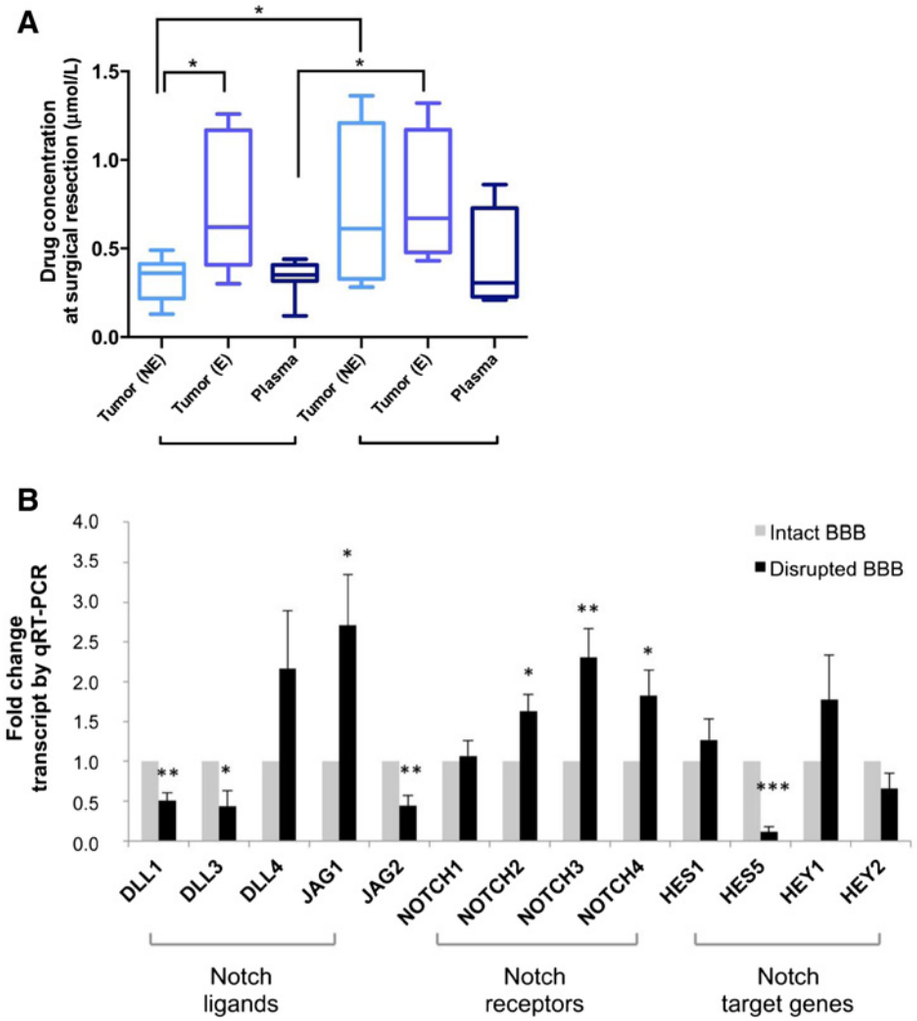

Figure 2.
Drug concentration levels and mRNA expression profile in enhancing and nonenhancing brain tissue. **A,** tumor drug concentration levels (ng/mg) after 7-day treatment ($n = 8$) and prolonged RO4929097 (RO) treatment ($n = 4$) are shown in areas of tumor tissue with intact (nonenhancing) and disrupted (enhancing) BBB, as distinguished by T1-weighted Gadolinium-enhanced MRI. **B,** mRNA expression profile in enhancing and nonenhancing brain tumor tissue of Notch pathway components; levels are normalized to 18S ($n = 6$). Values are expressed as mean ± SEM E, enhancing; NE, nonenhancing; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(nonenhancing) and disrupted BBB (enhancing), leading to tumor-to-plasma ratios that are similar to those measured in the enhancing regions after 7 days of treatment, averaging 2.7 in enhancing and 2.5 in nonenhancing brain tissue. However, the range of concentrations exhibited significant variability with one specimen (M09) reaching levels of approximately 1.3 μmol/L in nonenhancing tumor tissue (Supplementary Table S3). A notable distinction is that patients with a prolonged RO4929097 course had also received radiation treatment (average time since radiotherapy: 131 days; range: 57–357 days).

We further dissected the potential impact of RO4929097 treatment on Notch pathway components, using tumor tissue obtained from both areas displaying BBB breakdown (contrast-enhancing) and intact BBB (noncontrast-enhancing). Intraoperative tumor specimens were processed for quantitative mRNA analysis using a panel of primers against a range of Notch ligands, receptors, and gene targets. In comparison with areas of intact BBB, enhancing tissue showed significant downregulation of *DLL1*, *DLL3*, and *JAG2* ligands, as well as suppression of the downstream effector *HES5*, whereas *HES1* levels remained unchanged (Fig. 2B). Interestingly, there was also significant upregulation of several Notch receptors (Notch 2, 3, and 4), which may suggest activation of negative feedback loops due to the suppression of Hes5.

Effect of RO4929097 on proliferation, the Notch pathway, and tumor blood vessels

We next sought to validate whether the drug levels achieved in tumor tissue were sufficient to impact tumor cells proliferation. IHC analyses of the Ki-67 proliferation marker in matching tumor samples, obtained before and after 7 days of RO4929097 treatment, showed a trend toward a decrease in proliferation from 27.7% ± 7.3% to 13.6% ± 3.1%, albeit with marked interpatient variability (Fig. 3A and B and Supplementary Fig. S3).

RO4929097 potently inhibits the enzymatic cleavage event that the Notch receptor undergoes upon its activation, followed by release of NICD and its translocation to the nucleus (24). Therefore, we analyzed the expression of NICD in tumor cells in matching pre- and posttreatment glioblastoma tissue, via IHC. The number of NICD-positive cells decreased significantly from 21.9% ± 5.4% to 5.1% ± 1.3% after the initial presurgical 7-day treatment with RO4929097 (Fig. 3C and D), suggesting a state of Notch signaling inactivity or inhibition. Moreover, a notable difference in NICD expression was observed within tumor

vasculature (Fig. 3C, bottom). These data are suggestive of biologic activity of RO4929097 within tumor tissue.

Notch activity plays an important role in tumor angiogenesis but the role of Notch inhibition on glioma vascularity remains unclear (26–28). We therefore sought to determine the effect of RO4929097 treatment on glioma vessels through the analysis of available matching pre- and posttreatment tissues. IHC evaluation of tissues obtained before treatment (pre), at 7 days of RO4929097 (post) and at the time of recurrence after prolonged treatment (PostPP), shows no significant change in microvascular density, but a significant decrease in NICD-expressing vascular structures from 67.6% ± 5.2% (pre) to 30.0% ± 9.0% (post) and 24.2% ± 5.2% (PostPP) in matching samples (Fig. 3E and F). Expression of NICD in normal brain vessels was assessed in a set of normal brain controls and found to represent 10.1% ± 3.5% of blood vessels (Fig. 3E and F). Furthermore, vascular morphology in pretreatment tissue showed characteristic glomeruloid body formation, whereas in posttreatment tissue demonstrated a vascular phenotype that was more normalized, with largely monolayers of endothelial cells lining sometimes large but often more normal appearing vascular spaces (Fig. 3E). Taken together, these data suggest a modulation of glioma vasculature during RO4929097 treatment, and are consistent with the decreased perfusion observed on DCE-MRI.

Effect of RO4929097 alone and in combination with radiotherapy and temozolomide on the glioma stem cell population in organotypic slices

Our group and others have previously shown that inhibition of Notch signaling leads to a decrease in the number of putative $CD133^+$ GSC (11, 29). We have also demonstrated the importance of the vascular microenvironment in effecting the role of Notch inhibition, using tumor "organotypic" explants. Here, we established tumor explants from patients in whom adequate fresh tissue could be obtained during surgery ($n = 4$). Explants were treated for 10 days with RO4929097 (1 μmol/L) *in vitro*, following which there was a significant decrease in proliferation (assessed by Ki-67 quantification; Fig. 4A and B). Having demonstrated in previous studies synergistic effects between radiation and Notch inhibition, we focused on analyzing the impact of RO4929097, radiotherapy + temozolomide and radiotherapy + temozolomide + RO4929097 on cell viability in tumor explants; the latter condition being representative of the clinical trial treatment conditions (11). Only the triple combination (radiotherapy + temozolomide + RO4929097) had a significant attenuating effect on tumor cell viability, effecting a nearly 60% decrease compared with the control untreated explants (Fig. 4C). Finally, Notch activation in tumor cells is thought to be mediated by ligand-bearing endothelial cells and is greatest around tumor vasculature, an area thought to represent a stem cell niche (11, 30, 31). We therefore analyzed the proportion of GSC-like $CD133^+$ cells in the explants by FACS analysis. The data show a significant decrease in the proportion of $CD133^+$ cells after treatment with RO4929097 alone (Fig. 4D). Interestingly, neither the combination of radiotherapy + temozolomide (standard of care) nor the triple combination (radiotherapy + temozolomide + RO4929097) was associated with a further reduction in the $CD133^+$ population (Fig. 4D).

Gene expression analysis after treatment

Gene expression profiling was performed on a set of matched tissues obtained pretreatment and after the initial 7 days of drug therapy. In addition, 7 patients underwent surgery upon tumor recurrence while on prolonged drug treatment. To dissect gene expression patterns, we expanded our transcriptomal analysis via a customized RNA-Ampliseq panel including glioma lineage markers, Notch pathway components, and signaling mediators of angiogenesis and the EGFR pathway (Supplementary Fig. S4A and S4B). Results showed a trend toward downregulation of the primary Notch gene targets *HES5*, *HEY1*, and *HEY2* mostly in the recurrence group with prolonged RO 4929097 therapy, although differences did not reach statistical significance. Gene expression data in the recurrent group (Supplementary Fig. S4B) also demonstrated an increase in angiogenesis markers, as well as in *C/EBPB* (CCAAT/Enhancer Binding Protein Beta) and *STAT3*, genes considered master regulators of the mesenchymal phenotype in gliomas. Tumor recurrence in the setting of prolonged RO4929097 therapy therefore occurred in a background of low Notch activity, suggesting an "escape" from Notch dependence. The recurrent tumors exhibited upregulation of angiogenesis markers such as PECAM (CD31), angiopoietins, and VEGFA, suggesting a transition to a potential alternative Notch-independent neoangiogenesis (Supplementary Fig. S4B). Interestingly, the mesenchymal-associated genes C/EBPB and STAT3 had been downregulated after the presurgical 7-day RO4929097 treatment phase (Supplementary Fig. S4). Their reemergence at recurrence could represent a consequence of Notch inhibition, however, an evolution toward a more mesenchymal phenotype has also been reported in recurrent gliomas especially after radiation exposure (32, 33). The absence of recurrent controls, interpatient molecular tumor heterogeneity, and the limitation to two genes limits further conclusions.

Patient outcomes

Although not a study objective, and limited by the small sample size, phase I design, and the fact that most patients had tumor recurrence before initiation of treatment, we performed an exploratory analysis of survival. After a median follow-up of survivors of 24 months, the Kaplan–Meier median OS for glioblastoma patients was 21 months (95% CI, 12–28) and median PFS was 13 months (95% CI, 7–13). At the time of analysis, all patients with AA ($n = 4$) were alive (31+, 37+, 41+, and 53+ months). Two of them progressed at 31 and 46 months, respectively, while two other remained free of progression at 37+ and 41+ months.

Exploratory tissue correlates

To determine the potential association of tissue characteristics and patient survival, we conducted correlative analyses in an exploratory manner. Patients on whom both pre- and posttreatment tissue was available for IHC and molecular studies were divided into two subgroups ("favorable" and "poor" survival) based on a cut-off value of 14 months, chosen based on median survival in glioblastoma historic controls. The patient subgroup demonstrating favorable survival exhibited a substantial decrease in Ki-67 from 37.3% ± 10.7% at baseline to 8.5 ± 3.2% after 7 days of RO4929097, while the patients in the poor survival group experienced an increase in proliferation from 15.0 ± 2.3% to 20.4 ± 2.2% (Fig. 5A). Changes in NICD expression on vascular structures were also more significant in the group exhibiting better survival (22.5% ± 5.0% vs. 43.8% ± 9.0%; Fig. 5B). Interestingly, gene expression values of Notch targets were more consistently downregulated in the favorable survival subgroup compared with their poor survival counterparts (Fig. 5C). To exclude the

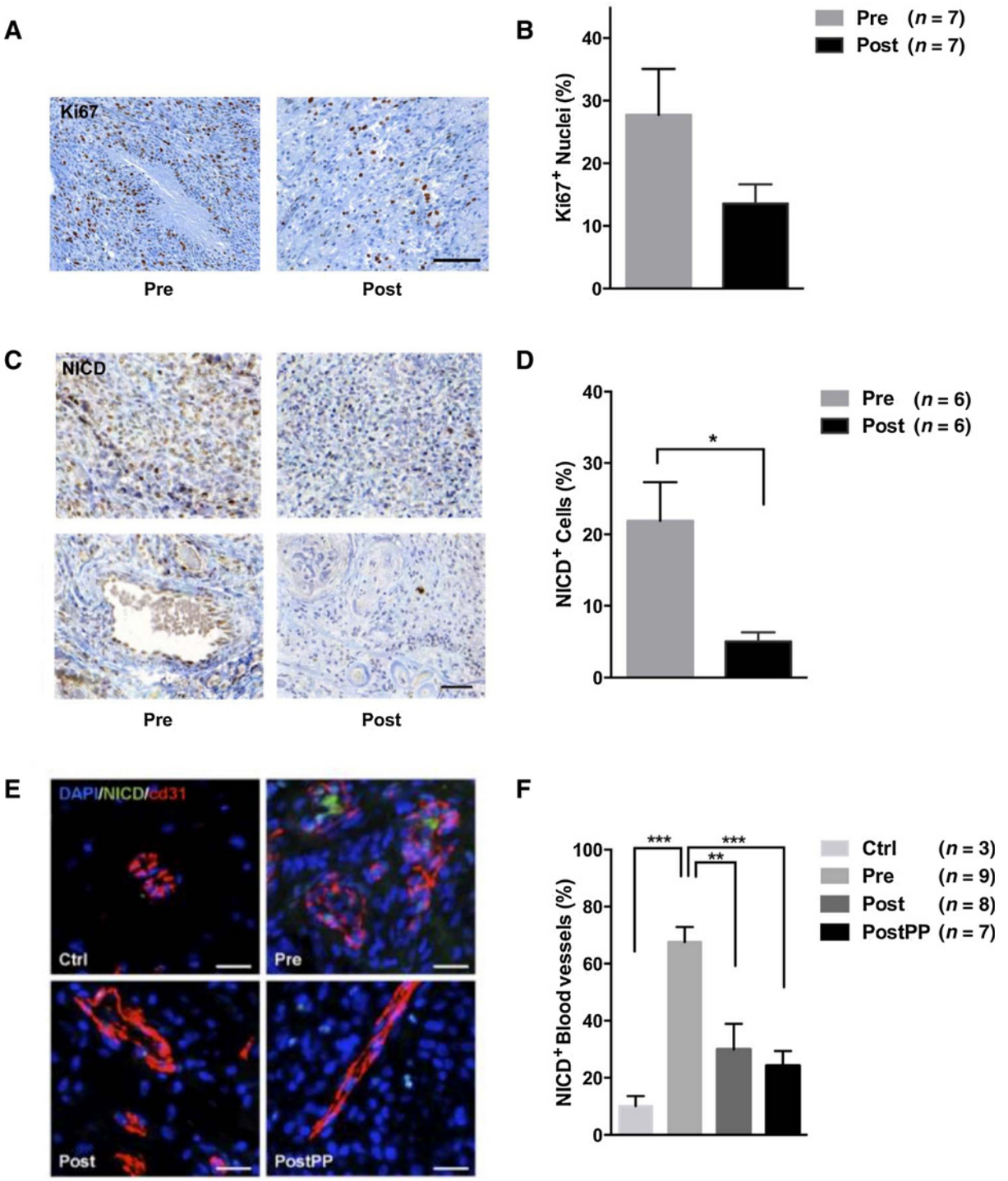

Figure 3.

Posttreatment effects of RO4929097 on proliferation, Notch receptor, and surrounding blood vessels. **A** and **B,** IHC and quantification of Ki-67 labeling index in matched pre- and posttreatment tumor tissue, before and after treatment ($n = 7$). **C,** NICD (nuclear intracellular domain of Notch) IHC of matched pre- and posttreatment glioblastoma patient samples. Bottom, NICD-positive cells in relation to vascularity. **D,** quantification of NICD$^+$ tumor cells after 7 days of RO4929097 treatment ($n = 6$). **E,** sections from normal brain (Ctrl), enrolled glioblastoma patients before (pre), after 7 days (post) and after multiple cycles (PostPP) of RO4929097 treatment were immunostained for NICD and vessel marker CD31. **F,** quantification of NICD$^+$ vascular structures as percent of total blood vessels in sections of normal brain tissue and glioblastoma samples before and after RO4929097 exposure. A minimum of 1,000 cells were counted for Ki-67 and NICD analysis; the entire tissue area was quantified to determine the ratio of NICD$^+$ vascular structures. Values are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

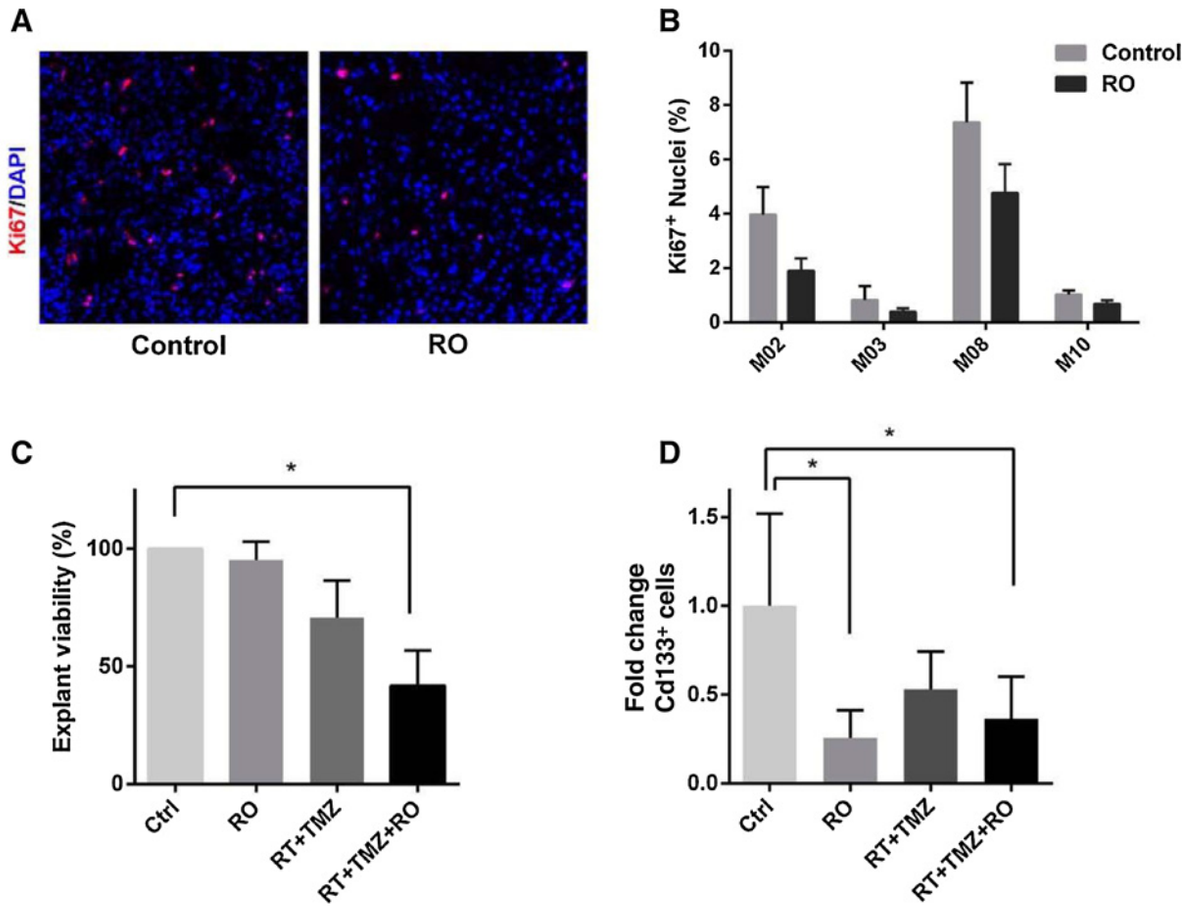

Figure 4.

Effect of RO4929097 treatment on proliferation, viability, and CD133$^+$ glioma stem cells in glioblastoma explants. **A,** immunofluorescent staining of Ki67 in organotypic explants before and after treatment with RO. **B,** quantification of Ki-67$^+$ nuclei in patient explants before and after drug treatment ($n = 4$). **C,** cell viability in tumor explants treated with RO4929097, radiotherapy, and temozolomide, and the triple combination, as measured by CellTiter Glo Luminescent Assay ($n = 5$). **D,** FACS analysis of cd133$^+$ cells in tumor explants after drug treatment ($n = 5$). Ctrl, control; RO, RO4929097; RT, radiotherapy; TMZ, temozolomide; *, $P < 0.05$.

possibility that these differences are due to variations in drug levels, average tissue drug levels in both favorable and poor survival groups were determined, showing no statistical differences in tumor tissue levels (0.15 ± 0.03 μmol/L vs. 0.19 ± 0.05 μmol/L; $P = 0.36$), and thus suggesting differential sensitivity to Notch inhibition among tumors. Although the number of available specimens is very limited, these data suggest that early tissue analysis (e.g., after 7 days of treatment) could have a predictive value as to the ability to achieve target inhibition and a biologic response.

Discussion

In this study, we report a phase I trial of oral gamma-secretase/Notch inhibitor RO4929097 in combination with standard treatment in newly diagnosed glioblastoma and WHO grade III AA. The trial design allowed for extensive exploratory studies, including neuroimaging and laboratory experiments evaluating drug brain penetration, target modulation, and potential mechanisms of resistance.

The combination of RO4929097, temozolomide, and radiotherapy was feasible and well tolerated. Toxicity frequencies were similar to those observed in previous trials of RO4929097 and temozolomide (19). The 20 mg daily dose was deemed the RP2D. Pharmacokinetic parameters of RO4929097 were in good agreement with data reported in phase I studies of RO4929097 given as a single agent or combined with cediranib (19, 23). Clinical investigations of the distribution of anticancer drugs into brain tumors are uncommon and it is uncertain how well preclinical models reflect drug uptake. To fully evaluate the potential of RO4929097 for the treatment of brain tumors, drug concentrations were determined in tissue resected from the contrast-enhancing regions of the tumor, representing tissue with a disrupted BBB, as well as nonenhancing regions in which the BBB is intact. While considerable variability among patients was observed, drug concentrations in the low micromolar range were achieved in both regions of the tumors. The observed intratumoral concentrations of the drug were comparable with its IC_{50} values against human brain cancer cell lines (13, 18). Interestingly, a differential expression profile of Notch ligands, receptors,

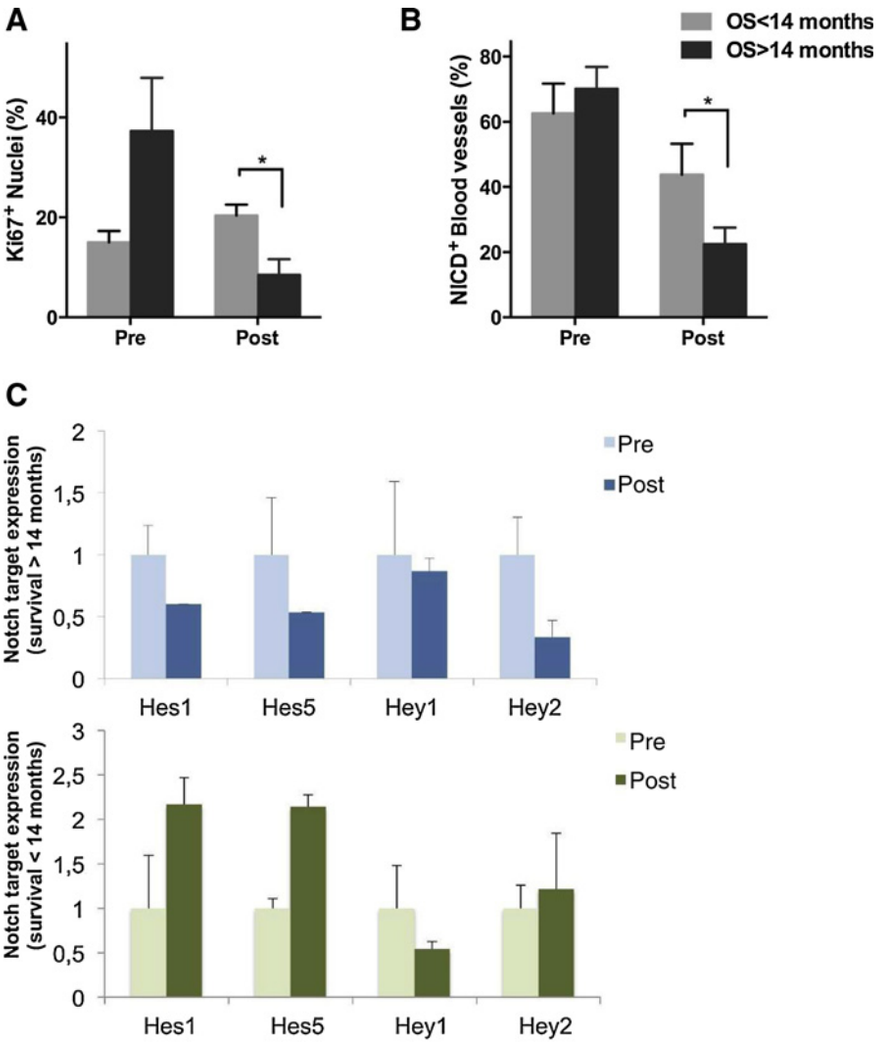

Figure 5.
Association of overall survival time with Ki-67 proliferation index, NICD+ vessel ratio, and Notch downstream target gene expression. **A** and **B,** Ki-67 proliferation index and NICD+ blood vessel ratios grouped according to patient survival groups ($n = 7$). **C,** expression of downstream Notch target genes (Hes1, Hes5, Hey1, and Hey2) by qRT-PCR in patients with poor and good overall survival times ($n = 5$). Values are expressed as mean ± SEM. *, $P < 0.05$; OS, overall survival.

and downstream targets was initially observed between enhancing versus nonenhancing areas, strengthening the hypothesis that the drug preferentially penetrated areas with BBB breakdown where it then produced a more potent, inhibitory effect on Notch targets. However, prolonged drug exposure seemed to bypass the BBB concern, as samples obtained at later times displayed similar drug concentrations in enhancing and nonenhancing areas. Heterogeneity of the tumor and adequately characterizing the integrity of the BBB in the region from where the tumor was resected are underlying concerns for studies such as this, as discussed in a recent review (33).

Previous data showed that radiation and Notch inhibition may act synergistically in impairing tumor survival (11, 13). In parallel to the clinical trial, we conducted experiments on tumor explants, which further support a role for the combination of radiation, temozolomide, and Notch inhibition. Analyses of glioblastoma organotypic explants showed a decrease in proliferation upon RO4929097 treatment (in comparison with IHC studies on parent tumor tissue), and an even more pronounced decrease in viability when exposed to triple therapy. In addition, the proportion of $CD133^+$ cells decreased after exposure to RO4929097, highlighting the role of the Notch pathway in maintenance of GSC. Armed with an appropriate sample size, tumor explants could be developed into a powerful screening platform with a predictive capacity for clinical response within a short time span, acting as a "functional biopsy." Tumor explants play a particularly important role in assessing tumor response whereby modulation of environmental factors, such as endothelial cells, is relevant. In the case of Notch inhibition, we have previously shown that the *in vitro* response of cell lines is often different from and less predictive of true Notch activity, in comparison to 3D explants which maintain endothelial cell integrity (11).

Our attempts to identify reliable markers of drug activity included quantitative IHC for NICD, the immediate product of gamma-secretase–mediated cleavage of the inner domain of the Notch receptor, as well as quantitative gene expression. The IHC data showed a significant decrease in NICD expression in matching specimens obtained before and after treatment. While intratumoral heterogeneity remains a concern, these data suggest that sufficient drug exposure and target modulation were achieved. In comparison, gene expression data were more challenging to interpret. Downregulation of Notch targets was inconsistent following one-week exposure to RO4929097, although samples obtained after prolonged treatment displayed a more pronounced

modulatory effect. Notably, *HES1*, a major gene target downstream of the Notch pathway remained essentially unchanged in some patients despite other evidence in support of pathway inhibition, such as the decrease in HES5 and in NICD. This raises the question on whether this could be a result of noncanonical, Notch receptor–independent activation in these patients, known to occur via a variety of signaling pathways such as hedgehog, FGF, Wnt, STAT, or BMP signaling attributed to the Activin receptor-like kinase (Alk)1 activity (34, 35).

The gene expression in tumor tissue obtained at later time points, after completion of radiotherapy, suggests that such tumors recurred in the context of persistently low Notch activity. Compared with the original tumors, recurrences exhibited significant neoangiogenesis as evidenced by a statistically significant increase in PECAM (i.e., CD31, a robust endothelial marker), as well as in the expression of angiopoietins. Coupled with the decrease in Notch activity in vascular structures seen upon initiation of therapy, these data would suggest that angiogenesis in the recurrent tumor developed independently of Notch signaling. Notch inhibition has also been reported to affect the biology of abnormal glioblastoma tumor vessels (26–28). Indeed, our DCE-MRI studies showed an early decrease in tumor blood perfusion upon RO4929097 treatment. This was associated with a reduction in tumor vessels expressing the cleaved form of the Notch receptor (NICD), as shown by IHC. Although prolonged treatment further reduced the number of NICD-positive blood vessels (achieving values similar to those of normal brain tissue), microvascular density was unchanged and gene expression data showed a marked increase in PECAM expression in these recurrent tumors. These data suggest that recurrent tumors switched to a Notch-independent angiogenic profile. Both the sustained activation of Hes1 and the neoangiogenesis probably represent aspects of a resistance phenomenon that develops in the context of sustained Notch inhibition. These findings also support the concomitant use of anti-VEGF agents in future trials (31, 35, 36).

This current study is limited by the inherent difficulties of translational research in human subjects, especially in phase I settings. Tissue availability was not comprehensive, particularly for samples from the time of initial biopsy, usually obtained in community hospitals and yielding small amounts of paraffin-fixed tissue from stereotactic procedures. Nonetheless, we attempted to optimize molecular analyses on all tissues in an effort to glean maximal information. Finally, while the data support the ability of GSIs to cross the BBB and modulate targets in the brain, tumor recurrence could not be avoided, suggesting a role for combination therapies. Drug levels could be further optimized, perhaps with the use of alternative agents with more favorable pharmacokinetic profiles as compared with RO4929097. In fact, for the time being, further development of RO4929097 has been halted, in a decision made by the manufacturer before the results of this study become available. Several other GSIs are in development and may provide viable alternatives for further exploring this approach.

In summary, we describe a proof-of-concept study within the scope of a phase I clinical trial investigating the use of gamma-secretase/Notch inhibition in combination with temozolomide and radiotherapy in newly diagnosed glioblastoma or anaplastic astrocytoma. This regimen was safe, well tolerated, and appears to be a promising addition in the effort to control these tumors. Correlative studies demonstrate the ability of this drug to achieve sufficient brain penetration, although higher doses and continuous administration may be beneficial. Tissue studies also provided insights into the mechanisms of action and resistance associated with Notch inhibition, confirming its effects on GSC populations and angiogenesis in humans, and supporting a potential synergism with radiotherapy and temozolomide. The study also highlights the tumor's ability to adapt to alterations in its microenvironment, thereby switching to a Notch-independent form of angiogenesis. These findings further endorse the necessity of targeting multiple signaling pathways concomitantly in gliomas, and highlight the biologic differences in targeting otherwise normal developmental pathways, as opposed to targeting specific mutations. It is clear that Notch inhibition, alone or in combination with radiation, is insufficient to fully control tumor progression; but it does demonstrate promise as a targeted biologic tool that counteracts tumor stem cell–like behavior by curbing self-renewal, and possibly angiogenesis. More effective antiproliferative agents and other environmental modulators will still be necessary as adjuncts.

Disclosure of Potential Conflicts of Interest

A.M.P. Omuro is a consultant/advisory board member for Bristol-Myers Squibb, Novartis, Oxigene, and Stemline. No potential conflicts of interest were disclosed by the other authors.

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References

1. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–96.
2. Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. N Engl J Med 2014;370:699–708.
3. Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R, et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. N Engl J Med 2014;370:709–22.
4. Singh SK, Clarke ID, Hide T, Dirks PB. Cancer stem cells in nervous system tumors. Oncogene 2004;23:7267–73.
5. Louvi A, Artavanis-Tsakonas S. Notch signalling in vertebrate neural development. Nat Rev Neurosci 2006;7:93–102.
6. Purow BW, Haque RM, Noel MW, Su Q, Burdick MJ, Lee J, et al. Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. Cancer Res 2005;65:2353–63.
7. Benedito R, Roca C, Sorensen I, Adams S, Gossler A, Fruttiger M, et al. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell 2009;137:1124–35.
8. Abravanel DL, Belka GK, Pan TC, Pant DK, Collins MA, Sterner CJ, et al. Notch promotes recurrence of dormant tumor cells following HER2/neu-targeted therapy. J Clin Invest 2015;125:2484–96.
9. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009;137:216–33.
10. Gordon WR, Vardar-Ulu D, Histen G, Sanchez-Irizarry C, Aster JC, Blacklow SC. Structural basis for autoinhibition of Notch. Nat Struct Mol Biol 2007;14:295–300.
11. Hovinga KE, Shimizu F, Wang R, Panagiotakos G, Van Der Heijden M, Moayedpardazi H, et al. Inhibition of notch signaling in glioblastoma targets cancer stem cells via an endothelial cell intermediate. Stem Cells 2010;28:1019–29.
12. Wang J, Wakeman TP, Lathia JD, Hjelmeland AB, Wang XF, White RR, et al. Notch promotes radioresistance of glioma stem cells. Stem Cells 2010;28:17–28.
13. Saito N, Fu J, Zheng S, Yao J, Wang S, Liu DD, et al. A high Notch pathway activation predicts response to gamma secretase inhibitors in proneural subtype of glioma tumor-initiating cells. Stem Cells 2014;32:301–12.
14. Fan X, Khaki L, Zhu TS, Soules ME, Talsma CE, Gul N, et al. NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. Stem Cells 2010;28:5–16.
15. Dantas-Barbosa C, Bergthold G, Daudigeos-Dubus E, Blockus H, Boylan JF, Ferreira C, et al. Inhibition of the NOTCH pathway using gamma-secretase inhibitor RO4929097 has limited antitumor activity in established glial tumors. Anti-Cancer Drug 2015;26:272–83.
16. Purow B. Notch inhibition as a promising new approach to cancer therapy. Adv Exp Med Biol 2012;727:305–19.
17. Olsauskas-Kuprys R, Zlobin A, Osipo C. Gamma secretase inhibitors of Notch signaling. Onco Targets Ther 2013;6:943–55.
18. Luistro L, He W, Smith M, Packman K, Vilenchik M, Carvajal D, et al. Preclinical profile of a potent gamma-secretase inhibitor targeting notch signaling with *in vivo* efficacy and pharmacodynamic properties. Cancer Res 2009;69:7672–80.
19. Tolcher AW, Messersmith WA, Mikulski SM, Papadopoulos KP, Kwak EL, Gibbon DG, et al. Phase I study of RO4929097, a gamma secretase inhibitor of Notch signaling, in patients with refractory metastatic or locally advanced solid tumors. J Clin Oncol 2012;30:2348–53.
20. LoConte NK, Razak AR, Ivy P, Tevaarwerk A, Leverence R, Kolesar J, et al. A multicenter phase 1 study of gamma-secretase inhibitor RO4929097 in combination with capecitabine in refractory solid tumors. Invest New Drug 2015;33:169–76.
21. Diaz-Padilla I, Hirte H, Oza AM, Clarke BA, Cohen B, Reedjik M, et al. A phase Ib combination study of RO4929097, a gamma-secretase inhibitor, and temsirolimus in patients with advanced solid tumors. Invest New Drug 2013;31:1182–91.
22. Strosberg JR, Yeatman T, Weber J, Coppola D, Schell MJ, Han G, et al. A phase II study of RO4929097 in metastatic colorectal cancer. Eur J Cancer 2012;48:997–1003.
23. Sahebjam S, Bedard PL, Castonguay V, Chen Z, Reedijk M, Liu G, et al. A phase I study of the combination of ro4929097 and cediranib in patients with advanced solid tumours (PJC-004/NCI 8503). Brit J Cancer 2013;109:943–9.
24. Richter S, Bedard PL, Chen EX, Clarke BA, Tran B, Hotte SJ, et al. A phase I study of the oral gamma secretase inhibitor R04929097 in combination with gemcitabine in patients with advanced solid tumors (PHL-078/CTEP 8575). Invest New Drug 2014;32:243–9.
25. Krop I, Demuth T, Guthrie T, Wen PY, Mason WP, Chinnaiyan P, et al. Phase I pharmacologic and pharmacodynamic study of the gamma secretase (Notch) inhibitor MK-0752 in adult patients with advanced solid tumors. J Clin Oncol 2012;30:2307–13.
26. Li JL, Sainson RC, Shi W, Leek R, Harrington LS, Preusser M, et al. Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth *in vivo*. Cancer Res 2007;67:11244–53.
27. Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, et al. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature 2006;444:1032–7.
28. Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature 2006;444:1083–7.
29. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature 2004;432:396–401.
30. Stockhausen MT, Kristoffersen K, Poulsen HS. The functional role of Notch signaling in human gliomas. Neuro-oncology 2010;12:199–211.
31. Zhu TS, Costello MA, Talsma CE, Flack CG, Crowley JG, Hamm LL, et al. Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH ligands that nurture self-renewal of cancer stem-like cells. Cancer Res 2011;71:6061–72.
32. Carro MS, Lim WK, Alvarez MJ, Bollo RJ, Zhao X, Snyder EY, et al. The transcriptional network for mesenchymal transformation of brain tumours. Nature 2010;463:318–25.
33. Pitz MW, Desai A, Grossman SA, Blakeley JO. Tissue concentration of systemically administered antineoplastic agents in human brain tumors. J Neuro Oncol 2011;104:629–38.
34. Pourquie O. Vertebrate segmentation: from cyclic gene networks to scoliosis. Cell 2011;145:650–63.
35. Woltje K, Jabs M, Fischer A. Serum induces transcription of hey1 and hey2 genes by alk1 but not notch signaling in endothelial cells. PLoS One 2015;10:e0120547.
36. Takebe N, Nguyen D, Yang SX. Targeting notch signaling pathway in cancer: clinical development advances and challenges. Pharmacol Ther 2014;141:140–9.