ZEB1 induces N-cadherin expression in human glioblastoma and may alter patient survival

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Abstract. The present study investigated the expression of epithelial-mesenchymal transition (EMT)-related factors zinc finger E-box-binding homeobox 1 (ZEB1), cadherin-1 (CDH1), cadherin-2 (CDH2) and the cell cycle modulating kinase cyclin-dependent kinase 1 (CDK1) in human glioblastoma (GBM) compared to normal brain tissue, as well as whether the levels of expression were associated with the overall and progression-free survival of the GBM patients. In 44 GBM and five normal brain tissue specimens, the expression levels of ZEB1, CDH1, CDH2 and CDK1 were evaluated by real-time PCR and immunostaining, and the results were correlated with clinical data. The expression levels of all investigated genes as detected by immunostaining were significantly higher in the GBM when compared to the normal brain tissues. There was no influence on survival. A linear correlation between ZEB1 and CDH2 and CDK1 expression was observed in GBM. Moreover, ZEB1 was involved in EMT (e.g., signaling in human GBM) and high ZEB1 levels were linked to an aberrant cell cycle processing, marked by CDK1 overexpression.

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Abbreviations: GBM, glioblastoma; EMT, epithelial-mesenchymal transition; IHC, immunohistochemistry; MET, mesenchymal-epithelial transition; MGMT, O^6 methylguanine-DNA-methyltransferase; MRI, magnetic resonance imaging; 5-ALA, 5-aminolevulinic acid; NBT, normal brain tissue; IRS, immunoreactivity score; qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; OS, overall survival; PFS, progression-free survival; ZEB1, zinc finger E-box-binding homeobox 1

Key words: cancer, glioblastoma, epithelial to mesenchymal transition, ZEB1, gene expression, tumor progression, cell cycle, CDK1, molecular oncology

Introduction

Glioblastoma (GBM) is the most frequent intrinsic brain tumor in adults (1). Despite intense research, the prognosis is still fatal with a mean overall survival (OS) of 12 to 16 months (2-4). Maximal treatment includes gross total tumor resection followed by concomitant radiochemotherapy and temozolomide (5,6). Glioblastoma treatments are based on systemic therapeutic approaches, which highlights the lack of specific targeted therapies for GBM patients and the need for better molecular understanding of the underlying mechanisms of glioma genesis and progression. One of these mechanisms includes epithelial-mesenchymal transition (EMT), a complex process allowing cells with epithelial characteristics to gain mesenchymal properties due to highly regulated changes in gene expression (7). While epithelial-like cells show a polarized subtype and are likely to engage in intracellular adhesion, mesenchymal differentiated cells present with altered polarization, higher migratory capacity and stem cell properties (8-10). This transition is valuable in processes such as embryogenesis and wound healing, but it leads to increased tumor progression, chemoresistance and mitotic progression (11). This process can occur in reverse [i.e., mesenchymal-epithelial transition (MET)], and cells are able to dynamically shift between transitional stages (12,13). Cells with a more epithelial phenotype express higher levels of E-cadherin coded by the cadherin-1 (CDH1) gene, a cellular membrane glycoprotein that assists cell adhesion and membrane stability (9,14). A typical mesenchymal marker, in contrast, is N-cadherin, coded by CDH2, which is associated with migratory capacity and loss of cell polarity (11). The decrease in E-cadherin expression, which is linked to increased N-cadherin expression, is a crucial indicator of EMT called the cadherin switch (11,13).

Previous studies have exhibited that EMT signaling in various cancer cells (e.g., colorectal carcinoma and breast cancer) leads to stem-like properties, induced autophagy, aggressive behavior and metastatic progression (15-17). As GBMs are of glial rather than epithelial origin, not every aspect of EMT applies to these tumors. Nonetheless, evidence suggests that glioma cells can change their morphological phenotype and genetic signature from a more epithelial-like to a more mesenchymal-like character (11); the term 'EMT-like

process' describes this mechanism. In GBM, EMT-like behavior is associated with invasion, tumor progression and therapy resistance (18). Iser *et al* (11) discuss a link between astrocyte-glioma interaction via EMT-inducing factors, and other studies postulate that glioma cells gain stem cell properties by transitioning to a mesenchymal state (18).

The transcription factor zinc finger E-box-binding homeobox 1 (ZEB1) is an important inducer of EMT in several malignancies, including GBM (19). Despite heterogeneous reports on the regulatory function of EMT in GBM, ZEB1 expression is associated with higher grades of malignancy, tumor progression and invasion (20-22). Regarding the expression of E-cadherin in human GBM, contradictory data exists, with some authors describing overexpression in GBM and others reporting low expression (23,24). Camand *et al* (25) reported lower N-cadherin expression levels in GBM compared to normal brain tissue (NBT), whereas other studies have described overexpression and correlation with higher grades of malignancy (26,27).

The present study was conducted to examine the expression of E-cadherin and N-cadherin, as well as the central EMT-induction factor ZEB1 and the marker of cell cycle upregulation cyclin-dependent kinase 1 (CDK1) in human GBM compared to NBT. Furthermore, the researchers investigated whether the expression of those genes is related to progression-free survival (PFS) and OS.

Materials and methods

Patient collective and tissue specimens. Forty-four patients who underwent tumor resection for a supratentorial GBM in the neurosurgical department of the University Hospital of Giessen, Germany between 2006 and 2015 were included. All patients were diagnosed with GBM, IDH-wild-type. The Institute of Neuropathology of the University Hospital Giessen (Germany) provided information on the O^6 methylguanine-DNA-methyltransferase (MGMT) promotor methylation status, as well as paraffin-embedded tissue sections of all patients. Follow-up records including age at diagnosis, sex, date of surgery, treatment protocols, magnetic resonance imaging (MRI) data and time of death were available. For all patients, intraoperatively obtained tissue from the 5-aminolevulinic acid (5-ALA)-positive tumor area was stored in frozen nitrogen. As a control, the researchers used five paraffin-embedded sections of normal human brain tissue (NBT) provided to their institution by the Institute of Neuropathology and four kryosample-derived brain tissue, provided by the Institute of Pathology at the University of Salzburg, Austria.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Giessen, Germany (AZ 07/09). Written informed consent was obtained from all patients before enrollment in the study.

Gene expression analysis by qPCR. Tissue was thawed and RNA from the tumor specimen was isolated using the RNEasy kit (Qiagen GmbH) according to the manufacturer's protocol. The NanoDrop ND-1000 spectrophotometer was used to photometrically evaluate the RNA concentration and purity (Thermo Fisher Scientific, Inc.), and samples with an extinction ratio of E260/280 >2 were used for further processing. Transcription to cDNA was performed using the QuantiTect reverse transcription kit (Qiagen GmbH).

For quantitative real-time polymerase chain reaction (qPCR), the TaqMan gene expression master mix and gene expression assays were used according to the manufacturer's protocol. For amplification, StepOne real-time PCR system (Applied Biosystems/Thermo Fisher Scientific, Inc.) was used. Analysis was conducted manually in triplicates, and dimensionless expression values were calculated via the ΔCT method. The customized TaqMan gene expression assays were HS00611018_m1 for ZEB1, HS00938778_m1 for CDK1, HS01023894-m1 for CDH1 (E-cadherin), HS00983056-m1 for CDH2 (N-cadherin) and Hs99999903_Actβ for Actin-β (all from Thermo Fisher Scientific, Inc.).

Immunostaining. All GBM specimens and five NBT sections were deparaffined and, after preheating, immunohistochemistry (IHC) was performed with the DCS SuperVision 2 kit (DCS Innovative Diagnostik-Systeme), following the manufacturer's instructions. The researchers used an anti-ZEB1 monoclonal mouse antibody (ab180905) in a dilution of 1:500, an anti-CDK1 monoclonal rabbit antibody (ab183550) in a dilution of 1:500, anti-N-cadherin monoclonal mouse antibodies (ab98952) in a dilution of 1:2,000 and anti-E-cadherin polyclonal rabbit antibodies (ab15148) in a dilution of 1:100 (all from Abcam). Human colon sections were used as a positive control for CDK1, human lung cancer tissue for ZEB1, human skin tissue for E-cadherin and human heart-sections for N-cadherin.

Regarding quantification staining intensity, ZEB1, CDK1 and E-cadherin were scored from 0 = no staining to 3 = intense staining and multiplied by the ratio of stained to unstained cells. For the cytoplasmic N-cadherin, only the staining intensity was scored as above. For all specimens, a dimensionless immunoreactivity score (IRS) defined the staining intensity.

Statistics. Statistical analysis was performed with SPSS (version 24) (IBM Corp.). A t-test followed by ANOVA, a Kruskal-Wallis test and a Mann-Whitney U test evaluated expression analysis. For survival analysis, groups with gene expression above and below the median gene expression were defined, and OS and PFS were calculated with the Kaplan-Meier method and log-rank test. A Pearson test analyzed correlation between the investigated genes. Results with $P<0.05$ were defined as significant.

Results

Patient characteristics and epidemiological data. The study included 44 patients with the diagnosis of IDH-wildtype GBM. Of the patients, 68.18% (n=30) were male and 31.82% (n=14) were female. Mean age (SD) at diagnosis was 63.8 (±11.5) years. All patients were treated by gross total resection of the tumor followed by concomitant radiochemotherapy and temozolomide maintenance therapy according to the Stupp-protocol (2). In 54.5% (n=24) of the patients, the MGMT promotor was methylated, and in 45.5% (n=20) of the patients,

Table I. Clinical characteristics and epidemiological data for all 44 investigated patients with GBM.

Patient characteristics (N=44)	
Mean age (SD) at diagnosis (years)	
Total	63.8±11.5
Males	64.3±12.4
Females	62.9±9.5
Sex n, (%)	
Male	30 (68.18)
Female	14 (31.82)
Molecular characteristics, n (%)	
IDH-1-wild-type	44
MGMT-promotor methylated	24 (54.5)
MGMT-promotor not methylated	20 (45.5)
Median PFS (months)	
Total	6.56±8.47
MGMT-promotor methylated	9.17
MGMT-promotor not methylated	4.73
Median OS (months)	
Total	15.8±20.2
MGMT-promotor methylated	13.74
MGMT-promotor not methylated	9.17

GBM, glioblastoma; PFS, progression-free survival; OS, overall survival.

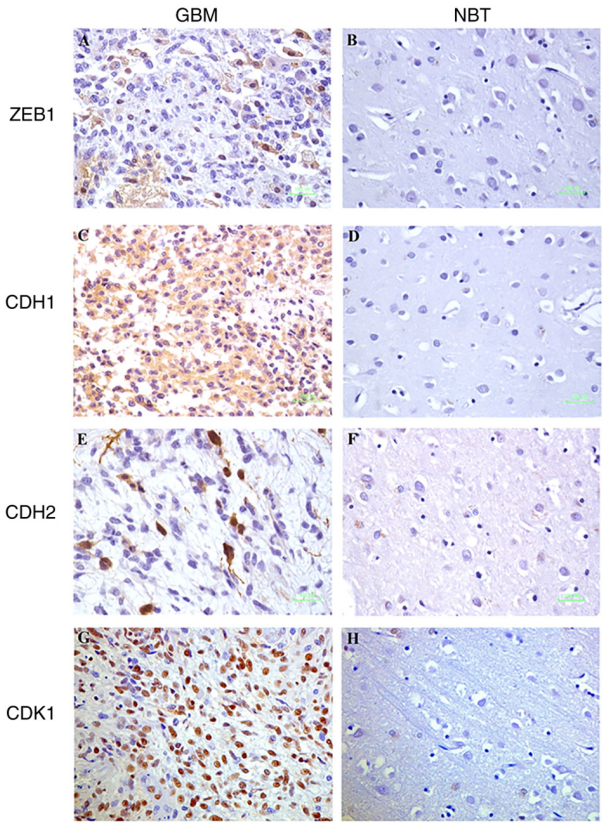

Figure 1. (A) CDH1 expression in GBM. (B) CDH1 expression in NBT. (C) CDH2 expression in GBM. (D) CDH2 expression in NBT. CDK1 was stained with higher intensity in (E) human GBM than in (F) NBT. ZEB1 expression was also significantly higher in (G) GBM than in (H) NBT. Although nuclear staining was observed no quantification was performed during the original investigations. As the project was already closed, no retroactive quantification was possible. The green lines indicate a measurement of 1 μm; further insertion of a scale bar is unfortunately not possible due to the used software. CDHI, cadherin-1; CDH2, cadherin-1; GBM, glioblastoma; NBT, normal brain tissue; CDK1, cyclin-dependent kinase 1; ZEB1, zinc finger E-box-binding homeobox 1.

it was not methylated. Median progression-free survival (PFS) in the patient collective was 6.56±) 8.47) months and the median OS was 15.8 (±20.2) months (Table I).

CDH1 (Fig. 1A), CDH2 (Fig. 1C), CDK1 (Fig. 1E) and ZEB1 (Fig. 1G) were overexpressed in human GBM compared to these levels in the NBT specimens (Fig. 1B, D, F and H).

ZEB1, E-cadherin, N-cadherin and CDK1 had significantly higher protein levels in all tested GBM specimens compared to NBT specimens. ZEB1 had an IRS of 21.71 (±6.96) in GBM vs. 3 (±3.94) in NBT specimens (P<0.001) (Fig. 2). The E-cadherin expression level in GBM was at 4.95 (±4.23) and 0.4 (±0.55) in NBT specimens (P<0.001). For N-cadherin, the IRS was 1.84 (±0.52) in GBM, compared to 0.8 (±0.45) in the NBT specimens (P=0.001). CDK1 was also overexpressed in human GBM with an IRS of 5.84 (±4.1) while the IRS in the NBT specimens was 1 (±0.71; P<0.001 (Fig. 2).

Regarding mRNA levels, similar results were observed. The gene expression level of *ZEB1* was 0.81 (±1.98) in GBM vs. 0.7 (±0.98) in NBT specimens. The expression level of *CDH1* coding for E-cadherin was 0.46 (±2.03) in GBM and 0.03 (±0.05) in the NBT specimens, while *CDH2* coding for N-cadherin was expressed at a level of 0.95 (±1.67) in GBM vs. 0.55 (±0.87) in the NBT specimens. However, none of these results reached statistical significance. Only the expression of *CDK1* was significantly higher in GBM tissue with an expression value of 1.12 (±5.14) in GBM compared to 0.0014 (±0.0005) in the NBT specimens (P=0.001) (Fig. 3).

No difference was observed in the expression level of CDH1, CDH2, CDK1 or ZEB1, nor in the corresponding proteins when comparing MGMT methylated and non-methylated tumor specimens.

Survival analysis. For all investigated genes, PFS and OS were analyzed for all patients and in relation to the MGMT-promotor methylation status. Neither for the total collective of the investigated GBM patients nor in the MGMT-positive or GBM-negative patients was a significant difference noted in the OS or PFS for patients with tumors with high expression levels of ZEB1, CDH1, CDH2 and CDK1.

Nevertheless, there was a trend toward a longer PFS and OS in patients with ZEB1 expression below the median compared to patients with ZEB1 expression above the median. Patients with higher CDH1 expression had a trend toward a longer PFS and OS compared to patients with lower CDH1 expression, but the effect only reached significance for PFS in the subgroup of MGMT-notmethylated tumors with a mean PFS of 4.37 and MGMT-negative tumors with CDH1 expression below the median compared to 5.56 months in MGMT-negative tumors with CDH1 expression above the median (P=0.005).

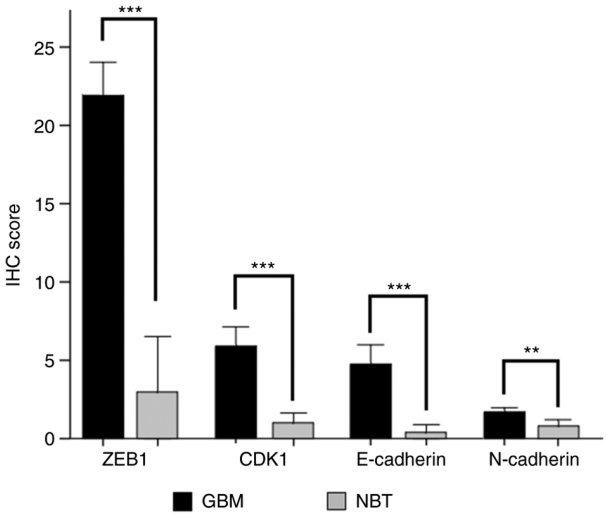

Figure 2. The IHC-score of ZEB1 (***P<0.001), CDK1 (***P<0.001), E-cadherin (***P<0.001) and N-cadherin (**P=0.001) were significantly overexpressed in human GBM compared to NBT specimens. CDK1, cyclin-dependent kinase 1; ZEB1, zinc finger E-box-binding homeobox 1; GBM, glioblastoma; NBT, normal brain tissue.

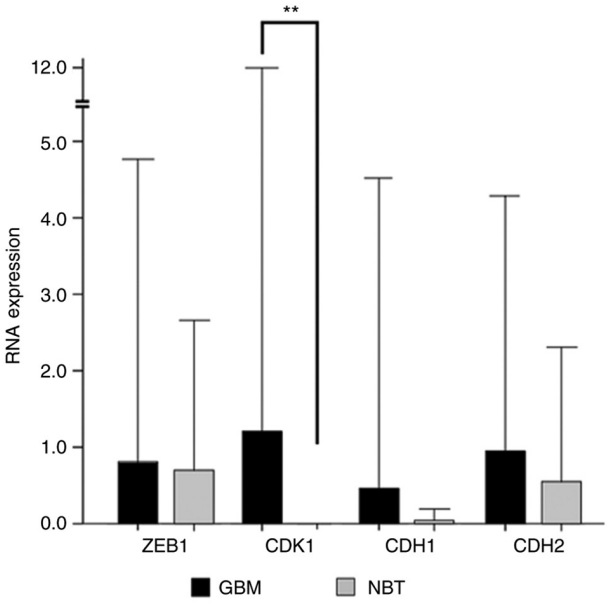

Figure 3. Expression of *ZEB1*, *CDK1*, *CDH1* and *CDH2* in GBM compared to NBT at the mRNA level. Only *CDK1* (**P=0.001) exhibited significantly higher expression in the GBM compared to the NBT specimens. *CDH1*, cadherin-1; *CDH2*, cadherin-1; GBM, glioblastoma; NBT, normal brain tissue; *CDK1*, cyclin-dependent kinase 1; *ZEB1*, zinc finger E-box-binding homeobox 1.

Regarding CDK1 expression, a slight trend toward a shorter PFS and OS for patients with CDK1 expression above the median was observed independently of MGMT-promotor methylation status.

Correlation of expression levels of the investigated genes and proteins. To investigate whether there is a correlation between the expression levels of the proteins, Pearson's analysis was performed. Overexpression of ZEB1 was associated with higher expression of CDH2/N-cadherin at the mRNA level with r=0.347 (P=0.03) and at the protein level with r=0.349 (P=0.01), suggesting a linear correlation. Similar high expression levels of ZEB1 corresponded to high CDK1 protein expression levels (r=0.363; P=0.01), although this could not be confirmed at the mRNA level. High CDK1 expression was associated with higher CDH2/N-cadherin mRNA expression (r=0.336; P=0.04). Only for the mRNA level was high CDH1 expression correlated with higher CDH2 expression (r=0.345; P=0.015) (Fig. 4).

Discussion

The present study demonstrated that zinc finger E-box-binding homeobox 1 (ZEB1), E-cadherin and N-cadherin were overexpressed in human glioblastoma (GBM) compared to normal brain tissue (NBT) specimens, and that their expression was independent from the MGMT promotor methylation status. In the patient collective, no differences in overall survival (OS) or progression-free survival (PFS) related to the expression of ZEB1, CDH1, CDH2 or CDK1 were observed. However, higher ZEB1 expression was correlated with higher levels of CDH2/N-cadherin and CDK1, suggesting a link between ZEB1 overexpression and a more mesenchymal phenotype with aberrant cell cycle processing.

ZEB1 belongs to a family of transcription factors characterized by two zinc finger clusters and a homeodomain that enable the molecule to bind specific DNA sequences. Interacting with several binding partners (i.e., co-transcription factors), ZEB1 is able to upregulate and downregulate the transcription of several genes. Via this mechanism, ZEB1 leads to downregulation of cadherin-1 (CDH1) and upregulation of cadherin-2 (CDH2) (i.e., cadherin shift) a central hallmark of epithelial-mesenchymal transition (EMT) in carcinoma cells (28).

In the literature, there is evidence that ZEB1 is involved in EMT induction, tumor progression and therapy resistance in GBM (18). Siebzehnrubl *et al* report that ZEB1 knockdown in a GBM mouse model led to downregulation of EMT signaling and increased chemosensitivity, leading to improved survival (22). Additionally, irradiation was reported to decrease ZEB1 expression and thereby EMT-related gene expression, leading to better patient survival rates (29). Previous studies have shown that ZEB1 promotes EMT by inducing the cadherin shift in human GBM and other cancers (11,30-33). In contrast to other malignancies, the typical hallmark of EMT (i.e., the switch from higher E-cadherin/CDH1 expression in an epithelial phenotype to higher N-cadherin/CDH2 expression in a mesenchymal phenotype) is not necessarily observed in GBM (11). In this study's dataset, a linear correlation between CDH1 and CDH2 expression was observed, although ZEB1 expression was only correlated to CDH2 expression. Various reports support this observation that the classical cadherin shift does not apply to gliomas (10,12,25). Due to the non-epithelial origin of glial tumors having a different gene expression pattern, authors have proposed the term 'EMT-like' or 'glial to mesenchymal transition' (GMT) (11).

Many malignancies harbor mutations that lead to aberrant cell cycle progression and thereby proliferation and

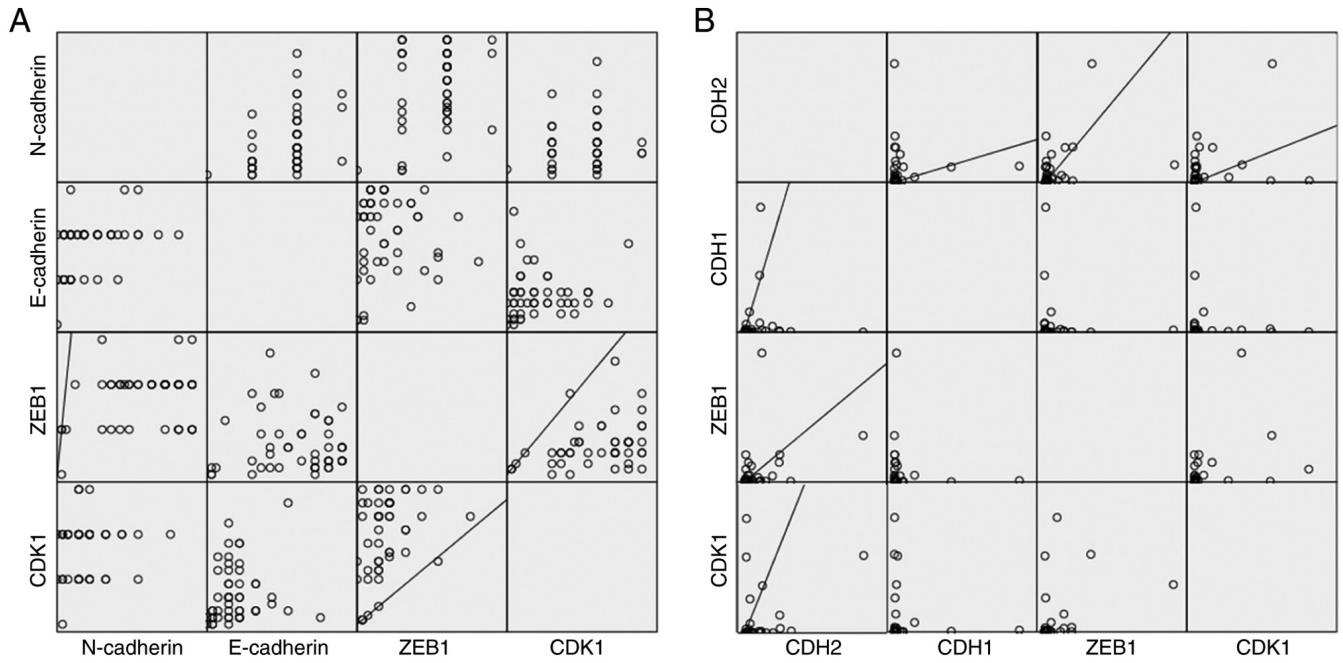

Figure 4. Correlation matrix of all investigated genes at the protein (A) and at the mRNA expression level (B).

tumor progression (34). Cyclin-dependent kinase 1 (CDK1) is a serine/threonine protein kinase that regulates the entry in mitosis. It is more highly expressed in cells with aberrant proliferation patterns and overexpressed in human GBM (35). Cancer cells with CDK1 overexpression tend to undergo faster tumor progression and higher proliferation rates (35,36).

In alignment with the literature, the CDK1 expression at the protein level was significantly higher in GBM than in NBT specimens in this study. The researchers also observed a linear correlation between N-cadherin/CDH2, ZEB1 and CDK1 expression. These findings imply that higher N-cadherin expression, which is characteristic for a mesenchymal phenotype, is also associated with aberrant proliferation and progression.

In conclusion, ZEB1, E-cadherin, N-cadherin and CDK1 are overexpressed in human GBM compared to NBT. ZEB1 expression levels correlate with N-cadherin and CDK1 expression levels. These findings lead to the conclusion that ZEB1 is a relevant regulator of EMT and cadherin shift in human GBM. This corresponds to CDK1 overexpression, which indicates aberrant cell cycle progression and is associated with an aggressive tumor phenotype. ZEB1, as a promotor of this cell signaling, is therefore a relevant target for further research and specific therapeutic approaches for GBM.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author.

Authors' contributions

Conceptualization of the study was accomplished by MAK. JN, ST and FPS were responsible for the methodology, software and statistical analyses. Investigation and data curation were accomplished by FH, HG, ST and EU. Writing and original draft preparation was the responsibility of HG. Writing, review and editing were conducted by MAK, EU and FPS. Supervision and project administration were the responsibility of MAK. All authors have read and agreed to the published version of the manuscript. HG and MAK guarantee the authenticity of the raw data collected in the study.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of University Hospital Giessen (AZ 07/09).

Patient consent for publication

Patient consent for publication was obtained.

Competing interests

The authors declare that they have no competing or conflicting interests. MAK, HG, JN, FPS, FH, EU and ST confirm disclosing all financial and non-financial competing interests for myself and on behalf of my co-authors.

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