VOLUME 32 · NUMBER 9 · MARCH 20 2014

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Cytogenetic Prognostication Within Medulloblastoma Subgroups

David J.H. Shih, Paul A. Northcott, Marc Remke, Andrey Korshunov, Vijay Ramaswamy, Marcel Kool, Betty Luu, Yuan Yao, Xin Wang, Adrian M. Dubuc, Livia Garzia, John Peacock, Stephen C. Mack, Xiaochong Wu, Adi Rolider, A. Sorana Morrissy, Florence M.G. Cavalli, David T.W. Jones, Karel Zitterbart, Claudia C. Faria, Ulrich Schüller, Leos Kren, Toshihiro Kumabe, Teiji Tominaga, Young Shin Ra, Miklós Garami, Peter Hauser, Jennifer A. Chan, Shenandoah Robinson, László Bognár, Almos Klekner, Ali G. Saad, Linda M. Liau, Steffen Albrecht, Adam Fontebasso, Giuseppe Cinalli, Pasqualino De Antonellis, Massimo Zollo, Michael K. Cooper, Reid C. Thompson, Simon Bailey, Janet C. Lindsey, Concezio Di Rocco, Luca Massimi, Erna M.C. Michiels, Stephen W. Scherer, Joanna J. Phillips, Nalin Gupta, Xing Fan, Karin M. Muraszko, Rajeev Vibhakar, Charles G. Eberhart, Maryam Fouladi, Boleslaw Lach, Shin Jung, Robert J. Wechsler-Reya, Michelle Fèvre-Montange, Anne Jouvet, Nada Jabado, Ian F. Pollack, William A. Weiss, Ji-Yeoun Lee, Byung-Kyu Cho, Seung-Ki Kim, Kyu-Chang Wang, Jeffrey R. Leonard, Joshua B. Rubin, Carmen de Torres, Cinzia Lavarino, Jaume Mora, Yoon-Jae Cho, Uri Tabori, James M. Olson, Amar Gajjar, Roger J. Packer, Stefan Rutkowski, Scott L. Pomeroy, Pim J. French, Nanne K. Kloosterhof, Johan M. Kros, Erwin G. Van Meir, Steven C. Clifford, Franck Bourdeaut, Olivier Delattre, François F. Doz, Cynthia E. Hawkins, David Malkin, Wieslawa A. Grajkowska, Marta Perek-Polnik, Eric Bouffet, James T. Rutka, Stefan M. Pfister, and Michael D. Taylor

Processed as a Rapid Communication manuscript. Listen to the podcast by Dr Robinson at www.jco.org/podcasts

Author affiliations appear at the end of this article.

Published online ahead of print at www.jco.org on February 3, 2014.

Support information appears at the end of this article.

D.J.H.S., P.A.N., and M.R. contributed equally to this work. S.M.P. and M.D.T. are co-senior authors.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Michael D. Taylor, MD, PhD, Hospital for Sick Children, 555 University Ave, Suite 1503, Toronto, ON M5G-1X8 Canada; e-mail: mdtaylor@sickkids.ca.

0732-183X/14/3209w-886w/$20.00

DOI: 10.1200/JCO.2013.50.9539

ABSTRACT

Purpose
Medulloblastoma comprises four distinct molecular subgroups: WNT, SHH, Group 3, and Group 4. Current medulloblastoma protocols stratify patients based on clinical features: patient age, metastatic stage, extent of resection, and histologic variant. Stark prognostic and genetic differences among the four subgroups suggest that subgroup-specific molecular biomarkers could improve patient prognostication.

Patients and Methods
Molecular biomarkers were identified from a discovery set of 673 medulloblastomas from 43 cities around the world. Combined risk stratification models were designed based on clinical and cytogenetic biomarkers identified by multivariable Cox proportional hazards analyses. Identified biomarkers were tested using fluorescent in situ hybridization (FISH) on a nonoverlapping medulloblastoma tissue microarray (n = 453), with subsequent validation of the risk stratification models.

Results
Subgroup information improves the predictive accuracy of a multivariable survival model compared with clinical biomarkers alone. Most previously published cytogenetic biomarkers are only prognostic within a single medulloblastoma subgroup. Profiling six FISH biomarkers (*GLI2*, *MYC*, chromosome 11 [chr11], chr14, 17p, and 17q) on formalin-fixed paraffin-embedded tissues, we can reliably and reproducibly identify very low-risk and very high-risk patients within SHH, Group 3, and Group 4 medulloblastomas.

Conclusion
Combining subgroup and cytogenetic biomarkers with established clinical biomarkers substantially improves patient prognostication, even in the context of heterogeneous clinical therapies. The prognostic significance of most molecular biomarkers is restricted to a specific subgroup. We have identified a small panel of cytogenetic biomarkers that reliably identifies very high-risk and very low-risk groups of patients, making it an excellent tool for selecting patients for therapy intensification and therapy de-escalation in future clinical trials.

J Clin Oncol 32:886-896. © 2014 by American Society of Clinical Oncology

INTRODUCTION

Medulloblastoma, the most common malignant childhood brain tumor, is an embryonal tumor with a peak incidence in early childhood. Current therapy entails surgical resection, craniospinal irradiation, and high-dose chemotherapy. Risk stratification is based primarily on clinical variables, with high-risk patients identified as having leptomeningeal metastases at presentation and/or an incomplete resection.[1-3] Unfortunately, most survivors are left with long-term disabilities secondary to the disease and treatment.[4-6] Clinicians have hypothesized that improved patient prognostication could enable therapy intensification in high-risk patients and therapy de-escalation to maximize quality of life in lower-risk patients.

Numerous publications have attempted to identify biomarkers to either support or supplant clinical risk stratification.[2,7-14] Mutations of specific genes such as *CTNNB* and *TP53* have shown prognostic significance.[15-19] Additional candidates include medulloblastoma-overexpressed genes such as *TRKC*, *ERBB2*, and *FSTL5*.[20-25] Several DNA copy-number aberrations have also been purported as biomarkers, although the results have often been conflicting.[15,26-48] These aberrations are summarized in Table 1. Few of these putative molecular biomarkers have been validated in prospective clinical trials.

It is now recognized that medulloblastoma is a collection of heterogeneous entities with disparate demographics, transcriptomes, genetics, and clinical outcomes.[2,28,32,49-60] According to international consensus, the principle subgroups of medulloblastoma are WNT, SHH, Group 3, and Group 4.[52] Because earlier prognostic biomarker studies did not account for these subgroups, we hypothesized that some of the disparate biomarker findings could have resulted from differential subgroup representation among studies. Several previously reported biomarkers were in fact enriched within a specific subgroup of the disease (eg, monosomy 6 in WNT tumors, *MYC* amplification in Group 3 tumors). In cases where a biomarker is prognostic across all medulloblastomas, but the prognostic impact is driven by a single subgroup, we suggest that the marker be designated as subgroup driven. These surrogate markers are replaceable by subgroup status. In cases where a biomarker is variably or not effective across the spectrum of medulloblastomas but is valid only within a specific subgroup, we suggest that it be designated as subgroup specific. Such biomarkers are prognostically informative only within specific medulloblastoma subgroups.

To determine whether subgroup affiliation and cytogenetic biomarkers could support or supplant clinical variables for prognostication in patients with medulloblastoma, we assembled an international discovery cohort of 673 medulloblastomas through MAGIC (Medulloblastoma Advanced Genomics International Consortium), for which we had both clinical follow-up and whole-genome copy-number data. To begin, we identified subgroup-specific copy-number aberrations (CNAs) and integrated them with clinical variables to develop subgroup-specific risk models based on the discovery cohort. To validate our models and ensure that our technique was generalizable to routine pathology laboratories, we then studied a panel of six cytogenetic biomarkers (*GLI2*, *MYC*, chromosome 11 [chr11], chr14, 17p, and 17q) using interphase fluorescent in situ hybridization (FISH) on a formalin-fixed paraffin-embedded (FFPE) medulloblastoma tissue microarray (TMA) that included 453 medulloblastomas treated at a single center and did not overlap with the discovery cohort.

Our analysis of > 1,000 patients with medulloblastoma clearly demonstrates that subgroup affiliation can improve prognostication with clinical variables and that a majority of published molecular biomarkers are relevant only within a single subgroup. The combination of clinical variables, subgroup affiliation, and six cytogenetic markers analyzed on FFPE tissues can achieve an unprecedented level of prognostic prediction for patients that is practical, reliable, and reproducible.

PATIENTS AND METHODS

Tumor Material and Patient Characteristics

A discovery set of 673 medulloblastoma samples with clinical follow-up was acquired retrospectively from 43 cities around the world. These samples

Table 1. Previously Reported Prognostic Molecular Markers in MB

Marker	Previous Studies		Our Study				
	Cohort	Prognosis	Validated	MB (*P*)	SHH (*P*)	Group 3 (*P*)	Group 4 (*P*)
1q gain	MB[26,27]	Poor	No	.61	.59	**.018**	.33
Chr2 gain	SHH[30]	Poor	No	.16	.66	.17	.49
3q gain	MB,[32] SHH[32]	Poor	No	.14	.20	.80	—
6q gain	MB[31]	Poor	No	.61	.30	.94	.19
Chr6(q) loss	MB[15,31,34]	Good	SGD	**.002**	.90	.73	—
10q loss	MB,[32,35] SHH[30]	Poor	SGS	**.012**	**.001**	.23	.082
17p loss	MB,[26,32,36-40] SHH,[30,32] Group 4[32]	Poor	SGS	**.003**	**.011**	**.030**	.37
17q gain	MB,[31,32,35,40] SHH,[30] Group 3,[32] Group 4[32]	Poor	SGS	.095	—	**.049**	.72
Iso17q	MB[31,35,38,41]	Poor	SGS	**.005**	—	**.008**	.81
CDK6 amplification	MB[32,40]	Poor	No	.51	.36	.17	.55
GLI2 amplification	SHH[30]	Poor	SGS	**< .001**	**.001**	—	—
MYC amplification	MB[31,42-46]	Poor	SGS	**< .001**	—	**.011**	.37
MYCN amplification	MB,[31,44] SHH,[32] Group 4[32]	Poor	SGS	.92	**.002**	—	.24
OTX2 amplification	MB[47]	Poor	No	.61	—	.46	.77

NOTE. Bold font indicates significance; — indicates event not observed at sufficient frequency (n ≤ 1).
Abbreviations: chr, chromosome; iso, isochromosome; MB, medulloblastoma (across all subgroups); SGD, subgroup driven; SGS, subgroup specific.

were copy-number profiled on the Affymetrix SNP6 platform (Santa Clara, CA) to identify potential biomarkers.[28] An independent validation set of 453 samples with clinical follow-up on a TMA was analyzed using FISH as previously described.[53] Tumors were classified based on signature marker expression into molecular subgroups as previously described[61]; additional tumors were classified based on cytogenetic aberrations using standard conditional probability models. Subgroup affiliation was not available for 162 discovery samples. The validation set additionally included 50 WNT tumors not on the TMA. Details on clinical data are listed in Data Supplement 1, along with the availability of clinical and cytogenetic data. Nucleic acid isolation, TMA construction, and β-catenin mutation analysis were performed as previously described.[28]

Prognostic Biomarker Identification

Cytogenetic events and CNAs were identified as previously described in the discovery set.[28] Subsequent to biomarker discovery, cross validation was performed to estimate the reproducibility of the candidates in an independent cohort, with multiple-hypothesis correction. Additionally, sample size estimates for prospective trials of the biomarkers were calculated based on the observed hazard ratios. Additional details are available in Data Supplement 1.

Statistical Analyses

Patient survivals were analyzed using the Kaplan-Meier method. The predictive values of biomarkers were assessed through time-dependent receiver operating characteristic analyses. Details of the survival analyses and risk model selections are available in Data Supplement 1.

RESULTS

Prognostic Significance of Clinical Variables Within Medulloblastoma Subgroups

Many prior medulloblastoma biomarker studies were limited by sample size. Our study included 1,126 patients with medulloblastoma (673 discovery plus 453 validation patients; Data Supplement 1), which is more than double the sample size of any prior medulloblastoma biomarker study, and it is one of few studies to include a validation cohort (Data Supplement 2). Although the discovery cohort accumulated by MAGIC consists of medulloblastomas gathered from 43 different treating centers from around the world, the subgroup-specific outcomes mirror those previously published, with good outcomes for patients with WNT, poor outcomes for those with Group 3, and intermediate outcomes for those with SHH and Group 4 medulloblastomas (Data Supplement 2), suggesting that the discovery cohort was a representative sample (Data Supplement 1).

To assess long-term survivors, patients with WNT medulloblastoma were observed for up to 10 years, and only two deaths were observed among 53 patients, both resulting from tumor recurrence (Fig 1A; Appendix Fig A1A, online only; Data Supplement 1). Among those with SHH tumors, there were significantly better outcomes among adult patients as compared with children or infants (Fig 1B; Appendix Fig A1B, online only). Infants with Group 4 tumors had significantly worse outcomes than children or adults (Fig 1B; Appendix Fig A1B, online only), suggesting that radiation therapy is critical in the treatment of Group 4 medulloblastoma. There was no consistent association between sex and prognosis in any of the four subgroups (Data Supplement 1). Desmoplastic histology indicated a more favorable prognosis than classic histology, which was more favorable than anaplastic histology among SHH tumors (Data Supplement 1). Large-cell/anaplastic histology was prognostically significant for Group 3 medulloblastomas in the discovery cohort but not in the validation cohort.

Metastatic status was not prognostic for patients with WNT tumors; however, macroscopic metastasis (M2/M3) was consistently associated with poor survival in all non-WNT subgroups, although the clinical effect was modest among patients with Group 4 disease (Fig 1C; Appendix Fig A1C, online only). Although the prognostic significance of M0 disease as compared with M2/3 disease was convincing across SHH, Group 3, and Group 4 subgroups, the prognostic significance of isolated M1 disease (presence of tumor cells in cerebrospinal fluid) was less clear (Fig 1C; Appendix Fig A1C, online only; Data Supplement 1). Isolated M1 disease was not consistently associated with poor prognosis in the discovery or validation cohort for any subgroup, which may be the result of small sample sizes. There were no CNAs in any of the subgroups that were associated with an increased risk of leptomeningeal dissemination (Data Supplement 1). Overall, many clinical biomarkers continued to exhibit prognostic significance when medulloblastoma was analyzed in a subgroup-specific fashion (Data Supplement 1).

Subgroup and Metastatic Status Are the Most Powerful Predictive Prognostic Biomarkers

Multivariable survival analyses were conducted to examine the relative predictive value of clinical variables and subgroup affiliation. Stepwise Cox regressions revealed that subgroup affiliation significantly contributed to multivariable survival prediction, on top of a regression model already parameterized by sex, age, metastatic status, and histology (Data Supplement 2). Furthermore, Cox proportional hazards models parameterized with both clinical biomarkers and molecular subgroups achieved higher accuracy in time-dependent receiver operating characteristic analyses (Data Supplements 1 and 2). In isolation, each biomarker had modest prediction accuracy (Data Supplement 2) compared with the complete multivariable model (Data Supplement 2). In the complete model, the removal of metastatic status and subgroup led to the greatest decreases in predictive accuracy (Data Supplement 2). Taken together, these results suggest that subgroup affiliation and metastatic status are the most important predictive biomarkers and that they make nonredundant contributions to the prediction of survival. We conclude that combining both clinical and molecular biomarkers can enhance prediction of patient survival.

Subgroup Specificity of Published Molecular Biomarkers

Several cytogenetic biomarkers have been associated with patient survival across medulloblastoma, but their prognostic value has seldom been assessed in the context of medulloblastoma subgroups (Table 1). Monosomy for chromosome (chr) 6 is associated with improved survival across medulloblastoma in toto (Fig 2A; Data Supplement 1). However, the prognostic value of chr6 loss can be completely attributed to its enrichment in WNT medulloblastomas (Fig 2B; Data Supplement 1), because loss of chr6 has no prognostic value among patients with WNT or non-WNT tumors when compared with their respective controls with balanced chr6. We suggest that monosomy 6 is a subgroup-driven biomarker; its prognostic significance is driven by its enrichment in a particular subgroup, and it thus holds no further significance in subgroup-specific analysis. Furthermore, these results would add a note of caution to using monosomy 6 as the lone diagnostic criterion for WNT medulloblastoma, because it was also observed in non-WNT medulloblastomas (seven [14%] of 49

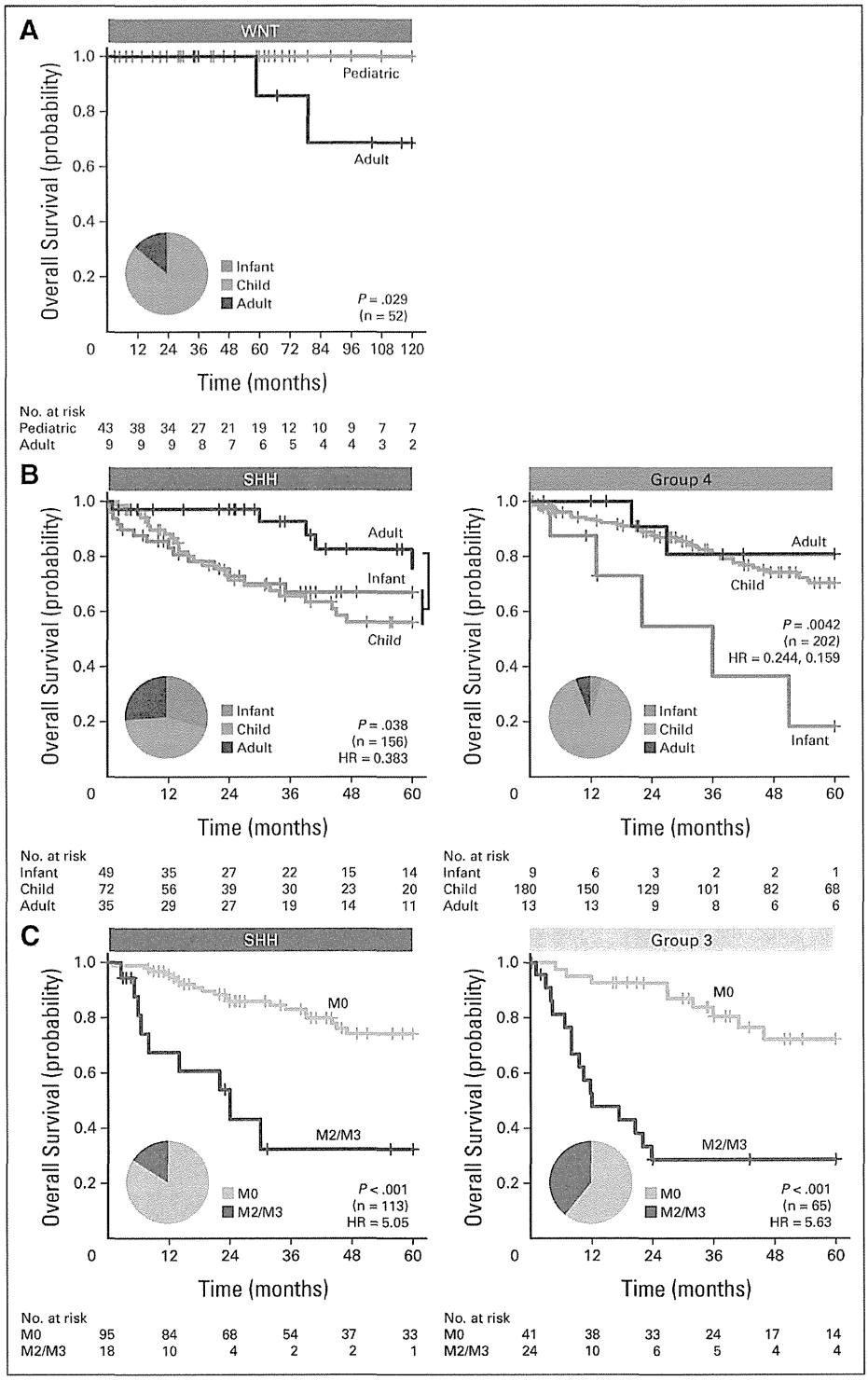

Fig 1. (A) Ten-year overall survival curves for WNT medulloblastoma by age group. (B) Overall survival curves for age groups within SHH and Group 4 subgroups (infant, age < 3 years; child, age 3 to < 16 years; adult, age ≥ 16 years). (C) Overall survival curves for metastatic status for SHH and Group 3 subgroups. Numbers below *x*-axis represent patients at risk of event; statistical significance evaluated using log-rank tests; hazard ratio (HR) estimates derived from Cox proportional hazards analyses.

monosomy 6 medulloblastomas were not in WNT subgroup), and monosomy 6 was only present in 42 (79%) of 53 WNT tumors. The prognostic role of isochromosome (iso) 17q has been controversial; for our cohort in toto, iso17q was a statistically significant predictor of poor outcome (Fig 2C). However, subgroup-specific analysis demonstrated that iso17q was highly prognostic for Group 3 but not for Group 4 medulloblastoma (Fig 2D), indicating that it is a subgroup-specific molecular biomarker. Similarly, although 10q loss was a

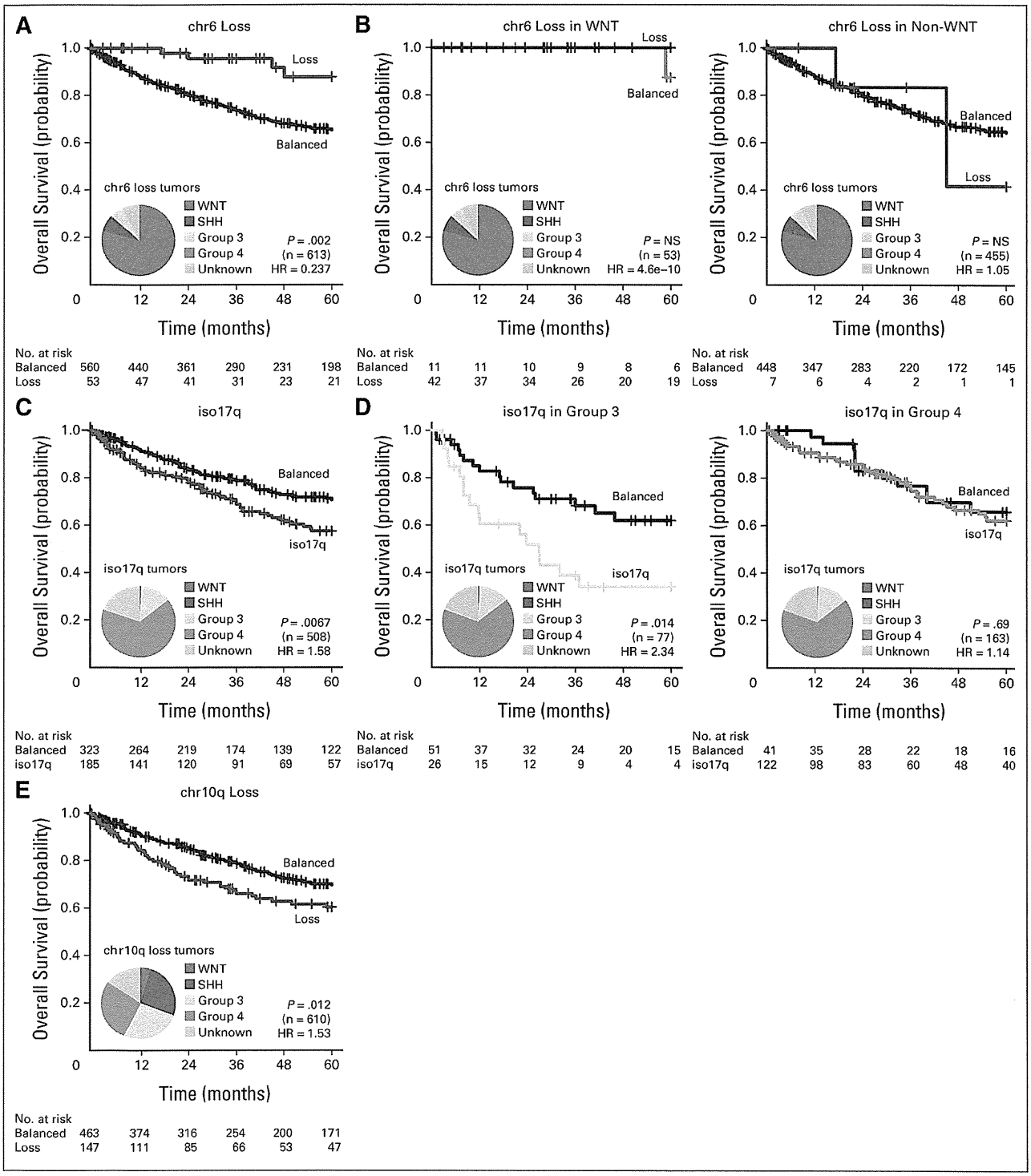

Fig 2. (A) Overall survival curves and frequency distribution of chromosome 6 (chr6) status across entire cohort. (B) Overall survival curves for chr6 status in WNT and non-WNT medulloblastomas. (C) Overall survival curves and frequency distribution of isochromosome 17q (iso17q) across entire cohort. Patients with broad gain or loss of chr17 excluded. (D) Overall survival curves for iso17q status in Group 3 and Group 4 subgroups. (E) Overall survival curves for chr10q status across entire cohort. HR, hazard ratio; NS, not significant.

modestly significant predictor of poor outcome across medulloblastomas (Fig 2E), its prognostic power was limited to SHH tumors in a subgroup-specific analysis (Appendix Figs A2A and A2B, online only). We conclude that determination of subgroup affiliation is crucial in the evaluation and implementation of molecular biomarkers for patients with medulloblastoma (Table 1; Data Supplement 1), because some putative biomarkers are merely enriching for a specific subgroup (ie, subgroup driven), whereas most others are significant only within a specific subgroup (ie, subgroup specific).

Patients With SHH Tumors Can Be Stratified Into Three Distinct Risk Groups

We identified 11 CNAs that were prognostically significant in our SHH medulloblastoma discovery set (Appendix Figs A3A to A3D, online only; Data Supplement 1) in univariable survival analyses. Given the considerable number of candidates, the reproducibility of the identified biomarkers was assessed through cross validation to facilitate candidate prioritization, and the sample sizes required for prospective trials were estimated for future studies (Data Supplement 1). Specific amplifications but not broad gains encompassing *GLI2* or *MYCN* were associated with poor prognosis (Appendix Figs A3A and A3B, online only; Data Supplement 1). Loss of chr14q conferred significantly inferior survival (Appendix Fig A3C, online only). There was no minimal region of deletion on chr14 in patients with SHH tumors (Data Supplement 1), and recent medulloblastoma resequencing efforts have not identified any recurrent single-nucleotide variants on chr14 in SHH medulloblastoma.[28,54,56,57,62] The presence of chromothripsis (ie, chromosome shattering) was associated with worse survival in those with SHH tumors (Appendix Fig A3D, online only).[17]

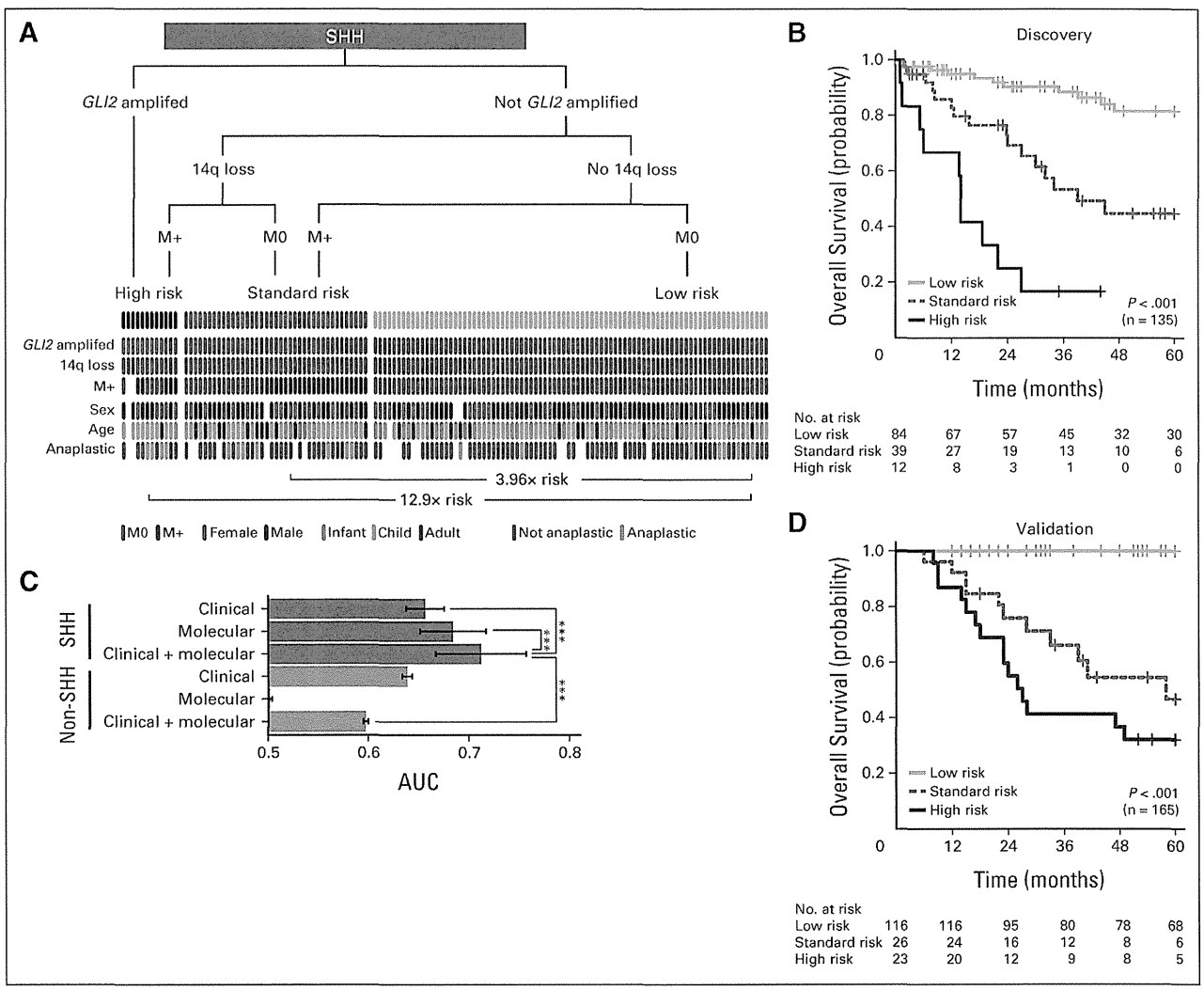

Fig 3. Clinical prognostication of patients with SHH medulloblastoma. (A) Risk stratification of SHH medulloblastomas by molecular and clinical prognostic markers. Decision tree, with events plot depicting status of molecular and clinical markers across risk groups below. (B) Overall survival curves for SHH risk groups. (C) Average time-dependent areas under the curve (AUCs) for risk groups stratified using only clinical or molecular markers or both. Risk stratification regimens applied to SHH and non-SHH medulloblastomas. ***$P < .001$ by Friedman rank sum tests. (D) Survival curves for SHH risk groups in validation cohort. Survival differences evaluated by log-rank tests; hazard ratio estimates derived from Cox proportional hazards analyses.

To integrate the individual biomarkers into a risk stratification model, multivariable Cox analyses were performed on all significant biomarkers. Through multiple stepwise regression procedures, a consensus set of biomarkers was selected for inclusion in the model in an unbiased manner. The proposed risk stratification scheme represents the model that was most consistent with available data in the discovery cohort, from among many possible alternatives (Figs 3A and 3B; Data Supplement 1). *GLI2* amplification, 14q loss, and leptomeningeal dissemination identified high- and standard-risk patients. Specifically, *GLI2* amplification alone identified patients with poor prognosis (Figs 3A and 3B; Data Supplement 1). Absence of these markers defined a low-risk group of patients who exhibited survivorship reminiscent of patients with WNT tumors. Importantly, none of the covariates, particularly age and anaplastic histology, could explain the survival differences observed among risk groups (Figs 3A and 3B; Data Supplement 1). Direct application of the proposed risk stratification scheme on the independent validation cohort yielded distinct survivorship rates for the three risk groups, thereby validating the model (Fig 3D).

Two additional stratification schemes were constructed using only clinical biomarkers or only cytogenetic markers; however, the proposed model, which combines both types of biomarkers, yielded the highest accuracy (Fig 3C; Data Supplement 1). Furthermore, the accuracy of the combined risk model was drastically reduced when applied across patients with non-SHH tumors, further underscoring the importance of taking subgroup into consideration during risk stratification. We conclude that by using two molecular biomarkers (*GLI2* and 14q FISH) and metastatic status, we can practically and reliably predict prognosis for patients with SHH medulloblastoma.

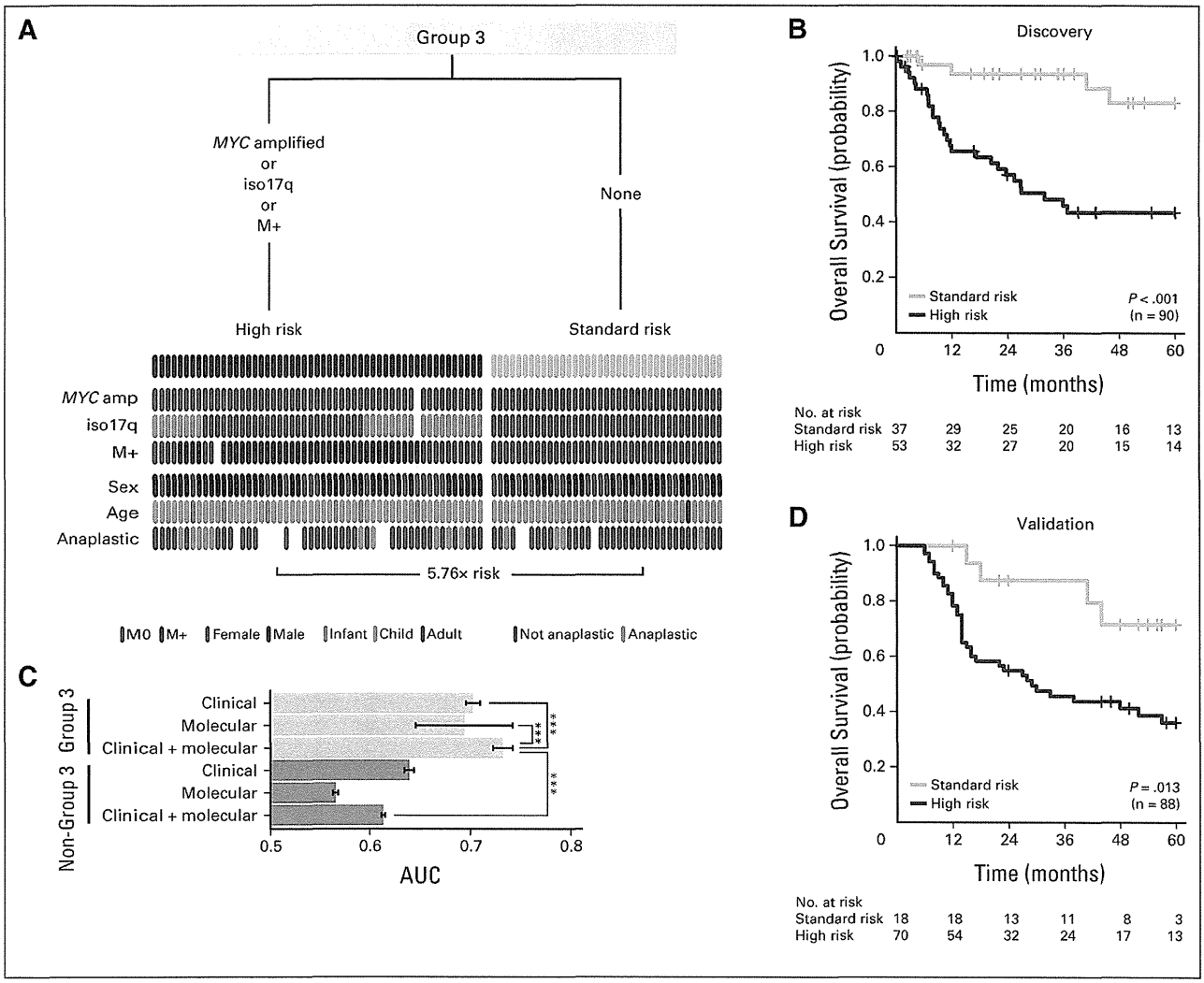

Fig 4. Clinical prognostication of patients with Group 3 medulloblastoma. (A) Risk stratification of Group 3 medulloblastomas by molecular and clinical prognostic markers. Decision tree, with events plot depicting status of molecular and clinical markers across risk groups below. (B) Overall survival curves for Group 3 risk groups. (C) Average time-dependent areas under the curve (AUCs) for risk groups stratified using only clinical or molecular markers or both. Risk stratification regimens applied to Group 3 and non–Group 3 medulloblastomas. ***$P < .001$ by Friedman rank sum tests. (D) Survival curves for Group 3 risk groups in validation cohort. Survival differences evaluated by log-rank tests; hazard ratio estimates derived from Cox proportional hazards analyses. Iso, isochromosome.

Metastatic Status, Iso17q, and MYC *Amplification Identify High-Risk Patients With Group 3 Medulloblastoma*

In patients with Group 3 tumors, iso17q and *MYC* amplification remained the only cytogenetic markers associated with poor survival (Appendix Figs A4A and A4B, online only), whereas chr8q loss and chr1q gain were the only good prognosis markers (Appendix Fig A4C, online only; Data Supplement 1). In multivariable survival analyses, patients with metastasis, iso17q, or *MYC* amplification represented the high-risk group (Figs 4A and 4B). Critically, absence of these markers identified a population of patients with Group 3 tumors with favorable prognosis. The risk groups were not associated with any clinical covariates, including age (Figs 4A and 4B; Data Supplement 1). Consistent with the findings in patients with SHH tumors, optimal risk stratification of those with Group 3 tumors required the use of both clinical and molecular prognostic markers, which have little prognostic value outside of Group 3 (Fig 4C; Data Supplement 1). Our proposed risk stratification scheme was validated on the nonoverlapping validation cohort using three molecular biomarkers (*MYC*, 17p, and 17q FISH) and metastatic status (Fig 4D).

Identification of a Low-Risk Group of Patients With Metastatic Group 4 Medulloblastoma

Patients with Group 4 tumors with whole-chromosome loss of chr11 or gain of chr17, in addition to 10p loss, exhibited better survival under univariable Cox models (Appendix Fig A5A, online only; Data Supplement 1). There was no cytogenetic marker associated with poor prognosis (Data Supplement 1). Specifically, neither *MYCN* gain nor amplification was associated with poorer survival in those with Group 4 tumors, in stark contrast to patients with SHH tumors, reinforcing

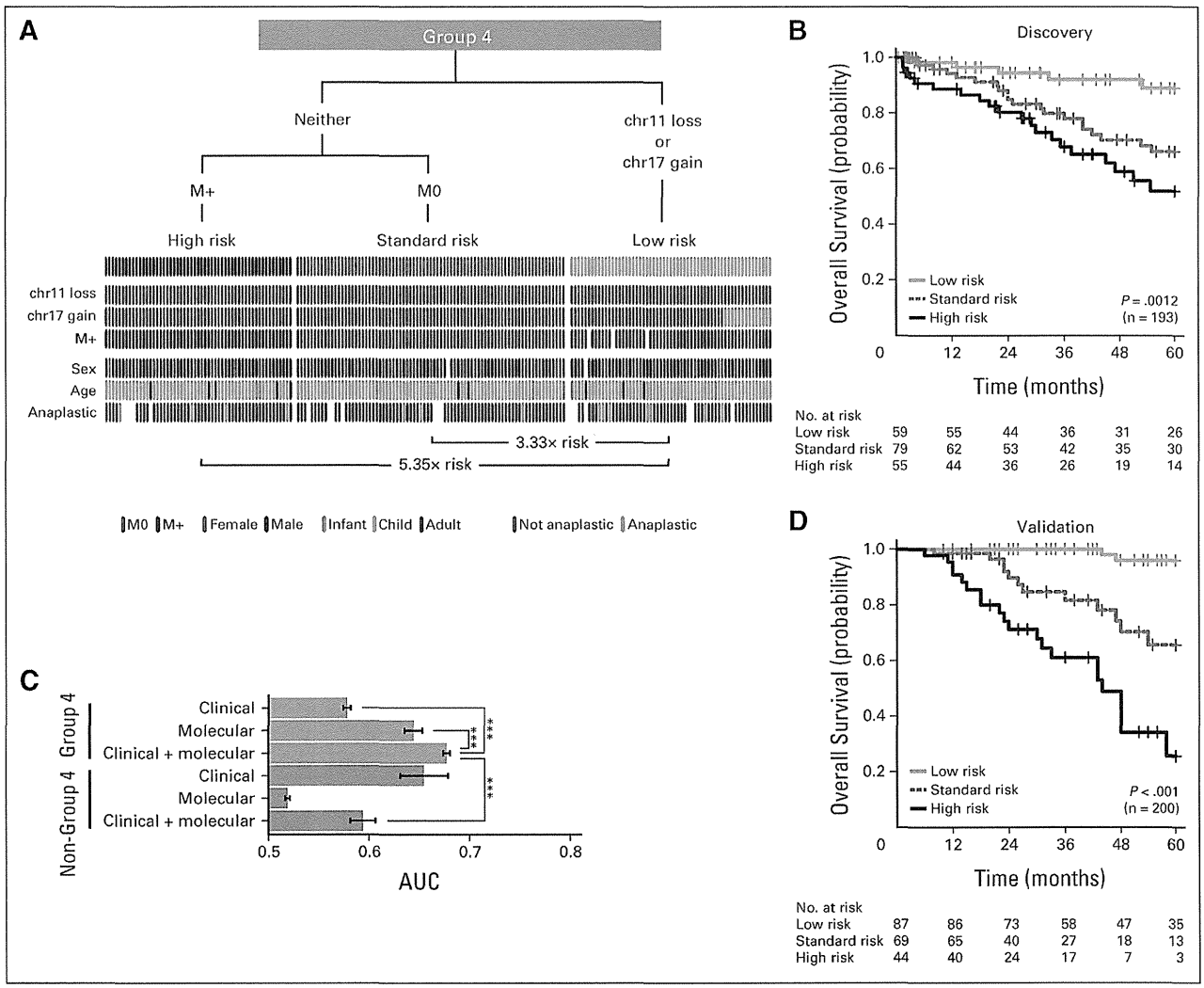

Fig 5. Clinical prognostication of patients with Group 4 medulloblastoma. (A) Risk stratification of Group 4 medulloblastomas by molecular and clinical prognostic markers. Decision tree, with events plot depicting status of molecular and clinical markers across risk groups below. (B) Overall survival curves for Group 4 risk groups. (C) Average time-dependent areas under the curve (AUCs) for risk groups stratified using only clinical or molecular markers or both. Risk stratification regimens applied to Group 4 and non–Group 4 medulloblastomas. ***$P < .001$ by Friedman rank sum tests. (D) Survival curves for Group 4 risk groups in validation cohort. Survival differences evaluated by log-rank tests; hazard ratio estimates derived from Cox proportional hazards analyses. Chr, chromosome.

the distinction in their underlying biology (Appendix Fig A5B, online only; Data Supplement 1). Similarly, none of the cytogenetic biomarkers identified for patients with Group 3 tumors (eg, iso17q) had any prognostic value for those with Group 4 tumors (Data Supplement 1). After unbiased model selection, the consensus set of biomarkers resulted in a risk stratification scheme in which leptomeningeal dissemination identified high-risk patients with Group 4 tumors, except in the context of chr11 loss or chr17 gain (Figs 5A and 5B). The biology underlying chr11 loss was not apparent, because there was no obvious minimal common region of deletion (Data Supplement 1), nor were there any recurrent single-nucleotide variants on chr11 reported. Patients with Group 4 tumors with either chr17 gain or chr11 loss, irrespective of metastatic status, exhibited excellent survivorship in both the discovery and validation cohorts (Figs 5B and 5D), and the survival differences were not explainable by covariates (Data Supplement 1). Consistent with other subgroups, the risk stratification model using both clinical and molecular biomarkers achieved the highest accuracy (Fig 5C). Critically, the cytogenetic biomarkers identified low-risk patients with Group 4 tumors who would be otherwise designated as high risk by evidence of metastasis and/or anaplastic histology; this finding could not be extrapolated to patients with SHH or Group 3 medulloblastoma (Figs 5A to 5C; Data Supplement 1). We conclude that through the use of three molecular biomarkers (chr11, 17p, and 17q FISH) and metastatic status, we can reliably predict the prognosis of patients with Group 4 medulloblastoma.

DISCUSSION

Current consensus identifies the existence of four major subgroups of medulloblastoma, with excellent prognosis for those with WNT tumors, intermediate prognosis for those with SHH and Group 4 tumors, and poor prognosis for those with Group 3 tumors.[32,52] However, early evidence suggests clinical heterogeneity within these core subgroups.[7,30,63] Practical and reliable prognostication of risk could allow for therapy intensification in high-risk children to improve survival and de-escalation of therapy in low-risk children so as to avoid the significant complications of therapy. However, the majority of published medulloblastoma biomarker studies included only small cohorts of patients, were not validated on nonoverlapping cohorts, and were performed in the presubgrouping era. Our prognostic study of 1,123 medulloblastomas, using techniques (eg, FISH) compatible with FFPE tissues, has identified clinically applicable risk stratification for SHH, Group 3, and Group 4 medulloblastomas.

We have demonstrated that medulloblastoma subgroup affiliation is significantly more informative for predicting patient outcome than existing clinical variables and that by incorporating subgroup status with conventional clinical parameters for risk stratification, the accuracy of survival prediction can be dramatically improved. Moreover, we have proposed, tested, and validated novel subgroup-specific risk stratification models incorporating both clinical and molecular variables. These models performed robustly both in the discovery cohort consisting of heterogeneously treated groups of patients and in a nonoverlapping validation cohort of patients treated at a single institution according to standardized treatment protocols. Because we do not have detailed treatment information for patients in the discovery cohort, it is possible that treatment protocols (type, duration, or intensity) could have affected our results. We suggest that this possibility can only be eliminated through examination of our stratification model in a sufficiently large prospective cohort. Although our study used single-nucleotide polymorphism arrays or interphase FISH on FFPE sections, it is possible that other approaches such as array comparative genomic hybridization could also be used to determine the copy-number status of the six markers.[64] Through the incorporation of current clinical variables, subgroup affiliation, and our six copy-number prognostic markers, as detailed in Data Supplement 1, rapid prognostication is feasible in the setting of a regular hospital neuropathology laboratory, making it a clinically utile technique. Because both subgrouping assays and prognostic FISH markers will need to be performed in a Clinical Laboratory Improvement Amendments–approved laboratory, we suggest that these assays be adopted and optimized in most major neuro-oncology centers, whereas smaller centers may consider sending tissues for analysis at larger centers. Our findings demonstrate the utility of incorporating tumor biology into clinical decision making and offer a novel perspective on risk stratification using FISH applicable on paraffin sections; thus, they could be translated immediately into routine clinical practice.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: David J.H. Shih, Paul A. Northcott, Marc Remke, Andrey Korshunov, Stefan M. Pfister, Michael D. Taylor
Provision of study materials or patients: Jennifer A. Chan, Linda M. Liau, Xing Fan, Robert J. Wechsler-Reya, Anne Jouvet, Carmen de Torres, Roger J. Packer
Collection and assembly of data: All authors
Data analysis and interpretation: David J.H. Shih, Paul A. Northcott, Marc Remke, Andrey Korshunov, Vijay Ramaswamy, Yuan Yao, Robert J. Wechsler-Reya, Erwin G. Van Meir, Stefan M. Pfister, Michael D. Taylor
Manuscript writing: All authors
Final approval of manuscript: All authors

REFERENCES

1. Gajjar A, Chintagumpala M, Ashley D, et al: Risk-adapted craniospinal radiotherapy followed by high-dose chemotherapy and stem-cell rescue in children with newly diagnosed medulloblastoma (St Jude Medulloblastoma-96): Long-term results from a prospective, multicentre trial. Lancet Oncol 7:813-820, 2006

2. Northcott PA, Korshunov A, Pfister SM, et al: The clinical implications of medulloblastoma subgroups. Nat Rev Neurol 8:340-351, 2012

3. Lannering B, Rutkowski S, Doz F, et al: Hyperfractionated versus conventional radiotherapy followed by chemotherapy in standard-risk medulloblastoma: Results from the randomized multicenter HIT-SIOP PNET 4 trial. J Clin Oncol 30:3187-3193, 2012

4. Edelstein K, Spiegler BJ, Fung S, et al: Early aging in adult survivors of childhood medulloblastoma: Long-term neurocognitive, functional, and physical outcomes. Neuro Oncol 13:536-545, 2011

5. Palmer SL, Hassall T, Evankovich K, et al: Neurocognitive outcome 12 months following cerebellar mutism syndrome in pediatric patients with medulloblastoma. Neuro Oncol 12:1311-1317, 2010

6. Lafay-Cousin L, Bouffet E, Hawkins C, et al: Impact of radiation avoidance on survival and neurocognitive outcome in infant medulloblastoma. Curr Oncol 16:21-28, 2009

7. Remke M, Hielscher T, Korshunov A, et al: FSTL5 is a marker of poor prognosis in non-WNT/non-SHH medulloblastoma. J Clin Oncol 29:3852-3861, 2011

8. Remke M, Hielscher T, Northcott PA, et al: Adult medulloblastoma comprises three major molecular variants. J Clin Oncol 29:2717-2723, 2011

9. Dubuc AM, Northcott PA, Mack S, et al: The genetics of pediatric brain tumors. Curr Neurol Neurosci Rep 10:215-223, 2010

10. Castelo-Branco P, Choufani S, Mack S, et al: Methylation of the TERT promoter and risk stratification of childhood brain tumours: An integrative genomic and molecular study. Lancet Oncol 14:534-542, 2013

11. Northcott PA, Jones DT, Kool M, et al: Medulloblastomics: The end of the beginning. Nat Rev Cancer 12:818-834, 2012

12. Pfister SM, Korshunov A, Kool M, et al: Molecular diagnostics of CNS embryonal tumors. Acta Neuropathol 120:553-566, 2010

13. Ramaswamy V, Northcott PA, Taylor MD: FISH and chips: The recipe for improved prognostication and outcomes for children with medulloblastoma. Cancer Genet 204:577-588, 2011

14. Northcott PA, Rutka JT, Taylor MD: Genomics of medulloblastoma: From Giemsa-banding to next-generation sequencing in 20 years. Neurosurg Focus 28:E6, 2010

15. Ellison DW, Onilude OE, Lindsey JC, et al: Beta-catenin status predicts a favorable outcome in childhood medulloblastoma: The United Kingdom Children's Cancer Study Group Brain Tumour Committee. J Clin Oncol 23:7951-7957, 2005

16. Thompson MC, Fuller C, Hogg TL, et al: Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. J Clin Oncol 24:1924-1931, 2006

17. Rausch T, Jones DT, Zapatka M, et al: Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with TP53 mutations. Cell 148:59-71, 2012

18. Pfaff E, Remke M, Sturm D, et al: *TP53* mutation is frequently associated with *CTNNB1* mutation or *MYCN* amplification and is compatible with long-term survival in medulloblastoma. J Clin Oncol 28:5188-5196, 2010

19. Tabori U, Baskin B, Shago M, et al: Universal poor survival in children with medulloblastoma harboring somatic *TP53* mutations. J Clin Oncol 28: 1345-1350, 2010

20. Hernan R, Fasheh R, Calabrese C, et al: ERBB2 up-regulates S100A4 and several other prometastatic genes in medulloblastoma. Cancer Res 63:140-148, 2003

21. Gilbertson RJ, Perry RH, Kelly PJ, et al: Prognostic significance of HER2 and HER4 coexpression in childhood medulloblastoma. Cancer Res 57:3272-3280, 1997

22. Gilbertson RJ, Pearson AD, Perry RH, et al: Prognostic significance of the c-erbB-2 oncogene product in childhood medulloblastoma. Br J Cancer 71:473-477, 1995

23. Eberhart CG, Kratz J, Wang Y, et al: Histopathological and molecular prognostic markers in medulloblastoma: C-myc, N-myc, TrkC, and anaplasia. J Neuropathol Exp Neurol 63:441-449, 2004

24. Grotzer MA, Janss AJ, Phillips PC, et al: Neurotrophin receptor TrkC predicts good clinical outcome in medulloblastoma and other primitive neuroectodermal brain tumors. Klin Padiatr 212:196-199, 2000

25. Segal RA, Goumnerova LC, Kwon YK, et al: Expression of the neurotrophin receptor TrkC is linked to a favorable outcome in medulloblastoma. Proc Natl Acad Sci U S A 91:12867-12871, 1994

26. McCabe MG, Bäcklund LM, Leong HS, et al: Chromosome 17 alterations identify good-risk and poor-risk tumors independently of clinical factors in medulloblastoma. Neuro Oncol 13:376-383, 2011

27. Lo KC, Ma C, Bundy BN, et al: Gain of 1q is a potential univariate negative prognostic marker for survival in medulloblastoma. Clin Cancer Res 13: 7022-7028, 2007

28. Northcott PA, Shih DJ, Peacock J, et al: Subgroup-specific structural variation across 1,000 medulloblastoma genomes. Nature 488:49-56, 2012

29. Northcott PA, Nakahara Y, Wu X, et al: Multiple recurrent genetic events converge on control of histone lysine methylation in medulloblastoma. Nat Genet 41:465-472, 2009

30. Northcott PA, Hielscher T, Dubuc A, et al: Pediatric and adult sonic hedgehog medulloblastomas are clinically and molecularly distinct. Acta Neuropathol 122:231-240, 2011

31. Pfister S, Remke M, Benner A, et al: Outcome prediction in pediatric medulloblastoma based on DNA copy-number aberrations of chromosomes 6q and 17q and the *MYC* and *MYCN* loci. J Clin Oncol 27:1627-1636, 2009

32. Kool M, Korshunov A, Remke M, et al: Molecular subgroups of medulloblastoma: An international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, group 3, and group 4 medulloblastomas. Acta Neuropathol 123:473-484, 2012

33. Clifford SC, O'Toole K, Ellison DW: Chromosome 1q gain is not associated with a poor outcome in childhood medulloblastoma: Requirements for the validation of potential prognostic biomarkers. Cell Cycle 8:787, 2009

34. Clifford SC, Lusher ME, Lindsey JC, et al: Wnt/wingless pathway activation and chromosome 6 loss characterize a distinct molecular sub-group of medulloblastomas associated with a favorable prognosis. Cell Cycle 5:2666-2670, 2006

35. Korshunov A, Remke M, Werft W, et al: Adult and pediatric medulloblastomas are genetically distinct and require different algorithms for molecular risk stratification. J Clin Oncol 28:3054-3060, 2010

36. Batra SK, McLendon RE, Koo JS, et al: Prognostic implications of chromosome 17p deletions in human medulloblastomas. J Neurooncol 24:39-45, 1995

37. Park AK, Lee SJ, Phi JH, et al: Prognostic classification of pediatric medulloblastoma based on chromosome 17p loss, expression of MYCC and MYCN, and Wnt pathway activation. Neuro Oncol 14:203-214, 2012

38. Lamont JM, McManamy CS, Pearson AD, et al: Combined histopathological and molecular cytogenetic stratification of medulloblastoma patients. Clin Cancer Res 10:5482-5493, 2004

39. Gilbertson R, Wickramasinghe C, Hernan R, et al: Clinical and molecular stratification of disease risk in medulloblastoma. Br J Cancer 85:705-712, 2001

40. Mendrzyk F, Radlwimmer B, Joos S, et al: Genomic and protein expression profiling identifies CDK6 as novel independent prognostic marker in medulloblastoma. J Clin Oncol 23:8853-8862, 2005

41. Pan E, Pellarin M, Holmes E, et al: Isochromosome 17q is a negative prognostic factor in poor-risk childhood medulloblastoma patients. Clin Cancer Res 11:4733-4740, 2005

42. Aldosari N, Bigner SH, Burger PC, et al: MYCC and MYCN oncogene amplification in medulloblastoma: A fluorescence in situ hybridization study on paraffin sections from the Children's Oncology Group. Arch Pathol Lab Med 126:540-544, 2002

43. Ellison DW, Kocak M, Dalton J, et al: Definition of disease-risk stratification groups in childhood medulloblastoma using combined clinical, pathologic, and molecular variables. J Clin Oncol 29:1400-1407, 2011

44. Ryan SL, Schwalbe EC, Cole M, et al: MYC family amplification and clinical risk-factors interact to predict an extremely poor prognosis in childhood medulloblastoma. Acta Neuropathol 123:501-513, 2012

45. Scheurlen WG, Schwabe GC, Joos S, et al: Molecular analysis of childhood primitive neuroectodermal tumors defines markers associated with poor outcome. J Clin Oncol 16:2478-2485, 1998

46. Bien-Willner GA, López-Terrada D, Bhattacharjee MB, et al: Early recurrence in standard-risk medulloblastoma patients with the common idic(17)(p11.2) rearrangement. Neuro Oncol 14:831-840, 2012

47. Adamson DC, Shi Q, Wortham M, et al: OTX2 is critical for the maintenance and progression of Shh-independent medulloblastomas. Cancer Res 70:181-191, 2010

48. Korshunov A, Remke M, Kool M, et al: Biological and clinical heterogeneity of MYCN-amplified medulloblastoma. Acta Neuropathol 123:515-527, 2012

49. Schwalbe EC, Williamson D, Lindsey JC, et al: DNA methylation profiling of medulloblastoma allows robust subclassification and improved outcome prediction using formalin-fixed biopsies. Acta Neuropathol 125:359-371, 2013

50. Northcott PA, Dubuc AM, Pfister S, et al: Molecular subgroups of medulloblastoma. Expert Rev Neurother 12:871-884, 2012

51. Dubuc AM, Morrissy AS, Kloosterhof NK, et al: Subgroup-specific alternative splicing in medulloblastoma. Acta Neuropathol 123:485-499, 2012

52. Taylor MD, Northcott PA, Korshunov A, et al: Molecular subgroups of medulloblastoma: The current consensus. Acta Neuropathol 123:465-472, 2012

53. Northcott PA, Korshunov A, Witt H, et al: Medulloblastoma comprises four distinct molecular variants. J Clin Oncol 29:1408-1414, 2011

54. Jones DT, Jäger N, Kool M, et al: Dissecting the genomic complexity underlying medulloblastoma. Nature 488:100-105, 2012

55. Kool M, Koster J, Bunt J, et al: Integrated genomics identifies five medulloblastoma subtypes with distinct genetic profiles, pathway signatures and clinicopathological features. PLoS One 3:e3088, 2008

56. Parsons DW, Li M, Zhang X, et al: The genetic landscape of the childhood cancer medulloblastoma. Science 331:435-439, 2011

57. Robinson G, Parker M, Kranenburg TA, et al: Novel mutations target distinct subgroups of medulloblastoma. Nature 488:43-48, 2012

58. Schwalbe EC, Lindsey JC, Straughton D, et al: Rapid diagnosis of medulloblastoma molecular subgroups. Clin Cancer Res 17:1883-1894, 2011

59. Ellison DW, Dalton J, Kocak M, et al: Medulloblastoma: Clinicopathological correlates of SHH, WNT, and non-SHH/WNT molecular subgroups. Acta Neuropathol 121:381-396, 2011

60. Gibson P, Tong Y, Robinson G, et al: Subtypes of medulloblastoma have distinct developmental origins. Nature 468:1095-1099, 2010

61. Northcott PA, Shih DJ, Remke M, et al: Rapid, reliable, and reproducible molecular sub-grouping of clinical medulloblastoma samples. Acta Neuropathol 123:615-626, 2012

62. Pugh TJ, Weeraratne SD, Archer TC, et al: Medulloblastoma exome sequencing uncovers subtype-specific somatic mutations. Nature 488: 106-110, 2012

63. Cho YJ, Tsherniak A, Tamayo P, et al: Integrative genomic analysis of medulloblastoma identifies a molecular subgroup that drives poor clinical outcome. J Clin Oncol 29:1424-1430, 2011

64. Bourdeaut F, Grison C, Maurage CA, et al: MYC and MYCN amplification can be reliably assessed by aCGH in medulloblastoma. Cancer Genet 206:124-129, 2013

Affiliations

David J.H. Shih, Marc Remke, Vijay Ramaswamy, Betty Luu, Yuan Yao, Xin Wang, Adrian M. Dubuc, Livia Garzia, John Peacock, Stephen C. Mack, Xiaochong Wu, Adi Rolider, A. Sorana Morrissy, Florence M.G. Cavalli, Claudia C. Faria, Stephen W. Scherer, Uri Tabori, Cynthia E. Hawkins, David Malkin, Eric Bouffet, James T. Rutka, and Michael D. Taylor, Hospital for Sick Children; David J.H. Shih, Marc Remke, Vijay Ramaswamy, Yuan Yao, Xin Wang, Adrian M. Dubuc, John Peacock, Stephen C. Mack, and Michael D. Taylor, University of Toronto, Toronto; Boleslaw Lach, McMaster University, Hamilton, Ontario; Jennifer A. Chan, University of Calgary, Calgary, Alberta; Steffen Albrecht, Adam Fontebasso, and Nada Jabado, McGill University, Montreal, Quebec, Canada; Paul A. Northcott, Andrey Korshunov, Marcel Kool, David T.W. Jones, and Stefan M. Pfister, German Cancer Research Center; Stefan M. Pfister, University Hospital Heidelberg, Heidelberg; Ulrich Schüller, Ludwig-Maximilians-University, Munich; Stefan Rutkowski, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; Karel Zitterbart, Masaryk University School of Medicine; Karel Zitterbart and Leos Kren, University Hospital Brno, Brno, Czech Republic; Toshihiro Kumabe and Teiji Tominaga, Tohoku University Graduate School of Medicine, Sendai, Japan; Young Shin Ra, University of Ulsan, Asan Medical Center; Ji-Yeoun Lee, Byung-Kyu Cho, Seung-Ki Kim, and Kyu-Chang Wang, Seoul National University Children's Hospital, Seoul; Shin Jung, Chonnam National University Research Institute of Medical Sciences, Chonnam National University Hwasun Hospital and Medical School, Chonnam, South Korea; Peter Hauser and Miklós Garami, Semmelweis University, Budapest; László Bognár and Almos Klekner, University of Debrecen, Medical and Health Science Centre, Debrecen, Hungary; Shenandoah Robinson, Boston Children's Hospital; Scott L. Pomeroy, Harvard Medical School, Boston, MA; Ali G. Saad, University of Arkansas for Medical Sciences, Little Rock, AR; Linda M. Liau, David Geffen School of Medicine, University of California Los Angeles, Los Angeles; Joanna J. Phillips, Nalin Gupta, and William A. Weiss, University of California San Francisco, San Francisco; Robert J. Wechsler-Reya, Sanford-Burnham Medical Research Institute, La Jolla; Yoon-Jae Cho, Stanford University School of Medicine, Stanford, CA; Giuseppe Cinalli, Ospedale Santobono-Pausilipon; Pasqualino De Antonellis and Massimo Zollo, University of Naples; Massimo Zollo, Ceinge Biotecnologie Avanzate, Naples; Concezio Di Rocco and Luca Massimi, Catholic University Medical School, Rome, Italy; Michael K. Cooper and Reid C. Thompson, Vanderbilt Medical Center, Nashville; Amar Gajjar, St Jude Children's Research Hospital, Memphis, TN; Simon Bailey, Janet C. Lindsey, and Steven C. Clifford, Newcastle University, Northern Institute for Cancer Research, Newcastle upon Tyne, United Kingdom; Erna M.C. Michiels, Pim J. French, Nanne K. Kloosterhof, and Johan M. Kros, Erasmus Medical Center, Rotterdam, the Netherlands; Xing Fan and Karin M. Muraszko, University of Michigan Medical School, Ann Arbor, MI; Rajeev Vibhakar, University of Colorado Denver, Aurora, CO; Charles G. Eberhart, Johns Hopkins University School of Medicine, Baltimore, MD; Maryam Fouladi, University of Cincinnati, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; Michelle Fèvre-Montange and Anne Jouvet, Université de Lyon, Lyon; Franck Bourdeaut, Olivier Delattre, and François F. Doz, Institut Curie and Institut National de la Santé et de la Recherche Médicale U830; François F. Doz, University Paris Descartes, Paris, France; Ian F. Pollack, University of Pittsburgh School of Medicine, Pittsburgh, PA; Jeffrey R. Leonard and Joshua B. Rubin, Washington University School of Medicine, St Louis Children's Hospital, St Louis, MO; Carmen de Torres, Cinzia Lavarino, and Jaume Mora, Hospital Sant Joan de Déu, Barcelona, Spain; James M. Olson, Fred Hutchinson Cancer Research Center, Seattle, WA; Roger J. Packer, Children's National Medical Center, Washington, DC; Erwin G. Van Meir, Emory University School of Medicine, Winship Cancer Institute, Atlanta, GA; Wieslawa A. Grajkowska and Marta Perek-Polnik, Children's Memorial Health Institute, Warsaw, Poland; and Claudia C. Faria, Hospital de Santa Maria, Lisbon, Portugal.

Support

Supported by a Canadian Institutes of Health Research (CIHR) Clinician-Scientist Phase II Award (M.D.T.); the Pediatric Brain Tumor Foundation (M.D.T., J.T.R.); the Terry Fox Research Institute (M.D.T.); Grant No. CA159859 from the National Institutes of Health (M.D.T., R.J.W.-R., W.A.W.); Genome Canada; Genome British Columbia; the Ontario Institute for Cancer Research; Pediatric Oncology Group Ontario; the family of Kathleen Lorette; the Clark H. Smith Brain Tumor Centre; Montreal Children's Hospital Foundation; the Sonia and Arthur Labatt Brain Tumor Research Centre, Hospital for Sick Children; the Chief of Research Fund; the Cancer Genetics Program; the Garron Family Cancer Centre; B.R.A.I.N. Child; CIHR Grant No. ATE-110814; CIHR Institute of Cancer Research Grant No. AT1-112286; C17; the TÁMOP-4.2.2.A-11/1/KONV-2012-0025 Project (cofinanced by the European Union and European Social Fund); the Frederick Banting and Charles Best Canada Graduate Scholarship (D.J.H.S.); the Dr Mildred Scheel Foundation for Cancer Research fellowship (M.R.); and Grant No. MH CZ-DRO FNBr 65269705 from University Hospital Brno (K.Z.).

Acknowledgment

We thank Susan Archer for technical writing and Stéphanie Reynaud for excellent assistance.

Appendix

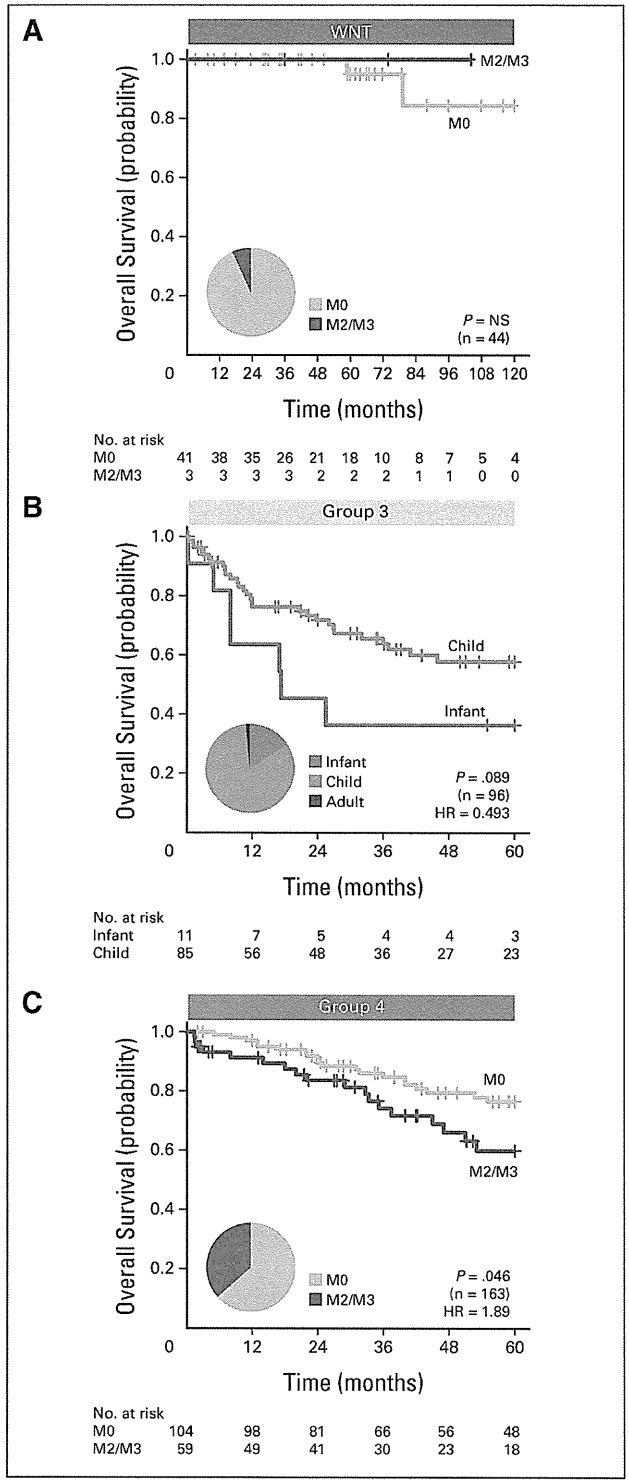

Fig A1. (A) Ten-year overall survival curves for WNT medulloblastoma by metastatic status. (B) Overall survival curves for age groups within Group 3 subgroup (infant, age < 3 years; child, age 3 to < 16 years). (C) Overall survival curves for metastatic status for Group 4 subgroup. Numbers below *x*-axis represent patients at risk of event; statistical significance evaluated by log-rank tests; hazard ratio (HR) estimates derived from Cox proportional hazards analyses. NS, not significant.

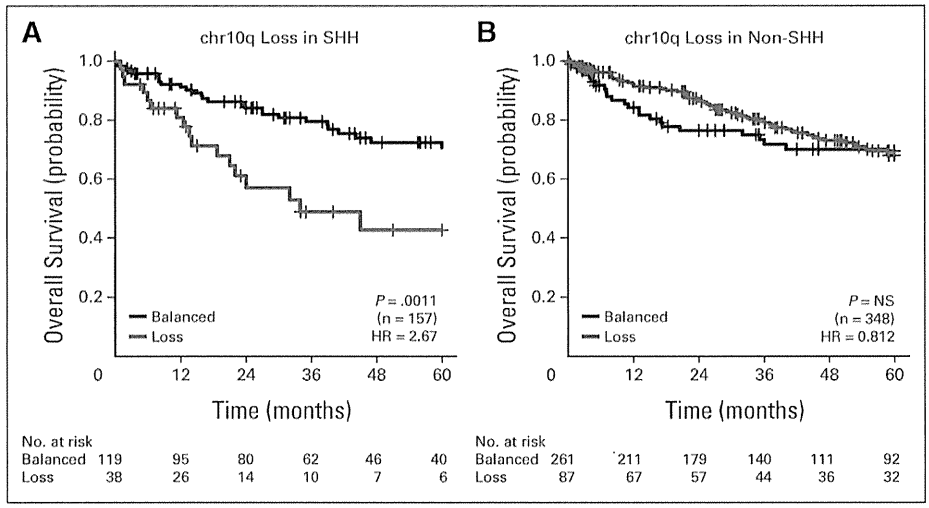

Fig A2. Overall survival curves for chromosome 10q (chr10q) status in (A) SHH and (B) non-SHH medulloblastomas; survival differences evaluated by log-rank tests; hazard ratio (HR) estimates derived from Cox proportional hazards analyses. NS, not significant.

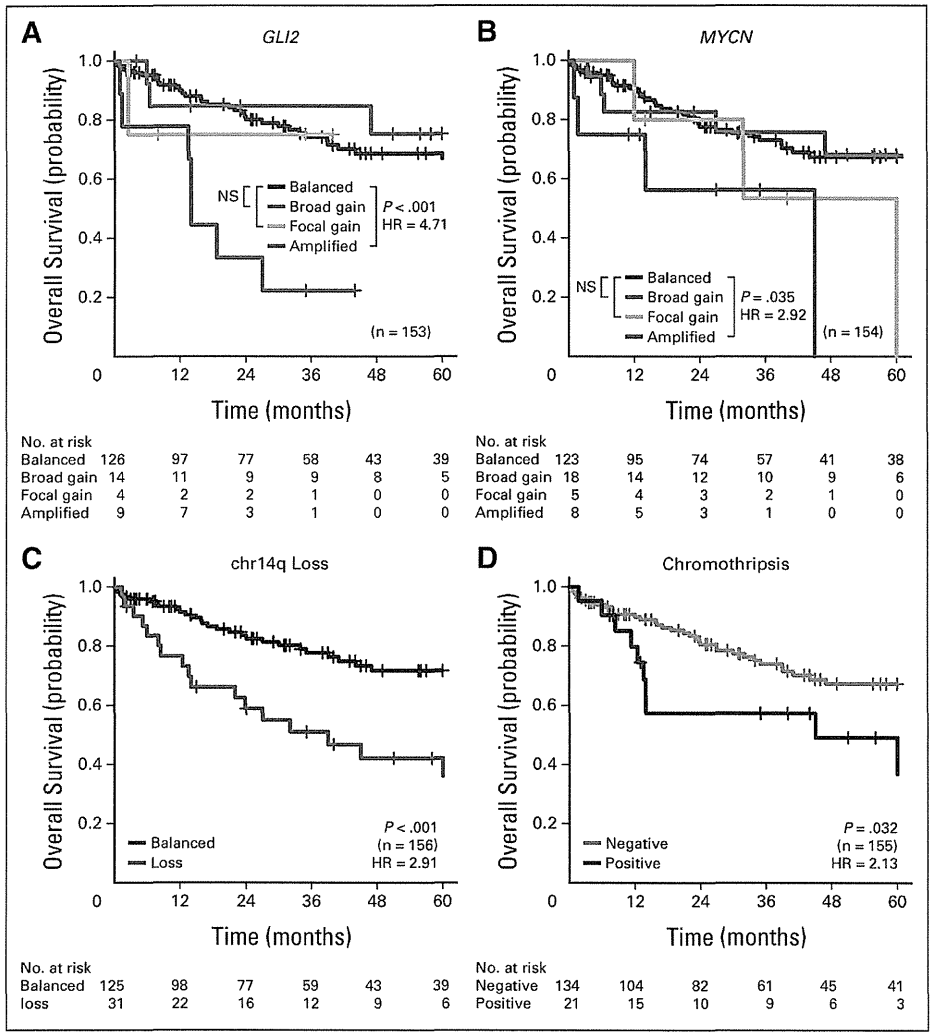

Fig A3. Clinical prognostication of patients with SHH medulloblastoma. Overall survival curves for (A) *GLI2* copy-number status, (B) *MYCN* copy-number status, (C) chromosome 14q (chr14q) status, and (D) chromothripsis status. HR, hazard ratio; NS, not significant.

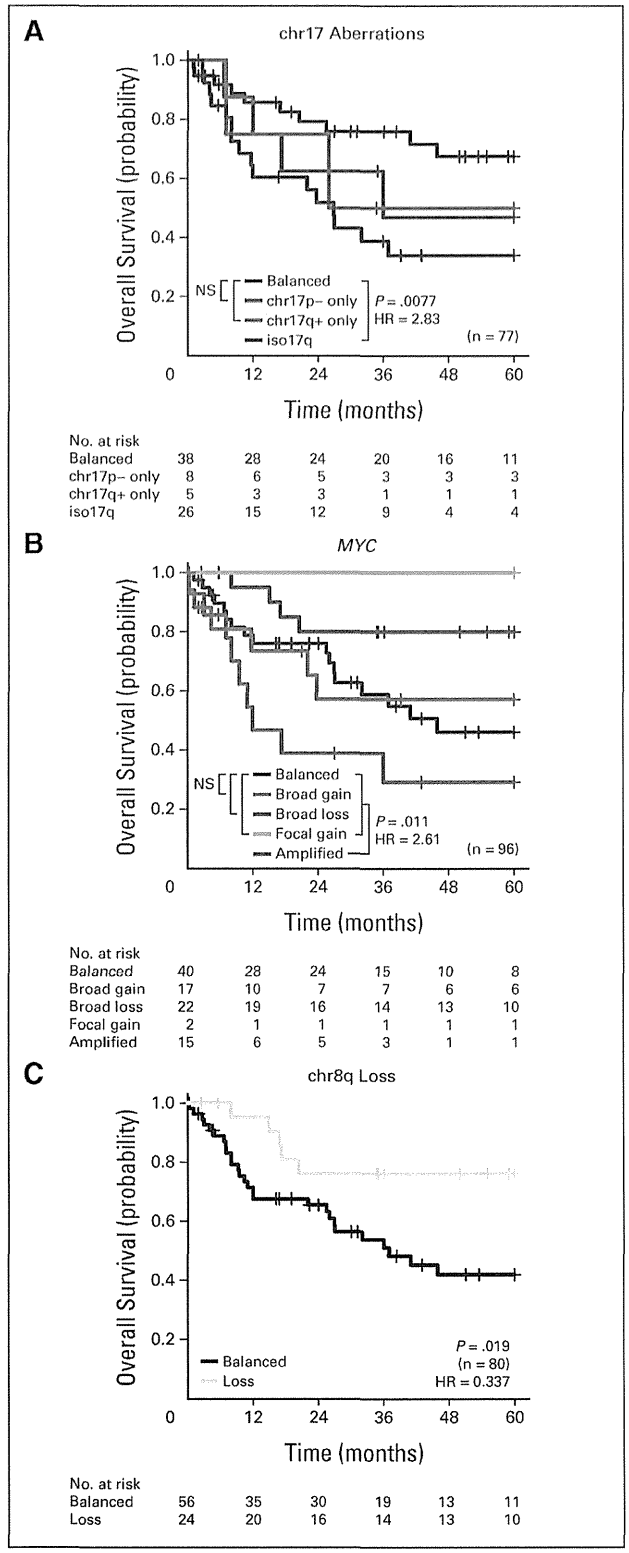

Fig A4. Clinical prognostication of patients with Group 3 medulloblastoma. Overall survival curves for (A) chromosome 17 (chr17) copy-number aberrations, (B) *MYC* copy-number status, and (C) chr8q status. HR, hazard ratio; iso, isochromosome; NS, not significant.

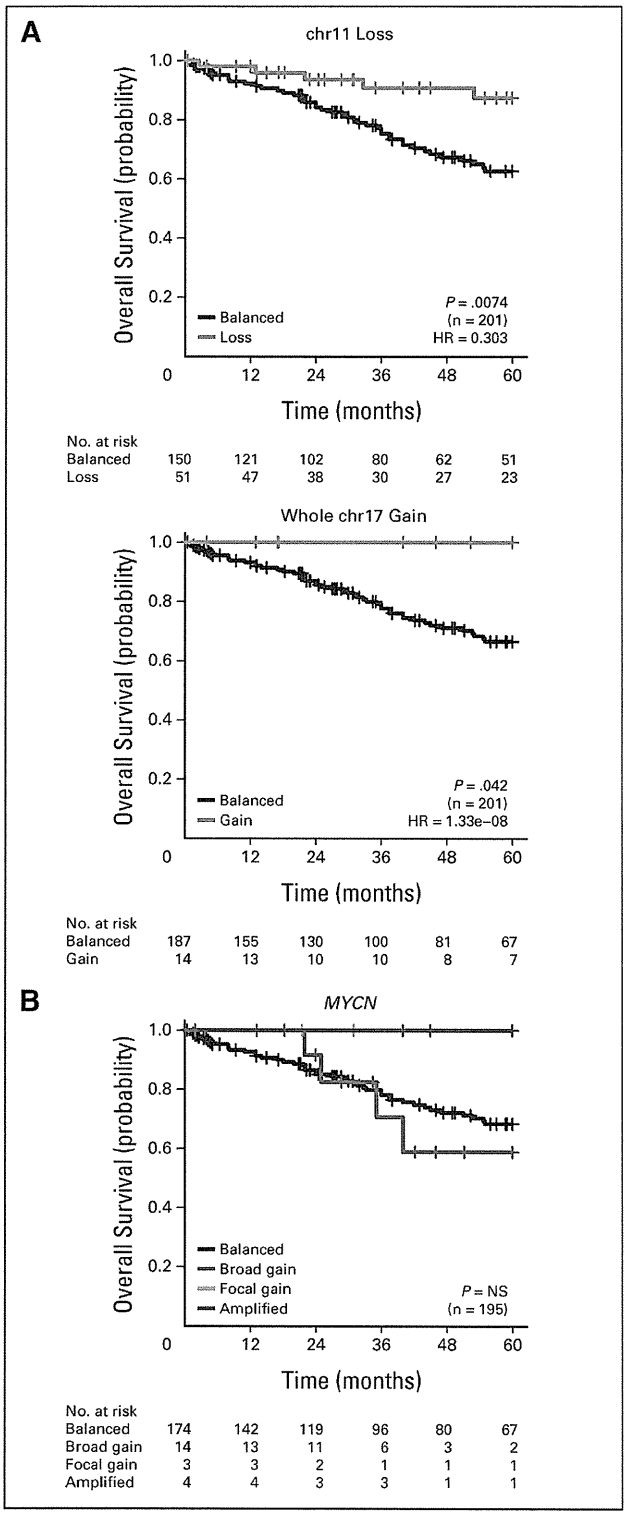

Fig A5. Clinical prognostication of patients with Group 4 medulloblastoma. Overall survival curves for (A) chromosome 11 (chr11) status and whole chr17 status and (B) *MYCN* copy-number status. HR, hazard ratio; NS, not significant.

J Neurosurg 120:1288–1297, 2014

Rapid and sensitive intraoperative detection of mutations in the *isocitrate dehydrogenase 1* and *2* genes during surgery for glioma

Laboratory investigation

Masayuki Kanamori, M.D., Ph.D.,[1] Atsuo Kikuchi, M.D., Ph.D.,[2] Mika Watanabe, M.D., Ph.D.,[3] Ichiyo Shibahara, M.D., Ph.D.,[1] Ryuta Saito, M.D., Ph.D.,[1] Yoji Yamashita, M.D., Ph.D.,[4] Yukihiko Sonoda, M.D., Ph.D.,[1] Toshihiro Kumabe, M.D., Ph.D.,[5] Shigeo Kure, M.D., Ph.D.,[2] and Teiji Tominaga, M.D., Ph.D.[1]

Departments of [1]Neurosurgery and [2]Pediatrics, Tohoku University Graduate School of Medicine; [3]Department of Pathology, Tohoku University Hospital, Sendai; [4]Department of Neurosurgery, Miyagi Cancer Center, Natori, Miyagi; and [5]Department of Neurosurgery, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan

Object. Intraoperative diagnosis is important in determining the strategies during surgery for glioma. Because the mutations in the *isocitrate dehydrogenase 1* and *2* (*IDH1* and *IDH2*) genes have diagnostic, prognostic, and predictive values, the authors assessed the feasibility and significance of a simplified method for the intraoperative detection of *IDH1* and *IDH2* gene mutations.

Methods. Rapid DNA extraction, amplification with conventional polymerase chain reaction (PCR) or co-amplification at lower denaturation temperature PCR (COLD-PCR), and fluorescence melting curve analysis with adjacent hybridization probes were performed for the intraoperative detection of *IDH1* and *IDH2* mutations in 18 cases of suspected nonneoplastic lesions and low- and high-grade gliomas and in 3 cases of radiation necrosis.

Results. DNA extraction for detection of the mutation took 60–65 minutes. The results of this assay showed complete correlation with that of Sanger sequencing. The sensitivity for detection of mutations in a background of wild-type genes was 12.5% and 2.5% in conventional PCR and COLD-PCR, respectively. The diagnosis of glioma was established in 3 of 5 cases in which definitive diagnosis was not obtained using frozen sections, and information was obtained for the discrimination of glioblastoma or glioblastoma with an oligodendroglioma component from anaplastic glioma or secondary glioblastoma. This assay also detected a small fraction of tumor cells with *IDH1* mutation in radiation necrosis.

Conclusions. These methods provide important information for establishing the differential diagnosis between low-grade glioma and nonneoplastic lesions and the diagnosis for subtypes of high-grade glioma. Although tumor cells in radiation necrosis were detected with a high sensitivity, further investigation is necessary for clinical application in surgery for recurrent glioma.
(http://thejns.org/doi/abs/10.3171/2014.3.JNS131505)

Key Words • **glioma** • **intraoperative molecular diagnosis** • ***IDH1*** • ***IDH2*** • **oncology**

Intraoperative diagnosis is important in determining the strategies to use during surgery for glioma. For example, in the differential diagnosis for low-grade glioma and nonneoplastic lesions, information on the sensitivity to radiation therapy and chemotherapy and the presence of viable tumor cells in recurrent glioma tissue influence the extent of the resection to be performed. Although the rates of diagnostic accuracy of intraoperative frozen-section and cytolytic smear-based examinations are 97.2% and 89.8%, respectively,[21,22] intraoperative diagnosis sometimes cannot give objective answers to these issues.

Recently, somatic mutations have been identified in the *isocitrate dehydrogenase 1* and *2* (*IDH1* and *IDH2*) genes in adult gliomas.[1,20,27] These mutations are frequently found in astrocytic and oligodendroglial tu-

Abbreviations used in this paper: COLD = co-amplification at lower denaturation temperature; FMCA = fluorescence melting curve analysis; IDH = isocitrate dehydrogenase; PCR = polymerase chain reaction; Tc = denaturation temperature; Tm = melting temperature.

This article contains some figures that are displayed in color online but in black-and-white in the print edition.

mors at a very early stage of gliomatogenesis, such as in WHO Grade II and III astrocytic and oligodendroglial tumors, and in secondary glioblastomas, but not in primary glioblastoma.[1,18,20] Thus, these mutations may have diagnostic value for differentiating low-grade glioma from nonneoplastic lesions, non-CNS tumors, or reactive gliosis[3,8] and for differentiating anaplastic glioma from primary glioblastoma or radiation necrosis.[3] Additionally, the mutations have been shown to be prognostic[1,20,25] in high-grade gliomas and we can expect them to be present after radiation therapy and chemotherapy.[11,26,29] Based on these findings, we considered that the intraoperative detection of these mutations has potential for clinical application.

The mutations in *IDH1* and *IDH2* are restricted to analogous codons, exon 4 at codon 132 and exon 4 at codon 172, respectively. Various methods have been reported to detect these mutations, including pyrosequencing,[5] immunohistochemistry with antibody specific to mutant IDH1 R132H protein,[3,4] enrichment of low-frequency mutant sequence with co-amplification at lower denaturation temperature polymerase chain reaction (COLD-PCR) combined with high-resolution melting[2] or with deep sequencing,[15] and conventional PCR, followed by fluorescence melting curve analysis (FMCA) with adjacent hybridization probes (HybProbe, Roche Applied Science),[8] which provide faster and more sensitive detection than conventional direct sequencing with the Sanger method. These methods allow rapid and sensitive detection of DNA from frozen or formalin-fixed tissues, but application to intraoperative detection of *IDH1* and *IDH2* mutations has not been demonstrated.

The COLD-PCR technique has been reported to be a simple method for identifying low-frequency mutations in the *TP53*, *KRAS*, and *EGFR* genes.[13] During the PCR process, a critical denaturation temperature (Tc) occurs at a temperature lower than the melting temperature (Tm). Below Tc, the PCR efficiency drops abruptly. The Tc is dependent on the DNA sequence, and DNA amplicons differing by a single nucleotide have different amplification efficiencies, with the PCR denaturation temperature set to Tc. Because most mutations found in the *IDH1* gene are Tc-reducing mutations, COLD-PCR with a denaturation temperature lower than the Tc of the wild-type sequence and higher than that of the mutant sequence should amplify only the mutant sequence,[3] thus enriching the low-frequency mutant sequence.

In this study, we developed systems based on conventional PCR and COLD-PCR, followed by FMCA with HybProbe, to achieve rapid, sensitive, and specific intraoperative detection of the *IDH1* and *IDH2* mutations and examined the feasibility and provision of useful information during surgery for glioma.

Methods

Tissues for Validation

We have previously reported the detection of mutations of *IDH1* and *IDH2* genes in glioma, based on Sanger sequencing analysis of DNA extracted with a QIAamp DNA Mini Kit (Qiagen).[27] To confirm concordance between the standard method for sequencing analysis and conventional PCR, followed by FMCA with HybProbe from rapidly extracted DNA, we analyzed 14 tumors previously examined for mutation status: 5 gliomas with *IDH1* mutation, including R132H (CGT→CAT) in 3, R132G (CGT→GGT) in 1, and R132S (CGT→AGT) in 1; 2 gliomas with *IDH2* mutation at R172 K (AGG→AAG); and 7 gliomas without mutations in the *IDH1* or *IDH2* gene. To determine the sensitivity for detection, we examined a dilution series with 50%, 25%, 12.5%, 5%, 2.5%, 1%, 0.25%, and 0% of DNA from the R132H mutation in the *IDH1* gene in a DNA sample from a tumor with the wild-type *IDH1* gene. These DNA samples were amplified with conventional PCR and COLD-PCR, followed by FMCA with HybProbe. Each experiment was performed in triplicate.

Intraoperative Detection of Mutation

Intraoperative detection of *IDH1* and *IDH2* mutations was performed in 18 patients who have undergone cytoreductive surgery or biopsy for intraparenchymal tumors since May 2012. This study was conducted with the approval of the ethics committee of Tohoku University School of Medicine, and written informed consent was obtained from all patients.

Detection of Mutation in Radiation Necrosis

Oligodendroglial tumors carrying the *IDH1* mutation develop recurrent disease with viable tumor cells rather than undergo radiation necrosis.[12] To assess the significance of intraoperative detection of *IDH1* mutation in recurrent cases for differentiation of recurrence from radiation necrosis and reactive astrocytes, we simulated the intraoperative diagnosis in such cases by using DNA rapidly extracted from stored frozen tissues from 3 rare cases diagnosed as radiation necrosis based on small fractions of tumor cells or reactive astrocytes using H & E staining of formalin-fixed, paraffin-embedded sections after treatment of glioma carrying the *IDH1* mutation.

Histological Processing for Intraoperative Detection of Mutation

Tumor specimens (10–20 mg) were cut into two pieces. One piece was immediately frozen in liquid nitrogen, followed by rapid extraction of DNA. The other piece was processed for intraoperative and postoperative histological examination. The intraoperative histological diagnosis was established by the same neuropathologist (M.W.) using current WHO criteria,[14] based on H & E staining.

Rapid DNA Extraction, Amplification, and FMC

DNA from the snap-frozen tissues was extracted with UltraClean Tissue & Cells DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the manufacturer's protocol. The amplification and detection of *IDH1* and *IDH2* mutations was performed using LightCycler 1.5 (Roche Applied Science), as described previously with some modifications.[3,9] Briefly, the loci flanking codon 132 of the *IDH1* gene and codon 172 of the *IDH2* gene were

amplified with conventional PCR. The forward and reverse primer sequences for the *IDH1* gene were 5'-CGG TCTTCAGAGAAGCCATT-3' and 5'-GCAAAATCACA TTATTGCCAAC-3'. For mutation detection, HybProbes complementary to the wild-type allele were designed. The HybProbe sequences for the *IDH1* gene were 5'-CCCCGG CTTGTGAGTGGATGGGTAAAACCTA-Fluorescein-3', and 5'-LC Red 640-CATCATAGGTCGTCATG CTTAT-Phosphate-3'. The forward and reverse primer sequences for the *IDH2* gene were 5'-TTCCGGGAG CCCATCAT-3' and 5'-TGCCCAGGTCAGTGGAT-3'. For mutation detection, HybProbes complementary to the wild-type allele were designed. The HybProbe sequences for the *IDH2* gene were 5'-CGTGCCTGCCAATGGTGA-Fluorescein-3', and 5'-LC Red 640-GGCTTGGTCCAG CCAGGGACTAGG-Phosphate-3' (Nippon Gene Research Laboratories). Extracted DNA (20 ng) was amplified with AmpliTaq Gold (Applied Biosystems) in a glass capillary tube in a 20-μl reaction volume. The conventional PCR conditions for the amplification of *IDH1* and *IDH2* genes were as follows: 95°C for 10 minutes, 40 cycles of PCR consisting of denaturation at 95°C for 15 seconds, and annealing and extension at 58°C for 30 seconds.

Alternatively, the same locus of the *IDH1* gene was amplified with COLD-PCR to achieve more sensitive detection. The same reaction mixture was amplified with COLD-PCR as follows: 95°C for 10 minutes and 10 cycles of PCR consisting of denaturation at 95°C for 15 seconds and annealing and extension at 58°C for 30 seconds; then 30 cycles at 79°C for 15 seconds and annealing and extension at 58°C for 30 seconds.

Postamplification FMCA with HybProbe was performed by gradual heating of samples at 0.1°C/sec from 40°C to 95°C. Fluorescence melting peaks were built by plotting the negative derivative of the fluorescent signal corresponding to the temperature (−dF/dT) with LightCycler software 4.1, as described previously.[8]

We confirmed the results of intraoperative detection of the mutations in *IDH1* and *IDH2* genes by Sanger sequence analysis and immunohistochemistry for IDH1 R132H protein as mentioned below.

Immunohistochemical Examination

The primary antibody used in this study was mouse monoclonal antibody for anti–IDH1 R132H protein (Dianova; 1:400). Pathological materials were immediately fixed in 10% buffered formalin (Wako Pure Chemical Industries, Ltd.) at room temperature for several days. Thick paraffin sections (2 μm) were deparaffinized, rehydrated, and incubated in 0.3% hydrogen peroxide in 100% methanol for 10 minutes at room temperature to block endogenous peroxidase. Antigen retrieval was accomplished by microwaving for 5 minutes in citric acid buffer (2 mmol/L citric acid and 9 mmol/L trisodium citrate dihydrate, pH 6.0). After blocking of nonspecific binding, slides were incubated with primary antibodies overnight at 4°C. Sections were treated with biotinylated anti–mouse immunoglobulin G for 30 minutes at room temperature, followed by peroxidase-conjugated streptavidin for 30 minutes at room temperature, using the Histofine kit (Nichirei Biosciences, Inc.). Sections were developed with diaminobenzidine solution (0.01 M 3.3'-diaminobenzidine in 0.05 M Tris-HCl buffer, pH 7.6, and 0.006% hydrogen peroxide). Nuclei were counterstained with hematoxylin.

Results

Detection of Wild-Type and Mutant IDH1 *and* IDH2 *Genes With Conventional PCR, Followed by FMCA With HybProbe*

DNA extraction required 15 minutes, and amplification and detection were completed within 50 minutes. A single peak, representing wild-type allele, was detected in the DNA of 7 tumors with wild-type *IDH1* and *IDH2* genes (Fig. 1A and E). All heterozygous mutations were successfully detected with different Tm from that of wild-type allele in 5 tumors with IDH R132H, 1 tumor with IDH1 R132G, and 1 tumor with R132S mutation (Fig. 1B–D). Similarly, mutation of the *IDH2* gene was detected under the same PCR conditions (Fig. 1F). Dilution analysis detected as low as 12.5% of the *IDH1* mutation (R132H) against a background of the wild-type allele (Fig. 2A).

Detection of Mutant Sequence of IDH1 *Gene With COLD-PCR and FMCA With HybProbe*

To enhance the sensitivity of detection for the *IDH1* gene, we applied COLD-PCR in the amplification steps. Amplification with COLD-PCR and detection with FMCA with HybProbe was completed within 45 minutes. Dilution analysis showed this assay detected 2.5% of the mutated allele (R132H) against a background of the wild-type allele (Fig. 2B).

Intraoperative Detection of Mutant Sequence From Surgical Specimen

During cohort periods, 18 cases were enrolled in this study. Mutation of the *IDH1* gene was detected in 10 of 18 cases. The results of intraoperative detection for *IDH1* and *IDH2* are shown in Table 1. These results completely corresponded with those of Sanger sequencing analysis and immunohistochemistry for IDH1 R132H protein. All mutations detected were IDH1 R132H. There were no false-positive or false-negative results.

Detection of IDH1 *and* IDH2 *Mutations in Low-Grade Glioma*

During the cohort period, the morphological diagnosis based on frozen-section examination was a low-grade glioma in 3 cases and a nonneoplastic lesion difficult to differentiate from low-grade glioma in 5 cases. The diagnoses of low-grade glioma were based on findings such as slightly increased cellularity, nuclear atypia, and uneven distribution of nuclei. A summary of the intraoperative diagnoses using frozen sections; final diagnoses using formalin-fixed, paraffin-embedded sections; and intraoperative detection of *IDH1* and *IDH2* gene mutations is shown in Table 1 (Cases 1–8). In the 5 cases of low-grade glioma and nonneoplastic lesions that were difficult to differentiate, detection of the *IDH1* gene mutation led to an intraoperative definitive diagnosis of low-grade glioma