Role of LIM and SH3 Protein 1 (LASP1) in the Metastatic Dissemination of Medulloblastoma

Christopher Traenka, Marc Remke, Andrey Korshunov, Sebastian Bender, Thomas Hielscher, Wolfram Scheurlen, Thomas G.P. Grunewald, Andreas von Deimling, Andreas E. Kuluzik, Guido Reifenberger, Michael D. Taylor, Peter Lichter, Elke Butt, and Stefan M. Pfister

Abstract

Medulloblastoma is the most common malignant pediatric brain tumor and is one of the leading causes of cancer-related mortality in children. Treatment failure mainly occurs in children harboring metastatic tumors, which typically carry an isochromosome 17 or gain of 17q, a common hallmark of intermediate and high-risk medulloblastoma. Through mRNA expression profiling, we identified LIM and SH3 protein 1 (LASP1) as one of the most upregulated genes on chromosome 17q in tumors with 17q gain. In an independent validation cohort of 101 medulloblastoma samples, the abundance of LASP1 mRNA was significantly associated with 17q gain, metastatic dissemination, and unfavorable outcome. LASP1 protein expression was analyzed by immunohistochemistry in a large cohort of patients (n = 207), and high protein expression levels were found to be strongly correlated with 17q gain, metastatic dissemination, and inferior overall and progression-free survival. In vitro experiments in medulloblastoma cell lines showed a strong reduction of cell migration, increased adhesion, and decreased proliferation upon LASP1 knockdown by small interfering RNA-mediated silencing, further indicating a functional role for LASP1 in the progression and metastatic dissemination of medulloblastoma. Cancer Res; 70(20); 8003-14. ©2010 AACR.

Introduction

Brain tumors and leukemias comprise the most common cause of cancer-related mortality in children with medulloblastoma being the most common malignant brain tumor in this age group (1). The presence of leptomeningeal dissemination at diagnosis detected either macroscopically by craniospinal imaging or microscopically by cerebrospinal fluid cytology, is a powerful prognostic marker in medulloblastoma (2, 3).

Molecular signaling pathways involved in the pathogenesis of standard-risk medulloblastoma such as the WNT and sonic hedgehog (SHH) signaling cascades are well-established (4–6), whereas the molecular biology underlying intermediate and high-risk medulloblastoma remains elusive. Aberrant activation of WNT signaling, typically caused by activating CTNNB1 mutations (and rarely APC or AXIN), followed by nuclear accumulation of CTNNB1 protein as first described by Ellison and colleagues (7), is now commonly accepted as the hallmark genetic event for standard (or low) risk medulloblastoma (8–10). The WNT subgroup of tumors may be cytogenetically identified by the presence of monosomy 6, which is exclusively found in tumors of this subset (4, 6, 8, 11). A second distinct subgroup of standard-risk patients identified by transcriptome studies (4–6) is molecularly characterized by aberrant SHH activation, and is typically driven by underlying mutations in key regulators of the pathway such as PTCH1 or SUFU (12–14). Collectively, these two subgroups account for approximately 35% to 40% of medulloblastomas. Significantly, these subgroups typically lack chromosome 17 aberrations (4, 5), which however is the most frequent cytogenetic event in medulloblastoma (11, 15–17).

DNA copy number aberrations of chromosome 17, including deletion of 17p and gain of 17q, are typically found in the presence of isochromosome 17q (i17q), the hallmark cytogenetic abnormality of high-risk medulloblastoma (4, 5, 11).
However, the driver gene(s) targeted by this cytogenetic aberration remain unidentified.

We previously showed in a large cohort of patients (n = 340) that isolated gain of chromosome 17q (without concomitant loss of 17p as observed in i17q cases) is associated with a poor prognosis, whereas isolated 17p deletion is not (11). We therefore decided to pursue the biological role of 17q gain in medulloblastoma. When investigating 28 pairs of primary and recurrent tumors, we found that 5 out of 13 tumors (38%), the primaries of which were characterized by a balanced chromosome 17, showed acquired gain of 17q in the relapsed tumors (18). These findings suggest that gain of chromosome 17q is a critically important aberration for medulloblastoma progression including metastatic dissemination in a large subset of these relapsed tumors.

Prior attempts to identify medulloblastoma oncogenes in 17q have suggested ERBB2 (19), BIRC5 (20), and PPM1D (16). As none of these genes were highly ranked in our screening against 17q balanced tumors, we hypothesized that additional candidate oncogenes in 17q should be considered.

In the present study, we identified LASP1 as one of the most differentially expressed and functionally relevant genes on 17q when comparing tumors with and without 17q copy number gains. The LIM and SH3 domain protein LASP1 (previously named MLN50) was initially identified from a cDNA library of breast cancer metastases (21). LASP1 mRNA is detected ubiquitously at low basal levels in all normal human tissues (22), but the protein is highly overexpressed in more than 55% of metastatic human breast cancer (23) and ovarian cancer (24). In a recent case control study, LASP1 expression in mammary carcinomas correlated significantly with tumor size and nodal positivity (25). The genomic locus of LASP1 at 17q12 is targeted by copy number gain or amplification in 20% to 30% of human breast cancers (26). Silencing of LASP1 by RNA interference in various cell lines resulted in a strong inhibition of cell migration and proliferation, with cell cycle arrest in G2-M phase (23, 24). These observations imply a dual function of LASP1 as both a structural scaffold at focal adhesions, and as a signal transducer conveying information from the cell surface to the nucleus (22).

To assess the role of LASP1 in medulloblastoma, we analyzed the prognostic value of LASP1 protein expression by immunohistochemistry on a tissue microarray representing sections from 207 clinically and histologically well-annotated samples from uniformly treated medulloblastoma patients. Our findings in the primary tumors, together with functional studies in medulloblastoma cell lines, provide strong evidence for an important role of LASP1 in the progression and metastatic dissemination of medulloblastoma.

Materials and Methods

Tumor material and patient characteristics

Nucleic acid isolation. Nucleic acids were extracted from tumor samples and cell lines as previously described (11). A detailed description is given in the Appendix.

Transcriptome analysis and microarray data analysis

Expression profiling and microarray image analysis were carried out as outlined in Supplementary Materials and Methods.

cDNA synthesis and quantitative real-time PCR

Oligo-d(t) primed cDNA synthesis of 1 μg total RNA was performed by using Superscript II Reverse Transcriptase (Invitrogen) as described in the protocols of the manufacturer. Each cDNA sample was analyzed in triplicate by quantitative real-time PCR on an ABI PRISM 7700 PCR System (Applied Biosystems) using ABsolute SYBR Green ROX Mix (ABgene) according to the instructions of the manufacturer. Gene expression was normalized to three endogenous housekeeping genes (SDHA, HPRT1, and LMNB1), which were constantly expressed in several expression experiments comparing subsets of primary medulloblastoma samples, medulloblastoma cell lines, and control tissues. Amplification of genomic DNA was excluded by intron-spanning primers and adequate test PCRs. Relative gene expression levels were calculated as described by Pfaffl (27) with the adaptation of three used housekeeping genes. Primer sequences are available in Supplementary Table S1.

Fluorescence in situ hybridization

Interphase fluorescence in situ hybridization analysis for chromosome 17 was performed on a medulloblastoma tissue microarray containing 207 tumor samples and 10 samples of nontumoral cerebellar tissues as controls (11). A commercial probe set delineating the loci of interest was used: 17p13.3/LIS1 (spectum orange) and 17q21/RARA (spectrum green; Vysis). Evaluation criteria and cutoffs were applied as described (11).

Immunohistochemistry

A primary antibody to LASP1 (28) was applied at a concentration of 10 μg/mL. Evaluation of immunostaining was performed in a semiquantitative manner and cases with clear evidence of (cytoplasmic as well as nuclear) immunostaining were considered immunopositive. Therefore, the analysis of our immunohistochemistry experiments was only performed by a positive versus negative evaluation of stained cores.

Cell lines and cell culture conditions

Medulloblastoma cell lines DAOY (purchased from American Type Culture Collection) and UW228-2 (kindly provided by S. Clifford, Newcastle, United Kingdom) were grown at 1 × 105 cells/mL at 37°C under 5% CO2 atmosphere in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum and 1% streptomycin/ampicillin (Invitrogen). D283 medulloblastoma cell line (purchased from American Type Culture Collection) was grown in α-MEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum and 1% streptomycin/ampicillin. Cells were cultured until homogeneous morphology of cells was reached and up to a maximum of four passages because LASP1 belongs to a group of several differentially expressed proteins that are upregulated after later passages (29).
Immunofluorescence

For immunofluorescence microscopy, cells were grown until homogenous morphology at a maximum of 70% confluence on glass chamber slides (for D283, cells were seeded on collagen-coated glass chamber slides) fixed in 4% (w/v) paraformaldehyde in PBS, permeabilized with 0.1% (w/v) Triton X-100 in PBS, and then stained with affinity-purified LASP1 antibody (diluted 10 μg/mL; ref. 25) followed by secondary Cy3-labeled anti-rabbit antibody (1:500; Dianova).

Small interfering RNA preparation and transfection

LASP1 knockdown was done using a mix of two small interfering RNA (siRNA) constructs targeting the LASP1 sequences 5′-AAG GTG AAC TGT CAT GAT AAG-3′ (bases 49–69; named A in Supplementary Fig. S1) and 5′-AAG CAT GCT TCC ATT GCG AGA-3′ (bases 80–100; named C in Supplementary Fig. S1; ordered from Dharmaco) A total of three different LASP1 siRNAs were tested alone or in combination in all cell lines. Knockdown efficiency of siRNA constructs was best for constructs A and C and independent of 17q status in cell lines without (DA0Y) and with 17q gain (D283; Supplementary Fig. S1). Similar results were obtained for UW228-2. Nontargeting siRNA no. 5 from Dharmaco was used as a control. Cells were plated at a density of 2 × 10^5 cells/flask, grown for 24 hours at a confluence of 30% to 50% and transfected with 2.5 μg of siRNA in reduced serum medium OPTI-MEM-I (Life Technologies, Paisley, United Kingdom) according to the manufacturer’s protocol. After 4 hours of incubation at 37°C, the transfection medium was replaced with 5 mL of routine cell culture medium and incubation was continued for 42 hours. For control cells, either 20 μL of HiPerfect or 2.5 μg of control siRNA were used. For adhesion and migration experiments, the cells were synchronized by starving overnight in basal medium with 0.5% FCS. At least three independent experiments were performed for each cell line, and representative results are shown. LASP1 knockdown was controlled by Western blots in all experiments.

Western blot analysis

For Western blotting, cells were lysed in Laemmli sample buffer. Equal amounts of protein, according to cell count, were resolved by 10% SDS-PAGE. After blotting on a nitrocellulose membrane (Schleicher & Schuell) and blocking with 3% nonfat dry milk (Bio-Rad) in 10 mmol/L Tris (pH 7.5), 100 mmol/L of NaCl, and 0.1% (w/v) Tween 20, the membrane was first incubated with the self-generated antibody against LASP1 (diluted 1 μg/mL; ref. 25) or with filamentous actin (diluted 1:5,000, Sigma-Aldrich) followed by incubation with horseradish peroxidase–coupled goat anti-rabbit IgG (Bio-Rad; diluted 1:5,000), and visualized by enhanced chemiluminescence (Amersham Biosciences). Quantification of autoradiography signals was carried out by densitometry using the ImageJ software (NIH).

Cell proliferation

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)–based CellTiter96 AQ Non-Radioactive Cell Proliferation Assay (Promega). Details are given in the Appendix.

Adhesion assay

To assess cell adhesion, 48-well plates were coated with 10 μg/mL of fibrinogen (Sigma) diluted in PBS and 0.1% bovine serum albumin overnight at 4°C. Cells were washed in serum-free medium, resuspended at a concentration of 5 × 10^5 cells/mL and 200 μL were seeded. Cells were allowed to attach for 2 hours at 37°C. Nonadherent cells were removed by gentle washing with PBS. Attached cells were fixed in 4% (w/v) paraformaldehyde for 10 minutes and then stained with 0.5% (w/v) crystal violet (in 2% ethanol, filtered with 0.45 μm pore size) for 20 minutes followed by washing three times with PBS. The blue dye was eluted in 10% acetic acid for 10 minutes, and the absorbance was measured at 595 nm on a plate reader. Adhesion assays were performed in 3 independent experiments, each with 6 replicates.

Cell migration

The migration assay was performed using a modified Boyden chamber assay (Transwell chambers, Corning Star). Details are given in the Appendix.

Statistical analysis

Median duration of follow-up was calculated according to Korn (30). Estimation of survival time distributions was performed using the Kaplan-Meier method. For comparisons of two survival curves, the log rank test was used. Comparisons of patient characteristics between LASP1<sub>high</sub> and LASP1<sub>low</sub> mRNA expression and between LASP1<sub>high</sub> and LASP1<sub>low</sub> protein expression were performed using Fisher’s exact test. Student’s t test was applied to determine the effects of siRNA knockdown on cell proliferation, adhesion, and migration. Signaling pathway impact analysis (SPIA) algorithm was used to identify relevant pathways upon LASP1 knockdown experiments (31). Spearman’s rank correlation coefficient was assessed to describe the relationship between 17q gain and LASP1 protein expression. Gender, age, M-stage, histology, 17q gain, and LASP1 protein expression were included in the multivariate Cox proportional hazards regression model as possible prognostic factors for overall survival. A result was considered significant at P ≤ 0.05. Statistical computations were performed with the statistical software environment R, version 2.9.0. Exact confidence intervals for Spearman’s rank correlation coefficient were calculated using StatXact software version 6.

Results

Differentially expressed genes in tumors with and without 17q gain

To identify novel oncogenes on chromosome 17q, the most frequent region of chromosomal gain in medulloblastoma, we hybridized total RNA from 10 equally represented medulloblastoma samples with 17q gain against 10 medulloblastoma samples with diploid 17q. Likely due to gene-dosage effects, the mean log 2 ratio of all genes...
on 17p was negative, whereas the mean expression ratio of 17q genes was positive (Fig. 1A). The top 50 upregulated genes on 17q in tumors harboring 17q gain are listed in Supplementary Table S2. Among the top 10 genes, we found several candidate genes of potential interest with respect to medulloblastoma biology, including CDK5R1, a brain-specific activator of cyclin-dependent kinase 5 and GNGT2, a guanine-nucleotide exchange factor known to
Figure 2. A, representative LASP1 immunohistochemistry with negative, predominantly nuclear or cytoplasmic staining in medulloblastoma (×200). B, high protein expression in tumors with 17q gain and metastatic disease (M1–3). Immunohistochemistry was performed on a tissue microarray with 207 medulloblastoma samples of uniformly treated children. C, progression-free (PFS) and overall survival (OS) rates estimated from the time of diagnosis using the Kaplan-Meier method for LASP1 high and LASP1 low expressing tumors as assessed by immunohistochemistry. The numbers at the bottom indicate the number of patients at risk in each molecular subgroup at a certain time point.
be involved in retinal phototransduction signaling and known to be activated in high-risk medulloblastoma (4, 5). Most interestingly, one probe covering the transcriptional start site of LASP1, a well-established driver of metastatic dissemination (21), showed a very strong upregulation (log 2 ratio = 3.1). A second clone for LASP1 mapping to the 3′ part of the 3′-untranslated region also showed a marked upregulation; albeit at lower levels (log 2 ratio = 1.1) than observed for the first LASP1 clone (Fig. 1A). Notably, expressing profiling data in an independent cohort of 103 medulloblastomas performed on a different platform (Affymetrix) in a different laboratory (Hospital for Sick Children, Toronto, Canada) confirmed that LASP1 mRNA expression was significantly associated with 17q status (P < 0.001; Supplementary Fig. S2).

**LASP1 mRNA expression correlates with 17q status, M stage, and patient outcome in medulloblastoma**

Because we had previously established a role for 17q gain in medulloblastoma dissemination (11), we decided to primarily focus on LASP1 as a candidate oncogene on chromosome 17q based on its known role in metastatic dissemination. Therefore, we validated our microarray results by quantitative real-time PCR in an independent cohort of 101 medulloblastoma samples for which the DNA copy number status of 17q was available. Patient data in association with LASP1 mRNA expression are shown in Supplementary Table S3. As anticipated from our microarray data, we found a very strong association between LASP1 expression, 17q status (P < 0.001; Fig. 1B, left), and metastatic disease at the time of diagnosis (P = 0.002; Fig. 1B, right). To follow an unbiased approach for correlation with survival data, median LASP1 expression was used as a cutoff between LASP1high and LASP1low expressing tumors. Doing so, we found a statistically significant difference between LASP1high and LASP1low expressing tumors for overall survival (P = 0.05; data not shown) using the Kaplan-Meier method, whereas progression-free survival (PFS) was not significantly different between these two groups (P = 0.21).

**LASP1 protein expression in medulloblastoma**

LASP1 protein expression was subsequently evaluated in 207 medulloblastoma samples on a medulloblastoma tissue microarray by immunohistochemistry. Among them, 100 (48%) samples were immunonegative (Fig. 2A, left), whereas the remaining 107 (52%) tumors showed strong and homogeneous LASP1 expression, with a predominantly

**Table 1.** Patient characteristics in the cohort investigated by immunohistochemistry and fluorescence in situ hybridization

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients</th>
<th>LASP1 negative</th>
<th>LASP1 positive</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4</td>
<td>24</td>
<td>14 (58%)</td>
<td>10 (42%)</td>
<td>0.12</td>
</tr>
<tr>
<td>4–18</td>
<td>165</td>
<td>74 (45%)</td>
<td>91 (55%)</td>
<td></td>
</tr>
<tr>
<td>&gt;18</td>
<td>18</td>
<td>12 (67%)</td>
<td>6 (33%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>76</td>
<td>40 (53%)</td>
<td>36 (47%)</td>
<td>0.38</td>
</tr>
<tr>
<td>Male</td>
<td>131</td>
<td>60 (46%)</td>
<td>71 (54%)</td>
<td></td>
</tr>
<tr>
<td>Metastatic stage</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M0</td>
<td>144</td>
<td>83 (58%)</td>
<td>61 (42%)</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>20</td>
<td>10 (50%)</td>
<td>10 (50%)</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>13</td>
<td>2 (15%)</td>
<td>11 (85%)</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>30</td>
<td>5 (17%)</td>
<td>25 (83%)</td>
<td></td>
</tr>
<tr>
<td>Histologic subtype</td>
<td></td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Desmoplastic/MBEN</td>
<td>9</td>
<td>9 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Classic</td>
<td>176</td>
<td>85 (48%)</td>
<td>91 (52%)</td>
<td></td>
</tr>
<tr>
<td>Large cell/anaplastic</td>
<td>22</td>
<td>6 (27%)</td>
<td>16 (73%)</td>
<td></td>
</tr>
<tr>
<td>Level of resection</td>
<td></td>
<td></td>
<td></td>
<td>0.68</td>
</tr>
<tr>
<td>Gross total resection</td>
<td>93</td>
<td>43 (46%)</td>
<td>50 (54%)</td>
<td></td>
</tr>
<tr>
<td>Subtotal resection</td>
<td>114</td>
<td>57 (50%)</td>
<td>57 (50%)</td>
<td></td>
</tr>
<tr>
<td>17q status</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Balanced</td>
<td>108</td>
<td>69 (64%)</td>
<td>39 (36%)</td>
<td></td>
</tr>
<tr>
<td>Copy number gain</td>
<td>99</td>
<td>31 (31%)</td>
<td>68 (69%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>207</td>
<td>100 (48%)</td>
<td>107 (52%)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: MBEN, medulloblastoma with extensive nodularity.
*Fisher’s exact test.
membranous and cytoplasmic distribution (Fig. 2A, middle). Only a small proportion of tumors exhibited nuclear positivity (Fig. 2A, right). All desmoplastic medulloblastomas were immunonegative for LASP1 (Table 1). Samples positive for LASP1 were significantly overrepresented in tumors with 17q gain detected by fluorescence in situ hybridization analysis (Fig. 2B, left; Spearman’s correlation coefficient \( r = 0.32; P = 0.0001 \)) and among patients with metastatic disease at diagnosis (Fig. 2B, right; Spearman’s correlation coefficient \( r = 0.32; P = 0.0001 \)). Interestingly, MYC or MYCN amplified tumors with extremely poor prognosis also show higher LASP1 protein expression as compared with molecular standard risk tumors (\( P = 0.02 \)). This finding further strengthens the role of LASP1 in molecular high-risk tumors.

Survival analysis by Kaplan-Meier estimate revealed a significant association of LASP1 immunoreactivity with diminished progression-free survival (\( P = 0.001 \)) and overall survival (\( P < 0.001 \); Fig. 2C). Most notably, in a multivariate Cox proportional hazards regression model including variables known to be most strongly associated with overall survival, LASP1 protein expression was an independent prognostic marker besides 17q gain and anaplastic histology (Table 2; \( P = 0.03 \)).

In addition, we analyzed tissues from 11 pairs of primary and recurrent tumors from the same patients. From these, six were strongly immunopositive for LASP1 in the primary tumor and all six maintained immunopositivity in the recurrent tumor. From five patients, however, in which no LASP1 protein expression was detectable in the primary tumor as assessed by immunohistochemistry, two patients showed strong LASP1 expression in the recurrent tumor, further strengthening the role of LASP1 in medulloblastoma recurrence and disease progression (Supplementary Fig. S3).

**In vitro inhibition of LASP1 attenuates the malignant phenotype of medulloblastoma cell lines**

To functionally assess the role of LASP1 in medulloblastoma, we used three well-established medulloblastoma cell lines: DAOY, UW228-2, and D283. The latter cell line is commonly used as an *in vitro* model for metastasized medulloblastoma and is known to harbor an i(17q). The adherent cell lines, DAOY and UW228-2, showed a strong accumulation of LASP1 in the focal adhesion contacts and an intense colocalization with filamentous actin (Fig. 3A). D283 cells, an adherent/suspension cell line with a known abnormal expression of neurofilaments (32), showed minor spreading and reduced focal contacts. LASP1 localization with filamentous actin was less definite but clearly observed at the leading edges of cells (Fig. 3A).

All cell lines were transfected with two combined siRNAs against LASP1, a control siRNA, or just treated with the transfection reagent HiPerfect. Interestingly, a significant inhibition of proliferation was observed visually (Fig. 3B) and by MTT assay in these cell lines upon LASP1 silencing (Fig. 3C, left). siRNA-mediated knockdown was confirmed to cause a profound reduction of LASP1 protein abundance in all cell lines with maximum silencing observed after 48 hours (Fig. 3C, right).

Because LASP1 has been shown to have a functional role in cell motility and tumor dissemination in other tumor entities, we investigated cell migration and adhesion in medulloblastoma cell lines before and after LASP1 silencing by using a modified Boyden chamber and a fibronectin adhesion assay. We observed a strong reduction in migratory potential (Fig. 4A) and an enhanced adhesion in all medulloblastoma cell lines upon LASP1 silencing (Fig. 4B). Similar results regarding adhesion, migration, and proliferation were obtained with single siRNA treatment (Supplementary Fig. S4).

In an attempt to characterize the molecular networks influenced by LASP1, we performed transcriptome analysis comparing differentially expressed mRNAs upon LASP1 knockdown using two independent siRNA clones (A, C) in two medulloblastoma cell lines with (D283) and without (DAOY) copy number gain of 17q. Notably, *LASP1* mRNA was the most strongly downregulated transcript, thus confirming the specificity of knockdown experiments. Based on previously reported roles in cell adhesion, up-regulation of *CEACAM1, NRCAM, KLHL28*, and *VASP* upon LASP1 knockdown was of particular interest (Supplementary Table S4). By contrast, downregulation of the following transcripts coding for proteins implicated in invasion and migration was notable: *MMP19, ADAM9, TPM3*, and *CAPZA2*.

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**Table 2. Multivariate Cox proportional hazards regression model for overall survival**

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR for OS (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.70 (0.87)</td>
<td>0.12</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4</td>
<td>0.43 (0.19–0.99)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>4–18</td>
<td>0.70 (0.17–2.85)</td>
<td>0.62</td>
</tr>
<tr>
<td>M stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>0.58 (0.20–1.71)</td>
<td>0.33</td>
</tr>
<tr>
<td>M1</td>
<td>0.66 (0.20–2.14)</td>
<td>0.49</td>
</tr>
<tr>
<td>M2</td>
<td>1.02 (0.44–2.37)</td>
<td>0.97</td>
</tr>
<tr>
<td>M3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histologic subtype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large cell/anaplastic</td>
<td>5.53 (2.52–12.11)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Desmoplastic/MBEN</td>
<td>1.01 (0.12–8.33)</td>
<td>0.99</td>
</tr>
<tr>
<td>17q status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Balanced status</td>
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<td></td>
</tr>
<tr>
<td>Copy number gain</td>
<td>5.98 (2.52–14.19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LASP1 immunoreactivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>2.24 (1.06–4.75)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Abbreviations: HR, hazard ratio; OS, overall survival; CI, confidence interval; MBEN, medulloblastoma with extensive nodularity.
(Supplementary Table S5). By using the top 100 differentially expressed genes for SPIA pathway annotation, “actin cytoskeleton regulation” (GNA13, ITGB1, and PPP1CC) and “focal adhesion” (VEGF, ITGB1, and PPP1CC) were among the most frequently affected cellular functions (Supplementary Table S6).

These findings establish a critical role for LASP1 in the proliferation and migration of medulloblastoma cells and...
suggest that LASP1 is a functionally relevant 17q oncogene upregulated in intermediate and high-risk medulloblastomas carrying an isochromosome 17q.

**Discussion**

Medulloblastomas had previously been considered a single entity. However, molecular studies conducted over the last 5 years have shown that medulloblastoma is a molecularly heterogeneous disease (4–6). Genome-wide transcriptome analysis has consistently revealed at least four distinct biological subentities with unique underlying pathophysiological mechanisms, demographics, and clinical outcomes. A unifying clinical feature of the two (5, 6) or three (4) molecular subgroups that are not characterized by WNT or SHH pathway activation is the high incidence of metastatic dissemination via the surrounding cerebrospinal fluid, and one of the unifying molecular characteristics observed in these tumors are chromosome 17 aberrations classifying them as high-risk medulloblastomas (9–11).

In a comparison of primary and recurrent medulloblastoma samples from 28 patients, we previously made the interesting observation that acquired isolated gains of 17q are a common genetic feature in relapsed medulloblastoma (18). Based on this observation, we attempted to identify novel oncogenes on 17q that could account for the selective growth advantage of tumor cells carrying this aberration, and explain their promigratory and proproliferative phenotype.

In this study, we identified LASP1 as a very promising candidate oncogene in medulloblastoma based on its overexpression in tumors harboring gain of 17q, as compared with medulloblastomas with a balanced karyotype.

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**Figure 4.** A. LASP1 knockdown inhibits cell migration. Medulloblastoma cells transfected with LASP1 siRNA and control siRNA were seeded in modified Boyden chambers and incubated for 4 h. Migrating cells were fixed with paraformaldehyde and stained with crystal violet. The absorbance was measured. Bars, SEM; *, *P < 0.001 versus control; Student’s t test. B. Increased adhesion in LASP1-depleted cells. Medulloblastoma cells were seeded in 48-well plates coated with fibronectin and incubated for 2 h. Cells were fixed with paraformaldehyde and stained with crystal violet. Absorbance was measured at 595 nm. Bars, SED; ***, *P < 0.001 versus control. LASP1 knockdown efficiency was controlled by Western blot.
on chromosome 17. This is mechanistically very intriguing, as LASP1 overexpression has been previously found to drive metastasis in breast cancer and ovarian cancer (23–25), both of which are also characterized by frequent gain of 17q in late stage disease (33, 34).

The clinical implication of our findings in medulloblastoma is further underlined by the strong association between LASP1 mRNA and protein expression within the presence of metastatic disease, and its role as an excellent prognostic marker as identified by immunohistochemistry in more than 200 primary medulloblastoma samples. As anticipated from our previous finding that 17q gain is frequently acquired during medulloblastoma progression, we noted strong LASP1 protein expression in two out of five recurrent medulloblastoma samples in which no expression was observed in the patient matched primary tumor. These findings are consistent with the attributed biological functions of LASP1 in primary tumors, including the regulation of cell migration and proliferation (21, 23–26, 35).

Supporting our observations in primary tumors, we found a striking phenotype upon siRNA-mediated silencing of LASP1 in three well-established medulloblastoma cell lines. In addition to a marked reduction in cell proliferation, we observed a profoundly impaired propensity of medulloblastoma cells to migrate through a filter membrane in combination with a substantially increased tumor cell to matrix adhesion upon LASP1 knockdown. These in vitro observations were nicely complemented by the identification of deregulated genes and signaling pathways upon LASP1 knockdown. Notably, KEGG pathways such as “actin cytoskeleton regulation” and “focal adhesion” were among the most frequently affected cellular functions as determined by SPIA analysis.

Since its identification in breast tumor cells (21), LASP1 was found to be upregulated in a number of other cancers [ovarian (24); kidney (36); and prostate (37)] and was suggested to be a negative prognostic marker. A recent study identified LASP1 as a novel p53 transcriptional target in hepatocellular carcinoma (38). Inactivating p53 mutations led to increased LASP1 expression and to a more aggressive hepatocellular carcinoma phenotype. Nevertheless, not all p53-mutated cell lines showed increased LASP1 expression (39). Interestingly, mRNA expression levels of p53, downstream intermediates and prominent target genes were not altered upon LASP1 knockdown in medulloblastoma cell lines (Supplementary Table S4–6).

In invasive breast cancer cells, LASP1 expression was inversely correlated with prostate-derived Ets factor, a transcription factor known to repress a variety of genes that are possibly involved in oncogenesis (40). However, interdependence of prostate-derived Ets factor and LASP1 expression in breast cancer cell lines remains controversially discussed (39, 41). None of the hallmark genes involved in metastatic cascades in breast cancer such as NM23, DLC1, DLRG1, KAI1, RHOGDID2, KISS1, and RKIP (reviewed in ref. 42) seem to be deregulated upon LASP1 knockdown in medulloblastoma. Thus, underlying molecular mechanisms might be essentially different in metastatic dissemination of medulloblastoma cells through the cerebrospinal fluid with LASP1 playing a central role in both.

Interestingly, EZRIN (EZR), a cytoskeletal cross-linker that connects the oncogenic stemness marker CD44 with the cytoskeleton (43–45), has recently been proposed to participate in medulloblastoma cell migration in concert with other cytoskeletal proteins. Notably, EZR silencing in medulloblastoma cell lines showed a similar phenotype with suppressed migration and invasion as LASP1 knockdown. However, in contrast with LASP1 silenced medulloblastoma cells, EZR knockdown led to reduced cell adhesion (46).

Since indirect interaction of LASP1 and EZR via the promigratory scaffolds KRP1 and PALADIN was recently reported (47, 48) and because LASP1 physically interacts with the important transcription factors ZYXIN and LPP that in turn promote prometastatic transcriptional signatures, it is tempting to speculate that LASP1 exerts part of its function as a signal transducer embedded in a novel signaling pathway and as a structural focal adhesion protein (22, 49).

We do not yet understand whether LASP1 expression is regulated by oncogenes or whether LASP1 itself acts as a transcription factor regulating oncogenes and proteins involved in cell migration. However, compared with normal tissue, tumor cells display an increased rate of LASP1-positive nuclei (24, 39), supporting the hypothesis that, next to its physiologic role as a scaffolding protein involved in cell motility, LASP1 may serve as a transcriptional cofactor involved in processes of cell proliferation and metastasis in malignant cells. Further studies will help to define the role of LASP1 in cancer development and metastasis.

In conclusion, we have identified LASP1 on 17q12 as a novel candidate oncogene with effects on metastatic dissemination and disease progression of medulloblastoma with high potential to serve as a powerful biomarker for outcome prediction in future prospective studies. Attempts to tackle signal transduction pathways that are driving tumor cell motility and invasiveness with targeted therapies will be an important step towards disease control in metastatic medulloblastoma, and LASP1 represents an excellent novel candidate oncogene for future targeted therapy approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Role of LIM and SH3 Protein 1 (LASP1) in the Metastatic Dissemination of Medulloblastoma

Christopher Traenka, Marc Remke, Andrey Korshunov, et al.

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