Epigenetic subclassification of meningiomas based on genome-wide DNA methylation analyses

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Meningiomas are among the most common intracranial tumors and are mostly curable by surgical resection. However, some populations of meningiomas with benign histological profiles show malignant behavior. The reasons for this inconsistency are yet to be ascertained, and novel diagnostic criteria other than the histological one are urgently needed. The aim of the present study is to subclassify meningiomas from the viewpoint of gene methylation and to determine the subgroup with malignant characteristics. Thirty meningiomas were analyzed using microarrays for 6157 genes and were classified into three clusters on the basis of their methylation status; these were found to be independent of the histological grading. One of the clusters showed a high frequency of recurrence, with a marked accumulation of methylation in a subset of genes. We hypothesized that the aggressive meningiomas universally share characteristic methylation in certain genes; therefore, we chose the genes that strongly contributed to cluster formation. The quantified methylation values of five chosen genes (HOXA6, HOXA9, PENK, UPK3A and IDGBP1) agreed well with microarray findings, and a scoring system consisting of the five genes significantly correlated with a high frequency of recurrence in an additional validation set of 32 patients. Of particular note is that three cases with malignant transformation already showed hypermethylation at histologically benign stage. In conclusion, a subgroup of meningiomas is characterized by aberrant hypermethylation of the subset of genes in the early stage of tumorigenesis, and our findings highlight the possibility of speculating potential malignancy of meningiomas by assessing methylation status.

Introduction

Meningiomas account for 24–30% of primary intracranial tumors with clinical malignancy, principally predicted by histological grading based on World Health Organization (WHO) criteria. Grade I meningiomas are considered benign and curable by appropriate surgical resection, grade II (intermediate grade/atypical) meningiomas have a high recurrence rate reaching 40% per 5-year period and grade III (anaplastic) show the worst prognosis (1–3). However, some grade I meningiomas show early or frequent recurrence similar to higher-grade meningiomas, despite their benign histological profile. To resolve this discrepancy, in 2007, the WHO revised their criteria based on morphological findings, emphasizing on mitotic activity and brain invasion (4,5); however, so far, there has been no interobserver and interinstitutional uniformity in the pathological diagnosis. One of the reasons for this inconsistency is the histological similarities between tumors in each grade, as high-grade meningiomas are generally considered to progress from lower-grade meningiomas (6). Furthermore, in some grade I meningiomas, recurrent tumors clearly display a histologically benign profile with no discernable difference from non-recurrent types. The Simpson grade is a clinical grading scale used to classify the extent of postoperative residual tumor, and it has been used as a predictor for recurrence. However, the Simpson grade is highly subjective, with its predictive outcome varying among surgeons. These facts indicate the limitations of using a histological and surgical classification system to grade tumors. There is, thus, an urgent need for a non-histological subclassification or marker system that reflects potential malignant characteristics of meningiomas.

The biological marker SI00A5 (7), the loss of NDRG2 (8) and the suppression of NF2 homologs and interacting proteins such as DAL-1 and TSCL1 (9,10) have been linked with the aggressiveness of meningiomas. In recent years, epigenetic alterations represented by gene methylation have drawn attention as one of the most compelling research subjects. The reasons for this are that various epigenetic alterations occur in the early stages of tumorigenesis (11) and are potentially reversible by epigenetic-regulating agents. However, biomarkers to predict the potential aggressiveness of histologically benign meningiomas have not been conclusively established; only the promoter hypermethylation of TIMP3 has been reported as a marker of malignancy in grade III meningiomas (12). However, since gene methylation occurs as a result of diverse alterations in widespread loci of the genome in various subtypes of tumors, comprehensive genome-wide DNA methylation analysis is warranted.

In the present study, we subclassified histologically benign and intermediate-grade meningiomas into three subgroups based on a genome-wide methylation analysis. The subclassifications were found to correlate well with clinical recurrence/progression but not with classical predictors, e.g. histological grades, Simpson grades and age. Furthermore, we selected five hub genes from hypermethylated genes in the clinical malignant cluster and established a scoring system. The simplified scoring system showed high reproducibility in the validation samples. Our findings indicated the presence of subgroups with potential malignant characteristics in conjunction with methylation status and the possibility of prediction by the methylation quantification in several genes.

Materials and methods

Collection of tumor and control tissues

Forty-three samples of frozen meningioma tissue (WHO grades I and II) were retrospectively randomly selected. After DNA quality check, 20 samples of WHO grade I and 10 of WHO grade II meningiomas were finally chosen. The clinical information of the patients is shown in accordance with reporting recommendation for tumor marker prognostic studies criteria (13) in Supplementary Table 1 (available at Carcinogenesis online). The validation set consisted of 32 formalin-fixed paraffin-embedded samples from our affiliated hospitals as a validation set (Supplementary Table 2, available at Carcinogenesis online). The validation set consisted of...
13 tumors classified as WHO grade I and 19 tumors classified as WHO grade II. There was no bias between the two sample sets in terms of clinical features, such as age, Simpson grade, gender and postoperative treatments.

Tumor recurrence/progression was evaluated by magnetic resonance imaging at the 3-month intervals on the basis of Response Evaluation Criteria In Solid Tumors guideline version 1.1.

Genomic DNA extraction and bisulfite treatment
Genomic DNA was isolated from 20–25 mg of fresh-frozen samples or five slices (10 μm per slice) of formalin-fixed paraffin-embedded samples using QIAamp DNA Mini Kit (QIAGEN, Tokyo, Japan). Deparaffinization using xylene and ethanol was carried out for formalin-fixed paraffin-embedded samples before DNA extraction. Extracted DNA (1 μg) was then subjected to bisulfite conversion using EpiTect Bisulfite Kit (QIAGEN) and diluted to 10 ng/μl before polymerase chain reaction amplification.

Methylated CpG island amplification microarrays
Global analysis by methylated CpG island amplification microarrays (MCAMs) using genomic DNA from meningiomas and control specimens was carried out.

MCAM was performed according to methods described previously (14). Briefly, 2 μg of genomic DNA was digested with 100 U Methyl-sensitivity restriction endonuclease Smal (New England Biolabs, Ipswich, MA) for 8 h at 20°C. This step was repeated. Subsequently, the DNA was digested with 20 U methylation-insensitive restriction endonuclease Xmal for 9 h at 37°C. Digested DNA (500 ng) was ligated to adaptors. After filling in the overhanging ends of the ligated DNA fragments at 72°C, DNA was amplified using 100 pmol of RMCA24 primer under the following reaction conditions: 95°C for 3 min, 25 cycles of 1 min at 95°C and 3 min at 77°C. The products were labeled with Cy5 (red) for tumor samples and Cy3 (green) for control samples using a random-primed Klenow polymerase reaction (Invitrogen, Carlsbad, CA) at 37°C for 3 h. The labeled samples were then hybridized to the custom-made 4 × 44K human promoter array containing 15 134 probes corresponding to 6157 unique genes (Agilent, Santa Clara, CA) in the presence of Cot-1 DNA for 40 h at 65°C. After the arrays were washed according to the manufacturer’s protocol, they were scanned on an Agilent scanner using Feature Extraction software (Agilent).

Quantification of promoter methylation by bisulfite pyrosequencing
To validate the results of MCAMs, we quantified methylation rate by bisulfite pyrosequencing. Primers for bisulfite pyrosequencing were designed by PSQ assay design (Biotage AB, Kungsgatan, Sweden). For all genes, the reproducibility and quantitative capability were confirmed using SssI methylase-treated DNA (New England Biolabs) as a positive control, peripheral blood DNA as a negative control, both these types of DNA mixed in equal quantities and sample DNA. The primer sequences are listed in Supplementary Table 3 (available at Carcinogenesis online). The bisulfite pyrosequencing was carried out according to methods described previously (15,16). The methylation rates at different CpG sites measured by pyrosequencing were averaged to represent the methylation levels of these genes. The conditions for polymerase chain reaction products were confirmed by electrophoresis with 3% agarose gel. In our previous articles, genes with methylation levels >15% were considered methylation positive because lower values could not easily be distinguished from background (17,18).

Analyses of MCAMs and statistical tools
The ratio of tumor signal to control signal calculated from the MCAM results was described as the signal ratio. To avoid an artificial effect of excess value, signal ratios >10 and control signals <600 were corrected to 10 and 600, respectively. A signal ratio with ≥2 increment was considered hypermethylation on the basis of our previous study (16), and the reliability of this criterion in the present study was confirmed using bisulfite pyrosequencing technique. The hierarchical cluster analysis for 6157 genes was performed using Cluster 3.0 software based on an agglomerative hierarchical clustering algorithm (http://rana.lbl.gov/EisenSoftware.htm), and the heat map was constructed using Java TreeView.

Consensus clustering has recently been used in many biomedical studies (19) because it can provide statistical stabilities of the identified clusters. Within the consensus clustering, k-means clustering with the Euclidean distance metric was used as the basic clustering option. For k ranging from 2 to 5, the k-means clustering was run >10 000 iterations with the subsampling ratio of 0.95. The consensus matrix for the purpose of visualization and cluster identification, hierarchical clustering with the Euclidean distance metric and the complete linkage option was applied to the estimated consensus matrix. The identified clusters were validated and confirmed using consensus cluster dependence factor plot analysis (20). The analysis of variance between clinical features and clusters was performed by the Kruskal–Wallis test using PASW Statistic (SPSS, Chicago, IL), and the Games–Howell test was added if a feature was significantly different among clusters. The significance of P value in each test was determined at P < 0.05.

To identify the genes showing significant differences among clusters, we calculated the P value by the Kruskal–Wallis test using the statistical software R (R foundation, Vienna, Austria), and we chose genes satisfying P < 0.01. To avoid false positives with genes with low signal ratios, the genes commonly methylated in more than one-third of any three clusters were selected according to our previous study (17). In addition, the pathway analysis using GeneSpring GX (Agilent) was performed for further refinement of genes.

Results
Quality assurance of MCAMs
MCAMs were performed by a two-color method matched to the same-gender control samples, and the reliability of MCAMs and the criteria for it were assured before data analyses. The dye swap experiment using the same sample (case #3) showed good reproducibility with a high determination coefficient (R² = 0.819; Figure 1A). Subsequently, to validate our criterion for hypermethylation (≥2 increment of the signal ratio) in this study, a signal ratio of five arbitrarily selected genes (RECS, CHAD, HIF3A, UPK3A and SPOCK2) in MCAMs was compared with the quantitative methylation values of the genes in bisulfite pyrosequencing. The quantitative capability and reproducibility of bisulfite pyrosequencing in these five genes had been previously confirmed using SssI methylase-treated DNA, peripheral blood DNA and tumor samples (data not shown). The positive test of methylation in bisulfite pyrosequencing was defined as 15% in agreement with previous studies (17,18,21,22) because lower values could not easily be distinguished from background. In addition, the methylation level of the five genes in normal dura matter was 13–15% (data not shown). As shown in Figure 1B, our criterion based on signal ratio correlated well with the actual quantitative values and showed acceptable sensitivity and specificity (89 and 86%, respectively).

MCAMs classification correlates with recurrence in meningiomas
Thirty meningiomas were distributed bimodally according to the number of methylated genes (Figure 1C). The unsupervised hierarchical cluster analysis classified 30 meningiomas into three clusters (clusters 1–3) based on the similarity of the methylation pattern (Figure 1B). The mean number of hypermethylated genes (signal ratio >2) in samples classified into clusters 1–3 was 164.6, 323.4 and 345.6, respectively (cluster 1 versus clusters 2 and 3; P < 0.001). The heat map in Figure 1D displays the 198 significantly different genes among the clusters. Eight of nine samples with >350 hypermethylated genes shown in Figure 1C were clustered into cluster 1, whereas less methylated tumors with <200 methylated genes completely were classified into cluster 1. Due to the limited sample size, we conducted consensus clustering analysis in which k-means clustering was used as the base clustering method and the number of clusters k = 2–5 were examined. Supplementary Figure 1A, available at Carcinogenesis online, displays the dendograms and heat maps of the consensus matrix for k = 2–5. The figure indicates that the clustering stability increases for k = 2 and 3 but not for k = 4 and 5 (Supplementary Figure 1B, available at Carcinogenesis online). We examined the members of the three clusters identified in the consensus matrix (for k = 3) and found that they almost perfectly match with the original three clusters in Figure 1D (Supplementary Figure 1C, available at Carcinogenesis online).

Clinical features, including gender, age, WHO grade, Simpson grade, perifocal edema and recurrence, were statistically compared between the three clusters. The proportion of the WHO grades did not show a significant correlation with cluster formation (P = 0.417). Similarly, age, Simpson grade and perifocal edema did not correlate with the subclassification based on methylation status (P = 0.891, 0.863 and 0.374, respectively). In contrast, male and recurrence were significantly different among clusters (P = 0.001 and 0.004, respectively). Compared with cluster 1, male cases were significantly
involved in clusters 2 and 3 (\(P = 0.035\) and 0.001, respectively), and more interestingly, recurrent cases accumulated in cluster 3 compared with clusters 1 and 2 (\(P = 0.004\) and 0.081, respectively).

**A novel scoring system associated with meningioma recurrence/progression**

The list of 198 genes in total, with significant differences among clusters (\(P < 0.01\)) and accumulation of methylated samples in any of the clusters, is shown in Supplementary Table 4 (available at *Carcinogenesis* online). From these genes, to estimate the correlation with recurrence, 113 genes that were frequently methylated in cluster 3 were chosen and were applied to the pathway analysis. We selected seven hub genes, which regulate other genes (Supplementary Figure 2, available at *Carcinogenesis* online, black squares). The chosen genes were quantified by bisulfite pyrosequencing, and the genes with unstable polymerase chain reaction amplification before pyrosequencing were excluded. Finally, five genes (*HOXA6*, *HOXA9*, *PENK*, *UPK3A* and *IGF2BP1*) were retained.

The quantitative values of the five genes corresponded well with the results of MCAMs among clusters (Figure 2A). Here, we established
A scoring system on a scale of 5–15 points, comprising three stages depending on the methylation values of the five genes (Figure 2B). Score 1 category was defined as <15%, corresponding to the negative test of methylation. On the other hand, hypermethylation of 30% and more was categorized into score 3 because certain patients showed marked hypermethylation, indicating another phenotype (Figure 2A).

On the basis of this scoring system, a total score of >9 points showed the highest sensitivity and specificity for recurrence in 30 patients analyzed by MCAMs (Figure 2C: 80 and 90%, respectively). The progression-free survival curve of 30 patients divided by scores of 9 and above is shown in Figure 2D.

To validate the universal applicability of this scoring system in meningiomas, the system was applied to 32 additional meningiomas collected from other hospitals. As was found in the initially analyzed 30 cases, five genes demonstrated apparent hypermethylation in the recurrent group, although only UPK3A did not show any statistical significance (Figure 3A). The progression-free survival curve divided at point 9 or above in our scoring system showed significant differences in the validation samples as well (Figure 3B). It is imperative to perform a multivariate analysis to show that the methylation index is independent of other clinical variables such as grade, age and gender. We have combined the test and validation samples (62 cases in total) for multivariate analysis. As shown in Figure 3C, tumor progression was significantly correlated with scores ≥9 but not with age, WHO grade, gender or Simpson grade.

Interestingly, three patients experienced malignant transformations at the recurrence, and the characteristics of their hypermethylation patterns were already observed at the first surgery when they were diagnosed with grade I meningiomas (Figure 3D).

**Discussion**

For meningiomas, most of the previous studies on methylation markers have targeted mainly a few known genes associated with cell proliferation, mitogenesis or oncogenesis, such as maternally expressed gene 3 (MEG3) (23), RASSF1A (24), uPA (25) and the tissue inhibitor of metalloproteinase 3 (TIMP3) (12). TIMP3 methylation is the generally acknowledged epigenetic marker of meningioma progression; however, it chiefly occurs in grade III (anaplastic) meningiomas and to a lesser extent in grade I and II meningiomas (17 and 22%, respectively). Therefore, a crucial methylation marker to predict an aggressive subtype in the early stage of meningiomas remains to be established, despite a need for it. Moreover, the studies targeting single key genes always confront the problem of whether such genes are actually responsible for the formation of tumor characteristics per se or are a part of extensive alterations associated with carcinogenesis. These problems prompted us to consider the necessity of genome-wide analyses for methylation status to comprehend tumor characteristics. Here, we investigated the global methylation status of low-grade meningiomas with the aim of reclassification and compensation of the histological classification. MCAMs revealed the presence of three clusters in grade I and II meningiomas. Cluster 1 showed clinically benign courses, whereas cluster 3 was characterized by a high frequency of recurrence and/or progression and accumulated hypermethylation in this subset of genes. On the other hand, although cluster 2 showed interesting aspects of methylation, it did not correlate with any of the patients’ clinical features, except with gender, because of the low number of patients in this cluster. This cluster might be a completely different subgroup from the other two clusters. Further studies using a larger number of patient samples are needed to understand the implications of this subgroup. In addition, there are substantial differences in normal epigenetic patterns between genders. To exclude the possibility that the defining features of one cluster compared with another could be an artifact of gender biases, we have performed multivariate analysis in each gender separately in combined test and validation sample sets (62 cases in total). As shown in Supplementary Figure 3A (available at Carcinogenesis online), the effect with recurrence still held up even when separated into male and female groups. Moreover, patients with low scores (<9 points) experienced significantly longer progression-free survival in both genders (Supplementary Figure 3B, available at Carcinogenesis online).

Noushmehr et al. reported the presence of CpG island methylator phenotype (CIMP), a characteristic accumulation of promoter methylation...
into a subset of genes in the particular subtype of the tumors, in human glioblastomas and the possibility of detailed subclassification for the prediction of a good prognostic subgroup based on CIMP presence (26–28). In this study, MCAM data showed that samples were distributed into low/high methylation groups (bimodal distribution), suggesting the existence of CIMP in meningiomas (Figure 1C). Indeed, among nine tumors with >350 methylated genes (i.e. hypermethylated tumors), majority of the tumors (eight tumors) were classified into cluster 3, which correlated well with the tumor aggressiveness. In addition, we provided here five potent markers, which effectively predict CIMP in meningiomas. Additionally, as an important finding in the present study, there were early alterations and stabilities of methylation status in malignant transformation cases (Figure 3D). This suggests two theories: (i) biological alterations including DNA methylation might be acquired before histological changes or (ii) aberrant methylation might be a predictor of malignant transformation as well as recurrence.

The five genes chosen in the present study are potential epigenetic biomarkers, but their association with several hematopoietic or solid tumors is interesting as demonstrated in previous studies. For example, the homeobox (HOX) family is a target for silencing by DNA methylation and polycomb complexes (29–31). The comethylation of HOX genes, including HOX6, leads to the downregulation of protein expression and dysfunction as tumor suppression genes and is frequently identified in adult chronic lymphocytic leukemia and childhood acute lymphocytic leukemia (32). HOXA9 has been reported to be a methylation marker of ovarian tumors (33) and squamous cell lung carcinomas (34). Additionally, a CIMP-like accumulation of methylated genes, including HOXA9, is detected in aggressive mantle cell lymphoma (35). The HOX family tends to show comethylation even between different HOX clusters (32); however, only HOX cluster genes in 7p15.2, such as HOXA5, HOXA6, HOXA9 and HOXA11, were frequently methylated, whereas other HOX clusters (HOXB, HOXC and HOXD) showed low methylation among all samples in our study (data not shown). This suggests the correlation between concordant methylation in the HOXA cluster and tumorigenesis or character formation in meningiomas. On the other hand, PENK methylation is reported in pancreatic cancers (36), bladder cancers (37) and pulmonary adenocarcinomas (38). PENK tends to be detected as one of a subset of aberrant methylated genes (37,38), similar to our present results. UPK3A alterations are mainly reported in urothelia and neoplasms, but recently an aberrant methylation of UPK3A has also been shown to be associated with the distant metastasis in colorectal cancers (39). IGF2BP1 (IMP-1/CRDBP) is an RNA-binding protein that regulates messenger RNA localization, stability and translocation. The IGF2BP1 protein is actively expressed during embryogenesis and is also considered to play a role in tumorigenesis by stabilizing messenger RNAs of the c-myc oncogene and IGF2 in certain cancers (40). On the other hand, it is reported that the IGF2BP1 knockdown
by RNA interference increases the proliferation in leukemia K562 cells (41), and silencing by promoter methylation accelerates the growth of tumor cells and migration of human breast cancer cells (42). The biological implications of IGF2BP1 methylation and functioning mechanisms in tumor cells remain uncertain, and they might differ among tumors of different origins.

In conclusion, this is the first study indicating the presence of subgroups with aberrant accumulation of promoter methylation in WHO grade I and II meningiomas. These alterations correlate with high frequency of recurrence and may occur in the initial stage of histological changes such as malignant transformation. Our findings highlight the possibility of speculating individuals with malignant characteristics from low-grade meningiomas by quantifying methylation of certain genes.

Supplementary material
Supplementary Tables 1–4 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/.

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