Perspective

Benefits, limitations and opportunities of NOTCH inhibitors for treatment of glioma

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Abstract: High grade gliomas are associated with worse prognosis and treatment failure. In glioma, the anti-tumor effect of Notch inhibition in combination with the standard care of treatment has been shown *in vitro* and in pre-clinical research. Now, a phase 0/1 trial by Xu *et al.*, evaluated for the first time the efficacy of a previously available NOTCH inhibitor (RO4929097) in combination with standard treatment consisting of radiation therapy (RT) with daily temozolomide (TMZ) followed by adjuvant TMZ in patients with glioblastoma (GBM) or anaplastic astrocytoma (AA). Quite impressively the authors also studied the *in vivo* pharmacokinetics of RO4929097 and tumor perfusion in glioma prior to RT + TMZ and in resections using contrast-enhanced MRI and noted tumor progression in several patients during treatment. However, defining disease progression versus pseudoprogression (psPD) as a result of therapy-induced alteration of blood-brain-barrier (BBB) can be difficult. While sufficient drug target inhibition was achieved in the initial tumor, recurrence occurred. Surprisingly, recurrent tumors exhibited increased expression of angiogenesis markers despite low NOTCH activity contrasting the pro-angiogenic role of NOTCH. While the authors concluded a NOTCH-independent form of angiogenesis, it is possible that therapy-induced inflammation and necrosis resulted into break down of the BBB causing the upregulation of pro-angiogenic markers. Given the disappointing outcome of the sustained NOTCH inhibition, their effect could potentially be enhanced when used in conjunction with angiogenesis inhibitors. Importantly, in this study it was not stated if GBM tumors originated *de novo* (primary) or progressed from low grade glioma (secondary). This is crucial due to their different cell of origin and molecular profile, which can lead to different treatment response and outcome. Further investigating different scheduling to allow normal tissue recovery, optimizing the sequence of multimodal treatments, combined with patient selection and monitoring seems necessary to move NOTCH therapeutics forward.

Keywords: NOTCH; glioma; γ-secretase inhibitors (GSI); clinical; clinical trial; biomarker

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A multi-institute phase 0/1 trial spearheaded by Memorial Sloan Kettering Cancer Center and sponsored by the National Cancer Institute recently reported their findings on the use of a novel targeted drug for patients newly diagnosed with high grade gliomas 17/21 glioblastoma (GBM) and 4/21 anaplastic astrocytoma (AA). GBM is the most common and deadliest primary brain tumor. Surgery, and postoperative radiation therapy (RT) with concurrent

chemotherapy (temozolomide), has shown to prolong survival at the highest quality but still, the median overall survival is a dismal 14-months after initial diagnosis (1). Treatment failure is frequent and recurrent tumors are invariably unresponsive. Alternative therapies are sorely needed.

Increasing evidence indicates that a small subpopulation of tumor cells with properties of cancer stem cells (CSC) or glioma stem cells (GSC) are associated with high grade tumors with worse prognosis, treatment failure and recurrence (2,3). In preclinical models for glioblastoma, CD133+ (a CSC marker) cells demonstrate a high tumor-initiating capacity (4) are more radiation-resistant (5) and chemo-therapy resistant (6) compared to the bulk of CD133- glioma cells. To target and selectively kill these CSCs, the NOTCH signaling pathway has been proposed as an attractive therapeutic route due to its role in maintaining glioma stem cells (7,8). NOTCH receptors are type I transmembrane signaling molecules that upon ligand binding on adjacent cells are activated by a proteolytic cleavage in the membrane by the enzyme γ-secretase leading to the release of the NOTCH intracellular domain (NICD) that translocates to the nucleus and activates gene expression (9). γ-secretase inhibitors (GSI) effectively inhibit the NOTCH pathway in cancer (stem cells) in preclinical models as well as in clinical trials (10,11).

The MSK trial reported by Xu *et al*. in June's issue of Clinical Cancer Research evaluated the efficacy of a previously available GSI (RO4929097) in combination with standard RT concurrent with daily TMZ followed by adjuvant TMZ in a cohort of patients (n=21) with newly diagnosed GBM (grade IV) or AA (grade III) (12). All the grade IV patients (n=10) were candidates for surgery and received daily GSI for 7 days prior to the surgery. The study made use of a 3+3 trial design using dosages of 10, 15, and 20 mg, to establish a maximum-tolerated dose and used advanced techniques such as dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and liquid chromatography with mass spectrometry to determine the pharmacokinetic and chemical abundance of the drug in resected tumor explants/slices. Of particular interest and debate in this study are tissue samples taken from tumors in a subset of patients (n=7) who had radiological recurrence while on treatment.

This is the first study to report clinically, what has been shown *in vitro*, and in preclinical data (13), that NOTCH inhibition can have a modulating anti-tumor effect in patients with high-grade gliomas treated with standard of care. This observation is supported by their data that show in the tumor a decrease in perfusion, and vascular permeability from pharmacokinetic analysis; a down-regulation of NOTCH ligands DLL1, DLL3 and JAG2 and the downstream effector HES5; a decrease in proliferation based on Ki-67 immunohistochemistry; a decrease in the number of NICD1-positive cells, and finally, the observation of vascular normalization phenotype following seven days of drug administration.

Previously the MSK group had already demonstrated the synergistic effect of NOTCH inhibition in combination with radiation on tumor cell survival, and that in patient-derived explants the triple combination (GSI+RT+TMZ) had a synergistic effect on cell viability (14). This is in line with our own observations on synergistic interactions using the same treatment combination that was published shortly before the study at hand (13). In our study, we demonstrated that the triple combination prolonged survival in an orthotopic glioma mouse model when compared to the single or dual drug combination. A separate noteworthy result reported in both studies was that, as expected a significant decrease in the proportion of CD133+ cells occurred when exposed to the NOTCH inhibitor alone but this cell population did not synergistically, nor additively, reduce further when using the triple combination treatment. Further analysis in our study showed a further reduction in the expression of other stem cell markers such as *SOX2* and *nestin* (13). Therefore, to understand the relevant response to NOTCH inhibition in the context of standard of care treatment in future clinical investigation, it is of importance to understand if the treatment resistant population sensitive to NOTCH inhibitors is contained within the *CD133*, *SOX2* and *nestin* expressing population or if it is an as of yet undefined distinct population. The identification of these GSC may have improved prognostic and predictive value.

Although the authors of this phase 0/1 trial report that the combination of the NOTCH inhibitor (20 mg/day, daily) with the standard of care was well tolerated in patients, they do report a reversible toxicity profile consisting mainly of grade 3 and 4 hematologic toxicities. In other trials (Phase 1 and 2) using the same γ-secretase inhibitor (20 mg/day, 3 days on-4 days off) common grade 1 and 2 toxicities including diarrhea, hypertension, fatigue and nausea in metastatic melanoma (15), patients with refractory metastatic or locally advanced solid tumors (16-18) as well as metastatic colorectal cancer (19) were reported, while transient grade 3 toxicity was reported when administering higher doses of the NOTCH inhibitor (18). In the 7 of the

35 registered trials at https://clinicaltrials.gov/ that have reported results, 85% (99/117) of patients indicate adverse events while 21% (25/117) of patients reported severe adverse events while on trial. This could partially explain why Roche has ceased to produce this pipeline drug.

In spite of these cold facts, the number of exploratory studies that were conducted in this phase 0/1 have resulted in a number of beneficial data, including an appreciation for the pharmacokinetic and chemical abundance of the GSI drug in the tumor. Using intra-operative contrast enhanced MRI before and after resection and 7 days of RO4929097 treatment (n=11) the authors show that despite lower drug uptake in low contrast areas (intact blood brain barrier) therapeutic doses were obtained in all patients albeit target genes were more strongly attenuated in high contrast areas. Moreover, the tissue samples of the tumor at the time of radiological progression had similar levels of the drug as was observed during the initial surgery suggesting a long metabolic half-life of RO4929097. In line with that, other reports argued that using RO4929097 at high concentrations (more than 20 mg/day) result in significant reduction of steady-state drug level and as a result a lack of activity (18,19). While this is a limiting factor in dose escalation ability of this drug, the current finding indicates that underexposing patients to RO4929097 NOTCH inhibitor could be clinically significant in treatment of patients with RO4929097 in trials which have stopped due to dose-limiting toxicity and increase possibilities for combination treatments where toxicity is an issue.

Furthermore, the biological activity of RO4929097 within the tumor was determined by gene expression analysis of the NOTCH pathway as a result of I. the drug penetration (perfused *vs.* non-perfused area) and II comparison analysis on a set of tumor tissues obtained pre- and post-treatment. This data suggests a decrease in NICD1 and *HES5* (*NOTCH* target gene) expression while the *HES1*, *HEY1* and *HEY2* expression, well-known NOTCH target genes remained unchanged. While sufficient drug exposure and target inhibition were achieved, the authors argued that the tumor heterogeneity or the regions from where the tumor was resected could be a concern in interpreting the inconsistent target inhibition among patients.

Despite high NOTCH activity in the initial tumor, tumors at time of progression, exhibited a low NOTCH activity, which exhibited increased expression of angiogenesis markers (CD31 and VEGFA) contrasting the pro-angiogenic role of NOTCH in tumor vasculature (20). The authors conclude a NOTCH-independent form of angiogenesis in these treated gliomas. Given the disappointing outcome of the sustained NOTCH inhibition, this effect could potentially be blocked by targeting an additional pathway disrupter such as anti-angiogenesis drugs. Importantly, investigating the best sequence for combination modality treatment could increase the effectiveness of these therapies especially when using anti-angiogenesis drugs, which affects tumor oxygenation and perfusion. In this regard, an ongoing trial investigating the effect of RO4929097 NOTCH inhibitor in combination with Bevacizumab (anti-angiogenesis) in treatment of recurrent glioblastoma has been stopped after future development of the NOTCH inhibitor has been halted (ClinicalTrials.gov: NCT01189240). This is unfortunate, as preclinical studies have clearly demonstrated potential for such combination treatments when used correctly. Li *et al.*, demonstrated that expression of the NOTCH ligand DLL4 in human glioma induced resistance to anti-angiogenic treatments but that blocking NOTCH using GSI concomitant to Bevacizumab induced strong responses in a preclinical model (21).

Study design

Several questions remain however on the study design by Xu and colleagues. First the group of patients in this study included AA, WHO grade III, as well as GBM, WHO grade IV. GBM may arise *de novo* (primary GBM) or progress from low grade gliomas (secondary GBM). While these diseases are histologically similar, their molecular features and cell-of-origin are different. Within the group of newly diagnosed GBM for most tumors (12/17) it was not determined if these were primary GBM (IDH1/2 wt) or secondary GBM (mutated IDH1/2). This is significant as these subtypes have different treatment response and outcome (22). Clinical trials are ongoing to identify which concurrent treatment schedule is able to prolong overall survival of AA. Further analysis of these trials (NCT00626990) will define if the Stupp protocol will become standard treatment for AA as well.

In their study Xu *et al.*, observed tumor progression in seven patients at a median time of 181 days (range, 104–399 days) after initiation of NOTCH inhibition. This means that in some patients progression is defined during the temozolomide cycles following chemoradiation. One could argue that at this point in the treatment schedule real progression is sometimes difficult to differentiate

from pseudo-progression. Pseudoprogression (psPD) is an important imaging artefact occurring in 20–40% of primary GBM which reflect increased uptake of MRI-contrast agent as a consequence of radiotherapy induced necrosis and inflammation, causing breakdown of the blood-brain barrier. Importantly, MGMT methylated patients with psPD have a better prognosis than those with no psPD mostly likely because of higher TMZ dose to the tumor (23). Re-operation is not standard of care when psPD is suspected. These therapy induced alterations in the BBB, leading to psPD, in conjunction with the angiogenesis phenotypes of NOTCH inhibitors may (partly) be explained by the upregulation of pro-angiogenic markers as a consequence of treatment induced inflammation and necrosis despite significant NOTCH inhibition. Thus, the conclusion that gliomas switch to a NOTCH-independent form of angiogenesis is premature and may lead to flawed conclusions with negative consequences for the patient and it should be investigated in recurrent high grade gliomas.

Moreover, because NOTCH inhibitors alter tumor vasculature, this will also affect TMZ uptake in the tumor when used concurrently. Surprisingly while the study measured RO4929097 in biopsies from the low and high contrast tumors, TMZ drug levels were not reported in tumors. As this would significantly impact response, different scheduling's could be exploited to obtain better responses.

The site of tumor biopsy is crucial in the analysis of the trial data. Therefore, it is important to unequivocally define areas of low versus high contrast uptake, since this can be interpreted differently in AA *vs*. GBM. In AA, contrast enhancement defines areas of dedifferentiation, while low contrast enhancement is seen in areas of gliosis surrounding the dedifferentiated, highly proliferating tumor areas. In GBM, contrast enhancement is also seen in the highly proliferating rim of the tumor, while low contrast enhancement is seen in the necrotic areas, which are a negative predictor in untreated GBM but are also induced by treatment. Since it is not stated if both primary and secondary GBMs are included, it is not clear from the presented data if comparable tumor areas are analyzed in the different glioma entities.

Patients that received combined treatment of TMZ and RO4929097 also developed reversible grade III hematopoietic toxicities common to treatment with TMZ. In addition, patients developed ionic imbalances probably due to intestinal dysfunction, a well-known off target effect of GSI, such as RO4929097. Gastrointestinal side effects of GSI should be closely monitored, when combined with an orally administered drug, like temozolomide as this affects uptake. Therefore, it would be interesting to analyze the temozolomide concentration, as part of the pharmacokinetic studies of this trial, as mentioned above.

How to move forward?

Unfortunately, because of the discontinuation of the RO4929097 from Roche, and other NOTCH inhibitors from other manufactures, further clinical studies will need to occur with other agents that target NOTCH. These include monoclonal antibodies that target specific NOTCH receptors and DLL and JAG ligands as well as other small molecules (10,24). Further improvements may be obtained by different scheduling to allow normal tissue recovery and optimizing the sequence of application in multimodal setting to achieve optimal interaction with combination treatments. For example, NOTCH inhibitors block tumor cell proliferation which is disadvantageous when this occurs at the same time of ChemoRadiation; treatments that are most effective in proliferating cells. Other developments may come from tumor specific delivery or activation of GSI's.

The elegant study of Xu *et al*., highlights several important aspects that may explain the limited efficacy of γ-secretase inhibitors in clinical trials seen so far. In none of the current trials a prospective selection of patients has been performed to select those patients that are most likely to benefit from treatment. Xu *et al*. shows that while some NOTCH target genes predict prognosis (cut-off 14 months) those same target genes do not predict drug response nor is their expression affected by GSI treatment. One explanation could be that regulation of commonly used NOTCH target genes (i.e., HES, HEY) can also be regulated NOTCH-independent (25). Furthermore, Xu *et al*. observed *HES5* but not *HES1* downregulation in GSI-treated GBM. Consistent with findings by Tolcher *et al*., (18) in our preclinical study we only observed HES1 downregulation and not *HES5* (nor *HEY2*) suggesting that there are species and tissue specific differences which makes extrapolation difficult. Importantly, diagnostic, predictive and pharmacologic biomarkers to enable selection and monitoring of patients receiving anti-NOTCH therapeutics are limited. Patient stratification using such biomarkers might identify a sub-group of patients with more robust and durable responses that can be objectively and quantitatively assessed and adapted when needed. When these steps have been made NOTCH inhibitors deserve a second chance.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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