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# Microarray-Based Screening for Molecular Markers in Medulloblastoma Revealed *STK15* as Independent Predictor for Survival

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## ABSTRACT

**Medulloblastoma, a primitive neuroectodermal tumor of the cerebellum, is one of the most common central nervous system malignancies of childhood. Despite aggressive multimodal therapy, including surgery, irradiation, and chemotherapy, 5-year survival rates have only approached 50–60%. To identify potential candidate genes that predict for overall survival (OS), we performed a gene expression profiling analysis in 35 newly diagnosed medulloblastoma neoplasms. Subsequently, the nine most promising candidate genes were analyzed by immunohistochemistry and fluorescence *in situ* hybridization on tumor tissue microarrays representing a series of 180 tumors. We found 54 genes in which expression levels predicted for unfavorable survival in medulloblastoma. In line with the gene expression profiling analysis, a positive staining for STK15 ($P = 0.0006$), stathmin 1 ($P = 0.001$), and cyclin D1 ($P = 0.03$) was associated with an unfavorable OS, whereas cyclin B1, DAXX, Ki-67, MYC, NRAS, and p53 showed no statistical significant effect. In comparison to clinically defined parameters such as gender, age, metastatic stage, extent of tumor resection, application of chemotherapy, and tumor grade, positive staining for STK15 was identified as an independent prognostic factor for OS ($P = 0.026$). Moreover, additional gene copy numbers of *MYC* ($P = 0.003$) and *STK15* ($P = 0.05$) predicted for poor survival. The combination of gene expression profiling with tissue microarray experiments allowed the identification of a series of candidate genes that predicts for survival in medulloblastoma. Of the results highlighted by the various data analysis procedures, genes associated with cell proliferation (*cyclin D1*), transcription (*MYC*), and especially mitosis (*stathmin 1*, *STK15*) appear particularly intriguing with respect to medulloblastoma pathomechanism.**

## INTRODUCTION

Medulloblastoma is a highly malignant primitive neuroectodermal tumor of the cerebellum. It accounts together with supratentorial primitive neuroectodermal tumors for 20–25% of all pediatric brain tumors with a peak incidence of 7 years of age (1). Approximately 65% of medulloblastoma patients are male. Despite aggressive multimodal therapy, including surgery, irradiation, and chemotherapy, disease dissemination is common and 5-year survival rates have only approached 50–60% (2). Age < 3 years, metastases at presentation and partial surgical resection are clinical features that are generally associated with an adverse prognosis (2, 3). To optimize treatment strategies for medulloblastoma patients, a more precise understanding of the cellular and molecular basis of this disease is clearly necessary.

Most pediatric medulloblastomas have abnormal karyotypes, including aneuploidy, unbalanced translocations, and numerous structural rearrangements in the form of double-minute, ring, and marker chromosomes (4, 5). In 30–50% of patients, deletion or rearrangement of part of chromosome 17 have been described previously (6–8). In most cases, 17p is lost, and head-to-head apposition of both 17q arms occurs, which had been previously considered as isochromosome 17q (9). Medulloblastomas with loss of 17p have breakpoints either proximally to the *TP53* locus at 17p11.2 or distal to *TP53* at 17p13.3. In ~10% of medulloblastomas, gene amplifications were identified: the common amplified regions include the oncogenes *MYC* (8q24) and *MYCN* (2p24.1; Ref. 8, 10). Although the clinical significance of the majority of these abnormalities remains unclear, recent studies show that amplifications as well as high transcriptional levels of *MYC* are associated with an unfavorable survival outcome (10, 11).

Recently, new insight in the prediction of medulloblastoma outcome was obtained by gene expression profiling experiments. The expression of genes characteristic for cerebellar differentiation or coding for extracellular matrix proteins was correlated with favorable prognosis, whereas the expression of genes related to cell cycle control, multidrug resistance, and ribosomal biogenesis was associated with poor prognosis (12). Moreover, metastatic disease was associated with expression profiles, including *platelet-derived growth factor receptor* and genes that are involved in the *RAS*/*MAPK* signaling pathway (13).

In the present study, we performed an expression profiling analysis of 35 medulloblastoma samples to identify potential candidate genes that predict for overall survival (OS) of the patients. Additionally, we applied the approach of tumor tissue microarray (TMA) experiments (14), a modern high-throughput technology that allowed screening of 180 clinically well-defined medulloblastoma tumor specimens on a large scale. Nine promising candidate genes were analyzed for changes on the protein expression level by immunohistochemistry (IHC). In addition, fluorescence *in situ* hybridization (FISH) was performed for the detection of additional gene copies of four of them.

## MATERIALS AND METHODS

**Patient Characteristics.** All tumor specimens used in this study were collected at the Department of Neuropathology, NN Burdenko Neurosurgical Institute (Moscow, Russia) between January 1990 and March 2002. All diagnoses were confirmed by histological assessment of a tumor specimen obtained at surgery by at least two neuropathologists according to the WHO 2000 criteria. Because desmoplastic medulloblastomas are rare and its diagnosis is highly subjective, we included only nondesmoplastic medulloblastomas in our study. All medulloblastoma samples were additionally graded as classic and anaplastic according to the recent scheme presented by Eberhart and Burger (15). Medulloblastoma samples were designated as anaplastic when patterns of moderate or severe anaplasia were present.

Clinical information on the patients included date of birth, date of operation, extent of resection determined by postoperative neuroradiological reports (gross total resection, no visible tumor; near-total resection, >90% of tumor removed; subtotal resection, 51–90% of tumor removed), irradiation or chemotherapy, follow-up time, and outcome. Metastatic stage was studied by craniospinal neuroimaging and liquor cytopathology. Approval to link laboratory data to clinical data was obtained by the Institutional Review Board.

Table 1 shows the characteristics of patients with medulloblastoma included in the cDNA microarray ($n = 35$) and TMA experiments ($n = 180$). There was

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**Note:** K. Neben and A. Korshunov contributed equally to this work.

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an overlap of 30 samples, which were included in both studies. All patients in both series received craniospinal irradiation after surgery. The postoperative irradiation included 36 Gy to the craniospinal axis and a boost of 53–56 Gy to the posterior fossa. Although all patients enrolled in the cDNA microarray study were additionally treated with adjuvant chemotherapy, this was only applied to 112 patients (62.2%) included in the TMA study. Chemotherapy was routinely administered to all patients who were treated after 1995. The chemotherapy regimen comprises lomustine, cisplatin, and vincristine as published previously (2, 16).

On March 1, 2003, the 180 medulloblastoma patients included in the TMA study had a median follow-up time of 34 months (range, 3–130 months). There were 109 survivors (median OS, 67 months; range, 14–130 months) and 71 deaths (median OS, 34 months; range, 3–112 months). Sixty-two deceased patients exhibited disease dissemination along the neuroaxis, whereas the remaining 9 patients displayed isolated local tumor progression. The 35 medulloblastoma patients included in the cDNA microarray study had a median follow-up time of 24 months (range, 3–39 months).

**cDNA Microarray Experiments.** Samples from each primary tumor were taken at the time of initial operation, immediately frozen in liquid nitrogen, and then continuously stored at −80°C until usage for cDNA microarray experiments. To confirm the presence of viable cellular tumor (≥90% neoplastic cells), cryosections of each medulloblastoma sample (~5-μm thick) were stained with H&E before RNA extraction. Total RNA was isolated according to a protocol that applies both Trizol reagent (Invitrogen, Karlsruhe, Germany) and RNeasy Midi spin columns (Qiagen, Hilden, Germany), followed by Oligotex resin (Qiagen) purification of poly-A RNA (17–19). The integrity and purity of the total RNA was analyzed on a 1% agarose gel. Total RNA quality and concentration were determined by absorption spectroscopy between 220 and 320 nm (1 $A^{260}$ = 40 μg/ml single-stranded RNA).

For gene expression experiments, cDNA microarrays containing replicate spots of 4211 different gene specific fragments representing 2600 different genes with relevance to mitosis, cell cycle control, oncogenesis, or apoptosis were processed as described previously (17–19). The tumor RNA was cohybridized with commercially available universal human reference RNA (Stratagene, La Jolla, CA), derived from 10 human cancer cell lines. Approximately 1 μg of medulloblastoma and 1 μg of reference mRNA were labeled with Cy3 and Cy5, respectively, using the Omniscript Reverse Transcriptase kit (Qiagen) and hybridized with 10 μg of $C_0$t1 DNA, 30 μg of bovine liver tRNA, and 10 μg of oligo-dT nucleotides in an automated hybridization chamber (GeneTac; Genomic Solutions, Ann Arbor, MI). For all samples, we performed color switch experiments, where the tumor and reference DNA were labeled via Cy3- and Cy5-dUTP, respectively, and *vice versa*. Data sets for spots not recognized by the GenePix Pro 4.0 analysis software (Axon Instruments, Union City, CA) were excluded from additional considerations. Additionally, all remaining data sets were ranked according to spot homogeneity (as assayed by the ratio of median and mean fluorescence intensities), spot intensity, and the SD of log ratios for replicate spots. Those data points, ranked among the lower 20%, based on the criteria just described, were removed from the data set. For each hybridization, fluorescence ratios (Cy5/Cy3) were normalized by variance stabilization (20). To combine experiments with switched dye labeling, the ratios of one experiment were inverted and averaged with the corresponding spots on the second array. The raw data of the microarray experiments are available online.[5]

Table 1 *Characteristics of medulloblastoma patients that were included in the cDNA microarray (n = 35) and tissue microarray studies (n = 180)*

| | cDNA microarray study | Tissue microarray study |
|---|---|---|
| | No. of patients (*n*) | No. of patients (*n*) |
| Age (years) | | |
| <3 | 3 (9%) | 15 (8%) |
| 3–15 | 19 (54%) | 107 (60%) |
| ≥16 | 13 (37%) | 58 (32%) |
| Gender | | |
| Male | 22 (63%) | 118 (66%) |
| Female | 13 (37%) | 62 (34%) |
| Metastatic stage | | |
| $M_0$ | 20 (57%) | 137 (76%) |
| $M_{1-3}$ | 15 (43%) | 43 (24%) |
| Volume of resection | | |
| Gross total | 19 (54%) | 102 (57%) |
| Nongross total | 16 (46%) | 78 (43%) |
| Chemotherapy | | |
| Yes | 35 (100%) | 112 (62%) |
| No | 0 | 68 (38%) |
| Histology | | |
| Classic | 23 (66%) | 135 (75%) |
| Anaplastic | 12 (34%) | 45 (25%) |

Table 2 *Univariate analysis (log-rank test) of clinicopathological features for 180 medulloblastoma patients included in the tissue microarray study*

| Variable | No. of patients | 5-year survival | *P* |
|---|---|---|---|
| Age (years) | | | |
| <16 | 122 | 53% | 0.78 |
| ≥16 | 58 | 58% | |
| Gender | | | |
| Male | 118 | 52% | 0.12 |
| Female | 62 | 62% | |
| Resection | | | |
| Gross total | 102 | 60% | 0.23 |
| Nongross total | 78 | 54% | |
| M stage | | | |
| $M_0$ | 137 | 64% | 0.02 |
| $M_{1-3}$ | 43 | 37% | |
| Chemotherapy | | | |
| Yes | 112 | 72% | 0.008 |
| No | 68 | 32% | |
| Histology | | | |
| Classic | 135 | 67% | 0.03 |
| Anaplastic | 45 | 43% | |

**Generation of TMAs.** As previously described (21), H&E-stained sections were made from each paraffin block to define representative tumor regions. From these regions, two tissue cylinders with a diameter of 0.6 mm were obtained and arrayed into a recipient block using the tissue chip microarrayer (Beecher Instruments, Silver Spring, MD). The recipient block was subsequently cut into sections of 5-μm on Histobond-silanized glass slides (Marienfeld, Lauda-Koenigshofen, Germany), which were treated with 3-aminopropylmethoxysilane (Fluka, Seelze, Germany) before cutting to support adhesion of the tissue samples.

**IHC.** Paraffin sections were deparaffinized and rehydrated with graded ethanol. Antigen retrieval was performed by heating for 10 min in a microwave either in 10 mM sodium citrate or Tris-EDTA buffer. Endogenous peroxidase was inactivated by incubating the TMAs in 3% hydrogen peroxide. After blocking with normal serum, the sections were incubated with the primary antibodies at 4°C overnight. Antibodies against the following antigens were used: cMYC, clone 9-E11 (1:40): Novocastra (Newcastle upon Tyne, United Kingdom); cyclin B1, clone GNS1 (1:100), DAXX, clone H-7 (1:100), NRAS, clone F-155 (1:150), stathmin 1, clone FL149 (1:250), STK15, clone N20 (1:250): Santa Cruz Biotechnology (Santa Cruz, CA); cyclin D1, clone DSC6 (1:40), Ki-67, clone MIB-1 (1:100): DAKO (Glostrup, Denmark); and p53, clone BP53-11 (1:1000): Progene (Darra Queensland, Australia). For detection, TMAs were incubated for 30 min with biotinylated secondary antibody, and for 30 min with ABC Reagent (Vector Laboratories, Burlingame, CA). The slides were stained with 3,3′-diaminobenzidine (Linaris, Wertheim-Bettingen, Germany), counterstained with hematoxylin, and mounted. For negative controls, primary antibodies were substituted with commercially produced control reagents according to the manufacturers' instructions.

An assessment of IHC was performed for each tissue core in a semiquantitative manner as a percentage of stained cells or nuclei/specimen independently by two different experimenters who were blinded to clinical data. Diffuse cytoplasmic and/or membranous immunostaining of DAXX, NRAS, stathmin 1, and STK15 as well as nuclear expression patterns of cyclin B1, cyclin D1, cMYC, p53, and Ki-67 of >10% of cells was considered as overexpression. Therefore, the analysis of our IHC experiments was only performed by a positive *versus* negative evaluation of stained cores, which guaranteed a high reproducibility of our results.

**FISH.** Two-color FISH to the sections of the TMAs was performed using Spectrum Orange-labeled probes for *CCND1*, *MYC* [both Vysis (Downers Grove, IL)], *NRAS* (PAC RP5–1000E10), and *STK15* [PAC RP5-1167H4, both (RZPD German Resource Center, Berlin, Germany)], and Spectrum Green-

[5] Internet address: http://www.dkfz.de/kompl_genome/Other/medulloblastoma.zip



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Table 3 *Genes in which expression levels correlate with unfavorable survival in 35 medulloblastoma patients*

| Gene description (gene symbol) | Image ID | Hazard ratio | Lower 95% CI[a] | Upper 95% CI | $P$ | Expression level |
|---|---|---|---|---|---|---|
| v-Ras neuroblastoma RAS oncogene homologue (*NRAS*) | 272189 | 5.4 | 2.1 | 13.8 | 0.0002 | −1.6 ± 1.3 |
| Protein phosphatase 2, regulatory subunit A (*PPP2R1A*) | 770027 | 3.6 | 1.8 | 7.1 | 0.0003 | 1.5 ± 1.4 |
| Mitogen-activated protein kinase 6 (*MAPK6*) | 117238 | 3.4 | 1.7 | 7.0 | 0.0006 | 1.2 ± 1.7 |
| $O^6$-methylguanine-DNA methyltransferase (*MGMT*) | 114760 | 4.1 | 1.6 | 10.5 | 0.001 | 1.0 ± 1.3 |
| *N*-Ethylmaleimide-sensitive factor (*NSF*) | 48794 | 5.4 | 1.7 | 16.9 | 0.002 | 1.3 ± 1.5 |
| Cytochrome P450, subfamily IVB, polypeptide 1 (*CYP4B1*) | 724888 | 3.6 | 1.5 | 8.4 | 0.002 | 1.4 ± 1.5 |
| Interleukin 13 receptor, α 2 (*IL13RA2*) | 41648 | 3.5 | 1.5 | 8.3 | 0.002 | 1.2 ± 1.4 |
| Ras homologue enriched in brain 2 (*RHEB2*) | 380852 | 3.2 | 1.5 | 6.5 | 0.002 | −1.2 ± 1.7 |
| Death-associated protein 6 (*DAXX*) | 214957 | 4.1 | 1.5 | 11.4 | 0.004 | −1.1 ± 1.3 |
| Acetaldehyde dehydrogenase 1 (*ALDH1A1*) | 840887 | 4.0 | 1.4 | 12.0 | 0.004 | 1.2 ± 1.3 |
| Chemokine (C-X-C motif) ligand 3 (*CXCL3*) | 1556433 | 3.6 | 1.5 | 8.7 | 0.004 | 1.3 ± 1.4 |
| Myeloproliferative leukemia virus oncogene (*MPL*) | 2340423 | 3.0 | 1.4 | 6.3 | 0.004 | 1.0 ± 1.3 |
| Minichromosome maintenance-deficient 5 (*MCM5*) | 112115 | 2.7 | 1.3 | 5.6 | 0.004 | −1.7 ± 1.3 |
| LIM homeobox protein 1 (*LHX1*) | 773263 | 4.5 | 1.4 | 14.3 | 0.005 | 1.1 ± 1.4 |
| v-akt murine thymoma viral oncogene homologue 2 (*AKT2*) | 340655 | 3.2 | 1.4 | 7.5 | 0.005 | −1.1 ± 1.3 |
| Proteasome subunit β type 1 (*PSMB1*) | 487241 | 3.6 | 1.4 | 9.0 | 0.005 | 1.0 ± 1.5 |
| Serine/threonine kinase 15 (*STK15*) | 843097 | 2.6 | 1.0 | 6.8 | 0.005 | −1.3 ± 1.5 |
| Aldehyde oxidase 1 (*AOX1*) | 114043 | 3.9 | 1.5 | 10.6 | 0.007 | 1.2 ± 3.2 |
| Catenin (cadherin-associated protein), δ 1 (*CTNND1*) | 109331 | 2.9 | 1.3 | 6.1 | 0.007 | 1.2 ± 1.4 |
| Cyclin-dependent kinase inhibitor 2D (*CDKN2D*) | 360750 | 2.7 | 1.3 | 5.8 | 0.007 | 1.0 ± 1.3 |
| myc proto-oncogene protein (*MYC*) | 417226 | 3.9 | 1.3 | 11.4 | 0.008 | −1.1 ± 1.3 |
| Cyclin B1 (*CCNB1*) | 1032770 | 3.8 | 1.2 | 11.8 | 0.009 | −1.4 ± 1.4 |
| Rho GDP dissociation inhibitor (GDI) α (*ARHGDIA*) | 740628 | 3.6 | 1.2 | 10.5 | 0.01 | −1.5 ± 1.5 |
| Nuclear factor I/B (*NFIB*) | 264688 | 3.4 | 1.3 | 9.4 | 0.01 | 2.8 ± 2.1 |
| Stathmin 1/oncoprotein 18 (*STMN1*) | 366823 | 3.0 | 1.2 | 7.2 | 0.01 | 3.4 ± 1.8 |
| ets variant gene 6 (*ETV6*) | 111961 | 2.9 | 1.2 | 6.9 | 0.01 | 1.2 ± 1.5 |
| SRY (sex determining region Y)-box 4 (*SOX4*) | 669302 | 2.9 | 1.2 | 7.2 | 0.01 | 4.2 ± 2.5 |
| Topoisomerase (DNA) II α (*TOP2A*) | 50051 | 2.8 | 1.2 | 6.5 | 0.01 | 1.2 ± 1.4 |
| Nuclear factor of activated T cells (*NFATC3*) | 154655 | 2.8 | 1.3 | 6.0 | 0.01 | 1.1 ± 1.3 |
| Myeloid/lymphoid or mixed-lineage leukemia (*MLL*) | 705000 | 4.2 | 1.1 | 15.3 | 0.02 | 1.3 ± 1.5 |
| Gamma-aminobutyric acid A receptor, α 2 (*GABRA2*) | 21754 | 3.2 | 1.3 | 7.8 | 0.02 | −1.3 ± 1.4 |
| Thrombospondin 2 (*THBS2*) | 240592 | 3.0 | 1.2 | 7.9 | 0.02 | 1.1 ± 1.3 |
| Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, γ polypeptide (*YWHAG*) | 136970 | 2.8 | 1.1 | 7.1 | 0.02 | 1.2 ± 1.4 |
| Protein kinase, DNA-activated, catalytic polypeptide (*PRKDC*) | 267593 | 2.8 | 1.1 | 7.1 | 0.02 | −1.4 ± 1.4 |
| Mitogen-activated protein kinase 1 (*MAPK1*) | 111678 | 2.8 | 1.1 | 7.1 | 0.02 | 1.2 ± 1.4 |
| Inositol polyphosphate-5-phosphatase (*INPP5D*) | 754611 | 2.8 | 1.2 | 6.6 | 0.02 | 1.0 ± 1.3 |
| v-erb-b2 erythroblastic leukemia viral oncogene 3 (*ERBB3*) | 44708 | 2.8 | 1.2 | 6.4 | 0.02 | 1.3 ± 1.4 |
| v-rel reticuloendotheliosis viral oncogene homologue (*REL*) | 704548 | 2.7 | 1.1 | 6.6 | 0.02 | 1.1 ± 1.3 |
| Retinoblastoma binding protein 1 (*RBBP1*) | 490061 | 2.7 | 1.2 | 6.3 | 0.02 | −3.6 ± 1.6 |
| Cyclin D1 (*CCND1*) | 758525 | 2.7 | 1.1 | 6.6 | 0.02 | 1.1 ± 1.4 |
| Tumor necrosis factor receptor superfamily (*TNFRSF11A*) | 346544 | 2.6 | 1.1 | 6.1 | 0.02 | 1.0 ± 1.4 |
| Nuclear receptor subfamily 4, group A, member 2 (*NR4A2*) | 38452 | 2.6 | 1.2 | 6.0 | 0.02 | 1.4 ± 1.4 |
| Dual specificity phosphatase 9 (*DUSP9*) | 28678 | 2.6 | 1.1 | 6.2 | 0.02 | 1.1 ± 1.4 |
| TEA domain family member 2 (*TEAD2*) | 4634096 | 2.9 | 1.1 | 7.7 | 0.03 | −2.1 ± 1.7 |
| Tumor protein p53 (*TP53*) | 924383 | 2.8 | 1.1 | 7.2 | 0.03 | 1.2 ± 1.3 |
| Tubulin, γ 1 (*TUBG1*) | 114433 | 2.7 | 1.0 | 7.1 | 0.03 | 1.3 ± 1.4 |
| Neural cell adhesion molecule 1 (*NCAM1*) | 366842 | 2.7 | 1.1 | 6.9 | 0.03 | −1.2 ± 1.2 |
| Cyclin H (*CCNH*) | 730411 | 2.6 | 1.0 | 6.6 | 0.03 | 1.6 ± 1.5 |
| Nuclear factor related to κ B binding protein (*NFRKB*) | 131626 | 2.6 | 1.1 | 6.0 | 0.03 | 1.3 ± 1.5 |
| Cytochrome P450, subfamily IVA, polypeptide 11 (*CYP4A11*) | 120466 | 3.5 | 1.0 | 11.8 | 0.04 | 1.2 ± 1.6 |
| Antigen identified by monoclonal antibody Ki-67 (*MKI67*) | 108718 | 3.0 | 1.0 | 9.1 | 0.04 | 3.9 ± 1.8 |
| Kinesin family member (*KIF17*) | 1540579 | 2.9 | 1.0 | 8.5 | 0.04 | 1.0 ± 1.3 |
| PMS2 postmeiotic segregation increased 2 (*PMS2*) | 503115 | 2.8 | 1.0 | 8.1 | 0.04 | 1.1 ± 1.4 |
| Cyclin-dependent kinase 8 (*CDK8*) | 42880 | 2.6 | 1.0 | 6.8 | 0.04 | 1.1 ± 1.2 |

[a] CI, confidence interval; expression level, mean expression level (medulloblastoma samples *versus* reference RNA pool) ± SD.

labeled centromeric probes of the chromosomes 1, 8, 11, and 20 (Vysis). Pretreatment of slides, hybridization, posthybridization processing, and signal detection were performed as described previously (22).

Medulloblastoma samples showing sufficient FISH efficiency (> 90% nuclei with signals) were evaluated, with 100 intact nuclei scored for the number of signals. Tumor signals were scored as gains when at least 10% of cells showed three or more signals of the oncogene probe. Three to seven signals/cell were considered as low level gains, whereas eight or more signals as well as tight signal clusters were counted as high level gains. The signals of the centromeric probes were used to control adequate hybridization and to exclude artifacts.

**Statistical Analysis.** Primary end point of the study was OS. To identify single genes which expression levels were possibly predictive for OS, we applied genewise proportional hazards models. This approach takes into account that all patients have a different follow-up time. For each gene, the estimated hazard ratio and its 95% confidence interval were determined for an increase of expression levels from the lower to the upper quartile to obtain comparable results for all genes analyzed. A generalized likelihood ratio test was performed for each gene to determine whether these changes in gene expression in relation to OS were of statistical significance. We used a hazard ratio of >2.5 (*i.e.,* 5:2) as cutoff level to identify a set of genes associated with unfavorable OS, whereas a hazard ratio of <0.4 (*i.e.,* 2:5) was used to obtain genes predicting for favorable survival.

Survival probabilities were estimated by the method described by Kaplan and Meier. All genes analyzed by TMA that showed a statistically significant effect in the log-rank test (cyclin D1, stathmin 1, and STK15) were also tested in a multivariate proportional hazards regression model together with clinically defined variables such as age, gender, and metastatic stage. An effect was considered statistically significant if the $P$ was $\leq 0.05$. The statistical analyses were performed using the software package SPSS 11.5 (SPSS, Inc., Chicago, IL) as well as the software package R, version 1.7 (23).

## RESULTS

**Characterization of the Study Population.** To analyze whether clinicopathological characteristics predict for OS among all 180 patients included in the TMA study, we performed a statistical analysis



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Table 4 *Genes in which expression levels correlate with favorable survival in 35 medulloblastoma patients*

| Gene description (gene symbol) | Image ID | Hazard ratio | Lower 95% CI[a] | Upper 95% CI | $P$ | Expression level |
|---|---|---|---|---|---|---|
| Zinc finger protein 9 (*ZNF9*) | 768504 | 0.26 | 0.1 | 0.7 | 0.003 | 1.0 ± 1.7 |
| IFN regulatory factor 3 (*IRF3*) | 754199 | 0.27 | 0.1 | 0.7 | 0.004 | −1.7 ± 1.3 |
| SRY (sex determining region Y)-box 3 (*SOX3*) | 2017442 | 0.29 | 0.1 | 0.8 | 0.007 | −2.8 ± 2.1 |
| Basigin (*BSG*) | 740717 | 0.28 | 0.1 | 0.9 | 0.01 | −2.0 ± 2.9 |
| Protocadherin 1 (*PCDH1*) | 338817 | 0.31 | 0.1 | 0.8 | 0.01 | −5.9 ± 2.5 |
| Ribosomal protein L13a (*RPL13A*) | 590046 | 0.33 | 0.1 | 0.8 | 0.01 | −2.4 ± 2.9 |
| Matrix metalloproteinase 14 (*MMP14*) | 2015513 | 0.35 | 0.2 | 0.8 | 0.01 | −2.2 ± 2.2 |
| Cystatin B (*CSTB*) | 588093 | 0.37 | 0.1 | 0.8 | 0.01 | −3.5 ± 1.5 |
| Ephrin-A4 (*EFNA4*) | 251603 | 0.38 | 0.1 | 0.9 | 0.01 | 1.0 ± 1.8 |
| Flightless I homologue (*FLII*) | 813489 | 0.39 | 0.1 | 0.8 | 0.01 | −1.4 ± 1.6 |
| Alpha-fetoprotein (*AFP*) | 259793 | 0.39 | 0.1 | 0.8 | 0.01 | −4.0 ± 1.8 |
| Zinc finger protein 162 (*ZNF162*) | 770438 | 0.26 | 0.1 | 0.9 | 0.02 | −4.6 ± 2.7 |
| IQ motif containing GTPase activating protein 1 (*IQGAP1*) | 41500 | 0.28 | 0.1 | 0.9 | 0.02 | −3.7 ± 2.7 |
| Ribosomal protein S15 (*RPS15*) | 590802 | 0.31 | 0.1 | 0.9 | 0.02 | −2.8 ± 2.8 |
| Interleukin 11 receptor, α (*IL11RA*) | 811920 | 0.35 | 0.1 | 1.0 | 0.02 | 1.1 ± 1.4 |
| Rho GTPase-activating protein 5 (*ARHGAP5*) | 269753 | 0.35 | 0.1 | 0.8 | 0.02 | −1.1 ± 1.3 |
| CDC28 protein kinase 1 (*CKS1A*) | 298493 | 0.39 | 0.1 | 1.0 | 0.02 | −4.4 ± 1.7 |
| Thymosin, β 4, X chromosome (*TMSB4*) | 755444 | 0.39 | 0.1 | 0.9 | 0.02 | 1.0 ± 4.8 |
| HIV type I enhancer binding protein 2 (*HIVEP2*) | 609261 | 0.33 | 0.1 | 0.9 | 0.02 | −1.6 ± 1.5 |
| Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide (*YWHAZ*) | 41897 | 0.32 | 0.1 | 0.9 | 0.03 | −2.2 ± 1.4 |
| FK506 binding protein 8 (*FKBP8*) | 325311 | 0.33 | 0.1 | 0.9 | 0.03 | −5.9 ± 3.3 |
| Poly(rC) binding protein 2 (*PCBP2*) | 305082 | 0.29 | 0.1 | 1.0 | 0.04 | −7.7 ± 3.0 |
| CDC14 cell division cycle 14 homologue A (*CDC14A*) | 22306 | 0.33 | 0.1 | 1.0 | 0.04 | −2.1 ± 2.3 |
| TNFRSF1A-associated via death domain (*TRADD*) | 824836 | 0.34 | 0.1 | 1.0 | 0.04 | 1.2 ± 1.4 |
| Peroxiredoxin 1 (*PRDX1*) | 484685 | 0.36 | 0.1 | 1.0 | 0.04 | −1.6 ± 2.5 |
| Caspase 10, apoptosis-related cysteine protease (*CASP10*) | 121409 | 0.38 | 0.1 | 1.0 | 0.04 | −2.3 ± 1.7 |
| Protein tyrosine phosphatase, non-receptor type 9 (*PTPN9*) | 488664 | 0.39 | 0.1 | 0.9 | 0.04 | 1.1 ± 1.4 |

[a] CI, confidence interval; expression level, expression level (medulloblastoma samples *versus* reference RNA pool) ± SD.

(Table 2). Although metastatic stage at diagnosis and anaplastic tumor grade were identified as significant predictors for unfavorable OS, the application of adjuvant chemotherapy was associated with a favorable outcome. This finding suggests that a representative group of medulloblastoma patients was included in the study. In a multivariate model, only metastatic stage (hazard ratio = 2.35, $P = 0.03$), and the application of chemotherapy remained statistically significant (hazard ratio = −3.26, $P = 0.008$).

**Identification of Potential Candidate Genes Related to Medulloblastoma Survival.** To identify genes in which expression levels might be predictive for OS, a genewise proportional hazard model was applied (see "Materials and Methods"). A combination of relevance (hazard ratio) and significance (likelihood ratio test) was used to select a set of genes as possibly prognostic factors for OS. On the basis of their hazard ratios (>2.5) and *P*s ($P < 0.05$), we selected 54 genes with expression levels predicting for unfavorable survival (Table 3). Of this set of genes, *MKI67*, *SOX4*, and *stathmin 1* were the genes most abundantly expressed in medulloblastoma (>3-fold) in comparison to the human reference RNA. Functionally, the genes are involved in a variety of biological processes such as cell cycle control and proliferation (*cyclin B1*, *cyclin D1*, *CCNH*, *CDK8*, *CDKN2D*, *MCM5*, *MKI67*, *stathmin 1*, and *TOP2A*), as well as RAS/MAPK-signaling (*MAPK1*, *MAPK6*, *NRAS*, and *RHEB2*). Notably, several genes code for viral oncogene homologues (*NRAS*, *AKT2*, *MYC*, *ERBB3*, *REL*, and *MPL*). In addition, we selected 27 genes with expression levels predicting for favorable survival (hazard ratio < 0.4, $P < 0.05$; Table 4).

A hierarchical cluster analysis was performed using both sets of genes predicting for favorable and unfavorable OS to group patients according to their expression levels of these 81 genes (Fig. 1). The dendrogram shows four distinct clusters (Fig. 2). All but 2 samples of medulloblastoma patients who died within the first year after operation were found in cluster 1A, whereas 2 of the youngest patients of the nonsurvivors (2 and 4 years of age) were grouped into cluster 2B. These two clusters, 1A and 2B, include 10 of 12 patients with anaplastic histology and 12 of 15 patients with metastatic disease.

**Protein Expression of Potential Candidate Genes Tested on TMAs by IHC.** For additional analysis on TMAs by IHC, we selected nine promising candidate genes based on their potential involvement in medulloblastoma tumorigenesis from the set of genes associated with unfavorable survival in medulloblastoma (Appendix 1, Fig. 1). We found a positive immunostaining for DAXX in 64%, cMYC in 57%, STK15 in 53%, NRAS in 47%, cyclin B1 in 46%, stathmin 1 in 46%, cyclin D1 in 43%, Ki-67 in 43%, and p53 in 10% of samples. When the results of immunolabeling were analyzed with respect to the OS, we found a positive staining for cyclin D1, stathmin 1, and STK15 being associated with an unfavorable OS (Fig. 3, Table 5), whereas no statistical significance was obtained for cMYC, cyclin B1, DAXX, NRAS, Ki-67, and p53 staining. The expression of cyclin D1, stathmin 1, and STK15 did not correlate with the metastatic stage, age, or gender of the patients. To analyze whether cyclin D1, stathmin 1, and STK15 are independent prognostic markers for OS in medulloblastoma when criteria such as M stage, age, gender, extent of tumor resection, application of chemotherapy, and tumor grade were known, we performed a multivariate proportional hazards regression analysis (Table 6). Although the expression of STK15 ($P = 0.026$) was identified as independent prognostic marker, this did not apply for cyclin D1 ($P = 0.18$) and stathmin 1 ($P = 0.19$).

**Testing for Additional Gene Copy Numbers of Potential Candidate Genes on TMAs by FISH.** FISH analysis for additional gene copy numbers of *cyclin D1*, *MYC*, *NRAS*, and *STK15* yielded evaluable results in 100, 100, 89, and 58% of arrayed tumors (Table 7). None evaluable tumors were attributable to loss of individual samples during processing or lack of probe hybridization. High level gains of *MYC* were found in 6.7% of samples (Fig. 4*A*). These amplifications were of statistical significance for a worse survival prognosis (log-rank test, $P = 0.003$); all 12 medulloblastoma patients carrying high level gains of *MYC* died within 5 years after treatment (Fig. 4*B*). In addition, low level gains were found for *cyclin D1* in 4.4%, *MYC* in 7.8%, *NRAS* in 5.6%, and *STK15* in 32.5% of tumors (Table 6). In comparison to patients with two signals/cell, patients with increased gene copies of *STK15* (Fig. 4*C*) displayed an OS of only 37 *versus* 63% at 5 years (log-rank test,



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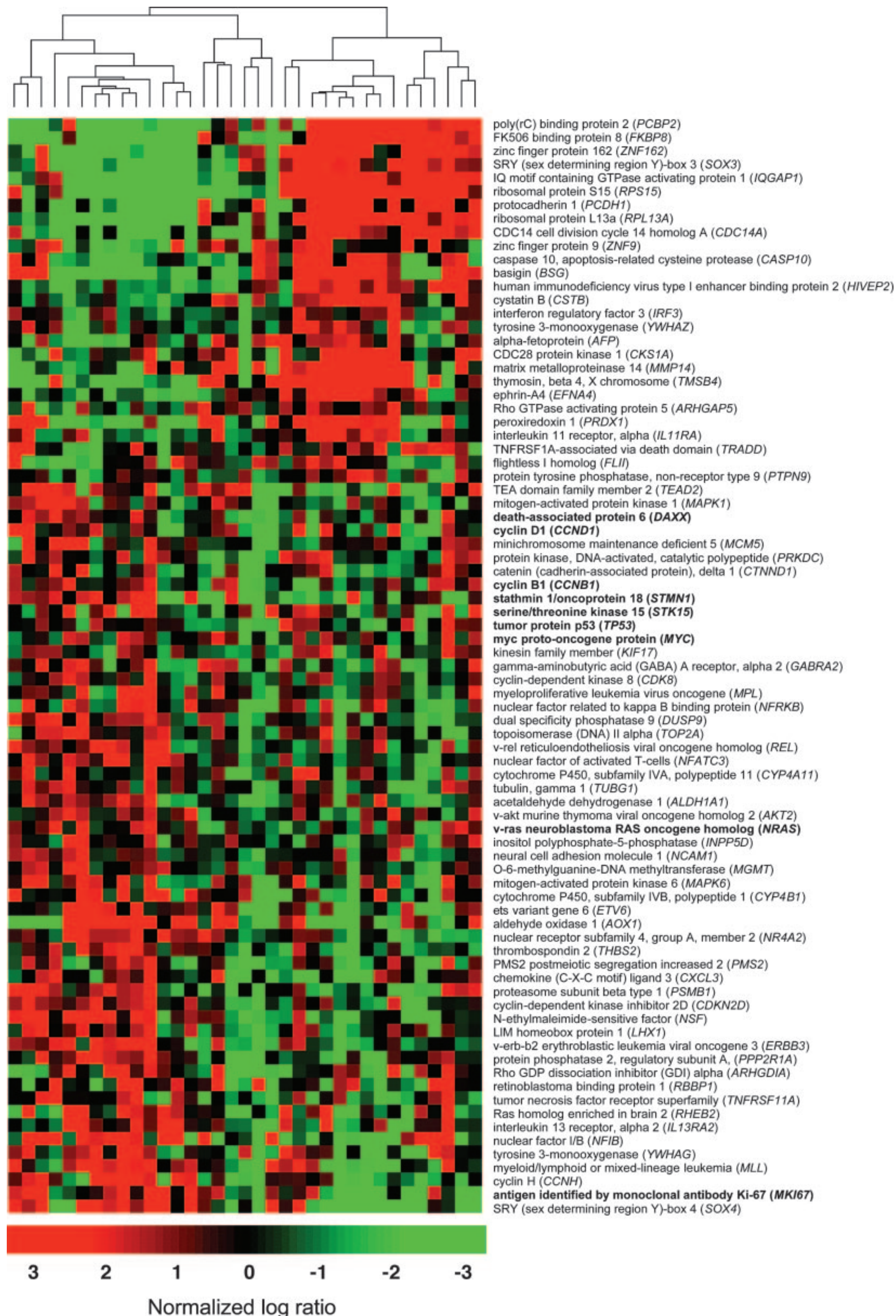


Fig. 1. Hierarchical cluster analysis of genes in which expression levels predicted for survival in 35 medulloblastoma patients. The genes were identified by a genewise proportional hazard model. A hazard ratio of >2.5 and *P* of the likelihood ratio test of <0.05 as cutoff levels were used to identify 54 genes associated with unfavorable OS, whereas a hazard ratio of <0.4 together with *P* of <0.05 was used to obtain 27 genes predicting for favorable survival. For illustration, each gene was normalized to the mean expression of all patients. Highly expressed genes are shown in *red*, whereas genes that are expressed at lower levels are displayed in *green*.

$P = 0.05$; Fig. 4*D*). Of these 104 tumors analyzed by FISH, 15 were also included in the gene expression profiling study. In average, the 6 tumors with additional gene copy numbers of *STK15* displayed 1.7-fold higher gene expression levels of *STK15* as compared with nine samples with a normal gene copy number count (Mann-Whitney *U* test, $P = 0.01$).

## DISCUSSION

The aim of the present study was the identification of potential candidate genes predicting for survival in medulloblastoma using the combined approach of cDNA and TMA technologies. We identified



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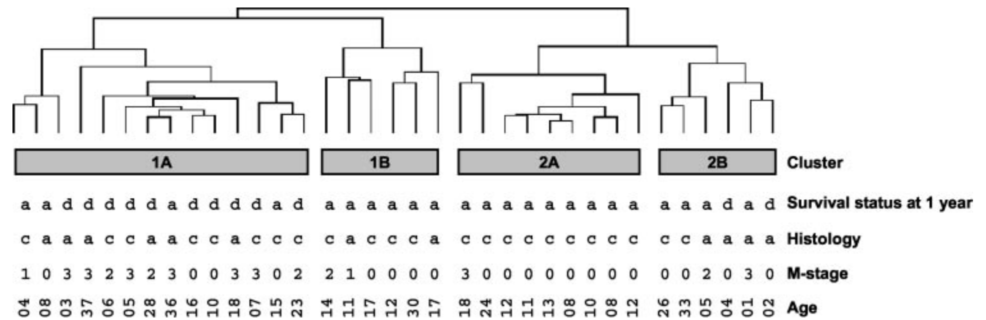


Fig. 2. Dendrogram from the hierarchical cluster analysis (Fig. 1) of genes, the expression levels of which predicted for favorable ($n$ = 27) and unfavorable ($n$ = 54) survival in 35 medulloblastoma patients. The dendrogram shows four major clusters. The survival status at 1 year (alive *versus* dead), histology (anaplastic *versus* classic), metastatic stage (M stage; 0–3), and patient age at diagnosis are indicated for each patient. All but 2 samples of medulloblastoma patients who died within the first year after operation clustered into cluster 1A, whereas two of the youngest patients who died (2 and 4 years of age) were grouped into cluster 2B.

81 genes in which expression levels correlated with OS. A hierarchical cluster analysis showed that most medulloblastoma patients with anaplastic histology and disseminated disease were grouped together with patients who died. In line with this finding, anaplastic and metastatic medulloblastomas have been correlated with genetic alterations as 17p loss and oncogene amplifications of *MYC* and *NMYC* that are associated with unfavorable survival (15, 24).

In detail, we identified 54 and 27 genes with expression levels predicting for unfavorable and favorable prognosis in medulloblastoma, respectively. The genes predicting for poor outcome comprise those that are involved in cell cycle activation and mitosis, DNA metabolism, or code for viral oncogenes. Some of these genes (*MYC*, *SOX4*, and *NRAS*) have been previously reported to be involved in medulloblastoma tumorigenesis and associated with unfavorable outcome (10, 11, 13, 25). Concordantly, the potential role of platelet-derived growth factor receptor and downstream activation of the RAS/MAPK signaling pathway for metastasis in medulloblastoma was outlined by MacDonald *et al.* (13). The list of unfavorable genes

Gene expression profiling

Tumor Tissue Microarray (IHC)

Fig. 3. Effect of STK15, stathmin 1, and cyclin D1 mRNA and protein expression on survival in medulloblastoma patients. High (*top quartile*) *versus* low (*bottom quartile*) levels of *STK15* (*A*), *stathmin 1* (*B*), and *cyclin D1* (*C*) were associated with an unfavorable survival as analyzed by cDNA microarray experiments ($n$ = 35). The protein expression of STK15 (*D*), stathmin 1 (*E*), and cyclin D1 (*F*) was analyzed in a large series of 180 patients on tumor tissue microarrays. STK15 (*G*), stathmin 1 (*H*), and cyclin D1 (*I*) were determined on the basis of immunohistochemical analysis in which tumors were classified as positive when >10% of the tumor cells were stained. In line with the results obtained by cDNA microarray experiments, we found a positive staining for STK15 (*D*), stathmin 1 (*E*), and cyclin D1 (*F*) associated with an unfavorable OS.



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also includes *MGMT*, which codes for the DNA repair protein $O^6$-methylguanine-DNA methyltransferase. Bacolod *et al.* (26) found that MGMT protein expression confers resistance to alkylating drugs in medulloblastoma-derived cell lines. The *MGMT*-mediated resistance to alkylating agents might be of importance because these drugs (*e.g.,* lomustine) have been included in treatment protocols for medulloblastoma patients (2, 16).

Our set of genes, predicting for favorable prognosis, comprises *TMSB4X*, of which, expression is closely related to cerebellar differentiation (27) and *PRDX1*, which has been recently identified as candidate for tumor suppression (28). Furthermore, we identified genes that have apoptosis-related functions (*CASP10* and *TRADD*), are involved in inflammatory and immune response processes (*IRF3* and *IL11RA*) or code for zinc finger proteins (*ZNF9* and *ZNF162*).

Gains of chromosomes 7 or 17q were described in up to 60% of all medulloblastoma samples (5, 8, 24). Interestingly, none of the genes associated with good prognosis were located on these chromosomal regions. In contrast, the set of genes predicting for adverse outcome included four genes located on chromosome 7 (*RHEB2*, *YWHAG*, *PMS2*, and *PMSB1*) and four genes on 17q (*ARHGDIA*, *NSF*, *TOP2A*, and *TUBG1*). This finding suggests that a cooperative selective growth advantage for medulloblastoma cases with alterations on chromosome 7 and 17q may exist, thus explaining the high frequency of aberrations involving both chromosomes along with the finding that high expression levels of genes located on both chromosomal regions correlated with an unfavorable survival.

In line with the results obtained by cDNA microarray experiments, a high expression of cyclin D1, STK15, and stathmin 1 on the protein level correlated with adverse prognosis in medulloblastoma. In contrast, no correlation between gene expression and IHC was found for cMYC, cyclin B1, DAXX, Ki-67, NRAS, and p53. To some extent, the discrepancies found for these genes on the protein and mRNA level might be related to different follow-up times of the patients included in the TMA and cDNA microarray studies (median follow-up time of 34 *versus* 24 months). On the other hand, the lack in correlation might be related to the short half-life time of some proteins. Formally, immunohistochemical heterogeneity of the tumor samples cannot be excluded, thus explaining the limitations of the TMA technology. Previous studies showed, however, that intratumor heterogeneity does not significantly affect the ability to detect clinicopathological correlations on TMAs, at least when two (as in our study) or more cores of each tumor are present on the TMAs (29). To see an influence on OS, the total amount of the accumulated proteins probably needs to exceed a certain cellular cutoff level. Therefore, additional studies are needed to determine robust standardized cutoff values for different antibodies to define protein overexpression in medulloblastoma.

Recently, *cyclin D1* was identified as an important mediator of Sonic hedgehog-induced proliferation in cerebellar granule cell precursors, which are a common target of transformation in medulloblastoma (30). Because 20–30% of sporadic medulloblastomas harbor mutation in *patched* and other elements of the Sonic hedgehog pathway (31), *cyclin D1* appears particularly intriguing with respect to medulloblastoma tumorigenesis. Both STK15 and stathmin 1 play an important role in the adequate segregation of chromosomes during mitosis. Mitotic defects might be a crucial event in medulloblastoma pathogenesis because aneuploidy is a common finding (5, 24). Stathmin 1 is a cytosolic phosphoprotein that is involved in cellular proliferation because its major function is to promote depolymerization of spindle microtubules. In Wilms tumors, *stathmin 1* was found highly expressed in more advanced stages of disease (32). In addition, high protein expression of stathmin 1 correlated with poor prognosis in breast cancer (33). Several studies showed that stathmin 1 is a

Table 7 *Frequency of low and high level gains for all the oncogenes analyzed by fluorescence in situ hybridization in medulloblastoma samples*

| Fluorescence *in situ* hybridization probe | No. of tumors analyzed | No. of low level gains | No. of high level gains |
|---|---|---|---|
| *Cyclin D1* | 180 | 8 (4.4%) | 0 |
| *MYC* | 180 | 14 (7.8%) | 12 (6.7%) |
| *NRAS* | 160 | 9 (5.6%) | 0 |
| *STK15* | 104 | 34 (32.7%) | 0 |

Table 5 *Univariate analysis (log-rank test) of cMYC, cyclin B1, cyclin D1, DAXX, Ki-67, NRAS, p53, stathmin 1, and STK15 expression analyzed by IHC[a] on tissue microarrays in 180 medulloblastoma patients*

| Variable | No. of patients | 5-year survival | *P* |
|---|---|---|---|
| cMYC (MYC) | | | |
| IHC positive | 102 | 56% | 0.57 |
| IHC negative | 78 | 53% | |
| Cyclin B1 (CCNB1) | | | |
| IHC positive | 83 | 54% | 0.31 |
| IHC negative | 97 | 55% | |
| Cyclin D1 (CCND1) | | | |
| IHC positive | 78 | 48% | 0.03 |
| IHC negative | 102 | 60% | |
| DAXX | | | |
| IHC positive | 115 | 59% | 0.24 |
| IHC negative | 75 | 50% | |
| Ki-67 (MKI67) | | | |
| IHC positive | 41 | 62% | 0.69 |
| IHC negative | 139 | 54% | |
| NRAS | | | |
| IHC positive | 84 | 57% | 0.51 |
| IHC negative | 96 | 52% | |
| P53 (TP53) | | | |
| IHC positive | 18 | 54% | 0.78 |
| IHC negative | 162 | 54% | |
| Stathmin 1 (STMN1) | | | |
| IHC positive | 82 | 42% | 0.001 |
| IHC negative | 98 | 68% | |
| STK15 | | | |
| IHC positive | 95 | 40% | 0.0006 |
| IHC negative | 85 | 70% | |

[a] IHC, immunohistochemistry.

Table 6 *STK15 protein expression is an independent prognostic factor for overall survival in 180 medulloblastoma patients*

The prognostic role of cyclin D1, STK15, and stathmin 1 expression was analyzed together with clinical parameters in a multivariate proportional hazards regression model.

| Variable | Hazard ratio | Lower 95% CI[a] | Upper 95% CI | *P* |
|---|---|---|---|---|
| Age (<16 *versus* ≥16 years) | 1.11 | 0.66 | 1.87 | 0.70 |
| Gender (male *versus* female) | 1.55 | 0.92 | 2.60 | 0.10 |
| M stage ($M_{1-3}$ *versus* $M_0$) | 1.62 | 0.96 | 2.73 | 0.07 |
| Resection (non-GTR *versus* GTR) | 1.14 | 0.72 | 1.93 | 0.65 |
| Chemotherapy (no *versus* yes) | 3.56 | 1.27 | 6.12 | 0.003 |
| Histology (anaplastic *versus* classic) | 1.41 | 0.82 | 2.38 | 0.15 |
| Cyclin D1 (positive *versus* negative) | 1.39 | 0.86 | 2.24 | 0.18 |
| Stathmin 1 (positive *versus* negative) | 1.42 | 0.84 | 2.40 | 0.19 |
| STK15 (positive *versus* negative) | 1.90 | 1.08 | 3.34 | 0.026 |

[a] CI, confidence interval; M stage, metastatic stage; GTR, gross total resection.



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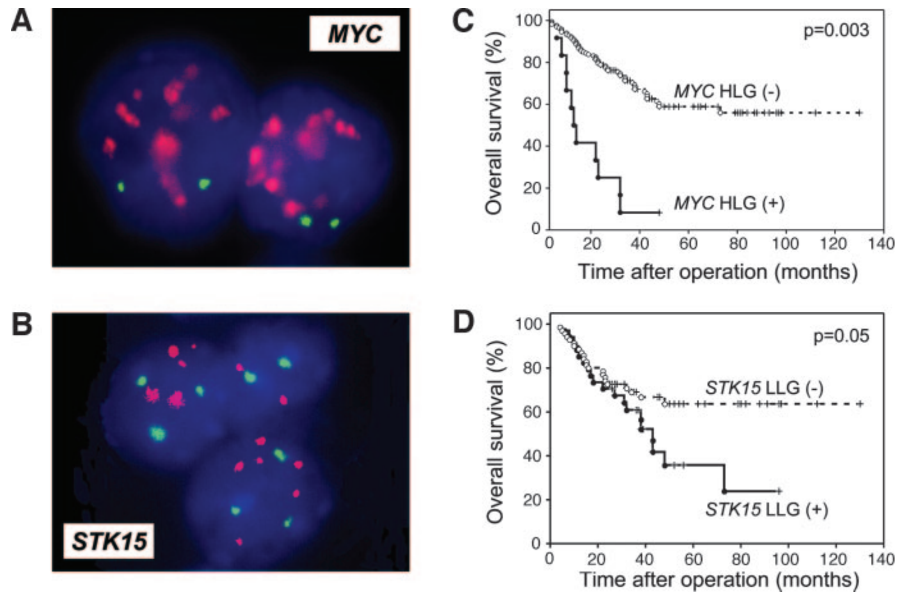


Fig. 4. Additional gene copy numbers of *MYC* and *STK15* detected by fluorescence *in situ* hybridization are correlated with an unfavorable survival in medulloblastoma patients (*C* and *D*). Examples of dual-color fluorescence *in situ* hybridization, demonstrating localization of the *MYC* (*A*) and *STK15* probes (*B*, *red signals*), as well as their corresponding centromeric probes of chromosome 8 (*A*) and 20 (*B*, *green signals*). High level gains (HLGs) of MYC (C) as well as low level gains of STK15 (*D*, LLGs) are predictors for an adverse outcome in medulloblastoma patients. Three to seven signals/cell were considered as LLGs, whereas eight or more signals as well as tight signal clusters were counted as HLGs.

potential therapeutic target because a *stathmin 1*-transfected lung cancer cell line increased the sensitivity to *Vinca* alkaloids (34), and antisense inhibition of *stathmin 1* expression showed a synergistic apoptotic effect in combination with paclitaxel treatment (35).

The *STK15* gene is localized on chromosome 20q13, a region amplified in a variety of human malignancies, including medulloblastoma (5). Additional gene copies of *STK15* were described in primary tumors of the breast (36), cervix (37), bladder (38), colon (39), and brain (40) and reported to be associated with aneuploidy and tumor transformation. Because recent studies showed that overexpression of *STK15* results in aberration of chromosomes and transformation of the cells, *STK15* might contribute to an increasing karyotypic instability and tumor progression in medulloblastoma (36).

When the prognostic significance of cyclin D1, STK15, and stathmin 1 protein expression was compared with clinically defined parameters, STK15 was identified as the only independent predictor for survival in medulloblastoma. Notably, STK15 expression was statistically more significant than the metastatic stage at diagnosis. This is in line with recent studies, showing that outcome predictors based on molecular markers are able to exceed well defined clinical parameters (3, 11, 12).

In the current study, high level gains of *MYC* as well as low level gains of *STK15* predicted for an unfavorable outcome of medulloblastoma patients. The frequency of *MYC* amplifications in the present study (6.7%) as well as their significance for survival is in line with previous studies (8, 10, 24), whereas additional gene copies of *STK15*, which we found in approximately one-third of medulloblastoma samples have not been described previously. In addition, low level gains of *STK15* were associated with a higher expression (1.7-fold) of this gene at the transcriptional level, suggesting a gene-dosis effect. Notably, increased gene copy numbers of *STK15*, as well as its high expression on the mRNA and protein level, correlated with an adverse outcome in medulloblastoma. Thus, *STK15* may serve as a potent molecular target for additional medulloblastoma treatment because the inhibition of *STK15* gene expression by antisense oligonucleotides resulted in the arrest of cell growth and increased apoptosis in cancer cell lines (41).

In summary, the combination of gene expression profiling with TMA technology seems to be a powerful approach for the identification of potent candidate genes predicting for survival in medulloblastoma. Of the results highlighted by the various data analysis procedures, genes associated with cell cycle control and mitosis like *cyclin D1*, *STK15*, and *stathmin 1* appear particularly intriguing with respect to medulloblastoma pathomechanism and might be promising targets to improve the cure rates in this disease.

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Microarray-Based Screening for Molecular Markers in Medulloblastoma Revealed *STK15* as Independent Predictor for Survival

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