Gliomas represent 40% of all primary central nervous system tumors diagnosed. Among them, glioblastoma multiformes (GBMs) are the most malignant, with a very poor survival time of approximately 15 months for most patients with this tumor. High-grade gliomas are the most common primary brain tumors in adults, and their malignant nature ranks them highly regarding cause of cancer death. Grading and identification criteria that can be used to provide information regarding tumor behavior include cell proliferation (cellularity and mitotic activity), nuclear atypia, neovascularization, and the presence of necrosis and/or apoptotic regions. Differences in molecular composition between tissue types or biomarkers can be used diagnostically to classify tumors and assess prognosis. Molecular markers have increasingly been used to assess and manage adult malignant
The most useful are markers that can predict response to certain therapies and guide clinical decisions. The most recent biomarkers are from genome-wide surveys associating somatic mutations with the risk of glioma development. Molecular biomarkers most commonly used to evaluate adult malignant gliomas from biopsy samples include 1p/19q co-deletion, methylation of the O\(^6\)-methylguanine–DNA methyltransferase gene promoter, alterations in the epidermal growth factor (EGF) receptor pathway and isocitrate dehydrogenase 1 and isocitrate dehydrogenase 2 gene mutations.\(^{2,4-9}\) Dozens of proteomics-based approaches have sought to find proteins that are unique to gliomas,\(^{10}\) but have been severely limited by issues of sample size, ability to detect low-abundance proteins, and reproducibility. Many of these studies have generated hundreds and even thousands of putative candidates, yet have not been able to follow them up with subsequent validation and characterization.

Via a bioinformatics method developed by our group,\(^{11-14}\) we conducted a global meta-analysis of approximately 18,000 microarray experiments from the National Center for Biotechnology Information database to identify gene sets consistently coexpressed across heterogeneous conditions. After identifying these gene sets, an automated, large-scale analysis of the peer-reviewed literature was conducted\(^{12,15}\) to identify genes that are consistently transcribed with established glioma-related genes, but which have themselves never been associated with gliomas in the literature. This process was used to identify ELTD1 (EGF, latrophilin, and 7 transmembrane domain-containing protein 1 on chromosome 1) as a novel gene that may be an important biomarker for the confirmation and detection of gliomas.

ELTD1 is not well characterized. Based on its sequence, ELTD1 is a member of the secretin family of G protein–coupled peptide hormone receptors and belongs to the EGF-7 transmembrane subfamily.\(^{15}\) Structurally, it contains a large extracellular domain with EGF-like repeats, a 7-transmembrane domain, and a short cytoplasmic tail.\(^{15}\) ELTD1 was first identified to be developmentally regulated in rat fetal and postnatal cardiomyocytes.\(^{15}\) ELTD1 has also been identified with its ligand dermatan sulfate in rheumatoid synovial tissue in rheumatoid arthritis patients.\(^{16}\) In more obscure roles, variations in ELTD1 have been thought to be a risk factor for cannabis use disorders,\(^{17,18}\) tick burden in cattle,\(^{19}\) and subcutaneous fat thickness.\(^{20}\) Of more importance to cancer, ELTD1 has been considered an endothelial marker in microvasculature.\(^{21}\) Our goal in this study was to determine whether ELTD1 could be used as a marker for glioma-related processes, and use immunohistochemistry (IHC) and molecular magnetic resonance imaging (MRI) to validate its presence in human and rodent gliomas. MRI is becoming one of the most commonly used techniques to provide information on brain tumor growth, vasculature, biochemical metabolism, and molecular changes in preclinical models, as MRI is the optimal imaging tool used in the diagnostic process for human gliomas.\(^{22}\) Molecular alterations can be assessed with the use of targeting magnetic resonance (MR) contrast agents, which can specifically indicate levels of cancer biomarkers that may be elevated in malignant tumors.\(^{22}\) The development of targeted imaging ligands attached to MRI contrast agents allows the in vivo evaluation of tumor biology, such as tumor cell apoptosis, angiogenic blood vessels, and the expression of specific tumor antigens or signaling pathways.\(^{23}\) Molecular imaging involves the coupling of a targeting moiety (antibody [Ab] or peptide targeted to a protein of interest) to a reporter molecule (eg, MRI contrast agent). Commonly used MRI contrast agents are gadolinium (Gd)-based compounds and iron oxide–based nanoparticles.

In this study, we identified ELTD1 as a putative glioma-associated marker via a bioinformatic method and experimentally validated its presence in both rodent and human gliomas via IHC and molecular MRI analyses in an F98 rodent glioma model. For IHC, ELTD1 was compared with traditional IHC markers for human gliomas including vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT-1), carbonic anhydrase IX (CAIX), and hypoxia inducible factor-1α (HIF-1α). ELTD1 expression in human gliomas was also evaluated from gene expression databases (Rembrandt, Erasmus, and The Cancer Genome Atlas [TCGA]) to establish whether this biomarker is differentially expressed in varying glioma grades.

**PATIENTS AND METHODS**

**Immunohistochemistry**

The human tissue sample portion of the study was conducted in compliance with the University of Utah Health Sciences Center Institutional Review Board. For IHC analysis, GBMs, anaplastic astrocytomas (AAAs), and anaplastic oligodendrogliomas (AOs) (high-grade gliomas: 50 patients, 21 female and 29 male; 40 GBMs: 6 AAs, 4 AOs) were compared with tumors classified as low-grade gliomas (21 patients: 10 female, and 11 male; 11 oligodendrogliomas: 10 low-grade astrocytomas (LGAs), including benign oligodendrogliomas. Abs to ELTD1 were available commercially (human specific Abs all used for IHC: all are rabbit polyclonal anti-human Abs; Cls-C40639 [LifeSpan Biosciences, Inc, Seattle, Washington]; NBP1-84775 [Novus Biologicals, Novus USA, Littleton, Colorado]; and PA1-32729 [Thermo Fisher Scientific Inc., Rockford, Illinois]; all human Abs were assessed and found to provide similar results; dilution was 1:500 and rodent Ab specific for both mouse and rat, recommended use for IHC and Western blotting: ETI (N:20); 546951; goat polyclonal anti-mouse, peptide mapping near the N-terminus of ETI [Santa Cruz Biotechnology, Inc, Santa Cruz, California; dilution was 1:100]). A lack of cross-reactivity between human and rodent Abs is illustrated in supplementary Ab data (see Appendix 1, Supplemental Digital Content 1, http://links.lww.com/NEU/A501. Lack of cross-reactivity between human and rat ELTD antibodies. IHC slides for human GBMs and rat F98 gliomas, in which human GBM and rat F98 tumor tissues were stained with either anti-human ELTD or anti-rat ELTD antibodies. Note only positive staining of human GBMs when stained with anti-human ELTD Ab or when rat F98 tumors are stained with anti-rat ELTD Ab. First slide is ×10 magnification, second slide is ×20 magnification, and third slide is ×40 magnification.) A toluidine blue (0.1%) counterstain was used (15 seconds). For human tissues, IHC was
performed using the Vectastain ABC Kit (Vector Laboratories, Burlingame, California). Negative controls were performed by replacing the primary Ab with nonimmune serum. Slides were examined using an Olympus BX41 microscope (Center Valley, PA, USA). Under ×200 (10 ocular × 20 objective) magnification, slides were scored by 2 investigators blinded to the specimen tumor grade and patient information. A score of 0 to 4 (0, 0-25%; 1, 25%-50%; 2, 50%-75%; 3, 75%-100%; 4, 100%) was assigned based on the number of cells stained in a given field. In previous studies we demonstrated that this method was very reproducible as demonstrated by good interrater reliability (P = .99, 95% confidence interval: 0.99-1.00) and intrarater reliability (P = .96; 95% confidence interval: 0.92-0.99). Each investigator reviewed the slide at low power and at random high-power fields independently of the other investigator when determining the IHC score. Scores of 2 to 4 were considered positive expression, whereas scores of 0 and 1 were considered negative expression.

For the rat F98 glioma tissues, IHC fluorescence staining was done for the endothelial cell marker, CD31 (fluorescein isothiocyanate–labeled donkey anti-mouse Ab). For ELTD1, a secondary Ab (Cy3-labeled donkey anti-goat [Jackson ImmunoResearch, Suffolk, England]) was used to detect the anti-ELTD1 Ab, and a fluorescein isothiocyanate (red)-labeled donkey anti-goat Ab to target the anti-CD31 (1:300, mouse monoclonal anti-rat; Dako Denmark, Glostrup, Denmark) Ab within the brain tissue. The nucleus was stained with 4′,6-diamidino-2-phenylindole (blue). Stained tissue slices were examined with a Nikon C1 confocal laser scanning microscope (Nikon Instruments, Melville, New York). Colocalization analysis was done using an Imaris Coloc module (version 6.4), and data were presented as the percentage of colocalization and the Pearson colocalization coefficient.  

**Synthesis of ELTD1 Nanoprobes**

The dextran-coated NH2 base iron oxide nanoparticle construct underwent conjugation with an ELTD1-specific Ab using a protocol previously reported by our group.  

20 mg of the activated Ab was conjugated to the activated polyethylene glycol nano particles and activated Ab. The Ab is activated with N-succinimidyl-S-acetylthioioace- tate to introduce a sulfhydryl group.  

**MRI Experiments**

In vivo MRI experiments on rats with F98 gliomas were carried out with the rats under general anesthesia (1%-2% isoflurane, 0.8-1.0 L/min O2). The MRI equipment used was a Bruker Biospec 7.0-T/30-cm horizontal-bore imaging spectrometer (Bruker BioSpin MRI GmbH, Ettlingen, Germany). Animals were imaged at 7 to 10 days after the cells were injected and then every 2 to 3 days until the desired volume of the tumor was obtained (75-150 mm3). Anesthetized (2% isoflurane) restrained rats were placed in a MR probe and their brains localized by MRI. Images were obtained using a Bruker S116 gradient coil (2.0 mT/m/A), a 72-mm quadrature multirung radiofrequency coil for radiofrequency transmission, and a rat head coil for radiofrequency signal receiving. MRI was performed for the purpose of determining the incidence, number, growth rate, and volume of each tumor for the F98 gliomas. Multiple 3D-MRI slices were taken in the transverse plane using a spin-echo multislice (repetition time, 0.8 seconds; echo time; 23 ms; 128 × 128 matrix; 4 steps per acquisition; 4 × 5-cm3 field of view; 1-mm slice thickness).

For determination of T2* values of the IO nanoprobes in gliomas, a multiple gradient echo method was used with the following parameters: TE (first echo) = 4 ms, echo spacing of 4-ms repetition time = 1500 ms, 10 echoes (TE = 4, 11, 18, 25, 32, 39, 46, 53, 60, 67 ms), 2 coronal (horizontal) slices, number of averages = 2, 256 × 256 matrix, 1-mm slice thickness with a spatial resolution of 0.137 mm/pixel, and an estimated total scan time of 10 minutes. T2* maps were generated from the multiecho data sets. Rat brains were imaged at 0 (pre-nanoprobe [cross-linked iron oxide (CLIO) anti-ELTD1 nanoprobe] or precontrast agent [control CLIO-IgG] administration), 10-minute intervals post-nanoprobe or IgG contrast agent injections for up to 3 hours. Rats were injected with a single intravenous dose via a tail vein catheter with either the anti-ELTD1 nanoprobe (anti-ELTD1 antibodies [goat anti-mouse] tagged with an IO-based contrast agent [CLIO-based] (200 µL/200 g rat; 1 mg Ab/kg; 0.05 mmol Fe3+/kg), or the normal rat IgG contrast control agent (same dose as anti-ELTD1 nanoprobe).

Multiple regions of interest (10 regions of interest within tumor and nontumor tissues) were selected (in various representative tumor and corresponding contralateral nontumor regions by 2 operators with interobserver agreement) from T2-weighted images and T2* maps to calculate relative changes in MR signal intensities and T2 values in F98 glioma-bearing rats administered either the CLIO-anti-ELTD1 nanoprobe (n = 3) or the CLIO-IgG contrast agent (n = 2). MR angiographic images were obtained as previously described.

**MRI Analysis**

A Mathemtica-based T2* program was used to crop the data/matrix to an area of interest, and it then fit each pixel of a designated image slice to the T2* relation: $I = I_0 + I_s \cdot \exp(TE/T2*)$, where $a$ is a constant determined by the FindFit function of Mathematica, $I$ is the intensity at time $t$, $T_s$ is the signal intensity at time 0, TE is the set of defined echo...
times, and $T2^*$ is the parameter of interest. The FindFit function of Mathematica optimizes the values not explicitly defined to produce the best possible fit. This method is applied to both pre- and postcontrast images. The percentage of difference is then taken from the fitted data/matrix using the following equation: % difference = [(after-before)/before] $\times$ 100. ArrayPlot graphical images are produced for the original cropped image, the fitted pre- and postcontrast image, and the percentage of difference of the fitted images. A contour plot is made using the percentage of difference data to highlight the regions where the greatest intensity change occurred. The contour plot is then overlaid on the original cropped image to provide a more visual display of where changes in signal intensity occurred.

**Prussian Blue Staining of the Nanoprobes**

Detection of the iron oxide–based nanoprobes in tissue cryosections was done using Prussian blue staining, which involves the treatment of sections with acid solutions of ferrocyanides. The ferric ion (+3) present in the iron oxide–based nanoprobes from tissue sections combines with the ferrocyanide and results in the formation of ferric ferrocyanide, visible as a blue pigment in bright-field imaging. Stained tissue slices were viewed and photographed with a Nikon Eclipse 800 microscope (Nikon Instruments).

**Gene Expression Analysis**

For the glioblastoma expression microarray analysis, raw Affymetrix .cel files were downloaded for TCGA (National Cancer Institute) (Cancer Genome Atlas; www.cancer.gov; 529 GBM samples), Rembrandt (National Cancer Institute Repository for Molecular Brain Neoplasia Data; www.rembrandt.ncl.nhs.uk; 229 total astrocytomas, of which 151 are GBMs), and Erasmus (National Center for Biotechnology Information Gene Expression Omnibus; GEO Series GSE16011; total of 187 astrocytomas, of which 159 are GBMs), as well as the corresponding clinical annotations for each. The .cel files were then processed using R and Bioconductor, using a custom chip algorithm implemented in R.

For mesenchymal and proneural gene signature definition, we used a composite of signatures from Phillips et al.59 and Verhaak et al.30 For a given tumor, the metagene mesenchymal and proneural signature scores were both calculated. Within a data set, the mesenchymal and proneural meta-scores were z-score–corrected to allow their comparison. Tumors were then assigned to one of the signatures based on the higher expressing metagene.

**Western Blot**

Frozen tissue was weighed, 200 mg was thawed in 1 mL red blood cell lysis buffer (Sigma-Aldrich, R7757, St. Louis, MO, USA) with protease inhibitors (Sigma, p8340), Na3V04 (1 mM), dithiothreitol (1 mM) and phenylmethanesulfonfluryl fluoride; 1 mM, then diced using surgical microscissors. Tissue was centrifuged at 1500 rpm, supernatant removed, and 500 µL lysis buffer containing proteases and phosphatase inhibitors added. Tissues were homogenized with a rotor-stator at 4°C for 1 minute, incubated on ice for 30 minutes with shaking, and then centrifuged (42 000g, 20 minutes, 4°C). The clear supernatant was transferred to a clean 1.5-mL tube. After determining the total protein concentrations, 40 µg of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using a Novex 4% to 12% gel (Invitrogen, Carlsbad, California), and transferred to polyvinylidene difluoride membranes. Western analysis was done using antibodies against ELTD1 (ETL (N-20): sc46951; Santa Cruz Biotechnology Inc). Secondary antibodies were labeled with horseradish peroxidase. The Supersignal West Pico chemiluminescent substrate kit (#34077; Thermo Scientific) was used for detection.

**Statistical Analyses**

Statistical differences in MR signal intensities and $T2^*$ relaxations, which indicated specific binding of the nanoprobes in glioma tissue, were analyzed in the treatment and control groups and in tumor and nontumor regions with an unpaired, 2-tailed Student $t$ test using commercially available software (InStat; GraphPad Software, San Diego, California). A $P$ value of $<.05$ was considered to indicate a statistically significant difference. For IHC scoring and ELTD1 expression, statistical differences were compared between groups using the Welch 2-sample $t$ test (unpaired, 2 sided), with $P$ values $<.05$ considered significant.

**RESULTS**

**Bioinformatics**

A global microarray meta-analysis (GAMMA) of all genes differentially expressed across 3651 human 2-color microarray experiments was conducted as previously described11 to identify gene–gene coexpression patterns that were consistent and specific across heterogeneous microarray experiments. The significance and reproducibility of the GAMMA predictions from the 2-color array data have since been corroborated by normalization31 and meta-analysis of 16 000 additional 1-color human microarrays.32 This “guilt-by-association” approach identifies gene sets that are likely to be associated in biologically relevant ways such as phenotype, disease, and genetic network. GAMMA has been used successfully to identify the mitotic role of a formerly uncharacterized gene called $C13orf3$ (now $Ska3$)33 and a role in coagulation for $CGorf105$ (now ADTRP)34 and to identify $OLF4$ as a novel neutrophil subset marker associated with granule secretion.35

With the GAMMA approach, genes are not analyzed directly, but the top 20 genes most consistently coexpressed with them are analyzed for what they have in common in the peer-reviewed literature using a large-scale computational analysis.12,14 This way, even if a protein has no known function, its function can be inferred. Then, using the Human Proteome Reference Database54 and other experimental sources on protein cellular localizations, we screened this list of predicted glioma-associated proteins for those that were extracellular or membrane bound because these proteins were thought to be ideal targets for molecular imaging probes and targeting therapies because they are more likely to be accessible to injected antibodies. Using our procedure, we identified membrane-bound proteins that have not yet been associated with gliomas, but whose expression consistently correlates with genes reported to be associated with gliomas. This circumvents a problem inherent in the lists of expressed genes derived by microarrays, which identify only those genes that are being actively transcribed at the time of the
Finally, we obtained increased confidence in the Endothelial growth factor, latrophilin, and 7 transmembrane-VOLUME 72 | NUMBER 1 | JANUARY 2013 |

ELTD1 scores are based on a combination of (1) how many genes out of the 20 top coexpressed analyzed genes showed associations with gliomas based on published reports and (2) their statistical significance based on random network simulations to estimate the probability that a set of equally frequent terms would be associated with gliomas. Only proteins with $P < .01$ significance were selected as potential candidates. A flow diagram of the GAMMA approach is illustrated in supplementary data (see Appendix 3, Supplemental Digital Content 3, http://links.lww.com/NEU/A503, which list GAMMA predicted associations for the ELTD1 gene; predicted associations that were tested in this study are shown in red).

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**Immunohistochemistry and Western Blot**

From human IHC assessment, ELTD1 was expressed in all gliomas where it was found to have an average IHC score of 2.7 ($\pm 1.16$) or more than 67% expressed in high-grade gliomas and an average IHC score of 2.05 ($\pm 1.07$) in low-grade gliomas or more than 33% expressed in low-grade gliomas (Figure 1), the difference being statistically significant ($P = .03$). The percentages of survival for the GBM, AA, and AO patients were 0, 0, and 50%, respectively. The percentages of survival for benign oligodendrogloma and LGA patients were 27.3% and 70.0%, respectively.

ELTD1 compared well with known glioma biomarkers, including VEGF, HIF-1$\alpha$, GLUT-1, and CAIX. The IHC average score for ELTD1 in high-grade gliomas was slightly lower than the average IHC scores for VEGF, HIF-1$\alpha$, GLUT-1, and CAIX (Figure 1A). In low-grade gliomas, ELTD1 had an IHC score that was less than those for VEGF, HIF-1$\alpha$, and CAIX (Figure 1A). Although the recently discovered glioma marker Brevican had 68% expression levels in high-grade glioma patients.
(Figure 1A), it was found to be the lowest for all biomarkers tested (Figure 1A). Brevican and the biomarker CAIX were not found to be significantly higher \((P = .19\) for both) when comparing high-grade with low-grade gliomas, whereas all other biomarkers (VEGF, HIF-1\(\alpha\), and GLUT-1) had significance levels of \(P = .01\), \(P = .02\), and \(P = .001\), respectively, including ELTD1 \((P = .03)\) and were significantly higher in high-grade compared with low-grade gliomas (Figure 1A).

Figures 2A and B depict representative IHC staining for ELTD1 in human GBMs and control brain tissues, respectively, indicating that GBMs (Figure 2A) had substantially higher levels (including both vascular and glioma cells). Figures 2C and D show representative IHC staining for ELTD1 in a rat F98 glioma model compared with contralateral brain tissue, respectively, with higher levels detected in glioma tissue (including both vascular and glioma cells) vs contralateral brain tissue (Figure 2D). Figure 2E is a representative Western blot of ELTD1 levels obtained from rat F98 glioma tissues (2 right lanes) compared with normal rat brains (4 left lanes), illustrating high levels of ELTD1 in tumor tissues.

Molecular MRI

With the use of molecular MRI and iron oxide–based nanoparticles, in vivo ELTD1 levels were detected in rat F98 gliomas (Figure 3). The ELTD1 probe was a dextran-coated iron oxide construct with an anti-ELTD1 Ab coupled to the dextran (Figure 3A). A representative T2* difference image overlaid on top of a T2-weighted morphological MR image is shown in Figure 3B. A corresponding MR angiography image and its difference image (2 hours after administration of the ELTD1 probe minus before injection of the probe) are shown in Figures 3C and 3D, respectively. Note the high signal intensity within the tumor region (see Figure 3B for morphological T2-weighted image). The percentage of change in T2* differences is shown dynamically within representative animals in Figure 3E, where only the glioma region from an F98 glioma–bearing rat administered the ELTD1 probe had a high percentage of T2* differences (12%-14%) compared with the contralateral region (6%-8%) in the same animal or the tumor or contralateral regions of a F98 glioma–bearing rat that was administered the nonspecific IgG probe (<6%). Corresponding quantitative T2* differences in the tumor region of F98 glioma–bearing tumors is shown in Figure 3F (where a T2* difference of 87.44 \(\pm\) 39.95 in an F98 tumor administered the ELTD1 probe (3 F98 glioma–bearing rats, 10 sample regions per rat, ie, 30 sampling regions in total) was significantly higher (~3-fold, \(P < .001\)) than that measured in the tumor region of the nonspecific IgG control, which was 26.87 \(\pm\) 35.48 (2 F98 glioma–bearing rats, 10 sample regions per rat, ie, 20 sampling regions in total).

Confirmation of the presence of the iron oxide–based anti-ELTD1 nanoparticles in an F98 glioma–bearing animal (Figure 4A; T2-weighted MR image) is shown in Figures 4Bi and 4Bii. Low levels of the iron oxide particles are also detected in the contralateral brain tissue of an animal administered the ELTD1 probe (Figure 4Ci and ii), as well as less probe within the glioma (Figures 4E and Figure 3F) or contralateral (Figure 4F) brain tissues of an F98 glioma–bearing animal (Figure 4D; T2-weighted MR image) administered the IgG contrast agent.

To establish whether ELTD1 was predominantly an endothelium-associated marker, excised F98 glioma and contralateral brain tissues exposed to the anti-ELTD1 probe were fluorescently labeled with an anti-CD31 Ab (targeted with a fluorescein isothiocyanate–labeled secondary Ab), and a secondary Cy3-labeled Ab against the anti-ELTD1 Ab used in the anti-ELTD1 probe (Figure 5). Colocalization images indicate that ELTD1 colocalized predominantly with endothelial cells (CD31) (yellow = red [CD31] + green [ELTD1]; Figures 5Bi and 5Ci; highlighted rectangular regions). Colocalization analysis indicated that the Pearson colocalization coefficients were 0.8089 (1.0000 would be 100% colocalization) (Figure 5Bii) and 0.7929 (Figure 5Ci) for ELTD1 and CD31 in glioma tissue, indicating a high association of ELTD1 with endothelial cells. There is also some evidence that indicates the slight presence of ELTD1 surrounding glioma cells that did not colocalize with CD31 (Figures 5Ci and 5Civ; highlighted circle in Figure 5Ci). Contralateral brain tissue also had low levels of ELTD1 that colocalized with CD31 (Figure 5Ai, highlighted regions; Pearson colocalization coefficient of 0.7149).

Gene Expression Analysis

A number of large public gene expression databases including multiple types and grades of gliomas have been established, including Erasmus, Rembrandt, and, more recently, TCGA (GBMs only). To determine whether mRNA levels of ELTD1 were related to glioma grade, survival, or tumor gene expression subtype, we performed analyses of data from these databases (as described in the Methods section). In the 2 databases that included different grades of gliomas (WHO II-IV), there was a very significant association of increased ELTD1 expression with higher grade (Figure 6). In addition, an analysis of survival using the Rembrandt database demonstrated that increased ELTD1 expression was associated with worse survival across glioma grades (ELTD1 Rembrandt Survival supplementary data; see Appendix 4, Supplemental Digital Content 4, http://links.lww.com/NEUA504). Increased ELTD1 expression is associated with worse prognosis across grades in gliomas [Rembrandt gene expression database; date of query 6/14/2012]. The probability of survival was worse for up-regulated ELTD1 expression compared with improved probability of survival in down-regulated ELTD1 expression. Total number of tumors = 343 (all glioma group), 173 up-regulated, 5 down-regulated, and 165 intermediate. Statistics [from the Rembrandt Web site] are log-rank \(P\) value [for significance of difference in survival between groups of samples]: up-regulated vs intermediate = 3.019854E-4; up-regulated vs down-regulated = 0.056211295; down-regulated vs intermediate = 0.2565988951; up-regulated vs all other samples = 1.396275E-4; down-regulated vs all other samples = 0.1333846694; and intermediate vs all other
FIGURE 2. Endothelial growth factor, latrophilin, and 7 transmembrane-containing 1 on chromosome 1 (ELTD1) levels are elevated in glioblastoma multiforme (GBM) and F98 rat gliomas. A, B, representative immunohistochemistry (IHC) staining for ELTD1 in GBM (Ai, x40 magnification; Aii, x10 magnification) and a normal (B, x10 magnification) human brain tissue. C, D, representative IHC staining for ELTD1 in a rat F98 glioma (Ci, x40 magnification; Cii, x10 magnification) and contralateral brain (Di, x10 magnification) tissues. Magnification bars for 100 μm are depicted on each image. White arrows indicate positive staining for ELTD1 predominantly in glioma tissue, and orange arrows indicate staining of ELTD1 associated with endothelial cells. E, Western blot for ELTD1 (85 kDa) obtained from rat F98 gliomas (F98) (2 right lanes) and normal (Control) brain (4 left lanes) tissue, illustrating high levels of ELTD1 in tumors.
In vivo levels of endothelial growth factor, latrophilin, and 7 transmembrane-containing 1 on chromosome 1 (ELTD1) are elevated in F98 rat gliomas, as determined by molecular magnetic resonance imaging after administration of an anti-ELTD1 probe. A, Illustration depicting the general design of the anti-ELTD1 superparamagnetic iron oxide–based probe (SPIO) used for in vivo molecular imaging, consisting of dextran-coated SPIO nanoparticles with amine functional groups for the conjugation of an anti-ELTD1 antibody. B, Horizontal image depicting regions (red) where probe accumulation was highest (obtained from T2* maps; glioma region outlined in gray dotted line). C, 3-dimensional magnetic resonance (MR) angiogram within the brain of an F98 glioma–bearing rat before administration of the anti-ELTD1 probe. Outlined region depicts cropped image region in B. D, MR angiogram difference image (post–anti-ELTD1 probe (180 minutes) pre-administration) after administration (via intravenous tail vein catheter) of anti-ELTD1-SPIO probe. Note middle cerebral artery with SPIO-based probe darkening of vasculature 3 hours after probe administration. Outline region depicts cropped image region in B. Elliptical regions depict darkened regions due to the presence of the ELTD1 probe. E, Graph indicating probe kinetics as determined by measuring the percentage of T2* differences in glioma and contralateral brain tissues within F98 glioma–bearing rat administered either the ELTD1 nanoprobe or an immunoglobulin G (IgG)-SPIO contrast agent. F, Histogram of T2* differences in the tumor regions of F98 glioma–bearing rats administered either the ELTD1 probe (n = 3, 10 regions of interest, ie, 30 samples) or the nonspecific IgG contrast agent (n = 2, 10 regions of interest, ie, 20 samples). The T2* difference was found to be statistically higher in the tumor region of the F98 tumor administered the ELTD1 probe compared with the IgG contrast agent.
samples = 8.958061E-4.) Analysis of survival within GBM tumors from these databases did not demonstrate significant survival association within grade IV tumors. However, we did find that when we analyzed ELTD1 expression as a function of tumor gene expression subtype within grade IV tumors, there was a potential association of higher ELTD1 expression in the mesenchymal vs proneural subtype, which was significant in the Rembrandt data set and showed a trend in the TCGA data set (Figure 7). Taken together, these data indicate that ELTD1 is potentially a strong biomarker of glioma grade and survival and may be preferentially associated with the mesenchymal subtype of GBM.

DISCUSSION

We have demonstrated that the differential presence of ELTD1 in gliomas compared with nondiseased regions could potentially serve alone or in combination with other glioma-specific biomarkers because it is detected in both human GBM and rodent models for gliomas. Despite current therapies, GBM is a devastating cancer, and the validation of more biomarkers for GBM could be beneficial in the diagnosis and therapeutic intervention of this disease. ELTD1, as shown in our human IHC data (Figures 1 and 2), fares well in comparison with more traditional IHC markers...
FIGURE 5. Endothelial growth factor, latrophilin, and 7 transmembrane-containing 1 on chromosome 1 (ELTD1) is strongly associated with endothelial cells. Fluorescence staining for CD31 (red; iii) and the ELTD1 probe (green; iv) in A. Contralateral brain tissue and (B, C) glioma tissue, 5 hours after administration of the anti-ELTD1 probe in an F98-glioma bearing rat. i, colocalization of CD31 and ELTD1 (yellow) indicate that most of the ELTD1 is endothelial cell associated (highlighted in rectangular regions); however, low levels are also detected surrounding glioma cells (Ci, highlighted in a circular region). Colocalization analysis images are shown in ii, where the Pearson coefficients for ELTD1 and CD31 were 0.8089 (Bii) and 0.7929 (Ci) in glioma tissue, and 0.7149 (Aii) in contralateral brain tissue. C, enlarged image (×2 zoomed image) of the glioma tissue. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). Magnification ×60. Magnification bars in Ai, Bi, and Ci are 50, 80, and 70 μm, respectively.
currently used to diagnose GBM. \(^{36}\) IHC staining in human high-grade gliomas (GBM + AAs + AOs) and low-grade gliomas (LGAs + benign oligodendrogliomas) indicated higher levels of ELTD1 for high-grade compared with low-grade gliomas. The level of ELTD1 was similar to currently investigated glioma markers including VEGF, HIF-1\( \alpha \), and GLUT-1. It is well-known that HIF-1\( \alpha \) is an important diagnostic marker and can be targeted for therapeutic intervention. \(^{24,37-43}\) ELTD1 also can be detected in an aggressive rodent model for gliomas. Within a rat F98 glioma model, ELTD1 levels from IHC assessment were found to be higher in glioma tissue; however, the contralateral tissue seemed to still have some staining for ELTD1 (Figures 2C and Figure 5A). Western blot data indicate high levels of ELTD1 compared with normal rat brain (Figure 2E). Lower levels of ELTD1 in normal brain tissue may be due to decreased vasculature (compared with a tumor). Human normal brain tissue had very low ELTD1 levels (Figure 2B) compared with extremely high levels in a GBM patient (Figure 2A).

Preclinical glioma models, induced by orthotopic (into native tumor sites) injection of primary tumor cells or tumor cell lines, represent the most frequently used in vivo cancer model systems for glioma research. \(^{23,44}\) F98 glioma cell lines were obtained from chemical induction as a result of administering ethylnitrosourea to pregnant rats, where the progeny developed brain tumors that were isolated, propagated, and cloned in cell culture. \(^{45}\) F98 gliomas are classified as anaplastic malignant tumors, which have an infiltrative pattern of growth, which is an attribute associated with human GBM. \(^{45,46}\) MRI techniques have been used by our group to demonstrate the aggressive nature of F98 gliomas. With the use of diffusion tensor imaging, we were able to demonstrate that the F98 glioma model is much more infiltrative than the rat C6 glioma model. \(^{47}\) With the use of MR angiography, we have shown that the F98 model predominantly uses preexisting blood vessels in tumor angiogenesis, but has longer and thicker new blood vessels compared with other glioma models. \(^{48}\) In this study in the F98 glioma model, we were also able to demonstrate, with the use of molecular MRI and an anti-ELTD1 probe, that substantial levels of ELTD1 are found in the tumor tissue of F98 glioma–bearing animals. ELTD1 was only found to be in high levels within glioma tissue with an approximately \(\sim 4\)-fold increase compared with contralateral brain tissue (Figure 3F). A decrease in T2 relaxation would indicate the presence of the anti-ELTD1 probe, which would be indicative of the presence of ELTD1. Colocalization images staining for ELTD1 and CD31 and subsequent analysis indicated that most of the ELTD1 detected by fluorescence confocal imaging was associated with endothelial cells (Figure 5). There is also some indication

**FIGURE 6.** Increased endothelial growth factor, latrophilin, and 7 transmembrane-containing 1 on chromosome 1 (ELTD1) expression is associated with higher grade in gliomas in both Rembrandt and Erasmus gene expression databases. ELTD1 expression was found to be significantly higher in grade IV gliomas compared with grade II gliomas in both Rembrandt (\(P < .001\)) and Erasmus (\(P < .001\)) databases. ELTD1 was found to be significantly higher in grade IV vs grade III in the Rembrandt database (\(P < .001\)), and higher in grade III gliomas vs grade II in the Erasmus database (\(P < .01\)).

**FIGURE 7.** Increased endothelial growth factor, latrophilin, and 7 transmembrane-containing 1 on chromosome 1 (ELTD1) expression is associated with mesenchymal (mes) phenotype in glioblastoma multiforme (GBM) (compared with the proneural [pn] subtype), as determined from the TCGA (mes = 275 and pn = 254) and Rembrandt (mes = 70 and pn = 55) gene expression databases. There was no significant differences in ELTD1 expression between mes and pn in the TCGA database (\(P > .05\)); however, there was a significant increase in the mes subtype (compared with the pn subtype) in the Rembrandt database (\(P < .001\)). For statistical analysis, a Welch’s 2-sample t test was used.
that ELTD1 may be expressed on some glioma cells at much lower levels. Therefore, any increase in ELTD1 will more than likely be associated with increased angiogenesis or neovascularization in gliomas. Decreased levels of ELTD1 in glioma cells compared with high levels in tumor vasculature from the in vivo data may reflect either predominant uptake by the endothelial cells before they reach the glioma cells or decreased uptake of the anti-ELTD1 probe in tumor tissue. IHC staining (Figure 2) indicates ELTD1 staining in tumor cell nuclei as well as around endothelial cells, indicating that the in vivo targeting may be restricted to the distribution of the probe to endothelial cells and only some tumor cells. Prussian blue staining for the anti-ELTD1 probe (Figure 4) seems to indicate intravascular staining based on the well-individualized pattern and distinct shapes, which could indicate an endothelial association and limited distribution of the probe to these cells. However, previous studies using the same probe construct to assess in vivo c-Met or VEGF receptor 2 (VEGFR2) levels in rat gliomas do not indicate that these probes only reach vascular cells, but do reach glioma cells, which would suggest that the ELTD1 probe has a preferred association with endothelial cells. To confirm ELTD1 expression with vascular endothelial cells, future experiments may also need to evaluate the levels of ELTD1 in association with the inhibition of neovascularization using antiangiogenic therapies (eg, bevacizumab or sunitinib). Additionally, verification of ELTD1 expression in neoplastic cells should be done, such as assessing EGF receptor gene amplification by fluorescence in situ hybridization colocalization with ELTD1-expressed cells.

It is interesting to note that the Human Protein Atlas shows very little positive staining for ELTD1 in malignant gliomas, but strong Ab staining for other cancers, such as thyroid cancer and malignant melanoma (http://www.proteinatlas.org/ENSG00000162618/cancer; accessed 07/24/12). This database is ideal for the initial determination of general expression levels of a particular protein that could indicate further study. However, more extensive studies, as we have done with the use of IHC staining for ELTD1 in numerous patient samples and in vivo expression levels of ELTD1 in a preclinical model, strongly suggest that malignant gliomas do have high levels of ELTD1. Also worthy of mention, according to SymAtlas (http://biogps.org/#goto=genereport&id = 170757; accessed 07/24/12), ELTD1 mRNA expression seems to be highest in hematopoietic stem cells as well as lung and common myeloid progenitor tissues. Whether hematopoietic stem cells are the source of neovascularization that we observe in gliomas would need to be further investigated.

From the gene expression results, we have also demonstrated that there was a strong association of ELTD1 expression with increasing grade (Figure 6). This results in a strong survival association when data across all grades (ELTD1 Survival supplementary data) are compared. However, there was not a survival association with expression level within GBM, suggesting that it is mainly a biomarker of grade. Alternatively, when we looked at GBM tumor subtype, it looked like there was a possible association with the mesenchymal subtype vs the proneural subtype that was significant in Rembrandt, but not in TCGA (Figure 7). It is reasonable to conclude that ELTD1 expression is a strong biomarker of grade (also supported by the IHC data), associated with survival across grades and may be increased in the mesenchymal subtype. ELTD1 expression and associated survival should, in the future, be evaluated by IHC via a glioma tissue microarray to confirm survival differences observed in Rembrandt.

Others have previously used molecular MRI to also assess neovascularization. For example, the expression of cell adhesion molecules, such as integrins, has been found to be up-regulated during tumor growth and angiogenesis, and αvβ3 expression, which has been correlated with tumor aggressiveness, can be measured by MRI with targeted paramagnetic-labeled cyclic arginine-glycine-aspartic acid peptides.\(^24,40\) In another study, within U87MG xenograft tumors in nude mice, arginine-glycine-aspartic acid-labeled ultrasmall superparamagnetic iron oxide probes were found to accumulate only within the neovascularature associated with tumors and not within tumor cells.\(^50\) Tumor angiogenesis was also monitored via the expression of CD105 in F98 tumor–bearing rats with the use of Gd-diethylentriaminepentaacetic acid (Gd-DTPA) liposomes targeted to CD105 (CD105-Gd-SLs) and MRI.\(^51\)

In our laboratories, MRI probes (either Gd or iron oxide based) have also been developed to monitor in vivo levels of molecular markers known to be overexpressed in malignant brain tumors, such as the angiogenic marker VEGFR2\(^26,28\); the tumor cell migration/invasion marker c-Met, a tyrosine kinase receptor for the scatter factor (also known as the hepatocyte growth factor)\(^27,28\); and the inflammatory marker inducible nitric oxide synthase (iNOS).\(^33\) With the use of a Gd-DTPA–albumin–anti-VEGFR2–biotin probe, regional differences in VEGFR2 levels were detected by MRI in vivo in a C6 glioma model, and probe specificity for glioma tissue, particularly in the peritumor and perinecrotic regions, was confirmed by tagging the biotin moiety of the probe in excised tissues with streptavidin–Cy3.\(^35\) The control nonspecific probe had rat IgG conjugated to the albumin instead of the VEGFR2 Ab. A similar result was obtained when an aminated dextran-coated iron oxide nanoparticles conjugated with an anti-VEGFR2 Ab was used in a C6 glioma model, where distribution of the probe was mainly in the peritumor and perinecrotic regions of the tumor.\(^26\) Confirmation of the presence of the nanoprobe was obtained by using Prussian blue stain for the VEGFR2-targeting iron oxide nanoparticles in excised tumor tissues.\(^26\) Both Gd- and iron oxide–based probes were also developed to characterize c-Met levels in C6 gliomas. c-Met is a tumor marker that is overexpressed in many malignant cancers, indicative of the invasive nature of a tumor. The distribution of c-Met was found to be more widely dispersed, but mainly concentrated in peritumor regions.\(^27,28\) As detected with a Gd-DTPA–albumin–anti-iNOS–biotin (anti-iNOS) probe, iNOS levels were found to vary in different rat glioma models, where the percentage of MRI signal intensity changes were highest in the C6 tumor compared with the RG2- and ethyl-nitrosourea-induced tumors.\(^35\) Dynamic kinetic monitoring of the
ant-i-iNOS probe indicated sustained uptake over 3 hours within tumor tissue regions and no specific uptake of a control Gd-DTPA–albumin-IgG-biotin contrast agent within tumors. Fluorescence imaging of the ant-i-iNOS probe by targeting the biotin moiety with streptavidin-Cy3 verified higher levels of probe uptake in C6 tumors vs RG2 gliomas, despite the increased perfusion and microvacularity detected in the RG2 tumors.

For this study, an iron oxide–based nanoparticle construct covalently bound to an anti-ELTD1 Ab was used to detect high levels of ELTD1 in the tumor regions of F98 glioma–bearing rats (Figures 4 and 5). Specificity of the ELTD1 probe seems to be associated with neovascularization.

CONCLUSION

The results presented strongly suggest that the associative analysis method used in this study was able to accurately identify ELTD1 as a glioma-associated biomarker, possibly due to increased angiogenesis. Both ex vivo and in vivo validation studies indicate that ELTD1 is a biomarker that can be used to confirm or detect the presence and grade of gliomas, particularly high-grade gliomas in humans, and that this biomarker may play an important diagnostic role in addition to currently used markers for gliomas, particularly as a histological marker for identifying vascular proliferation.

Disclosures

Supported by Oklahoma Medical Research Foundation, the National Institutes of Health (grant 5P20RR020143-07 to J. D. W.), and Oklahoma Center for the Advancement of Sciences and Technology (OCAST grant AR092-049 to R. A. T.). The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

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The authors have identified a novel tumor biomarker, ELTD1, for high-grade gliomas via a unique bioinformatics meta-analysis of approximately 18,000 publicly available gene expression profiles. Their protocol identified glioma-associated genes not previously reported in the literature. ELTD1 protein is preferentially expressed by tumor endothelial cells of high-grade gliomas. Antibody-based nanoprobes for ELTD1 were synthesized and showed to localize to an F98 rat glioma orthotopic model. Finally, ELTD1 expression was positively correlated with glioma grade in the Rembrandt and Erasmus databases, associated with decreased survival across all glioma grades, and associated with the mesenchymal GBM subclass. This work highlights the utility of comprehensive, unbiased screens for novel glioma biomarkers, for such work opens up new avenues of investigation.

Discovery of novel biomarkers such as ELTD1 may aid in determining more precise subtypes and histopathological classifications of gliomas that potentially have clinical significance in tumor biology and assessing therapeutic response. One crucial future study is to validate ELTD1 protein expression and associated change in patient survival via clinically annotated glioma tissue microarrays to confirm the survival differences observed in Rembrandt. The positive correlation of ELTD1 mRNA expression across increasing grades of glioma; its origins in the epidermal growth factor, 7-transmembrane subfamily; and localization expression to endothelial cells all suggest potential ELTD1 involvement in tumorigenic mechanisms, and further study could yield new therapeutic targets. Such novel biomarkers may also be useful for assessing therapeutic response, especially with newly available targeted therapies. In the era of personalized medicine, research dedicated to systematically characterizing predictive tumor biomarkers is increasingly useful for classifying tumor patients for designing efficient clinical trial assessment of new targeted therapies.

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Molecular biomarkers have increasingly been used to assess and manage cancer. In this article, the authors used a very "high-tech" methodology (advanced laboratory and imaging techniques + bioinformatic tools in humans as well as in animal models) to propose ELTD1 as a novel and potential glioma-associated biomarker. ELTD1 expression was found to be higher in high-grade than low-grade gliomas with a clear association with tumor grade and patient survival. Moreover, ELTD1 was validated as a specific marker of neoplastic angiogenesis, showing an extremely lower expression in normal brain tissue than in tumors.

From the technical point of view, the article is highly detailed, giving information for the reproducibility of the experimental design, even if, obviously, many tools described are not familiar to a large neurosurgical audience. Notwithstanding this, the article appears very interesting and informative for the neuroscientific community, and the authors should be commended for their results as well as for the experimental background (7T MR and bioinformatics applied in neuropathology).

It should be emphasized that new "biomarkers" are proposed daily in the scientific literature; however, just a few of them have been successfully translated into clinical practice. Our opinion is that finding and statistically demonstrating that a "biomarker" is a meaningful biomarker does not mean that a real clinically useful parameter has been found, but something like an "epiphenomenon" occurring along the pathology. The proposal of a protein as a biological biomarker or of a morphometric parameter as an image biomarker should undergo systematic validation, after the technical and statistical steps, in a more specific way. The proposed biomarkers should undergo a step-by-step hierarchical validation system before being proposed as clinically meaningful, in the same way as for drugs, from experimentation to clinical application, from bench to bedside. Moreover, it is our opinion that molecular biomarkers should be investigated in parallel with objective morphometric parameters; in the case of ELTD1, which is associated with neoangiogenesis, for example, the analyses should run in parallel with the morphometric analyses of the microvessels, in terms of number, density, and geometric complexity of the microvascular networks, which are geometrically different in physiological vs pathological states as well as in different types and subtypes of tumors. There is no doubt that further investigations could give ELTD1 the “dignity” to become a clinically meaningful biomarker, being added in the near future to the family of other glioma-specific biomarkers.