Expression of FoxM1 is required for the proliferation of medulloblastoma cells and indicates worse survival of patients

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Translational relevance

Medulloblastoma is the most common malignant brain tumor in childhood. It is related to unsatisfactory survival rates and the development of causal therapies is highly desired. We show here that expression of the transcription factor FoxM1 may serve as an independent prognostic marker which easily allows clinicians to individually adjust therapy strategies. We further show that FoxM1 is essential for the growth of medulloblastoma cells and may serve as therapeutic target. Last, the antibiotic Siomycin A proved to successfully downregulate FoxM1 and to inhibit tumor cell growth. Our studies may therefore serve as a basis for clinical trials including expression of FoxM1.
as a prognostic marker to stratify patients and Siomycin A as a novel drug to target medulloblastoma.

Abstract

Purpose: The transcription factor Forkhead box M1 (FoxM1) is a key regulator of cell cycle progression. It is involved in the development of multiple organs, and we have previously reported on its important role for the mitotic entry of cerebellar granule neuron precursors. Constitutive expression of FoxM1 is required for the growth of multiple cancer types. This study aimed to determine its role in medulloblastoma, the most frequent malignant brain tumor in childhood that can derive from cerebellar granule neuron precursors.

Experimental Design: We evaluated the expression of FoxM1 together with its prognostic value in two independent series of human medulloblastoma samples using immunohistochemistry (n=43) and gene expression arrays (n=193). The functional impact of FoxM1 expression was characterized by knockdown experiments in four human medulloblastoma cell lines, and the thiazole antibiotic Siomycin A was tested in order to downregulate FoxM1 and inhibit tumor cell growth.

Results: FoxM1 was highly expressed in all subtypes of medulloblastoma. Importantly, expression levels of FoxM1 significantly correlated with unfavorable clinical outcome in univariate analysis (p=0.0005), and FoxM1 was identified as an independent prognostic marker by multivariate analysis (p=0.037). Knockdown of FoxM1 in medulloblastoma cell lines resulted in a significant decrease of cell viability which was caused by a failure in mitotic spindle formation and caspase-dependent mitotic catastrophe. Siomycin A significantly inhibited the expression of FoxM1 and the growth of medulloblastoma cells.
Conclusions: FoxM1 may be used as an additional prognostic marker and may represent a potential novel target to treat patients suffering from medulloblastoma.

Introduction

Medulloblastoma is the most common malignant brain tumor in childhood with a five-year overall survival of 66% (1). State-of-the-art treatment combines surgery, craniospinal irradiation, and multiple chemotherapeutics with severe sequelae of toxic side effects (2-4). The development of novel treatment options is therefore urgently needed. Medulloblastoma is a heterogeneous disease with 6 subgroups that differ with respect to histology, molecular expression profiles, genetics and clinical outcome (5). One of these subgroups is associated with a constitutive activation of the Sonic hedgehog signaling pathway, which may for instance be caused by mutations of genes encoding the Hedgehog receptors Patched or Smoothened (6, 7). While the cellular origin of medulloblastoma is uncertain for most of the 6 subgroups, tumors associated with pathological Hedgehog signaling have recently been demonstrated to arise from cerebellar granule neuron precursors (8). During normal development, these precursors extensively proliferate due to a physiological stimulation by Sonic hedgehog (9) until they migrate away from their mitotic niche in the external granule cell layer and differentiate at postnatal stages (10). While the mechanisms of differentiation in cerebellar granule neurons are not yet clearly understood, it is evident that their cell cycle progression is dependent on FoxM1 (11), which is a target gene of Hedgehog signaling (12, 13).

FoxM1 (previously known as HFH-11B, MPP2 or Trident) is a member of the Forkhead family of transcription factors that share homology within the winged-helix/Forkhead DNA-binding domain (14, 15). FoxM1 is predominantly expressed in fetal tissues but its expression may be maintained in proliferating adult tissues (15,
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16). FoxM1 is a key regulator of the transition from G1 to S phase by activating genes required for cell cycle progression such as Cdc25B, cyclin B1, Aurora B kinase, and Polo-like kinase 1 (14, 17). FoxM1 is also essential for chromosome segregation (16, 18), and depletion of FoxM1 leads to a failure to enter S phase and impairs proper M-phase completion with subsequent mitotic catastrophe (15, 17, 19). Increased levels of FoxM1 of expression have been detected in many different types of human cancer such as basal cell carcinomas, breast cancer, glioblastomas, lung cancers, pancreas and prostate cancer (13, 19-22). Within this context, FoxM1 has not only been identified as a prognostic marker (22), but its expression has also been demonstrated to be required for the growth of lung cancer (20), prostate carcinomas (21) and breast cancer (19).

Previous work has demonstrated a crucial role for FoxM1 during the development of cerebellar granule neuron precursors as well as a pathologically maintained expression of FoxM1 in tumors arisen in ptc+/− mice, a well established mouse model for Sonic hedgehog associated medulloblastoma (11, 23). However, the relevance of FoxM1 in human medulloblastoma has not yet been investigated. We show here that FoxM1 is highly expressed in human medulloblastomas and that it significantly correlates with patient’s outcome. Blocking of FoxM1 in medulloblastoma cell lines by RNAi technology as well as by the chemical inhibitor Siomycin A leads to caspase-dependent mitotic catastrophe and growth inhibition. FoxM1 may therefore represent a potential target to treat medulloblastoma.

Material and Methods

Tissue samples and cell lines

A total of 43 formalin-fixed paraffin-embedded surgical tumor samples from patients with medulloblastoma were analyzed. Inclusion of patients in the study was unbiased.
and only dependent on the availability of sufficient tumor material and clinical follow-up data. Patients included 21 males (48.8%) and 22 females (51.2%). They were treated in the University Hospitals of Munich, Göttingen, Bremen, Hannover and Münster (all Germany). Nineteen children were treated in prospective multicenter trials as described: (1) HIT 2000 trial (n=9; stratification of risk-adapted treatment according to age and metastasis stage into 4 different treatment arms with combination of postoperative chemotherapy and/or radiotherapy and/or maintenance chemotherapy) (2) HIT'91 trial (n=6; randomized trial of postoperative chemotherapy followed by craniospinal radiotherapy versus postoperative craniospinal radiotherapy followed by maintenance chemotherapy) (24); (3) HIT-MED'99 (n=2; stratification of risk-adapted treatment according to age and metastasis stage into 4 different treatment arms); (4) HIT'88 trial (n=1; preradiation chemotherapy and radiotherapy) (25); (5) HIT'87 trial (n=1; systemic interval chemotherapy until craniospinal radiotherapy was applied at 3 years of age or at relapse) (26). The median age was 9.3 years (range, 0.6 – 45.6 years). The median follow-up of survivors was 53 months (range, 22.2 – 175.6 months). 16 patients succumbed to their disease, 25 were alive as of January 1st, 2011. The study included one anaplastic medulloblastoma, 25 medulloblastomas of classic histology, 14 medulloblastomas of desmoplastic histology and 2 medulloblastomas with extensive nodularity. One tumor was not classified. Detailed patient characteristics are given in Supplementary Table 1. Tumor diagnosis was established by standard light-microscopic evaluation of hematoxylin and eosin-stained sections and silver stains. Diagnoses were made independently by at least two neuropathologists based on the criteria of the latest WHO brain tumor classification (27). The human medulloblastoma cell line DAOY as well as HEK293T cells were purchased from the American Type Culture Collection. The human medulloblastoma cell line D425med was obtained from Dr. Darell Bigner (Duke University Medical Center, Dur-
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ham, NC, USA). The R300 and the UW228 human medulloblastoma cell lines were obtained from Dr. Michael S. Bobola (Children’s Hospital and Regional Medical Center, Seattle, WA, USA).

**Immunohistochemistry**

Paraffin-embedded tissue was sectioned, deparaffinised and rehydrated before heat-induced antigen retrieval was performed. Immunohistochemical staining was done using primary antibodies against FoxM1 (1:25, Santa Cruz Biotechnology, USA) and the HRP/DAB staining system (DAKO, Sweden) according to the manufactures specifications. Hemalun was used for nuclear counterstain. For immunofluorescent stainings, sections and fixed medulloblastoma cells from cell cultures were washed twice with PBS/0.1% TritonX-100 and then incubated in blocking buffer (I-Block™ Protein-Based Blocking Reagent, Applied Biosystems, Germany) for 30 min. Primary antibodies (FoxM1 1:25, Santa Cruz Biotechnology, USA; Ki67 1:500, Dako, Germany; Cyclin B1 1:200, Santa Cruz Biotechnology, USA) were diluted in blocking buffer and applied over night at 4°C. Next, cells were washed twice with PBS/0.1% TritonX-100 and incubated for another 30 min with a 1:500 dilution of fluorescent-labeled secondary antibodies (Invitrogen, USA) in blocking buffer. Cells were washed twice with PBS/0.1% TritonX-100, counterstained with 4′, 6-Diamidino-2-phenylindole (DAPI) and mounted in Immu-Mount (Thermo Shandon, USA). All images of tumor tissue were collected on an Olympus IX50 microscope. Images from cell culture experiments were taken using a confocal laser scanning microscope (Zeiss, Germany).

**Cell culture**

All cell lines were grown in DMEM medium containing 10% fetal calf serum and 1% glutamine at 37°C and 5% CO2 in a humidified atmosphere. Lentiviral particles for
cell line transductions were produced by transfecting HEK293T cells with plasmids containing sequences encoding for packaging and envelope proteins as well as for shRNAs using FuGENE6® (Roche, Germany). pLKO.1 vectors including FOXM1 shRNA as well as mock sequences were provided by the Broad Institute of Harvard and MIT (Cambridge, MA, USA). ShRNA sequences were GCACATCAACAA-TAGCCTAT (#1), GCCAATCGTTCTCTGACAGAA (#2), and CCGCAGGTATGCACGCGT (mock). Supernatant medium from transfected HEK293 cells including viral particles was harvested 48 h after transfection and stored at -80°C until use. Medulloblastoma cells were transduced with viruses overnight before cells were selected for those with successfully integrated shRNA sequences by treatment with 2 µg/ml puromycin for 48 h. Cell viability was measured using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays according to manufacturer’s instructions (Roche, Germany). For analysis of the spindle apparatus, DAOY medulloblastoma cells were transiently transfected with an αTubulin-GFP construct using the NanoJuice® transfection reagent kit (Merck, Germany) before knock down was performed. Finally, cells were seeded on glass-bottom plates (Mattek Corporation, USA) and incubated in a heated chamber at 37°C and 5% CO2 in a humidified atmosphere for 16 hours. Spindle morphology of the living cells was visualized with a confocal laser scanning microscope (Zeiss). The percentage of cells with mitotic spindle defects was determined by counting at least 200 mitotic cells from three different transfections for both negative control and FoxM1 shRNAs or control treated and Siomycin A treated cells. Mitotic cells with a regular bipolar spindle were defined as normal. Cells were scored as abnormal if they displayed more than two spindle poles. Siomycin A was obtained from the NCI-Chemotherapeutic Agents Repository (USA), solved in DMSO, and applied to the cells for 48 h prior to protein extraction or MTT assays.
Western blotting

Standard semi-dry Western blotting was carried out as described (11). 25 µg of each protein sample were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore, USA). The primary antibodies used were anti-FoxM1 (1:500; Santa Cruz Biotechnology, USA), anti-cleaved Caspase3 (1:1000, Cell Signaling Tech, USA), anti-Cyclin B1 (1:500; Santa Cruz Biotechnology, USA), anti-β-Actin (1:5000, Sigma, USA) and anti-β-Tubulin (1:5000, Sigma, USA). Peroxidase-conjugated secondary antibodies included goat anti-mouse (1:2000; Jackson Immunoresearch Laboratories, USA) and goat anti-rabbit (1:2000; Pierce Biotechnology, USA) antibodies. Blots were developed using enhanced chemiluminescence (Roche, Germany), according to the manufacturer’s instructions. Signals were quantified using the Image J software (NIH, USA).

Statistical analysis

For analysis of the expression data and cell viabilities, the Mann-Whitney-U-test was used to compare the median of two groups. To calculate the fraction of tumor cells labeled with antibodies against FoxM1 and Ki67, at least 500 tumor cells were counted for each tumor. Correlation of two paired data sets was done with the Spearman correlation. Fractions of mitotic cells with abnormal spindles in different experimental conditions were compared using the Fisher’s exact test. The Kaplan–Meier method was used to estimate survival, and log-rank-tests were used to compare survival between the respective groups. For all tests, a p-value<0.05 was considered significant. Analyses were performed using Prism 5 (Graphpad, USA) except
for multivariate analyses with Cox regression models that were done using PASW statistics 18 (IBM, USA).

Results

Expression pattern of FoxM1 in human medulloblastoma tumor samples

We have previously performed in situ hybridizations to demonstrate expression of FoxM1 in murine cerebellar tissue (11). In particular, we found that FoxM1 is expressed in granule cell precursors of the developing cerebellum as well as in medulloblastoma that arise in ptc+/− mice, but not in adult cerebellar tissue. In order to see to what extend FOXM1 is expressed in human medulloblastoma samples, we first reanalyzed recently published global gene expression arrays of 193 medulloblastoma tissue samples (5). As shown in Figure 1A, medulloblastomas express high levels of FOXM1 without significant differences between the distinct molecular subgroups (p=0.0536). With respect to the different histological subtypes, FOXM1 is similarly expressed in classic and nodular/desmoplastic medulloblastomas. However, large cell/anaplastic medulloblastomas, which are characterized by CMYC or NMYC amplification and worse survival (28), express higher levels of FOXM1 than other histological subtypes (p<0.001, Fig. 1B). Patients’ age did not correlate with the expression of FOXM1 (p=0.5785, Suppl. Fig. 1A). Next, we used antibodies raised against FoxM1 on sections from formalin-fixed, paraffin-embedded tumor material of 43 medulloblastoma cases to visualize expression of FoxM1 on a cellular level. Histopathological characteristics of the tumors and patients’ clinical data are provided in Supplementary Table 1. Expression of FoxM1 protein was detectable in all analyzed medulloblastomas and was restricted to the tumor cell nuclei (Fig. 1C). Tumors displayed a median expression of 6.95%, ranging between 2.44% and 30.7% of nuclei binding antibodies against FoxM1 (Fig. 1D). As shown in Figure 1C, FoxM1 expression in des-
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...nplastic medulloblastomas was predominantly found in internodular tumor areas, which are highly proliferative, but only to a lesser extent in nodules that are usually more differentiated. We therefore asked whether FoxM1 might be expressed only in Ki67+ tumor cells or whether expression of FoxM1 correlates with expression of Ki67 in medulloblastoma. As revealed by double immunofluorescent stainings, antibodies against FoxM1 may also bind to Ki67+ tumor cells (arrowheads, Fig. 1E), and overall expression of FoxM1 did not correlate with the expression of Ki67 in sections of human medulloblastoma tissue samples (n=43, rs=0.254; p=0.197, Fig. 1F). Similarly to the data generated from expression arrays, we did not find significant differences of FoxM1 expression in classic and nodular/desmoplastic medulloblastoma (p=0.64; Suppl. Fig. 1B). Also, FoxM1 protein expression was not dependent on the age of the patient (p=0.4082; Suppl. Fig. 1C).

To test the clinical relevance of FoxM1 expression levels in medulloblastomas, we analyzed the survival of patients according to the protein expression of FoxM1 in medulloblastoma tumors by using the Kaplan-Meier method. Clinical follow-up data was available for 41 patients, and patients were dichotomized into two equally large groups using the median FoxM1 expression (7.02%) as cutoff. The overall survival of patients with a high number of FoxM1+ tumor cells (n=20) was significantly worse than the survival of patients with a low number of FoxM1+ tumor cells (n=21; p=0.0005; Fig. 2A). The 5-year overall survival was better in medulloblastoma patients with low FoxM1 expressing tumors when compared with patients with high FoxM1 expressing tumors (FoxM1_low group, 84.8% versus FoxM1_high group, 42.8%).

In order to confirm these results in an independent cohort, we re-analyzed available micro-array data from 130 medulloblastoma patients with documented clinical follow-up (5). Here, patients were sorted on the basis of expression of FOXM1 and subsequently dichotomized on the basis of a series of FOXM1 expression levels. Log-rank
significance was calculated for each cutoff and the best $p$-value out of the sequence was used for further analysis. As shown in Figure 2B, patients suffering from tumors with high levels of $FOXM1$ ($n=44$) had a significantly worse outcome compared to patients with tumors that displayed only low levels of $FOXM1$ ($n=86$; log-rank test, $p=0.0055$). The same cohort of patients was originally used to identify a subgroup of patients with a distinct genetic fingerprint that drives poor clinical outcome (5). This “c1” subgroup is characterized genetically by $CMYC$ copy number gains and transcriptionally by enrichment of photoreceptor pathways and increased miR-183_96_182 expression. Further parameters that were previously found to predict the outcome of patients with medulloblastoma included Wnt pathway activation (29), large cell/anaplastic histology (28) and the presence of metastases (4). In order to evaluate whether the prognostic value of $FOXM1$ is independent of these characteristics, we performed uni- and multivariate analyses using a Cox regression model. As demonstrated by univariate Cox-regression analyses, expression of $FOXM1$ again turned out as a prognostic marker in patients with medulloblastoma ($p=0.008$), similar to LC/A histology ($p=0.008$) and a “c1” molecular profil ($p=0.009$, Fig. 2C). Although Wnt activation and metastatic disease did not reach statistical significance in univariate analyses of this series (Fig. 2C), they were included into the multivariate model to obtain estimates of Hazard ratios adjusted for them (Fig. 2D). Using this multivariate model, we found that expression of $FOXM1$ is an independent prognostic marker for patients with medulloblastoma (hazard ratio, 2.24; 95% confidence interval, 1.05-4.77; $p=0.037$; Fig. 2D).
Knock down of FoxM1 reduces medulloblastoma cell growth by induction of mitotic catastrophe

In order to unravel the functional impact of FoxM1 in medulloblastoma, we aimed to knock down *FOXM1* expression in 4 permanent human medulloblastoma cell lines (D425med, DAOY, R300, and UW228) and measured cell viability in control and in knockdown conditions. As shown in Figure 3A, all medulloblastoma cell lines expressed high levels of FoxM1 protein when transduced with lentiviral particles containing mock sequences. However, transduction with lentiviral particles containing two different shRNA sequences against *FOXM1* resulted in a dramatic decrease in FoxM1 protein expression, as measured by Western blot analysis (Figure 3A). Remaining levels of FoxM1 expression ranged between 9.23%-47.66% compared to protein levels of mock transduced cells. In order to investigate whether high expression of FoxM1 might be a general phenomenon associated with proliferating cells, we further analyzed FoxM1 expression in HEK293T cells, and found that this cell line express only very low levels of FoxM1 (Fig. 3A). Cell viability was measured using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromid (MTT) assays. Knock down of FoxM1 decreased medulloblastoma cell viability significantly (up to 35% of mock infected cells; *p*<0.05 for all medulloblastoma cell lines) in all four cell lines using both constructs (Fig. 3B). Interestingly, knock down of FoxM1 in HEK293T cells did not affect cell viability significantly (Fig. 3B). Mechanistically, Western blot analysis revealed that cleaved Caspase 3 was upregulated in all FoxM1-depleted medulloblastoma cell lines, but not in HEK293T cells with a knock down of FoxM1 (Fig. 3C). Upregulation of Caspase 3 may occur as a part of mitotic catastrophe (30) which had previously been described both for FoxM1-deficient cerebellar granule neurons (11) as well as for tumor cells with a knock down of FoxM1 (19). We therefore examined possible structural changes in cell spindle dynamics in dependence of FoxM1
expression. We visualized the spindle of DAOY medulloblastoma cells by transfecting cells with a αTubulin-GFP construct (Fig. 4A). Transduced cells were seeded on glass-bottom plates and imaged 16 h after transduction with knock down constructs using a confocal laser scanning microscope. We observed significantly more mitotic cells with an abnormal spindle (57.2% and 63.4%) when FoxM1 is depleted by two different knock down constructs (shRNA #1 and shRNA #2) compared to mock infected cells (21.2%) or untreated cells (no virus; 3.2%; Fig. 4B) (p<0.05). We further looked at the expression of Cyclin B1 in such cells since enhanced expression of Cyclin B1 as well as its translocation to the cell nucleus are indicators of a possible G2M arrest of the cells (31, 32). As demonstrated in Fig. 4C, knock down of FoxM1 resulted in a shift of Cyclin B1 to the nuclei of medulloblastoma cells and subsequent Western blot analyses revealed that overall expression of Cyclin B1 was clearly upregulated in FoxM1 deficient cells (Fig. 4D). Together, we suggest that knock down of FoxM1 in medulloblastoma cells results in mitotic catastrophe as shown by enhanced expression of cleaved caspased 3, nuclear translocation of Cyclin B1 and abnormal cell spindles.

**Siomycin A reduces expression levels of FoxM1 and recapitulates effects of FoxM1 knock down in medulloblastoma cells**

The antibiotic thiazole compound Siomycin A has previously been reported to represent a specific inhibitor of FoxM1 (33). We therefore tested whether Siomycin A was able to reduce levels of FoxM1 in medulloblastoma cells and hence to inhibit tumor cell growth. As shown in Figure 5A, treatment of human medulloblastoma cell lines D425med, DAOY, R300, and UW228 with Siomycin A resulted in a decrease of FoxM1 protein levels in all human medulloblastoma cell lines and in HEK293T cells. Treatment with 10 µM Siomycin A resulted in a down-regulation of FoxM1 protein
levels to 48.20% (mean) in medulloblastoma and HEK293T cells (Fig. 5A). Next, cell viability was determined using MTT assays and showed a dose-dependent Siomycin A-induced reduction of cell numbers in all treated human medulloblastoma cell lines (Fig. 5B). Interestingly, growth of HEK293T was not significantly affected by Siomycin A, suggesting that Siomycin A is not generally anti-proliferative or toxic (Fig. 5B). High concentrations of Siomycin A reduced cell viability below initial values (∆OD<0) in medulloblastoma cell lines, indicating a reduction of cells. We therefore assessed the expression of cleaved Caspase 3 in order to determine whether programmed cell death or mitotic catastrophe would play a role in this context, similar to the situation observed for lentiviral knock down of \textit{FOXM1} (Fig. 3). As demonstrated by Western blots shown in Figure 5C, Siomycin A induced upregulation of cleaved Caspase 3 in medulloblastoma cells, but not in HEK293T cells. Next, we analyzed the spindle formation in living tumor cells that were treated with Siomycin A. αTubulin-GFP transfected DAOY cells were seeded on glass-bottom plates and incubated in a heated chamber for 16 h. Untreated cells, cells treated with DMSO and cells treated with 5 µM Siomycin A were then imaged with a confocal laser scanning microscope. We observed a significant increase of mitotic cells with an abnormal spindle apparatus in Siomycin A treated cells (32.6%) as compared to DMSO treated cells (3.4%, \(p=0.049\), Fig. 6A, B). No significant difference was observed with respect to the number of abnormal spindles in cells with DMSO treatment and cells without any treatment. In order to determine whether these findings may have resulted from mitotic catastrophe of the cells we again looked for a possible G2M arrest of the cells. Similar to the cells with a knock down of FoxM1 (Fig. 4), cells treated with Siomycin A showed a translocation of Cyclin B1 to the nucleus (Fig. 6C) and an overexpression of Cyclin B1 as revealed by Western blots from Siomycin A-treated tumor cells (Fig.
6D). Taken together, our data suggest that Siomycin A is able to significantly down-regulate expression of FoxM1 and inhibits the growth of medulloblastoma cells.

Discussion

We report here that FoxM1 is highly expressed in human medulloblastoma and that it proved to be a prognostic marker both in a training set of 43 tumors that were analyzed by immunohistochemistry as well as in a test set of 130 tumors that were independently analyzed by expression microarrays. Functionally, our experiments demonstrate that FoxM1 is required for the growth of medulloblastoma cells and that it may be targetable by the thiazole antibiotic Siomycin A.

The clinical outcome of patients suffering from medulloblastoma is very heterogeneous with a 5-year event-free survival rate ranging from only 20-40% for infants with metastatic medulloblastoma to more that 80% for patients with non-metastatic medulloblastoma (34). Nevertheless, all of those tumors are considered WHO grade IV tumors and treated with radiation and multimodal chemotherapy. This may ideally result in the survival of patients with aggressive medulloblastoma, but it also causes severe long term side effects in “overtreated” patients with less aggressive tumors. It is therefore one of the big pathologist’s challenges to reliably identify more and less aggressive medulloblastomas and to facilitate the clinician’s decision for an appropriate treatment that is based on a tumor’s individual fingerprint. This idea in mind, recent advances have finally made it possible to identify aggressive medulloblastoma that are associated with a poor clinical outcome (35). However, current approaches to characterize medulloblastoma are mainly based on global gene expression arrays, and single prognostic markers that may be easily evaluated on formalin-fixed, paraffin-embedded material are rare. Nuclear expression of beta-catenin is one example for such a marker. It has, in recent years, been identified and confirmed to correlate...
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with relatively good outcome (36) and may therefore be implemented in future risk stratifications prior to treatment decisions (37). Expression of FoxM1 is easily detectable by antibody staining on paraffin sections and has proven as a predictive marker both in a smaller training set of medulloblastoma samples as well as in a larger test set. Our results are in line with previous reports on glioma (22), lung cancer (38), and malignant peripheral nerve sheet tumors (39) that have also suggested the expression of FoxM1 as a marker of poor clinical outcome.

Whereas the expression of FoxM1 is dispensable for the normal proliferation of granule neurons and for the overall development of the cerebellum (11), we show here that medulloblastoma cells undergo mitotic catastrophe and stop growing in the absence of FoxM1. These observations suggest that expression of FoxM1 is essentially required in cancer but may be compensated by other factors during normal cerebellar development, a major requirement for the treatment of children with drugs that suppress the expression of FoxM1. Siomycin A has been identified to specifically down-regulate the expression of FoxM1 (33) and is therefore discussed as a potential candidate to treat patients with tumors that express high levels of FoxM1 (40). Indeed, recent studies demonstrated that thiazole antibiotics such as Siomycin A can suppress tumor growth in a human breast cancer xenograft model and may have potential anticancer activities against breast cancer in vivo (41). It will therefore be interesting to see whether future studies may uncover similar effects in mouse models for medulloblastoma and in clinical studies.

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**Figure Legends**

**Figure 1: Expression pattern of FoxM1 in human medulloblastomas.** Analysis of microarray data from 193 medulloblastoma tumor samples showed high expression of *FOXM1* in all molecular subgroups of medulloblastoma (A) and significantly more *FOXM1* in the LC/A group (*p*<0.05) compared to all other histological groups (B). LC/A=large cell/anaplastic, CL=classic, N/D=nodular/desmoplastic, NOS=not otherwise specified. (C) H&E stainings of a desmoplastic and a classic medulloblastoma (upper left and upper right, respectively) and protein expression of FoxM1 in both histological subtypes (respective lower panels). Bars=500 µm (upper panels) and 100 µm (lower panels). (D) Quantification of FoxM1+ cells in medulloblastoma tumor sections (n=43) with the solid bar representing the median. (E) Immunofluorescent images of a human medulloblastoma showed expression of FoxM1 in Ki67+ (arrows) and Ki67- (arrowheads) tumor cells (bar=20 µm). (F) Overall levels of FoxM1 and Ki67 expression did not correlate in medulloblastomas (n=43, *p*=0.197).

**Figure 2: Prognostic impact of FoxM1 expression in medulloblastoma.** (A) Median fraction of FoxM1+ tumor cells was set as threshold to divide patients into FoxM1high (n=20) and FoxM1low (n=21). Patients with tumors expressing low levels of FoxM1 protein survived significantly longer than patients with tumors expressing high levels of FoxM1 protein (*p*=0.0005). (B) Survival analysis of a separate patients cohort (n=130) with *FOXM1* expression analyzed by microarrays. Patients were sorted on the basis of expression of *FOXM1* and subsequently dichotomized on the basis of *FOXM1* expression. For group separation, the log-rank significance was calculated. The best *p*-value out of the sequence was used to represent the final *FOXM1* ex-
expression cutoff. High expression of FOXM1 (n=44) was related to a significantly worse outcome compared to low expression of FOXM1 (n=86, \( p=0.0055 \)). (C) Univariate Cox regression analysis of the microarray data set (n=130) revealed that FOXM1 expression, LC/A histology and c1 subgroup are significantly correlated with worse clinical outcome. (D) Multivariate survival analysis of the microarray data set (n=130) demonstrating that FOXM1 expression was an independent prognostic factor (\( p=0.037 \)) with regard to molecular subgroups, histology, and M stage. CI denotes confidence interval.

**Figure 3: Effects of FoxM1 knock down on human medulloblastoma cell lines.**

(A) Human medulloblastoma cell lines D425med, DAOY, R300 and UW228 as well as HEK293T cells were transduced with two different knock down constructs for FoxM1 (shRNA #1 and shRNA #2) and resulting FoxM1 protein levels were measured and compared to control (Mock virus). \( \beta \)-Tubulin was used as loading control.

(B) Cell viability was significantly decreased in all medulloblastoma cell lines when FoxM1 was depleted. *, \( p<0.05 \); ***, \( p<0.001 \); n. s., not significant (C) Expression of cleaved Caspase3 (CASP3cleaved) was induced when FoxM1 was down-regulated in medulloblastoma cell lines. \( \beta \)-Actin was used as loading control.

**Figure 4: Knock-down of FoxM1 induced mitotic spindle catastrophe.** The medulloblastoma cell line DAOY was stable transfected with an \( \alpha \)Tubulin-GFP (\( \alpha \)-TUB-GFP) construct and seeded on glass-bottom plates (Mattek Corp., USA). Morphology of the spindle was visualized with a confocal laser scanning microscope (Zeiss, Germany) in wild-type (No virus), mock infected (Mock virus) and FoxM1siRNA infected (shRNA #1 and shRNA #2) cells. Scale bar for all images = 10 \( \mu \)m (A). (B) Quantification of mitotic cells showed a significant (\( p<0.05 \)) increase of cells with an abnormal spindle when FoxM1 was depleted with siRNA. *, \( p<0.05 \). (C) Immunofluorescent stainings of DAOY medulloblastoma cell line for Cyclin B1 (green) and DAPI. Cells
FoxM1 in medulloblastoma

showed nuclear localisation of Cyclin B1 when FoxM1 was down-regulated by shRNAs. Scale bar=20 µm. (D) Western Blot showed increased expression of Cyclin B1 when FoxM1 was down-regulated. Numbers indicate level of Cyclin B1 compared to control (Mock virus). β-Actin was used as loading control.

Figure 5: Treatment of human medulloblastoma cell lines with Siomycin A. (A) Human medulloblastoma cell lines D425med, DAOY, R300, and UW228 as well as HEK293T cells were treated with two concentrations of Siomycin A (Sio 5 µM and Sio 10 µM), and resulting protein levels were measured and compared to DMSO control (Control). β-Tubulin was used as loading control. (B) Cell viability was decreased dose-dependently with increasing concentrations of Siomycin A in all medulloblastoma cell lines, but not in HEK293T cells. (C) Expression of cleaved Caspase3 (CASP3cleaved) was induced when FoxM1 was down-regulated in medulloblastoma cell lines, but not in HEK293T cells.

Figure 6: Siomycin A induced mitotic spindle catastrophe. The medulloblastoma cell line DAOY was stably transfected with an αTubulin-GFP (α-TUB-GFP) construct and seeded on glass-bottom plates (Mattek Corp., USA). Morphology of the spindle was visualized with a confocal laser scanning microscope (Zeiss, Germany). Cells were imaged without treatment, with DMSO, or with 5 µM Siomycin A. Scale bar is 10 µm for all images (A). (B) Quantification of mitotic cells showed a significant (p<0.05) increase of cells with an abnormal spindle when FoxM1 was down-regulated with Siomycin A. *, p<0.05. (C) Immunofluorescent stainings of DAOY medulloblastoma cell line for Cyclin B1 (green) and DAPI. Cells showed nuclear localisation of Cyclin B1 when FoxM1 was down-regulated by Siomycin A. Scale bar=20 µm. (D) Expression level of Cyclin B1 was upregulated when cells were treated with Siomycin A compared to DMSO as control. Numbers indicate level of Cyclin B1 compared to control (DMSO). β-Actin was used as loading control.
Priller et al., Fig. 1

A. No variation of medians, $p=0.0536$

B.  

C. H&E

D.  

E.  

F. No correlation, $r_s=0.254; p=0.197$
Priller et al., Fig. 2
### Univariate survival analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXM1&lt;sup&gt;high&lt;/sup&gt; expression (reference: FOXM1&lt;sup&gt;low&lt;/sup&gt; expression)</td>
<td>2.67</td>
<td>1.30-5.49</td>
<td>.008</td>
</tr>
<tr>
<td>LC/A Histology (reference: all other)</td>
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<td>Molecular subgroup, MYC (c1) (reference: all other)</td>
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<td>M stage, M+ (reference: M0)</td>
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<td>Molecular subgroup, WNT (c6) (reference: all other)</td>
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<td>.05-2.54</td>
<td>.295</td>
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### Multivariate survival analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>p-value</th>
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<td>1.33</td>
<td>.52-3.35</td>
<td>.553</td>
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Table 1: Univariate Cox regression analysis of the microarray data set (n=130) revealed that FOXM1 expression, LC/A histology and c1 subgroup are significantly correlated with worse clinical outcome. Multivariate survival analysis of the microarray data set (n=130) demonstrated that FOXM1 expression was an independent prognostic factor (p=0.037) with regard to molecular subgroups, histology, and M stage. CI denotes confidence interval.
A

**FoxM1**

<table>
<thead>
<tr>
<th></th>
<th>Mock virus</th>
<th>shRNA #1</th>
<th>shRNA #2</th>
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<tbody>
<tr>
<td>D425med</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAOY</td>
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<tr>
<td>R300</td>
<td></td>
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<tr>
<td>UW228</td>
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</tr>
<tr>
<td>HEK293T</td>
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**β-Tubulin**

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<th>Mock virus</th>
<th>shRNA #1</th>
<th>shRNA #2</th>
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<tr>
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<td>R300</td>
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<tr>
<td>HEK293T</td>
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</tbody>
</table>

**Level of FoxM1 normalized to β-Tubulin**

- **D425med**
- **DAOY**
- **R300**
- **UW228**
- **HEK293T**

B

**Cell viability normalized to control [%]**

- **D425med**
- **DAOY**
- **R300**
- **UW228**
- **HEK293T**

C

**CASP3cleaved**

<table>
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<th>Mock virus</th>
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<th>shRNA #2</th>
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<tr>
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**β-Actin**

<table>
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</tbody>
</table>
Priller et al., Fig. 4

A

No virus | Mock virus

DAOY-dTUB-GFP

shRNA #1 | shRNA #2

B

Fraction of mitotic cells with abnormal spindle [%]

No virus | Mock virus | shRNA #1 | shRNA #2

C

No virus | Mock virus

DAOY-dTUB-GFP

shRNA #1 | shRNA #2

D

Cyclin B1

β-Actin

No virus | Mock virus | shRNA #1 | shRNA #2
Priller et al., Fig. 5

A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sio 5 µM</th>
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<td>FoxM1</td>
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<tr>
<td>β-Tubulin</td>
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Level of FoxM1 normalized to β-Tubulin

D425med | DAOY | R300 | UW228 | HEK293T

B

ΔOD265nm(48h-0h) - normalized to control

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<thead>
<tr>
<th></th>
<th>ΔOD265nm(48h-0h) - normalized to control</th>
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<tbody>
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C

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sio 5 µM</th>
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</thead>
<tbody>
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<td>CASP3 cleaved</td>
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D425med | DAOY | R300 | UW228 | HEK293T
Priller et al., Fig. 6

A

<table>
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<tr>
<th>No treatment</th>
<th>DMSO</th>
<th>Siomycin A</th>
</tr>
</thead>
</table>

DAOY-tUB-GFP

B

No treatment

DMSO

Sioymcin A

Fraction of mitotic cells with abnormal spindle [%]

C

<table>
<thead>
<tr>
<th>No treatment</th>
<th>DMSO</th>
<th>Siomycin A</th>
</tr>
</thead>
</table>

Cyclin B1

β-Actin

D

0.8 1.0 2.4

No treatment DMSO Siomycin A
Expression of FoxM1 is required for the proliferation of medulloblastoma cells and indicates worse survival of patients

Markus Priller, Julia Poschl, Leticia Abrao, et al.

Clin Cancer Res Published OnlineFirst September 14, 2011.