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ELAVL3: A NEW PROTEIN PROMOTING THE GROWTH OF PRONEURAL GLIOBLASTOMA ?

LAUSANNE, DECEMBRE 2016

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ABSTRACT

ELAVL3 is an RNA-binding protein, specifically expressed in terminally differentiated neurons. It is necessary for neuron differentiation and maintenance as well as for neuronal plasticity and memory. Bioinformatic analysis, based on The Cancer Genome Atlas data, showed that ELAVL3 expression is increased in gliobastoma multiforme (GBM) of the proneural subtype. It also revealed strong correlation between ELAVL3 and proneural signature gene expression. Those results suggest that the protein might be implicated in GBM development. However, because the role of ELAVL3 in malignant cells has not yet been investigated, it is not clear whether the increased protein expression is necessary for malignant cell tumoregenicity or if it is simply a byproduct of the expression of proneural genes. This study aims to assess the importance of the increased expression of ELAVL3 in proneural GBM stem cells (GSCs) and to clarify the putative role of the protein in the development of this malignancy.

First of all, the ELAVL3 expression was determined in GSCs grown both as spheroids and as differentiated adherent cells. Then, its expression was suppressed in GSCs. Those cells were then used to perform a proliferation assay and injected as xenografts into mice, in an attempt to better understand ELAVL3 function.

The results of the in vitro experiments showed that the ELAVL3 expression is higher in proneural GSCs compared to stem cells of the other subtypes, which is in agreement with the results of the bioinformatic data analysis. Proliferation assays also showed that non-ELAVL3 expressing cells have a lower proliferation rate than ELAVL3 expressing cells. Unfortunately, those results could not be confirmed by the results of the xenograft injections, due to the lack of data yielded by in vivo experimentation.

The results showed aincreased expression of ELAVL3 in proneural GBM stem cells that has never been described before. It also revealed involvement of the protein in cell proliferation, thus offering potential new perspectives regarding its function and its implication in tumorigenesis. Further studies will be needed to confirm these findings and to establish the exact function of ELAVL3 proneural glioblastoma.

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Figures and tables

INTRODUCTION

Glioblastoma multiforme (GBM), WHO grade IV glioma[1], is the most common type of primary central nervous system malignancy[2]. It also is the most lethal, with a median survival time that ranges from 12 to 18[3] months and a three-year survival rate a little over 7%[4;5]. The majority of GBM arise de novo, in patients with no identifiable risk factors. Those are known as primary glioblastoma. Secondary tumours arise from pre-existing low grade lesions, i.e. grade II or III gliomas. They are rare and only occur in about 10% of patients with GBM[3]. There is currently no curative treatment for GBM. Standard management is based on maximal surgical excision of the tumour, followed by radiotherapy with concomitant and adjuvant Temozolomide chemotherapy[2-5;6;7;8]. Because total surgical resection is impossible to achieve and because GBM does not always respond to medication or radiation, recurrences are frequent and occur in a vast majority of patients[4;5;8;9]. Nonetheless, this treatment combination is the most effective currently available and has been shown to increase survival time[4;7]. Rapid progression, invasiveness and heterogeneity on the macroscopic, microscopic and genetic levels are typical features of GBM[2;5;7], explaining both poor prognosis and current lack of cure.

Current WHO classification relies on histological features. However, it doesn't account for genetic abnormalities, even if they influence prognosis or response to treatment[3]. For example, MGMT promoter methylation has been associated with higher response rates to chemotherapy, whereas the absence of this mutation contributes to treatment resistance[10].
In an attempt to better characterize GBM, a recent study identified recurrent genomic abnormalities in gliobastoma cells, which allowed the creation of a new classification. Based on differences in gene expression profile GBM can now be divided into classical, mesenchymal, neural and proneural subtypes. Each subgroup was associated with distinct gene alterations and specific expression patterns. Proneural malignancies were shown to express higher levels of genes involved in oligodendrocytic development, such as PDGFRA, NKX2-2 and OLIG2, and proneural development genes, such as DCX, DLL3, ASCL1 and SOX family members, than malignancies from other subtypes. Moreover, this study showed that the genetic profile of malignancies influences treatment efficiency, for example standard combination of radiotherapy and chemotherapy has no effect on tumours of the proneural subtype[11].

The presence of cancer stem cells (CSCs) in GBM provides an additional explanation for treatment failure. GBM CSCs have been shown to be involved in resistance to radiotherapy and to chemotherapy[6;12;13], as well as in tumour maintenance and progression[10].

From a functional point of view, CSCs are proliferating, self-renewing cells that possess the ability to form the various types of differentiated cells composing the bulk of solid tumours. They are also capable of initiating tumour growth when transplanted[6;10;12;13]. Therefore, even if CSCs only account for a small percentage of malignant cells, they are thought to be the driving force of tumours.
The exact mechanisms CSCs use to maintain their stem state have not yet been identified. As far as GBM CSCs are concerned, a few elements have been shown to be involved in stemness maintenance, including tumour environment, niche factors, immune response, metabolism, genetics and epigenetics[6;10].

Gene expression may either be regulated at the transcriptional or at the post-transcriptional level. RNA-binding proteins (RBPs) play an essential role in post-transcriptional gene regulation (PTGR) by controlling various aspects of messenger RNA (mRNA) processing, including maturation, transport, degradation, stability and translation[14;15]. RBPs are defined as proteins interacting with coding or non-coding RNA, either directly, through RNA recognition motifs or indirectly, through ribonucleoprotein complexes[16]. Because these cellular pathways are essential for maintaining cell homeostasis, their disruption may lead to a number of diseases, including cancer. A few RBPs families have been shown to be directly involved in tumorigenesis[17], including the ELAVL/Hu family[18].

The ELAVL/Hu RBP family includes four highly conserved paralogs, namely ELAVL1 (HuA/HuR), ELAVL2 (HuB), ELAVL3 (HuC) and ELAVL4 (HuD). ELAVL1 is ubiquitously expressed, whereas ELAVL2, ELAVL3 and ELAVL4 are only expressed in terminally differentiated neurons [16;19;20;21]. Despite their differential expression, all members are involved in PTGR and share the same functions, which depend on the cellular compartment the proteins are expressed in. In the cytoplasm, they are responsible for target mRNAs stabilization and/or transcription activation by binding transcripts 3' UTRs containing AU-rich elements (AREs)[20;22;23]. However, in the nucleus they control pre-mRNA alternative polyadenylation and alternative splicing, by regulating the inclusion or exclusion of various exons[20; 24]. RNA interactions are mediated via RNA recognition motifs (RRMs). Each ELAVL protein possesses three RRMs, namely RRM1, RRM2 and RRM3[20;22], which share over 90% similarity between paralogs, but have different functions[24]. All four paralogs also carry a hinge region, located between RRM2 and RRM3. It contains localization sequences, both a nuclear localization sequence and a nuclear export sequence, which allow the proteins to travel from the cytoplasm to the nucleus and vice-versa[19;20].

The neuron-specific paralogs are involved in neuronal differentiation and maintenance[20;25] and are necessary for neuronal plasticity and memory[26;27]. ELAVL1 plays a role in cellular response to stress, such as DNA damage, by regulating the expression of genes involved in cell growth and proliferation[26]. However, it is not necessary for neuronal differentiation[20]. ELAVL1 is currently the only paralog that has been shown to be involved in the acquisition of a number of hallmarks of cancer[18]. The role of the other ELAVL/Hu proteins in tumorigenesis, if they play any, remains to be investigated.

Interestingly, RNA-sequencing data obtained from primary GBMs grown either as stem-cell-like spheroids compared to their differentiated adherent progeny revealed that ELAVL3 was one of the most up-regulated transcript in GSCs. Additionally, bioinformatic analysis based on TCGA data shows that the expression of ELAVL3 is increased in cells of the proneural subtype compared to other subtypes of GBM. This analysis also reveals strong co-expression between ELAVL3 and a few typical proneural genes and stem related genes, including SOX4, DCX, DLL3, ASCL1, NKX2-2, PDGFRA and OLIG2.

Those results hint that ELAVL3 might be implicated in glioblastoma tumorigenesis. However, because the role of ELAVL3 in malignant cells has not yet been investigated, it is not clear whether the protein expression is increased because it contributes to malignant cells tumorigenicity or if its over-expression is simply a by-product of the expression of pro-neural genes. This study aims to assess the importance of the increased expression of ELAVL3 in GSCs of the proneural subtype and to clarify the protein role in the development of this malignancy.

First the expression of all four ELAVL paralogs was measured in stem-cell-like spheroids and in differentiated adherent cells. The results showed that ELAVL3 is indeed the only paralog to be invariably overexpressed in spheroids compared to adherent cells. ELAVL3 was then knocked-out in spheroids using the CRISPR-Cas9 system. In vitro experiment revealed that ELAVL3-expressing cells proliferate faster than non ELAVL3-expressing cells, suggesting that ELAVL3 might somehow be involved in the development of GBM.

MATERIAL AND METHOD

Cells

Primary cell lines used in this study were previously described by Suva *and al.*[28] and Wakimoto *and al.*[29], including copy number alterations and tumour histopathology of primary tumours and xenografts. GSCs were grown in Neurobasal medium (Gibco) enriched with N2 supplement (Invitrogen), B27 supplement (Invitrogen), Glutamax (Gibco), human FGF and EGF (R&D Systems). 293T cells, HeLa cells and differentiated adherent cells derived from GSCs were grown in DMEM + GlutaMAX medium (Gibco) supplemented with 10% FBS (PAN-biotech) and non-essential amino acids (Gibco).

Quantitative real-time PCR

RNA was extracted from BT 4A, 4S, 8A, 8S, 11A and 11S cells lysate using miRCURY RNA isolation kit (Exiquon), according to manufacturer instructions. To produce cDNA, RNA templates, dNTPs and random primers were incubated at 65°C for 6 minutes. M-MLV reverse transcriptase (Promega), RNAsin ribonuclease inhibitor (Promega) and RT M-MLV 5x reaction buffer (Promega) were then added. Samples were incubated at 42°C for 50 minutes and at 70°C for 15 minutes. 18S ribosomal RNA and cyclophilin A (PPIA) were used as internal controls. 18S and PPIA probes (Thermo Scientific) were mixed with TaqMan universal PCR mastermix (Applied Biosystems), ELAVL paralogs primer sets with POWER SYBR Green mastermix (Applied Biosystems). Two different sets of primers were used for ELAVL3, in order to ensure the specificity of the amplified products. PCR reactions were performed three times for each sample. Primers specificity for their target sequence was assessed by running PCR products on a 2% agarose gel stained with GelRed nucleic acid gel stain (Biotium). The following primer sets were used: ELAVL1 F 5'- AAA TAC GTG ACC GCG AAG –3' and R 5'- CGC CCA AAC CGA GAG AAC A –3'; ELAVL2 F 5'- CAA CAC CCT GAA TGG ATT GAG A –3' and R 5'- TTT TTG GAA GTC CGC TGA CAT –3'; ELAVL3 (1) F 5'- ATG GTC ACT CAG ATA CTG GGG –3' and R 5'- CCA ACT TGC AGG ACT CGA TGT –3'; ELAVL3 (2) F 5'- CCT CAA ATT ACA GAC GAA GAC CA –3' and R 5'- GCT GAC GTA CAG GTT AGC ATC –3'; ELAVL4 F 5'- CGG TGC TAC GGA ACC GAT TAC –3' and R 5'- TTG TCC AGC CTG AAC CTC TGA –3'.

Lentiviral mediated over-expression of ELAVL3

ELAVL3 DNA sequence was amplified by PCR using a DNA template obtained from spheroids derived cDNA (MGH8) and the following ELAVL3 primers set: F 5'- CAC CAC GGT CAC TCA GAT ACT GGG GGC CAT G -3'; R 5'- CAT TCA CGC CTT GTG CTG TTT GCT GGT C -3'.

Denaturation temperature was set at 98°C, annealing at 58°C and extension at 72°C. PCR product was run on a 1% agarose gel stained with GelRed nucleic acid gel stain (Biotium). The band containing the putative ELAVL3 DNA sequence was cut and purified using JETQUICK gel extraction spin kit (Genomed), according to manufacturer instructions.

ELAVL3 DNA sequence was inserted into pENTR TOPO vector, in order to allow electroporation in Stbl3 chemically competent E. coli. Transformed cells were grown overnight on kanamycin agar plates. Single colonies were harvested and grown overnight as miniprep, in kanamycin LB medium. Plasmid was isolated using JETQUICK plasmid miniprep spin kit (Genomed), according to manufacturer instructions. Purified plasmid was sequenced using BigDye Terminator v1.1 cycle sequencing kit (Thermofisher), according to manufacturer instructions.

ELAVL3 DNA sequence was transferred to pLiv puro vector using Gateway LR clonase enzyme mix (Invitrogen), according to manufacturer instructions. Stbl3 chemically competent E. coli were transformed and grown overnight on ampicillin agar plates. Single colonies were harvested and grown overnight as miniprep, in ampicillin LB medium. Plasmid was isolated using JETQUICK plasmid miniprep spin kit (Genomed) and sequenced using BigDye Terminator v1.1 cycle sequencing kit (Thermofisher). In the absence of mutation, plasmid was grown overnight as maxiprep, in ampicillin LB medium and isolated using JETSTAR plasmid purification maxi kit 2.0 (Genomed), according to manufacturer instructions.

293T cells were incubated with DIMEM mixed with pLiv plasmid containing ELAVL3 DNA sequence, pMD2.G envelop plasmid, PCMV-ΔR8 packaging plasmid and FuGENE transfection reagent (Promega). The virus, contained in 293T culture medium, was harvested two days later. Medium was filtered through a 0.45µM filtration unit (Millipore), added to HeLa and BT4A cells, together with polybrene (6µg/ml), and incubated overnight. Infected cells were selected by adding puromycin (1mg/ml) two days after the infection for five days.

Gene knock-out using CRISPR-Cas9

The CRISPR-Cas system (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR-associated proteins) is part of the adaptive immune system of bacteria and archeons and provides protection against phages and viruses. When foreign DNA is detected, Cas protein is responsible for slicing it into smaller pieces. Those fragments, known as spacers, are then inserted between two CRISPR repeats and flanked by a protospacer adjacent motif (PAM) sequence. The spacers are used to generate an RNA guide, which allows Cas nuclease to recognize and cut out the target exogenous DNA sequence. [30;31]

CRISPR-Cas9[32] is a modified version of the CRISPR-Cas system, which was developed for genome editing in mammalian cells. It is made of Cas9, a CRISPR-associated protein containing two nuclease domains and of a single guide RNA (sgRNA), a combination of

CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA). The sgRNA includes a 20 nucleotides region specifically designed to recognize target genes. The annealing of the sgRNA to its complementary sequence and the recognition of PAM sequence by Cas9 allows the nuclease domains to generate a double strand break (DBS), which is either repaired by homologous recombination (HR) or non-homologous end joining (NHEJ). In this case, the lack of repair template favours DBS repair via NHJE pathway.[33;34] Because this mechanism is prone to error, an indel, i.e. insertion or deletion, is likely to be inserted and should either cause a frameshift mutation or the insertion of a stop codon. The alteration of the DNA sequence should lead to the production of a truncated protein without any biological activity.

Short guide RNAs were designed using a design software developed by Zhang Lab (http://tools.genome-engineering.org), which looks for potential sgRNA in target sequences and for potential mismatches in the rest of the genome. The results with the highest scores are the most specific and the less likely to cause off-target mutagenesis[35].

Four guide RNAs were chosen for ELAVL3, each with a score of 93 or higher.

Forward oligonucleotides were designed according to guide RNA sequences and reverse oligonucleotides according to their complementary sequences. Each sgRNA targets a different region of the ELAVL3 gene, in order to increase chances of creating an indel that will efficiently suppress all protein biological activity. The following sequences were used as short guide RNAs:

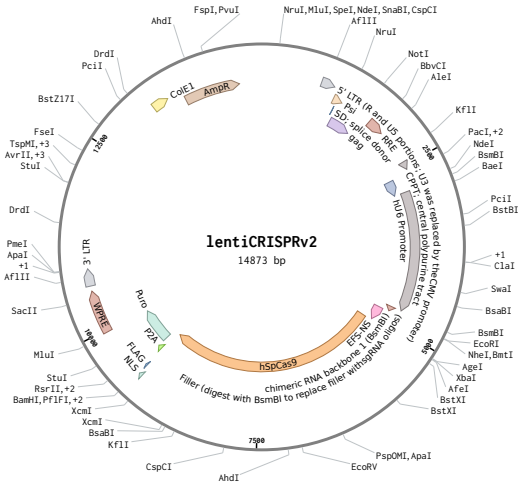

Figure 1, lentiCRISPR V2 structure

sgRNA 1	top strand	5'- CACC CGT GTA CAA CCT GTC ACC GG -3'
	bottom strand	5'- AAAC CCG GTG ACA GGT TGT ACA CG -3'
sgRNA 2	top strand	5'- CACC CGT AGC GCC GGG CGG ATG AC -3'
	bottom strand	5'- AAAC GTC ATC CGC CCG GCG CTA CG -3'
sgRNA 3	top strand	5'- CACC ACG TGA TGA TGC GGC CGT AC -3
	bottom strand	5'- AAAC GTA CGG CCG CAT CAT CAC GT -3'
sgRNA 4	top strand	5'- CACC ATG CTA ACC TGT ACG TCA GC -3'
	bottom strand	5'- AAAC GCT GAC GTA CAG GTT AGC AT -3'

CACC overhangs were added to the 5' extremity of each top strand and AAAC overhangs to the 5' extremity of each bottom strand, to allow oligonucleotides insertion in BsmBI-digested lentiCRISPR V2 vector. Oligonucleotides were inserted into lentiCRISPR V2 plasmid according to the protocol provided by Zhang lab[36].

Stbl3 chemically competent E. coli were transformed with CRISPR vector containing sgRNAs and grown overnight on ampicillin agar plates. Single colonies are screened by PCR, using the following primers: F 5'- GAG GGC CTA TTT CCC ATG ATT -3'; R1 5'- ACA GGT TGT ACA CGG GTG TTT C -3'; R2 5'- CGC TAC GGG TGT TTC GTC CTT T -3'; R3 5'- TAG CTC TAA AAC GGC CGC ATC ATC -3'; R4 5'- CAG GTT AGC ATG GTG TTT CGT CCT TTC C -3'. PCR products were run onto a 1,2% agarose gel. Cells expressing the plasmid were harvested and grown overnight as miniprep, in ampicillin LB medium. The plasmid was then purified and sequenced. In the absence of mutation, cells transformed with the CRISPR vector containing sgRNAs were grown overnight as maxiprep. The plasmid was then purified. 293T cells were incubated with a mix of DIMEM the purified plasmid, pMD2.G envelop plasmid, PCMV-ΔR8 packaging plasmid and FuGENE transfection reagent (Promega). The virus, contained in culture medium was harvested two days later and filtered through a 0.45µM filtration unit (Millipore). The filtrate was ultracentrifuged at 15'000 RPM for 1 hour and 30 minutes with a SW28 rotor (Beckman-Coulter). The supernatant was decanted and the pellet was resuspended by shaking for 2 hours at 4°C. It was then added to BT4S and BT8S cells, together with polybrene (6µg/ml), and incubated overnight. Infected cells were selected by adding puromycin (1mg/ml) two days after the infection.

Western blot

Cells were lysed using complete nuclear buffer (50mM Tris-HCL pH 7.5, 0.5M NaCl, 1% NP-40, 1% DOC, 0.1% SDS, 2mM EDTA, complete mini EDTA free protease inhibitor tablet (Roche)). Lysate was sonicated for 10 seconds, in order to fragment nucleic acids, and spined down for 10 minutes, in order to separate proteins from cellular fragments. 50ug of proteins were mixed with Laemmli sample buffer containing ß-mercaptoethanol (SB ß+) and water. Samples were loaded onto a 15% acrylamid gel (water, 30% acrylamid mix, 1,5M Tris-HCL pH 8.8 and 6.8, 10% SDS, 10% ammonium persulfate, TEMED), together with SB ß+ in empty wells and PageRuler protein ladder (Thermo Scientific). Migration was performed in cold running buffer (TRIS, glycine, SDS), by applying a current of 150V and 500mA. The acrylamid gel is then transferred onto a 0.22µM nitrocellulose membrane, in cold transfer buffer (TRIS, glycine), by applying a current of 250V and 250mA. The membrane is blocked for 1 hour in 5% milk. The membrane was rinsed in TBS-tween (TBST), incubated with the primary antibody for 1 hour at room temperature or at 4°C overnight and then with the secondary antibody for 1 hour at room temperature. The membrane was washed three times in TBST for 10 minutes

after each incubation. Primary antibody for ELAVL3: anti-ELAVL3/HuC (Ribonomics), purified from rabbit serum, diluted 1:500 in 5% milk in TBST; primary antibody for tubulin: anti-α-tubulin mAb (Calbiochem), purified from mouse serum, diluted 1:2.500 in 5% milk in TBST; secondary antibody for ELAVL3: polyclonal goat anti-rabbit immunoglobulins HRP (Dako), diluted 1:20.000 in 2.5% milk in TBST; secondary antibody for tubulin: ECL anti-mouse IgG, HRP-linked whole antibody (GE Healthcare), purified from sheep serum, diluted 1:5.000 in 2.5% milk in TBST. Membranes were washed with chemiluminescent substrates for HRP, SuperSignal west pico chemiluminescent substrate (Thermo scientific) for tubulin and WesternBright Sirius western blotting detection kit (Advansta) for ELAVL3. Images were acquired on a Fusion FX Instrument (Vilber Lourmat).

Proliferation assay

BT8S cells infected with sgRNAs 1, 2, 3 and 4 were mechanically dissociated and plated in 96-well low adherence plates. BT8S cells infected with CRISPR GFP were used as control. 3x 5000 cells were plated for each condition. The experiment was performed using Cell proliferation ELISA, BrdU (colorimetric) kit (Roche Life Science), according to manufacturer instructions. BrdU was added 24 hours, 48 hours and 72 hours after plating and removed 24 hours later. Plates were read at 370 nm and 420 nm.

Intracranial glioma cells injections

BT8S cells infected with sgRNA 2 and 3 were injected in mice. Cells infected with CRISPR GFP were used as control. Cells were mechanically dissociated and resuspended in Neurobasal medium (Gibco) without any supplementation. 1000 cells were injected in each mouse and 5 mice were injected for each condition. Injection site was 2 mm medial from the bregma, injection depth was 2,5 mm from the dura-mater.

RESULTS

Bioinformatic analysis

Bioinformatic analysis based on the Cancer Genome Atlas (TCGA) data, was performed in order to compare ELAVL3 expression in the four subtypes of GBM to its expression in a variety of different malignancies. Results show that ELAVL3 was expressed at higher levels in all four subtypes of GBM when compared to other malignancies. They also revealed that the highest expression of ELAVL3 occurred in tumours of the proneural subtype when compared to GBM of the classical, neural and mesenchymal subtypes (Figure 2).

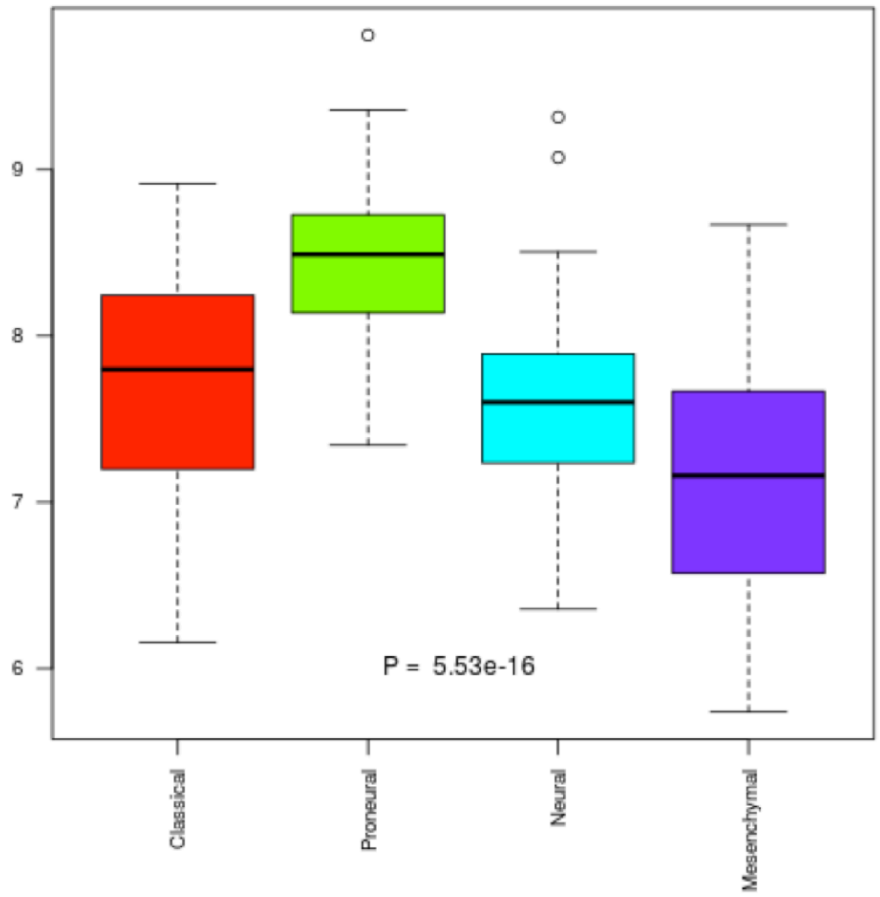

Figure 2, expression of ELAVL3 in the different molecular subtypes of gliobastoma multiform

We also analysed genes co-expressed with ELAVL3 and found a strong correlation between the expression of ELAVL3 and several proneural genes such as PDGFRA, of NKX2-2, of OLIG2, of DCX, of DLL3, of ASCL1 and of SOX 4 (Table 1).

Gene	Pearson correlation coefficient	P-value
PDGFRA	0.34	1.43^{E}-5
NKX2-2	0.67	0
OLIG2	0.53	1.27^{E}-12
DCX	0.72	0
DLL3	0.53	1.22^{E}-12
ASCL1	0.33	2.89^{E}-5
SOX4	0.55	1.92^{E}-13

Table 1, genes co-expressed with ELAVL3

Comparative expression of ELAVL paralogs by quantitative real-time PCR

RNA-sequencing of primary GBM revealed that the expression of ELAVL3 is increased in glioma stem cells when compared to their differentiated progeny. In order to confirm these observations, quantitative PCR was performed on cell lysates from three primary GBM cell cultures, namely BT4, BT8 and BT11, cultivated as stem-cell-like spheroids and as their differentiated non-tumorigenic adherent cells.

Results showed that the relative expression of ELAVL3 was higher in stem-cell like spheroids than in differentiated non-tumorigenic adherent cells (Figure 3).

This experiment also allowed comparison of the relative expression of the other members of the ELAVL family in stem cell-like spheroids versus differentiated non-tumorigenic adherent cells. Results show that apart from an increase of ELAVL2 expression in BT11 spheroids, there was no significant variation of ELAVL1, ELAVL2 or ELAVL4 expression in spheroids or in adherent cells of any of the studied tumours (Figure 3).

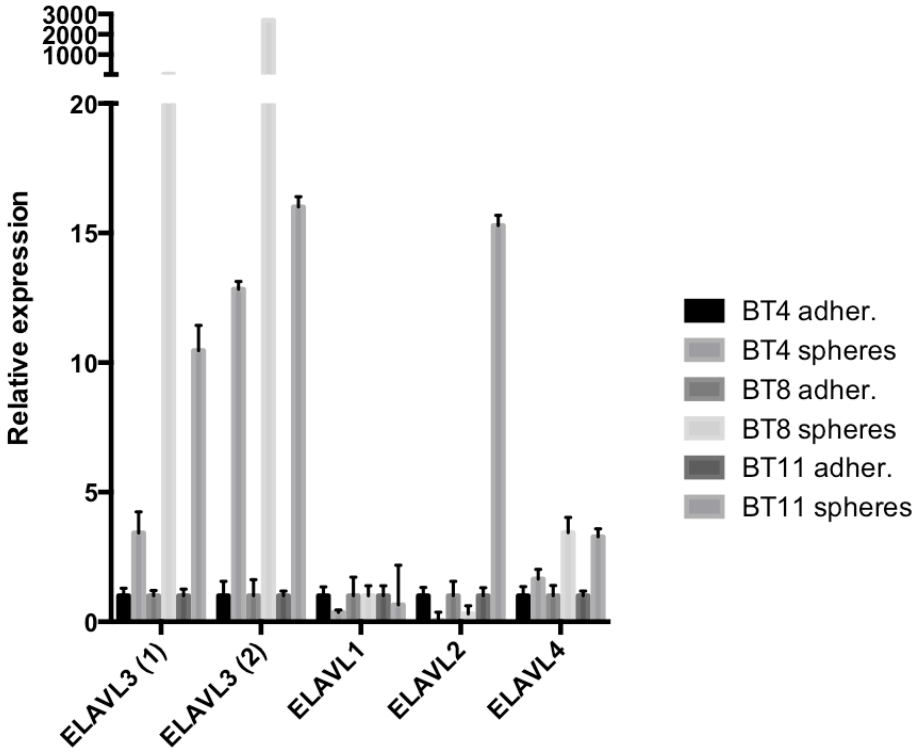

Figure 3, relative expression of ELAVL paralogs in spheroids compared to non-tumorigenic adherent cells

ELAVL3 over-expression

In order to evaluate the effect of ELAVL3 on cell differentiation, the protein expression was induced in HeLa cells and in adherent BT8 cells, where it is not physiologically expressed. Another batch of HeLa cells and adherent BT8 cells were infected with the empty plasmid, to allow comparison with cells in which ELAVL3 was over-expressed.

The first band that was recognized by the ELAVL3-specific antibody stopped migrating around 40 kDa, as indicated by the black arrow. The second band migrated a little lower, between 40 kDa and 35 kDa, as indicated by the red arrow. According to the human protein atlas, the molecular mass of ELAVL3 is 39.5 kDa[37]. It was therefore assumed that the lower band recognized by the antibody corresponds to ELAVL3 and that the upper band is a non-specific protein also recognized by the antibody.

The results of the western blot showed that the only cells in which ELAVL3 is physiologically expressed are BT8S. They also show that the over-expression of the protein in HeLa cells and in BT8A cells was successful, which appears obvious when comparing the ELAVL3-expressing cells to their respective empty counterparts (Figure 4).

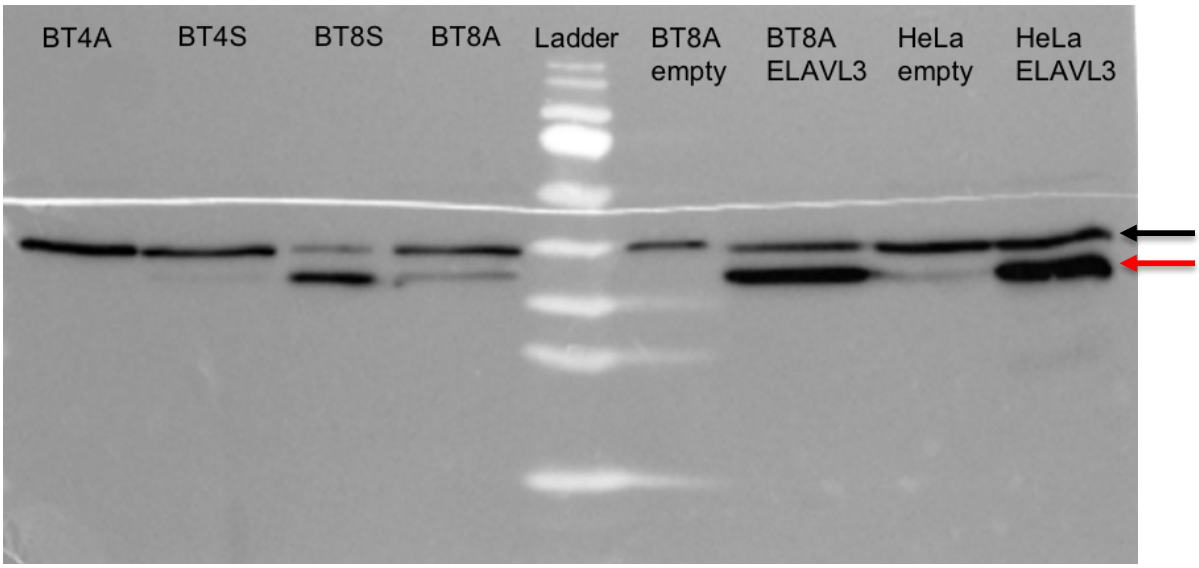

Figure 4, ELAVL3 over-expression in BT8A cells and HeLa cells

Microscopic observation revealed the formation of dendritic-like extensions in ELAVL3-expressing HeLa cells cultivated in non-neuronal medium, whereas non ELAVL3-expressing cells maintained their baseline phenotype. However, the protein over-expression appeared to be toxic for the cells, which died much faster than their non ELAVL3-expressing counterparts.

Proliferation assay

In order to clarify the role of ELAVL3 in proneural GBM, the protein expression was downregulated in BT8 spheroids. The knock-down was performed using the CRISPR-cas9 system and guide RNAs designed to target ELAVL3. Proliferation assays were then performed, to compare the proliferation rate of ELAVL3-expressing cells to that of ELAVL3-deficient cells. A western blot was performed in order to confirm the protein knock-down. The ELAVL3 specific antibody used was the same as the one used to confirm the over-expression. Just as in the previous experiment, the upper band, indicated by the black arrow, was assumed to be a non-specific protein recognized by the antibody and the lower band, indicated by red arrow, to be ELAVL3. The western blot results showed that ELAVL3 is expressed in the control cells, namely those infected with a CRISPR vector containing a GFP. They also showed that ELAVL3 is no longer expressed in cells infected with the CRISPR short guide RNAs 2 and 3, which were specifically designed to recognize ELAVL3 (Figure 5). This confirms that the protein knock-down was successful.

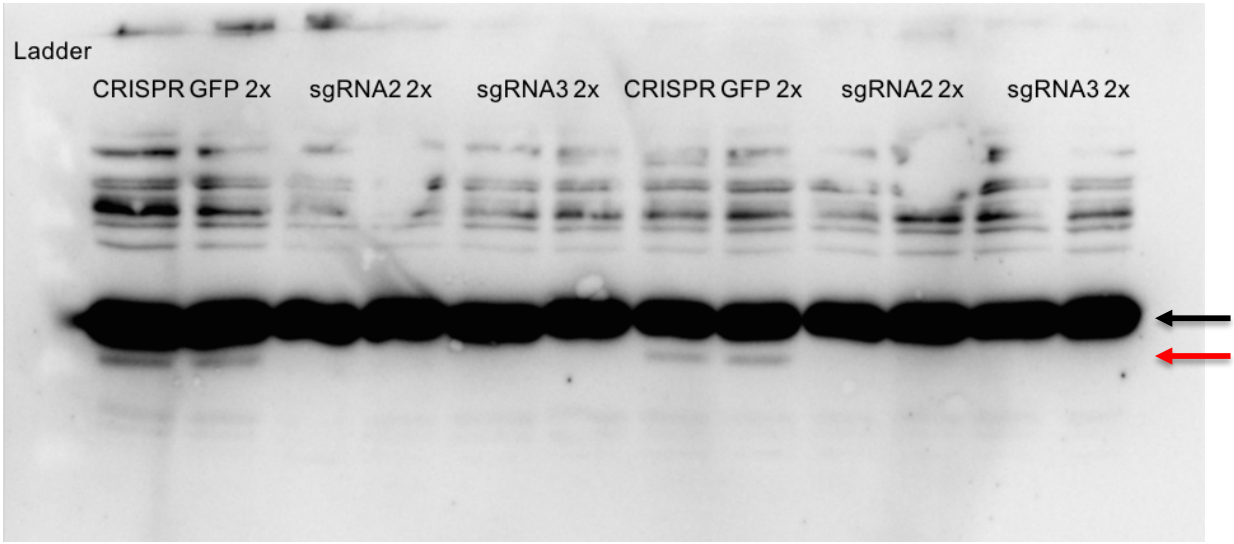

Figure 5, ELAVL3 knock-down in BT8S cells

Once the ELAVL3 knock-down was confirmed, the cells were used to perform a proliferation assay. Absorbance measurement were performed at 24, 48 and 72 hours.

The results showed higher levels of BrdU in control cells, infected with CRISPR-GFP, than in non ELAVL3-expressing cells, infected with guide RNAs 2 and 3, which translated to higher absorbance rates in control cells than in non-ELAVL3 expressing cells (Figure 6).

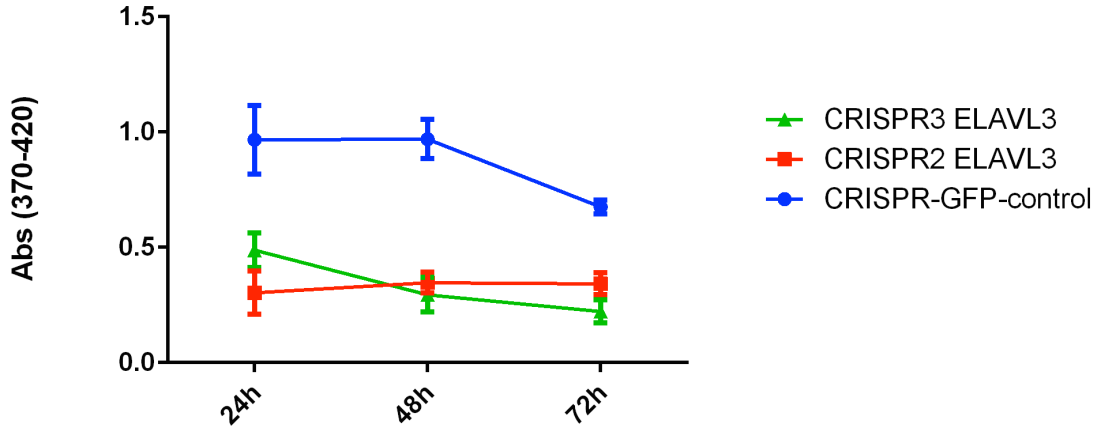

Figure 6, BT8S proliferation assay

Intracranial glioma cells injections

Mice were injected with non ELAVL3-expressing cells and ELAVL3-expressing cells. The aim was to compare survival rate between the two groups, in order to see whether ELAVL3 knock-down has an impact on GSCs behaviour, more specifically on their proliferation rate, and to correlate the results with those of in vitro experiments.

Moreover, the histology of both ELAVL3-expressing tumours and of non ELAVL3-expressing tumours is to be compared, in order to assess how the protein influences stem cells differentiation.

Over 6 months after the injections were performed, only one mice from the control group had died. The remaining mouse did not show any physical sign of tumour growth. Because the mice were asymptomatic for much longer than expected after receiving the xenograft, the experiment was terminated since it was unlikely to yield any usable data.

DISCUSSION

The first part of this study aimed to confirm the TCGA data analysis and to compare the expression of ELAVL3 in stem cell-like spheroids to its expression in differentiated non-tumorigenic adherent cells.

Quantification of ELAVL3 expression levels through real-time quantitative PCR revealed that the protein is expressed at higher levels in spheroids than in adherent cells. This is in agreement with the aforementioned results. Additionally, in vitro experiments showed that ELAVL 3 is the only neuron-specific ELAVL protein to be consistently up-regulated in tumorigenic cells. The fact that it is the only neuron-specific paralog to be over-expressed in this subtype of tumour hints that it may be involved in proneural GBM tumorigenesis.

The analysis also revealed that the increased protein expression specifically occurs in malignancies of the proneural subtype. However, there is not enough data to conclusively confirm the analysis results since there was no in vitro comparison of ELAVL3 expression in cells of the mesenchymal, neural and classical subtypes.

Finally, the analysis revealed that ELAVL3 expression correlates with the expression of oligodendrocytic development genes and of proneural development genes. Interestingly, those were also found to be expressed at higher levels in malignancies of the proneural subtype[11]. These results further suggest a distinctive role of ELAVL3 in proneural malignancies.

Altogether, the data yielded through statistical analysis and later confirmed through in vitro experiments led to the observation that ELAVL3 is overexpressed in GSCs of the proneural subtype and that its expression strongly correlates with the expression of proneural signature genes. Moreover, these findings have not yet been described in the literature and their relevance is not known. However, they do suggest that ELAVL3 is necessary for the development of proneural glioblastoma stem cells.

The second part of this study focused on investigating the significance of ELAVL3 expression in stem cell-like spheroids of the proneural subtype and on evaluating the impact of re-expressing the protein in cells where its expression was lost. Because ELAVL3 is overexpressed in GBM of the proneural subtype and given its association with tumours of this specific subtype, it was hypothesized that the protein could be involved in the development or in the differentiation of such tumours.

The over-expression of ELAVL3 in non-neuronal cells led to the acquisition of a neuron-like phenotype, with the growth of dendritic-like structures. Those were hypothesized to be neurites, since ELAVL3 and the other neuron-specific ELAVL paralogs thought to be necessary for neural differentiation and maintenance. This finding is in agreement with

previous experiments [38;39;40], which showed that expression of neuron-specific ELAVL proteins in non-neuronal cells induced morphological changes with the formation of neuronal structures, through stabilization of mRNAs transcripts involved in neural differentiation.

Proliferation assays allowed comparison between the proliferation rate of ELAVL3-expressing GSCs and ELAVL3-deficient GSCs. Results show that ELAVL3-expressing cells proliferate faster than non ELAVL3-expressing cells. Because ELAVL3 has been shown to stabilize and/or to activate transcription of target mRNAs by binding to their 3'UTRs[20;22;23], it could be hypothesized that ELAVL3 promotes the translation of transcripts necessary for cellular proliferation. Some targets of neuron-specific ELAVL proteins have been identified, one of them being an RBP known as Musashi1 (MSi1)[40;41]. The level of Msi1 expression has been correlated with GBM grade[42;43]. The protein has been shown to promote tumour growth through positive regulation of the Notch signaling pathway. Msi1 blocks the translation of the Numb protein, which inhibits the activation of the Notch pathway, thus promoting cell proliferation[41]. Moreover, this protein has also been implicated in the development of GBM resistance to chemotherapeutic agents by increasing the secretion of IL-6 through the Akt/PI3K signaling pathway[42]. Studies have found neuron-specific ELAVL proteins to stabilize Msi1 mRNA, thus diminishing its degradation and promoting its translation[41]. Positive regulation of the Notch pathway could be one the mechanisms that confers a proliferative advantage to ELAVL3-expressing malignancies.

Xenografting of ELAVL3-expressing and non ELAVL3-expressing GSCs in mice was performed to determine if observations obtained through in vitro experiments were also valid in vivo. BT8 spheroids were assumed to proliferate rapidly and to develop into aggressive tumours. Based on this assumption, it was decided to inject less cells than instructed by protocol. However, there was no tumour growth in either the control group or the ELAVL3 knock-down group, even after an extended period of time. One explanation could be that they were cultivated for a prolonged period of time, which led to the gradual loss of their tumorigenesis potential.

This study is limited by the fact that experiments were conducted on cells from one out of four GBM subtypes and on only three different proneural cell lines. The protein expression should be assed in cells of the mesenchymal, neural and classical subtypes in order to conclusively confirm results of the TCGA data analysis. It should also be evaluated in proneural GSCs from different cell lines, in order to determine if the findings in this study are reproducible. Another limitation is the lack of in vivo data, which prevents histological comparison of non ELAVL3-expressing tumours with ELAVL3-expressing ones.

CONCLUSION

In conclusion, this study reveals the existence of an increased expression of ELAVL3 in gliobastoma multiforme of the proneural subtype which hasn't been described before. It also offers new perspectives regarding the protein function and its implication in tumorigenesis.

A lot remains to be done as far as characterization of ELAVL3 role in glioblastoma stem cells is concerned. Further studies will be needed to better understand its biological function and to establish the exact nature of its involvement in malignancies of the proneural subtype. Identification target transcripts specific to ELAVL3 will also necessary to better understand how the protein influences cell metabolism and tumour development. It could also help selecting targets for new therapeutic agents.

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