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A pro-invasive role for the Ca²⁺-activated K⁺ channel KCa3.1 in malignant glioma

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Abstract

Glioblastoma multiforme (GBM) are highly motile primary brain tumors. Diffuse tissue invasion hampers surgical resection leading to poor patient prognosis. Recent studies suggest that intracellular Ca²⁺ acts as a master regulator for cell motility and engages a number of downstream signals including Ca²⁺-activated ion channels. Querying the REpository of Molecular BRAin Neoplasia DaTa (REMBRANDT), an annotated patient gene database maintained by the National Cancer Institute, we identified the intermediate conductance Ca²⁺-activated K⁺ channels, KCa3.1, being overexpressed in 32% of glioma patients where protein expression significantly correlated with poor patient survival. To mechanistically link KCa3.1 expression to glioma invasion, we selected patient gliomas that, when propagated as xenografts *in vivo*, present with either high or low KCa3.1 expression. In addition we generated U251 glioma cells that stably express an inducible knockdown shRNA to experimentally eliminate KCa3.1 expression. Subjecting these cells to a combination of *in vitro* and *in situ* invasion assays, we demonstrate that KCa3.1 expression significantly enhances glioma invasion and that either specific pharmacological inhibition with TRAM-34 or elimination of the channel impairs invasion. Importantly, after intracranial implantation into SCID mice, ablation of KCa3.1 with inducible shRNA resulted in a significant reduction in tumor invasion into surrounding brain *in vivo*. These results show that KCa3.1 confers an invasive phenotype that significantly worsens a patient's outlook, and suggests that KCa3.1 represents a viable therapeutic target to reduce glioma invasion.

Keywords

glioma; KCa channel; invasion; KCNN4; IK

Introduction

Glioblastoma multiforme (GBM) is the most malignant and most common among primary brain tumors, referred to as gliomas (Jemal et al., 2006; Huncharek and Muscat, 1998).

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Gliomas are notorious for diffusely invading the brain, making complete surgical resection rarely feasible. Residual cancer typically causes recurrence, explaining poor patient prognosis, with average life expectancies of only 12–15 months (Zalutsky, 2005). Common routes for invasion include blood vessels, white matter tracts, and the brain parenchyma (Sontheimer, 2004).

Signals and biological changes that regulate glioma invasion are not completely understood. However, cyclical, oscillatory changes in intracellular Ca^{2+} correlate with cell migration in both normal and malignant cells in the brain (Ronde et al., 2006). For example, during development, migrating cerebellar granule cells show NMDA-R-mediated intracellular Ca^{2+} oscillations which determine the velocity and direction of cell movement (Komuro and Rakic, 1993). Similar Ca^{2+} oscillations occur during the movements of gliomas are triggered by the activation of muscarinic acetylcholine receptors (Bordey et al., 2000) or Ca^{2+} -permeable AMPA receptors (Ishiuchi et al., 2002).

Ca^{2+} can affect migration in many ways, and our understanding is still evolving. However, it is widely accepted that actin polymerization and depolymerization at the leading and trailing edges of the cell, respectively, require changes in Ca^{2+} , and many cell-cell and cell-matrix interactions are regulated by changes in intracellular Ca^{2+} (For review see (Wei et al., 2012)). Recent investigations have added Ca^{2+} as a regulator of K^{+} and Cl^{-} ion channels, which are highly expressed in gliomas (Becchetti, 2011); for example the genetic knockdown of the CaMKII-regulated Cl^{-} channel/transporter ClC-3 impaired glioma invasion (Cuddapah and Sontheimer, 2010; Cuddapah et al., 2011). Ca^{2+} -activated channels could be useful because of their unique properties: they are able to respond to Ca^{2+} changes by allowing movement of ions, both of which are important during migration.

In the current study, we examined whether Ca^{2+} regulated ion channels contribute to glioma invasion. A search of the annotated genomic patient data in the REepository of Molecular BRAin Neoplasia DaTa (REMBRANDT) database identified the Ca^{2+} -activated K^{+} channel gene KCNN4 , which encodes for the intermediate conductance Ca^{2+} -activated K^{+} channel, KCa3.1 , as overexpressed in 32% of glioma patients and correlated with significantly shortened survival. Using patient-derived gliomas propagated in the flank of nude mice as well as glioma lines with genetic knockdown of KCa3.1 expression, we show that KCa3.1 confers an invasive phenotype and its ablation significantly reduces glioma invasion *in situ* and *in vivo*. Importantly, a highly selective pharmacological inhibitor for KCa3.1 , TRAM-34, is already in clinical trials for other diseases and could be used for the development of anti-invasive therapy for human gliomas.

Materials and Methods

Animals

SCID mice of both sexes older than 2 months were used following approved protocols according to the University of Alabama Institutional Animal Care and Use Committee.

Drugs and Solutions

Clotrimazole (10 μ M), puromycin (2 μ g/mL), and doxycycline (1 μ g/mL) were obtained from Sigma (Milwaukee, WI, USA), and TRAM-34 (1 μ M) from Tocris Biosciences.

For electrophysiology, the following recording solution was used (in mM): 130 Na gluconate, 5 KCl, 1 CaCl_2 , 32.5 HEPES, and enough glucose to achieve 320mOsm. Solution was brought to pH 7.4 with NaOH.

Internal pipette solution used during electrophysiology contained (in mM): 140 KCl, 1 MgCl_2 , 10 EGTA, and 10 HEPES. 4.9 mM CaCl_2 was added on the day of recording to achieve a free Ca^{2+} concentration of 150nM. Solution was brought to pH 7.2 with Tris Base.

The cutting solution used to make acute slices for *in situ* live invasion contained the following (in mM): 135 NMDG, 1.5 KCl, 1.5 KH_2PO_4 , 23 Choline-Bicarbonate, 25 D-Glucose, and 0.4 Ascorbic Acid. Following NMDG addition, pH was brought to 7.4 using HCl. Before cutting, 0.35 CaCl_2 and 1.75 MgCl_2 were added. Solution was bubbled with 95% O_2 / 5% CO_2 to reach pH 7.4.

The *in situ* invasion assay was performed in a modified artificial CSF (ACSF) solution containing (in mM): 125 NaCl, 3 KCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , and 25 D-Glucose. 2 CaCl_2 , 1 MgCl_2 , and 7% fetal bovine serum (FBS) were added the day of the experiment. Solution was bubbled with 95% O_2 / 5% CO_2 to reach pH 7.4.

Cell Culture

The U251 glioma cell line, along with other lines, were maintained in DMEM/F12 with 7% fetal bovine serum (FBS). Xenolines were maintained in the flank of nude mice as described previously (Giannini et al., 2005; Sarkaria et al., 2007). Before use, xenolines were maintained in culture in gliospheres using Neurobasal A media without FBS to prevent cell attachment to the flask.

Knockdown and Control shRNA

Knockdown of KCa3.1 was performed using TRIPZ-inducible lentiviral shRNA from Thermo Scientific. U251-GFP cells were transfected as described previously (Weaver et al., 2006) with anti-KCNN4 shRNA (construct V2THS_133694) and the non-silencing shRNA control. Cell lines were clonally grown and maintained with puromycin-containing media. A 4–6 day application of doxycycline (dox) was used to induce shRNA expression.

Electrophysiology

Whole-cell recordings were performed with an Axopatch 200B amplifier (Axon Instruments; Foster City, CA, USA) on coverslips plated for 3–7 days using pClamp 8.2 software (Axon Instruments) and standard recording techniques. A resistance of 3–5 M Ω was used for glass pipette tips. Series resistance was compensated to 80%, and cells were omitted if the resting potential was $< -25\text{mV}$ upon gaining access.

Immunostaining

Cultured cells were fixed using 4% PFA and blocked using 10% goat serum and 0.5% Triton X-100 in PBS. Primary antibodies were added overnight, and then slices were washed and secondary antibody (goat anti-mouse Alexa 488 (Invitrogen; Grand Island, NY, USA) at 1:500) applied. Slices were washed, then mounted on glass slides. The primary antibody KCa3.1 (Alomone, ATTO-488) was used at 1:250, and Alexa Fluor 546 Phalloidin (Invitrogen) was used at 1:100. Staining was imaged with a 40× air objective using the Olympus Fluoview FV1000 laser scanning microscope.

Western Blots

Western blotting was performed as described previously (Bomben and Sontheimer, 2008). Primary antibodies KCa3.1 (1:500) from Sigma, NaKATPase (1:500) from Millipore, and GAPDH (1:5000) from Abcam were used with secondary antibodies conjugated to horseradish peroxidase.

Data Analysis

Data were analyzed using OriginPro8.5, including significance of the Kaplan-Meier survival curve using a log-rank test. Other statistics were calculated using Graphpad software employing ANOVA or Student t-tests, as appropriate, to determine significance. All values are mean ± SEM unless otherwise stated.

In vitro Transwell Migration

8µm pore filters were coated with vitronectin (5µg/mL), and the nonadherent xenolines were plated with 7% serum to allow them to express the proteins necessary for adherence. The following day, all cells were lifted using 0.5 mM EGTA for 20 minutes. Cells were suspended in migration assay buffer (serum-free media with 0.1% fatty acid-free bovine serum albumin), and plated on top of the filters at a concentration of 100,000 cells/mL. Cells were allowed to adhere for 30 minutes before drug application, and 5 hours later, Crystal Violet was added to stop the migration and stain the cells. Using a Zeiss Axiovert 200M microscope with a 20× objective, five random images per filter were used to quantify the number of cells that migrated through the pore to the bottom of the filter.

In situ Live Invasion

Xenolines in gliospheres were plated with 7% serum to allow them to begin to express the proteins necessary to adhere to slices. The following day, 200µm acute slices were cut and recovered in ACSF for 45 minutes. While recovering, cells were lifted and xenolines were incubated with Cell Tracker Orange cellular dye for later visualization. Slices were then transferred to a modified ACSF solution with 5% FBS, and 80,000 cells were applied to the top of the slices. Cells were allowed to migrate for 5 hours before fixation with 4% PFA overnight. Slices were then mounted on glass slides, and Z-stack images were taken of cells migrating into the slice using a 40× air objective on the Olympus Fluoview FV1000 laser-scanning microscope.

For analysis, the z-axis was divided into 10 μm bins from the top of the slice and cell number determined for each bin. For comparison of datasets, both the average distance from the top of the slice and an exponential fit to the histogram data were computed. Both imaging and analysis were performed blinded.

***In vivo* Intracranial Glioma Implantation and Migration**

Surgery was performed on SCID mice under anesthesia with 2–5% isoflurane, half of them fed with a doxycycline diet food (Bio-Serv). After setup on a Stoelting stereotaxic apparatus, an incision was made in the scalp, and a hole in the skull was drilled 0.5mm anterior to bregma and 1mm to the left of midline. $1.25\text{--}5.0 \times 10^5$ U251 glioma cells with knockdown shRNA were implanted 2mm deep using a 10 μL SGE syringe and the World Precision Instrument Micro4 Microsyringe pump controller system. Mice were allowed 3 weeks for tumor growth before intracardiac perfusion with 4% paraformaldehyde and analysis of migration.

For analysis, 100 μm slices were made of the tumor-implanted brains, and the distance of cell migration from the tumor mass was analyzed using the Length function of AxioVision 4.6 software (Carl Zeiss) on a Zeiss Axiovert 200M. Analysis was made for each animal, and then averaged. Both imaging and analysis were performed blinded. Representative images of the tumors were captured using an Olympus Fluoview FV1000 laser-scanning microscope.

Results

Overexpression of KCa3.1 correlates with decreased patient survival

We hypothesized that Ca^{2+} -activated ion channels participate in regulation of glioma invasion, and specifically focused on Ca^{2+} -activated K^+ channels, given their importance in cell migration (Schwab et al., 2012) and other aspects of cancer biology (McFerrin et al., 2012; Ruggieri et al., 2012). This family of channels contains three pharmacologically and biophysically distinct classes, KCNMA1 (KCa1.1), KCNN1 (KCa2), and KCNN4 (KCa3.1).

We first examined whether any of these genes are dysregulated in patients with highly invasive gliomas. To answer this, we queried REMBRANDT, an annotated patient database maintained by NCI (National Cancer Institute, 2005), for Ca^{2+} -activated channels that might affect patient outcome, sampling all primary brain tumor types. Although this database lacks direct annotations for tumor invasiveness, it is able to provide survival rate based on gene expression levels; therefore we elected to use patient survival as a surrogate indicator. We then asked whether patient survival was reduced with gene upregulation, defined as increased gene expression in tumor samples 1.5 fold greater than non-tumor samples. Using this approach, we found that KCMA1/ KCa1.1, was only upregulated in a small number of patients (10.2%), and channel expression was not significantly correlated with patient survival. KCNN1/KCa2 is not commonly expressed in glioma cell lines (Weaver et al., 2006). Patient data shows upregulation of different KCa2 splice variants, yet gene expression failed to uniquely correlate with increased, decreased, or no change in patient survival. However, KCNN4, which encodes for the intermediate conductance or KCa3.1

channel, was upregulated in 32% of the patient samples where it correlated with a highly significant ($p=4.82e^{-6}$) decrease in patient survival (Fig. 1A). In light of the robust difference in patient survival, we elected to further investigate whether this difference in prognosis may be due to changes in glioma invasion.

Patient-derived glioma cell lines and xenolines maintain variable KCa3.1 expression

Since the REMBRANDT data suggests that patients show variable expression of KCa3.1, we next needed a clinically relevant model system in which to study the role of KCa3.1 in tumor invasion.

Published reports suggest that a number of glioma cells lines, including D54, U87, GL261, G115, and U251 express KCa3.1 (Catacuzzeno et al., 2011; Cuddapah et al., 2013; Sciacaluga et al., 2010; McFerrin et al., 2012). We used Western blot analysis for a side-by-side comparison of KCa3.1 expression for a number of established cell lines, using GAPDH expression as a loading control. As illustrated in Fig. 1B, these differed substantially regarding protein expression, with highest levels of expression observed in U251, and barely detectible expression in U87. Interestingly, U87 have been widely reported to be non-invasive, growing solid tumor masses, as compared to U251, which are quite invasive (Jacobs et al., 2011). Moreover, in U251 cells immunostained with specific antibodies to KCa3.1 labeled the leading edge of cells (Fig. 1C) often referred to as invadopodium.

Since established cell lines frequently alter their genotype and phenotype in culture, we elected to also screen patient tissues that had been propagated by ectopic implantation into the flank of a nude mouse. Previous studies have found that these cells, often referred to as “xenolines,” retain gene and protein expression found in the original patient samples (Giannini et al., 2005; Sarkaria et al., 2007). We found 4 patient xenolines that covered a spectrum of KCa3.1 expression on Western blot ranging from very low to very high expression (Fig. 1D). To ensure the presence of functional channels, we recorded activity using whole-cell patch-clamp electrophysiology. Specifically, we recorded from both the Jx12 and Jx39 cells and isolated the KCa3.1 currents by blocking Cl^{-} channels and KCa1.1 channels with gluconate and paxilline, respectively. Cells were held at $-40mV$ and stepped from $-100 mV$ to $+120 mV$ in $20mV$ increments. To isolate the current attributable to KCa3.1, we calculated a TRAM-34-sensitive current. The resulting drug-sensitive currents were normalized to their capacitance and plotted as a function of voltage to obtain an I–V curve. Fig. 1E shows average current density I–V plots ($n=4$ each). In agreement with our Western and immunostaining data, Jx12 cells show a prominent TRAM-34-sensitive current, whereas the Jx39 cells showed no evidence of a TRAM-34-sensitive current (Fig. 1E). Representative traces are presented in Fig. S1. These data provide us with two patient-derived xenolines that endogenously express either high (Jx12) or low KCa3.1 (Jx39), and these were used for further characterization of cell invasion *in vitro* and *in situ*.

Since cell invasion is likely to be affected by other differences in protein expression unrelated to KCa3.1, it was desirable to also investigate invasion in cells where only this channel was manipulated. To accomplish this, we utilized U251 cells and generated sister lines suppressing KCa3.1 expression with a doxycycline-inducible knockdown construct.

Scrambled shRNA served as a control. Induction of shRNA expression was confirmed by expression of red fluorescent protein (RFP). Western blotting was used to assess knockdown of KCa3.1, and multiple repetitions ($n > 3$) confirmed a decrease in protein in the knockdown line after 4–5 days dox application (Fig. 2A). The scrambled line with and without dox, and the knockdown line without dox had similar levels of KCa3.1 expression (Fig. 2A).

In order to confirm a functional loss of KCa3.1 using a more sensitive method, we performed whole-cell patch-clamp electrophysiology on knockdown and control cell lines. Using methods described previously, we isolated the TRAM-34 sensitive currents and created I–V plots. As seen in Fig. 2B, TRAM-34 sensitive currents in U251 cells containing the scrambled construct were comparable in the presence and absence of dox. However, when dox was added to the knockdown cell line, the current was abolished (Fig. 2C). Current densities were quantified at +120mV, showing a significant decrease in the knockdown cell line with dox (Fig. 2C). These results extend the previous findings, indicating a complete loss of functional KCa3.1 channels in the knockdown cells lines.

As a result we now have multiple ways to assess the role of KCa3.1 in glioma invasion, either using cell lines or xenolines with addition of specific inhibitors or using the dox-inducible U251 knockdown cells to selectively inhibit protein translation.

***In vitro* transwell migration is altered with decreased KCa3.1 function**

We hypothesized that expression of KCa3.1 facilitates cell volume changes required for cell invasion; if this is true, inhibition or knockdown of the channel should decrease migration only in cell lines that express the channel. We first used *in vitro* Transwell migration assays, which measure the chemotactic migration of cells across an 8 μ m filter. This assay requires cells to change their volume (Watkins and Sontheimer, 2011) a process previously shown to engage the activity of K^+ and Cl^- channels (Schwab et al., 2012). For quantification, we assessed the relative change in the number of cells that successfully migrated to the bottom of the filter after application of vehicle or the specific inhibitor of KCa3.1, TRAM-34.

As shown in Fig. 3, the two xenolines with high KCa3.1 expression, Jx12 and Jx14, showed a significant decrease in Transwell migration upon channel inhibition with TRAM-34 (25% for Jx12; Fig. 3A, and 30% for Jx14 ; Fig. 3B). By contrast, xenolines with low KCa3.1 expression were unaffected by TRAM-34 (Fig. 3C, D), suggesting that KCa3.1, if present, enhances cell migration *in vitro*.

Similarly, in the glioma cell line U251, inhibition of KCa3.1 using either TRAM-34 or clotrimazole significantly reduced Transwell migration by $25.7\% \pm 0.053$ ($n=5$, $p<0.05$), and $31.2\% \pm 0.051$ ($n=5$, $p<0.05$), respectively, normalized to vehicle control (Fig. 3E). To rule out non-specific drug effects, we repeated these studies using KCa3.1 knockdown cell lines. All control lines produced similar migration levels, yet cells with induced knockdown shRNA for KCa3.1 had a significant ~30% decrease in migration compared to the other controls (Fig. 3F).

Attenuation of KCa3.1 function inhibits *in situ* invasion

To mimic the spatial constraints invading gliomas encounter in the brain, we next used a brain slice invasion assay that preserves some environmental factors invading cells encounter including blood vessels, nerve tracts, and released factors. Here, glioma cells were seeded on top of an acute brain slice and the distance that cells migrate into the tissue was determined after 5 hours. For each slice we measured the distance of migration by counting the number of cells located in 10 μm sections, i.e. 1–10 μm deep, 11–20 μm deep, etc. The number of cells in each binned distance was plotted in histogram form and fit to an exponential decay function as previously described (Withers and Lee, 2006). The fit yielded a “migration constant” that is best interpreted as the migration distance achieved by at least 1/e or 1/3 of the cells.

Because their fluorescence enabled ease of visualization, we first determined the effect of TRAM-34 on migration of U251-GFP gliomas. There was a significant decrease in the average distance the cells migrated into the slice compared to vehicle ($p < 0.05$) (Fig. S2A). There was also a decrease in the number of cells that reached farther distances in the slice, as reflected by a decrease in the migration constant (Fig. 4A). Fig. 4B presents a 3D volume reconstruction of the z-projection for vehicle and TRAM-34 treated slices.

Migration of xenolines was similarly analyzed after application of TRAM-34, though it was necessary to first load cells with a fluorescent dye for visualization. These experiments show a decrease in the average distance of migration in the high KCa3.1 expressing Jx12 cells ($p < 0.05$) (Fig. S2C, Fig. 5A). By contrast, the Jx39 cells, which express low levels of KCa3.1, showed no change after TRAM-34 application in the average distance migrated (Fig. S2D) or migration constant (Fig. 5C). Representative images are shown in Fig. 5B, D.

We similarly examined the KCa3.1 knockdown cells to confirm that the observed effects were due to a loss of channel function rather than non-specific drugs. Using the Scrambled + Dox and Knockdown + Dox cell lines, we were able to confirm expression of the shRNA in specific cells using the presence of the RFP fluorescent marker, which is transcribed along with the shRNA after Dox application. Therefore, we analyzed only the RFP-positive cells, and found a significant reduction in the average distance of migration after KCa3.1 knockdown ($p < 0.05$) (Fig. S2B). Furthermore, there was a decrease in the migration constant (Fig. 4C), which can be visualized using 3D volume reconstruction (Fig. 4D). We also assessed migration of all cells in the four lines, Scrambled and Knockdown with and without Dox, and found an inhibition of cell migration only after KCa3.1 knockdown (Fig. S2E), indicating that KCa3.1 is involved in cell migration *in situ*.

KCa3.1 knockdown decreases *in vivo* migration

To determine if KCa3.1 expression also is necessary to support cell invasion *in vivo*, we examined intracranial glioma implants in SCID mice. We used U251 cells transfected with knockdown shRNA for KCa3.1 and fed mice with Dox-containing food to allow expression of the shRNA. As a control, mice were implanted with the KCa3.1 knockdown cells, but given normal mouse chow. A 5 day Dox pretreatment was given for both the mice and cells. Tumors were allowed to grow for 3 weeks, then tumor invasion was analyzed as described

previously for live invasion assays. There was a 30.9% decrease in the average distance that cells migrated away from the tumor mass in KCa3.1 knockdown cells compared to uninduced control cells (Fig. 6A). Furthermore, the number of cells that migrated long distances decreased, as reflected by a lower migration constant after loss of KCa3.1 (Fig. 6B). Fig. 6C shows representative images. These results support the conclusion that KCa3.1 expression enhances glioma invasion *in vivo*.

Discussion

A retrospective unbiased search of a patient genomic database identified that KCa3.1 overexpression correlated with decreased patient survival. Though KCa3.1 was first studied in regard to volume regulation in red blood cells (Gardos, 1958), it was later found in glioma cells and implicated in apoptosis regulation (McFerrin et al., 2012), and growth factor-induced *in vitro* chemotaxis (Cuddapah et al., 2013). In this study, we found variable expression of KCa3.1 in glioma cell lines and patient-derived lines using Western blotting, immunostaining, and electrophysiology (Fig. 1). Our study extends previous observations providing clear evidence that KCa3.1 is functionally expressed in some patient gliomas and, if expressed, enhances their invasive biology (Catacuzzeno et al., 2011; Cuddapah et al., 2013; Sciacaluga et al., 2010). When present, KCa3.1 inhibition or knockdown decreases migration in multiple cell lines using multiple methods of assessment, including *in vivo* tumor invasion. Other studies have implicated KCa3.1 in chemotactic *in vitro* migration, for example chemotaxis towards fetal calf serum, bradykinin, and CXCL12 (Catacuzzeno et al., 2011; Cuddapah et al., 2013; Sciacaluga et al., 2010). By contrast, our study did not use growth factors; hence the observed differences in migration suggest an intrinsic role for this channel in endogenous cell migration.

Ion channels as oncogenes

The idea that ion channels may be involved in cancer is by no means new, and in fact a number of studies of systemic cancers suggest that ion channel mutations are causative of the malignant transformations, and some channels commonly involved in cancer formation could be referred to as “oncochannels” (Huber, 2013). For example the voltage-gated K^+ channel family called “ether a go-go” or EAG is overexpressed in over 75% of all cancers and is sufficient to induce tumor growth *in vivo* when artificially expressed in non-cancerous cells (Camacho, 2006). Therefore, EAG has been proposed to be an oncogene. In glioma, other channels might produce similar effect, as patient studies show a high rate of ion channel/transporter mutation and expression changes in primary brain tumor samples (Masselli et al., 2012; Molenaar, 2011). KCa3.1 may play a role similar to EAG, as they are both K^+ channels, and could act in similar roles. Furthermore, heterologous expression of KCa3.1 has been shown to increase migration (Schwab et al., 2007). Though we have not shown that KCa3.1 is involved in the malignant transformation of gliomas, it does seem to enhance the malignant nature of the cells by facilitating migration.

Other KCa channels

Although KCa3.1 activity is involved in migration of glioma cells if expressed, some lines showed little to no functional expression of the channel. It is possible that this reflects a

decreased invasive capacity, or that other ion channels are engaged in the process. One possibility is compensation by other KCa channels, which we examined in our preliminary search of the genomic database REMBRANDT.

One channel of interest is the large conductance, or KCa1, channel, which has previously been implicated in glioma migration (Weaver et al., 2006). Though this channel is expressed in glioma lines, it is infrequently upregulated in the patient population (10.2%), and this upregulation is not correlated with changes in patient prognosis. Therefore, it is important to recognize that cell lines are not perfect models of human disease, and that studies should be ideally supplemented with patient data in order to confirm their relevance.

The small conductance KCa2 channel also yielded intriguing results. Though previous studies have not found evidence of KCa2 expression in cultured glioma cell lines (Weaver et al., 2006), upregulation of different subtypes were correlated with either increased or decreased patient survival. Because of the lack of specific inhibitors to subtypes and high levels of channel expression in neurons, we did not pursue the investigation of KCa2.

Mechanism of KCa3.1 in migration

Interestingly, we found that in the transwell migration assay, inhibition or knockdown of KCa3.1 decreased migration 25–30% regardless of cell line. This may indicate that KCa3.1 is utilized in migration in similar ways across many lines. The exact mechanism was not investigated specifically, but it is likely that these channels aid volume change required to traverse narrow pores. Volume changes require the coordinated efflux of K^+ and Cl^- ions acting as osmolytes, which moves cytoplasmic water across the cell membrane (Schwab et al., 2007; Becchetti, 2011). Movement of water is facilitated by the water channels aquaporins, which are expressed by glioma and can affect migration (McCoy and Sontheimer, 2007). These volume changes may be particularly important in gliomas, because the small size of the extracellular brain space provides a significant obstacle to cell movement (Schwab et al., 2012). Indeed, gliomas shrink 30–35% of their original volume as they invade *in vivo*, and this is highly regulated by Cl^- channels (Watkins and Sontheimer, 2011). In order to maintain electroneutrality, K^+ ions must accompany Cl^- , and both channels are likely activated by the same signal, such as Ca^{2+} increases. For example, both $ClC-3$ and KCa3.1 are activated by high levels of Ca^{2+} and facilitate migration (Cuddapah et al., 2013). They commonly colocalize on the cell membrane in lipid rafts, along with potential Ca^{2+} channels, the TRPC channels (Cuddapah et al., 2013). Furthermore, thorough studies of KCa3.1 in MDCK-F cells showed that the channel is important specifically for the retraction of the trailing edge of the cell, where it is activated by a gradient of Ca^{2+} and subsequently leads to efflux of water and local volume decrease (Schwab et al., 2012), a mechanism likely shared by many other cells including gliomas.

Potential KCa3.1 regulation in glioma invasion

Characterizing the Ca^{2+} signal for KCa3.1 activation is important, as it is likely highly specific (Stocker, 2004) and could be targeted therapeutically. One possible Ca^{2+} source influx via the stretch activated TRPC1 channels, as neuroblasts migrating through the rostral migratory stream utilize TRP channels as a source for KCa3.1 activation (Turner and

Sontheimer, 2013). Another possible source is the intracellular stores, as KCa3.1 activation during glioma apoptosis is inhibited by IP₃R blockade (McFerrin et al., 2012).

A likely trigger for internal Ca²⁺ increase is growth factor signaling. For example, bradykinin, which causes Ca²⁺ oscillations in glioma cells, can co-activate ClC-3 and KCa3.1 channels (Cuddapah et al., 2013). Inhibition of either channel caused a decrease in chemotactic migration towards bradykinin, and inhibition of both channels almost completely abolished cell migration. Importantly, KCa3.1 and ClC-3 colocalize to lipid rafts, placing them into functional domains and under the control of the same growth factor (Cuddapah et al., 2013). It is therefore likely that they work together to regulate migration through volume change, with internal Ca²⁺ acting as a master regulator to coordinate the activation of both channels.

Therefore, our overarching hypothesis is that growth or motogenic factors increase internal Ca²⁺ in the proximity of KCa3.1, and activate these channels in concert with Ca²⁺-regulated Cl⁻ channels. The resulting salt efflux leads to the release of cytoplasmic water and a decrease in cell volume that facilitates migration.

KCa3.1 as a therapeutic target

Data in the REMBRANDT database suggest an upregulation of KCa3.1 that correlates with a significant decrease in patient survival (Fig. 1A). This upregulation occurred in 32% of patients. However, this does not indicate that only 32% of patients would show a reduction in cell invasion if treated with KCa3.1 inhibitors, as even at “normal” levels KCa3.1 may aid cell migration. In this database, only a small percentage of patients, 17%, had >1.5× downregulated KCa3.1; therefore, most patients express some level of KCa3.1 and could benefit from drugs that interfere with KCa3.1 function. KCa3.1 inhibitors are in pre-clinical and clinical trials for multiple diseases, including sickle cell anemia, and if successful, could be adopted as experimental therapeutics for glioma patients (Wulff et al., 2000; Wulff et al., 2007).

However, cell migration is a complex process; therefore it is unlikely that targeting one ion channel could greatly impact patient survival. One solution is to simultaneously block multiple ion channels, for example supplementing KCa3.1 inhibition with blockade of another K⁺ channel could prevent compensation. Also, it is likely that K⁺ and Cl⁻ channels act in concert to affect cell volume and migration; therefore, blocking both could drastically decrease migration and improve survival. Further investigation is warranted to determine the therapeutic potential of ion channel in gliomas, specifically KCa3.1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main Points

- Using the NCI database, we found that upregulation of KCa3.1 correlates with poor prognosis in patients.
- Pharmacological inhibition or genetic knockdown of KCa3.1 decreases glioma migration, as investigated *in vitro* and *in situ* in multiple glioma cell types, including patient-derived xenograft-propagated tumors.
- Glioma invasion is also attenuated by shRNA knockdown of KCa3.1 in tumor cells implanted in mice *in vivo*.

Figure 1.

KCa3.1 expression in glioma. (A) In patient samples and clinical trials of all types of glioma, upregulation of KCa3.1 expression by 1.5 $\times$ is correlated with significantly decreased survival time, assessed using the REMBRANDT database. Upregulation occurs in 32% of patients (132 out of 413). $p=4.82 \times 10^{-6}$. (B) Common cultured glioma lines were analyzed via western blotting for KCa3.1 expression. Most cells lines had KCa3.1 protein, though expression was variable. GAPDH was used as a loading control. (C) U251 cells immunostained for KCa3.1 show expression on the membrane with high levels on the cells' leading edges. Scale bar 50 μm . (D) Four xenografts propagated in mouse flank assessed via western blot express different levels of KCa3.1, with Jx12 and Jx14 having higher expression than Jx22 and Jx39. GAPDH was used as a loading control. (E) Functional expression of KCa3.1 was confirmed via whole-cell patch-clamp electrophysiology. Jx12 cells present a TRAM-34 sensitive current, whereas Jx39 cells have no change in current after drug application.

Figure 2.

Knockdown of KCa3.1 channels in U251 cells. (A) KCa3.1 protein levels are reduced only after transfection of U251 cells with knockdown shRNA and application of dox to induce transcription, as seen using western blotting. NaKATPase was used as a loading control. (B, C) Functional presence of the channel was assessed via whole-cell patch-clamp electrophysiology. U251 cells transfected with scrambled (B) and knockdown (C) shRNA with and without dox application were held at -40 mV and a step of -100 to $+120$ mV was applied before and after TRAM-34 application. The resulting I–V curves for the TRAM-34 sensitive current were plotted, with no change in the Scr shRNA (B) and a loss of current in the KD + dox line (C). The current density was quantified at $+120$ mV, with a significant decrease in current in the after KCa3.1 knockdown. (Scr-Dox: 9.57 ± 3.14 , n = 8; Scr+Dox: 12.90 ± 2.90 , n = 5; KD-Dox: 23.48 ± 9.41 , n = 8; KD+Dox: 1.44 ± 0.54 , n = 5.) * = p<0.05.

Figure 3.

KCa3.1 is involved in migration *in vitro*. A transwell migration assay was used to assess *in vitro* migration in multiple glioma lines. (A, B) The high expressing xenolines Jx12 (A) and Jx14 (B) have attenuated migration after TRAM-34 application. (Jx12: Vehicle: 18.53 cells $\pm$ 1.32, TRAM-34: 13.87 cells $\pm$ 0.91, n=8.; Jx14: Vehicle: 13.01 cells $\pm$ 1.12, TRAM-34: 9.14 cells $\pm$ 1.08, n=6.) (C, D) The xenolines that have low KCa3.1 expression, Jx22 (C) and Jx39 (D), show no change in migration after TRAM-34 application. (Jx22: Vehicle: 35.29 cells $\pm$ 1.72, TRAM-34: 37.57 cells $\pm$ 2.18, n=5; Jx39: Vehicle: 15.64 cells $\pm$ 1.47, TRAM-34: 17.19 cells $\pm$ 1.53, n=5.) (E) Normalized migration through a transwell filter was significantly decreased after application of TRAM-34 and clotrimazole. (F) Knockdown of KCa3.1 decreases migration compared to control lines. (Scr-Dox: 87.4 cells $\pm$ 3.25, n=6 ; Scr+Dox: 93.39 cells $\pm$ 7.94, n=6; KD-Dox: 87.94 cells $\pm$ 4.68, n=6 ; KD+Dox: 61.84 cells $\pm$ 7.30, n=6.)

* = p<0.05.

Figure 4.

Loss of KCa3.1 function in U251 cells inhibits *in situ* invasion. (A, C) An *in situ* live invasion assay was analyzed by fitting an exponential decay function to the distance into the slice glioma cells migrated, with the bar graph indicating the number of cells at each depth into the slice, and the lines indicating the exponential decay fit. The migration constant and migration were decreased either after (A) TRAM-34 application (Vehicle: 11.87 ± 1.95 , $n=15$; TRAM-34: 9.61 ± 0.172 , $n=17$) or after (C) knockdown of KCa3.1 (Scr+Dox: 23.49 ± 2.97 , $n=16$; KD+Dox: 8.81 ± 0.248 , $n=17$). Migration constants are listed along with the SEM. (B, D) 3D volume reconstructions were performed on a z-projection of the cells into the slice after (B) vehicle or TRAM-34 or (D) knockdown. Scale bars = 20 μm .

Figure 5.

Inhibition of KCa3.1 blocks *in situ* migration in xenolines expressing the channel. (A) Jx12s, expressing high levels of KCa3.1, have decreased migration after inhibition of KCa3.1. (Vehicle: 18.54 ± 5.21 , $n=7$; TRAM-34: 9.68 ± 0.906 , $n=5$) (C) Jx39s, expressing low levels of KCa3.1, have no change with TRAM-34 application. (Vehicle: 9.06 ± 0.601 , $n=7$; TRAM-34: 10.19 ± 0.640 , $n=8$; $p<0.05$) (B, D) 3D volume reconstructions of the images are presented. Scale bars = 20 μm .

Figure 6.

Knockdown of KCa3.1 decreases *in vivo* migration. (A) There was a significant decrease in the average distance cells migrated away from the tumor mass after knockdown of KCa3.1 compared to control. (-Dox: 152.65 ± 13.75 , n=8; +Dox: 105.53 ± 10.43 , n=9.) (B) Exponential decay analysis shows a decrease in migration after KCa3.1 knockdown, and a decrease in the migration constant. (-Dox: 156.0 ± 22.90 , n=8; +Dox: 100.4 ± 13.54 , n=9.) (C) Representative images of the tumors mass with migratory cells. * = $p < 0.05$. Scale bar = 100 μm .