Epigenetic silencing of the kinase tumor suppressor *WNK2* is tumor-type and tumor-grade specific

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Both genetic and epigenetic mechanisms contribute to meningioma development by altering gene expression and protein function. To determine the relative contribution of each mechanism to meningioma development, we used an integrative approach measuring copy number and DNA methylation changes genomewide. We found that genetic alterations affected 1.9%, 7.4%, and 13.3% of the 691 loci studied, whereas epigenetic mechanisms affected 5.4%, 9.9%, and 10.3% of these loci in grade I, II, and III meningiomas, respectively. Genetic and epigenetic mechanisms rarely involved the same locus in any given tumor. The predilection for epigenetic rather than genetic silencing was exemplified at the 5' CpG island of *WNK2*, a serine-threonine kinase gene on chromosome 9q22.31. WNK2 is known to negatively regulate epidermal growth factor receptor signaling via inhibition of MEK1 (mitogen-activated protein kinase kinase 1), and point mutations have been reported in *WNK1*, *WNK2*, *WNK3*, and *WNK4*. In meningiomas, *WNK2* was aberrantly methylated in 83% and 71% of grade II and III meningiomas, respectively, but rarely in a total of 209 tumors from 13 other tumor types. Aberrant methylation of the CpG island was associated with decreased expression in primary tumors. *WNK2* could be reactivated with a methylation inhibitor in IOMM-Lee, a meningioma cell line with a densely methylated *WNK2* CpG island and lack of WNK2 expression. Expression of exogenous WNK2 inhibited colony formation, implicating it as a potential cell growth suppressor. These findings indicate that epigenetic mechanisms are common across meningiomas of all grades and that for specific genes such as *WNK2*, epigenetic alteration may be the dominant, grade-specific mechanism of gene inactivation. *Neuro-Oncology 11, 414–422, 2009 (Posted to Neuro-Oncology [serial online], Doc. D08-00170, November 11, 2008. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2008-096)*

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Meningiomas are the second most common primary tumor of the CNS.[1] They are thought to arise from neoplastic leptomeningeal cells lining the brain and spinal cord and are graded according to their histology using the WHO classification system.[2] The majority of meningiomas are benign (grade I) tumors that are usually cured by surgical resection. Atypical (grade II) and malignant (grade III) meningiomas account for 10% of cases and carry a worse

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prognosis due to their higher likelihood of recurrence and metastasis.[3]

The mechanism of meningioma development remains enigmatic from a genetic standpoint. Although mutations of the gene for the hereditary disorder neurofibromatosis type 2 (NF2) are a defining alteration in meningiomas, only 60% of sporadic tumors carry this abnormality.[4] Other genes involved in meningioma development have been sought, but identifying compelling candidates has proven challenging. One reason is that the complex chromosomal imbalances make positional cloning studies difficult.[5,6] However, another reason may be that other mechanisms, such as epigenetic alterations, may affect gene expression. This possibility is suggested by the apparent absence of any large genetic alterations in nearly 40% of meningiomas.[5,6] Loss of expression of components of the transforming growth factor-β signaling pathway distinguish high-proliferative from low-proliferative meningiomas,[7] suggesting a link between specific alterations in gene expression and meningioma cell proliferation.

Epigenetic mechanisms transcriptionally silence genes by methylating CpG islands within promoter regions,[8] by altering the acetylation or methylation status of local histones, or both. Because these modifications preserve the normal genomic sequence, conventional genetic methods are unable to detect aberrant methylation or histone modifications. Gene-specific assays have identified aberrant methylation on *CDKN2A*, *CDKN2B*, *DAPK*, *GSTP1*, *MGMT*, *p14*, *p17*, *RB1*, *THBS*, *NDRG2*, and *VHL* in meningiomas.[9–15] Although this single-gene approach demonstrates aberrant methylation in meningiomas, it is limited in that only a fraction of the 30,000 CpG islands are surveyed.[16]

To determine the extent of epigenetic involvement in meningiomas, larger-scale assessments of aberrant methylation are necessary. Restriction landmark genome scanning (RLGS) is a two-dimensional gel-based method for assessing the methylation status of thousands of CpG islands.[17] When combined with array comparative genomic hybridization (aCGH),[18] a high-resolution method of determining changes in copy number across the genome, the relative contributions of genetic and epigenetic mechanisms can be determined.[19,20] In this study, we hypothesized that epigenetic mechanisms play a significant role in meningioma development and sought to determine their relative contribution in each malignancy grade.

Materials and Methods

Primary Meningiomas, Leptomeninges, and Cell Culture

Primary tumor samples were obtained from the Neurological Surgery Tissue Bank at the University of California, San Francisco (UCSF), including ten grade I (meningothelial subtype), seven grade II, and nine grade III meningiomas. Nontumor leptomeninges were obtained from four autopsies and two intraoperative cases, all from unrelated patients with no known history of malignancy. All samples were obtained with informed consent, and their use was approved by the Committee on Human Research at UCSF.

We also used two human malignant meningioma cell lines, IOMM-Lee[21] and KT21-MG1.[22] Cells were plated at low density and maintained in Dulbecco's modified Eagle's–H16 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in 5% CO_2. For demethylation experiments, cells were treated with 5 μM 5-aza-2'-deoxycytidine (Sigma-Aldrich, St. Louis, MO, USA) for 3 days, with the medium and drug changed every 24 h. Transfection was performed using FuGene 6 (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions.

Array Comparative Genomic Hybridization

aCGH was performed as previously described.[18] Briefly, DNA from tumor and normal tissues were labeled with Cy3 and Cy5 dyes, respectively, and cohybridized to a microarray consisting of 2,413 mapped bacterial artificial chromosomes. The image was then captured and processed using custom software, and the mean $\log_2$ ratios of spots in triplicate were calculated and plotted for deletions and gains along each chromosome.

Restriction Landmark Genome Scanning

RLGS was performed as previously described for the *Not*I/*Eco*RV/*Hinf*I combination.[23] Twenty-two tumors (nine grade I, six grade II, and seven grade III) and four leptomeninges produced analyzable profiles as defined by minimal spot smearing and uniform spot intensity. From the thousands of RLGS spots within each profile, 691 were selected for comparison based on the criteria that each spot (1) was clearly defined, (2) had associated sequencing data, and (3) had consistent intensity across normal profiles.[24] Tumor profiles were compared against normal profiles by visual inspection of overlaid autoradiographs.

Bisulfite Treatment, PCR, and Sequencing

Meningiomas, leptomeninges, and cell-line DNA were treated with sodium bisulfite (Sigma-Aldrich) to convert the unmethylated cytosine bases to uracil, as previously described.[25] Touchdown PCR was done for 10 cycles starting at an initial annealing temperature of 68°C and decreasing by 2°C every two cycles to a final annealing temperature of 58°C for the final 30 cycles. The products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) vectors and then transformed into TOP10 competent cells (Invitrogen). Individual bacterial colonies were subject to PCR using vector-specific primers and then sequenced. The primers used to amplify the *WNK2* CpG island were as follows: for region I, forward 5'-TAG TTT GTT TAT TTT GTT TTG G-3' and reverse 5'-CTA AAC CTA ACA CGC CCA CCT AAC-3'; region II, forward 5'-TTT GTT AGT TAG TTA

GTT AG-3' and reverse 5'-CCT ACC CAA CCC TAA CCC AAA A-3'; region III, 5'-TTT GGG TTA GGG TTG GGT AG-3' and reverse 5'-TCT AAA AAC CCT AAA AAA AA-3'; region IV, forward 5'-GGG GTT TTT AGG GAT ATT TTA GTA GG-3' and reverse 5'-TCT ATA AAC CAA AAA CAC ACA AAC C-3'; and region V, forward 5'-TTT TGG TTT ATA GAG ATG GA-3' and reverse: 5'-CTT ACA AAA CAA AAA CAC CC-3'.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time Reverse Transcriptase PCR

Meningiomas, leptomeninges, and cell-line RNA were isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Sixteen meningiomas (six grade I, three grade II, and seven grade III) and three leptomeninges (three surgical and one autopsy) yielded intact RNA. RNA was treated with Turbo DNase (Ambion, Austin, TX, USA), and 800 ng was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (RT; Invitrogen), oligodeoxythymidylic acid, and random primers. Quantitative real-time RT-PCR was done with the Assays-on-Demand for *WNK2* (Hs00198403_m1; Applied Biosystems, Foster City, CA, USA) on an Opticon2 thermocycler (MJ Research, Waltham, MA, USA). Human *GusB* was used as a control for quantitation. Water and genomic DNA were used as negative controls.

Luciferase Reporter Gene Assay

Five promoter-reporter constructs were made by cloning different regions of the *WNK2* CpG island into *Bgl*II and *Hind*III restriction sites in the pGL3-Basic *luciferase* vector (Promega, Madison, WI, USA) and confirmed by sequencing (pGL3-WNK2). The pGL3-Basic vector was used as a basal level of luciferase activity, and the vector pGL3-Promoter containing the SV40 (simian virus 40) promoter was used as a positive control. IOMM-Lee, KT21-MG1, and HEK293T cells were transfected with 1 μg of each vector and cotransfected with 10 ng (1:100) of pRL-TK (Promega) expressing *Renilla* luciferase using FuGene 6 (Roche). After 48 h, firefly luciferase activity was measured using the Promega Dual-Luciferase Reporter Assay system and normalized against *Renilla* luciferase activity.

Expression Constructs

The partial *WNK2* cDNA from the HUGE Protein library was a gift from Dr. Nagase (Kazusa DNA Research Institute, Chiba, Japan).[26] The complete coding sequence was created by adding the missing 5' and 3' sequences with double-stranded oligonucleotides. The full-length cDNA was then cloned into pcDNA3.1/V5-His-TOPO (Invitrogen) linking the V5-His epitope to the carboxy-terminal end (pWNK2-V5) and confirmed by sequencing.

Colony Formation Assay

IOMM-Lee and KT21-MG1 were seeded and grown in a six-well tissue culture plate until 85% confluency was reached. Equimolar amounts of either pWNK2-V5 expression construct (3 μg) or pcDNA3.1/V5-His-TOPO empty vector (1 μg) was transfected using FuGene 6. One day after transfection, cells were split at 1:100 and exposed to 800 μg/ml G418 (Geneticin, an antibiotic used to select cells positive for WNK2 constructs; Invitrogen) for 10–14 days. Cells were fixed in 20% methanol and stained with crystal violet, and colonies with >50 cells were counted.

Immunofluorescence Staining of Ectopic Human WNK2 Expression in HEK293T and Meningioma Cells as Supplemental Experimental Procedures

Forty-eight hours after transfection, cells were washed with phosphate-buffered saline (PBS) and fixed with prechilled methanol/acetone (1:1 vol/vol) for 5 min, washed with PBS, and incubated for 30 min in blocking buffer containing 5% normal goat serum and 2.5% bovine serum albumin (BSA) in PBS. After incubation with V5 antibody (1:800; Invitrogen) at room temperature for 30–45 min, cells were washed twice with 1% BSA in PBS and incubated with secondary goat antimouse-rhodamine (1:50; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cells were counterstained with diamidinophenylindole, mounted with a cover slip, and viewed under a Zeiss fluorescence microscope.

Results

Genetic and Epigenetic Contributions to Meningioma Development

To determine the relative genetic and epigenetic contributions to meningioma development, we integrated data from RLGS and aCGH. From the thousands of RLGS spots within each profile, 691 were selected for comparison based on the criteria that each spot (1) was clearly defined, (2) had associated sequencing data, and (3) had consistent intensity across normal profiles.[24] Each RLGS spot represents a specific locus, and losses or decreases in spot intensity can be attributed to (a) homozygous methylation, if the decrease in intensity in tumor DNA is >70% relative to normal tissue and no deletion of the flanking loci is detected; (b) partial methylation, if the decrease in intensity is between 30% and 70% and no deletion of the flanking loci is detected; (c) deletion, if the decrease in intensity is between 30% and 70% and a deletion of the flanking loci is detected; (d) gain, if aCGH shows a gain of the flanking loci; (e) methylation and deletion, if the decrease in intensity is >70% and a deletion of the flanking loci is detected; or (f) methylation and gain, if the intensity decreases but the aCGH shows a gain of the flanking loci. These findings were categorized as epigenetic mechanisms (a and b), genetic mechanisms (c and d), and combined mechanisms (e and f).

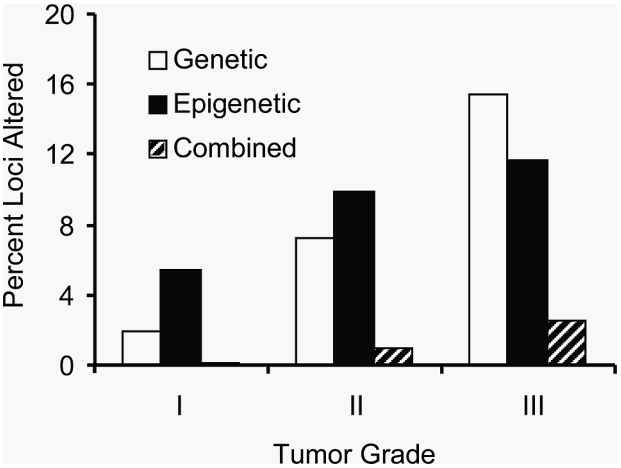

Fig. 1. Relative genetic and epigenetic contributions to meningioma development. The relative frequency of genetic and epigenetic alteration was analyzed in 22 human meningiomas (nine grade I, six grade II, and seven grade III). The 15,202 possible events (691 loci in each of 22 tumors) were attributed to one of the three categories as described in "Results."

From 15,202 possible events (at 691 loci in 22 tumors), 74.3%–92.5% of the loci were unaltered by either genetic or epigenetic mechanisms, respectively (Fig. 1). In grade I, II, and III meningiomas, genetic mechanisms affected 1.9%, 7.3%, and 13.3% of loci, epigenetic mechanisms affected 5.4%, 9.9%, and 10.3% of loci, and combined mechanisms affected 0.2%, 0.9%, and 2.1% of loci, respectively. It is likely that a proportion of genomic or epigenomic events were missed because of the limited resolution of our assays.

The CpG Island of WNK2 *Is Frequently Altered in Grade II and III Meningiomas, and the Alteration Is Tumor-Type Specific*

RLGS spots that are decreased in intensity or absent across multiple profiles may point to CpG islands that are frequent targets for genetic or epigenetic changes. We found that RLGS spot 1D05 was altered in a large proportion of high-grade tumors (Fig. 2A–C). Five of six (83%) grade II and five of seven (71%) grade III meningiomas showed decrease of spot intensity or complete spot loss, while only two of ten (20%) grade I meningiomas did. The flanking loci were retained by aCGH (see supplementary data, Fig. 1S), implying that the 1D05 locus was intact and that decrease or absence of spot intensity is a result of partial or homozygous methylation, respectively. In those tumors showing a change, homozygous methylation occurred in two of five (40%) grade II and four of five (80%) grade III meningiomas. RLGS spot 1D05 corresponds to the CpG island upstream of the gene encoding the protein kinase WNK2 on chromosome 9q22.31. When compared with 209 tumors from 13 other tumor types, the aberrant methylation of *WNK2* occurred mainly in meningiomas and adult gliomas, but rarely in the other tumor types (Fig. 2D). This result indicates that the epigenetic alteration of *WNK2* is tumor-type specific. In contrast, copy number alterations were not observed at the *WNK2* locus on 9q, though chromosome 9p deletions were common in meningiomas.

WNK2 *Is Frequently Affected by Aberrant Methylation in Grade II and III Meningiomas*

RLGS relies on the methylation-sensitive endonuclease *Not*I to differentiate between methylated and unmethylated CpG islands. Because isolated CpG dinucleotide methylation or polymorphisms within the restriction site can block endonuclease activity and mimic CpG island methylation, we performed bisulfite sequencing to dis-

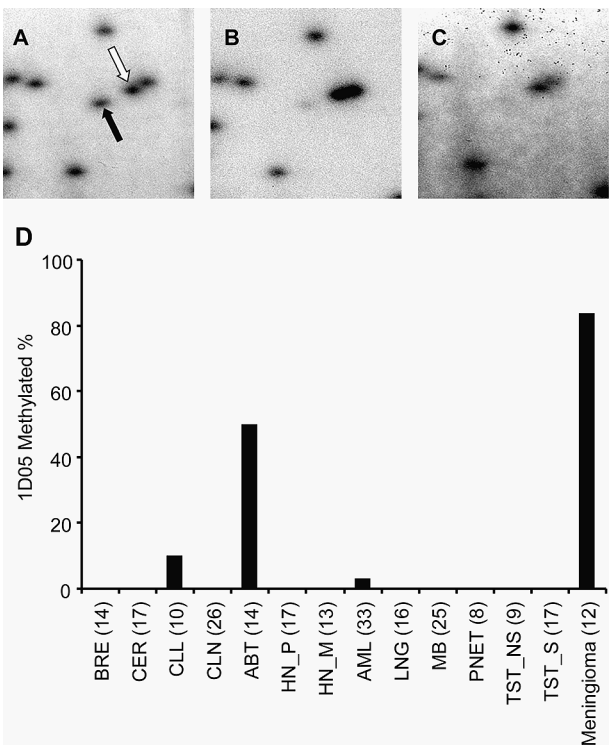

Fig. 2. RLGS spot 1D05 is frequently altered in higher-grade meningiomas, and the alteration is tumor-type specific. (A) A magnified section of an RLGS profile from normal human leptomeninges showing the presence of 1D05 (black arrow) and the adjacent spot, 1D06 (white arrow). (B and C) Representative RLGS sections from a grade II (B) and a grade III (C) meningioma showing loss of 1D05 but not 1D06. (D) The percentage of tumors with 1D05 methylation in 14 different types of tumors showed that the aberrant methylation of this locus is tumor-type specific. Abbreviations: BRE, breast carcinoma; CER, cervical carcinoma; CLL, chronic lymphocytic leukemia; CLN, colorectal carcinoma; ABT, adult brain tumors; HN_P, primary head and neck squamous cell carcinoma; HN_M, metastatic head and neck squamous cell carcinoma; AML, acute myeloid leukemia; LNG, non-small-cell lung carcinoma; MB, medulloblastoma; PNET, primitive neuroectodermal tumor; TST_NS, nonseminomatous testicular germ cell tumor; TST_S, seminomatous testicular germ cell tumor. Numbers in parentheses are numbers of samples for that tumor type.

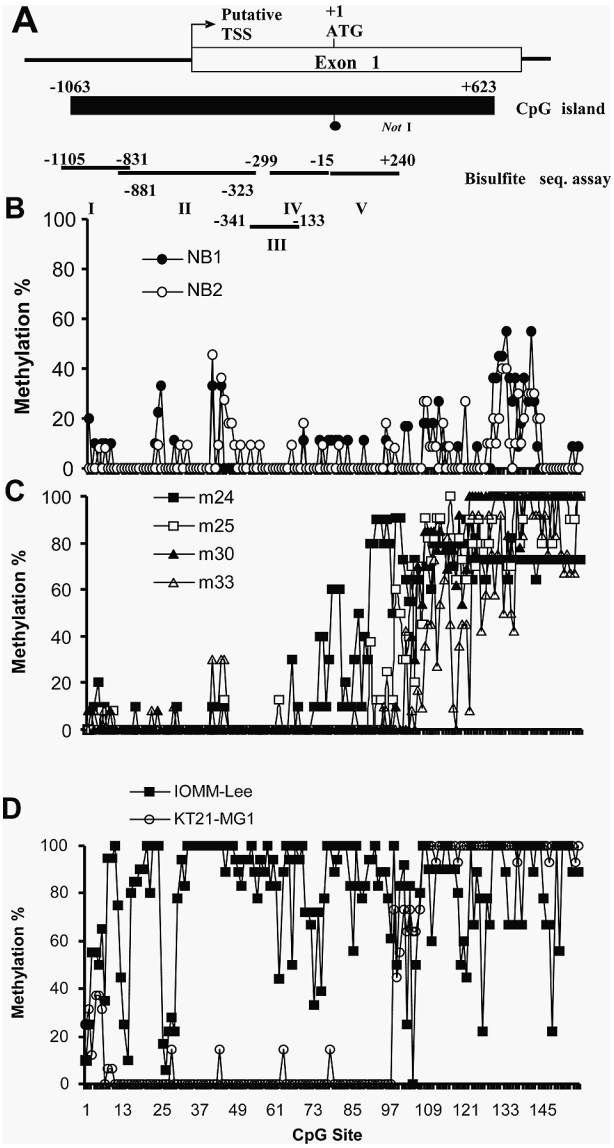

Fig. 3. *WNK2* is frequently affected by aberrant methylation in grade II and III meningiomas. (A) Schematic drawing showing the WNK2-associated CpG island and the location of five fragments for bisulfite sequencing analysis ("seq. assay"). The methylation status of individual CpG sites within this CpG island was calculated from clones of bisulfite sequencing analysis. An average of 11 clones (range, 7–20) were sequenced for each fragment. The numbers by each fragment represent the position of the fragment relative to the translation start site (ATG). (B–D) The methylation rate of these fragments in nontumor leptomeninges (NB1, NB2; B), grade II (m24, m25) and grade III (m30, m33) meningiomas (C), and cell lines (IOMM-Lee, KT21-MG1; D). CpG sites 1–22 were analyzed from fragment I; sites 23–97, from fragment II; sites 98–104, from fragment III; sites 105–120, from fragment IV; and sites 122–156, from fragment V.

criminate among these possibilities and to confirm and extend our methylation analysis. Five regions spanning the entire CpG island were sequenced following bisulfite treatment, the first four containing the putative promoter regions and the last containing the translational start site and the *Not*I restriction site (Fig. 3A). The level of methylation differed significantly between normal and tumor samples (Fig. 3B,C). Normal leptomeninges were comparatively unmethylated, while grade II and III meningiomas had methylation on nearly all CpG dinucleotides at the 3'-end of the CpG island and were consistently less methylated at the 5'-end of the promoter. These results confirmed and extended our previous finding that *WNK2* is a frequent target for homozygous methylation, and are similar to the pattern observed in gliomas.[27] The lack of aberrant methylation in the 5' region of the CpG island could indicate that methylation of this region is not required for silencing or that, similar to gliomas, histone deacetylation in the 5' region may syngerize with aberrant DNA methylation in the 3' region of the CpG island. We did not observe *WNK2* silencing in any of the tumors that lacked aberrant methylation in the 3' region, suggesting some contribution of this regional methylation to *WNK2* silencing. Bisulfite sequencing of two malignant meningioma cell lines, IOMM-Lee and KT21-MG1, showed distinctive methylation patterns. IOMM-Lee cells, a WNK2-nonexpressing meningioma cell line, had dense methylation across the entire CpG island, while in KT21-MG1, a WNK2-expressing cell line, dense methylation at the 3'-end tapered off in the 5'-end of the CpG island (Fig. 3D).

WNK2 Expression Is Significantly Down-Regulated in Primary High-Grade Meningiomas, and Demethylating Treatment Reactivates Expression in a Silenced Meningioma Cell Line

To determine whether aberrant methylation of the WNK2 CpG island is associated with changes in its expression, quantitative RT-PCR was performed on meningiomas and normal leptomeninges. WNK2 was highly expressed in all leptomeninges and in nearly all grade I meningiomas, except for m17 (Fig. 4A). In comparison, WNK2 expression was down-regulated in all grade II and III meningiomas examined. Of the two malignant meningioma cell lines, WNK2 was suppressed in IOMM-Lee, which is consistent with its methylation status. Treatment with 5-aza-2'-deoxycytidine, a chemical inhibitor of DNMT1 (DNA methyltransferase 1), reactivated WNK2 expression in this cell line. Conversely, WNK2 was expressed in KT21-MG1 and treatment with 5-aza-2'-deoxycytidine did not alter the level of WNK2 expression (Fig. 4B).

The 5'-End of the WNK2 *CpG Island Functions as a Promoter*

To determine whether the *WNK2* CpG island functions as a promoter in meningioma cells, as it does in gliomas,[27] we cloned five different fragments covering different regions of the CpG island into the pGL3-Basic vector (Fig. 5A). The resultant plasmids were transiently cotransfected with pRL-TK into IOMM-Lee, KT21-MG1, and HEK293T cells. The luciferase activities were compared with those from the pGL3-Basic vector (Fig. 5B). Plasmids 1, 3, and 5 showed strong promoter

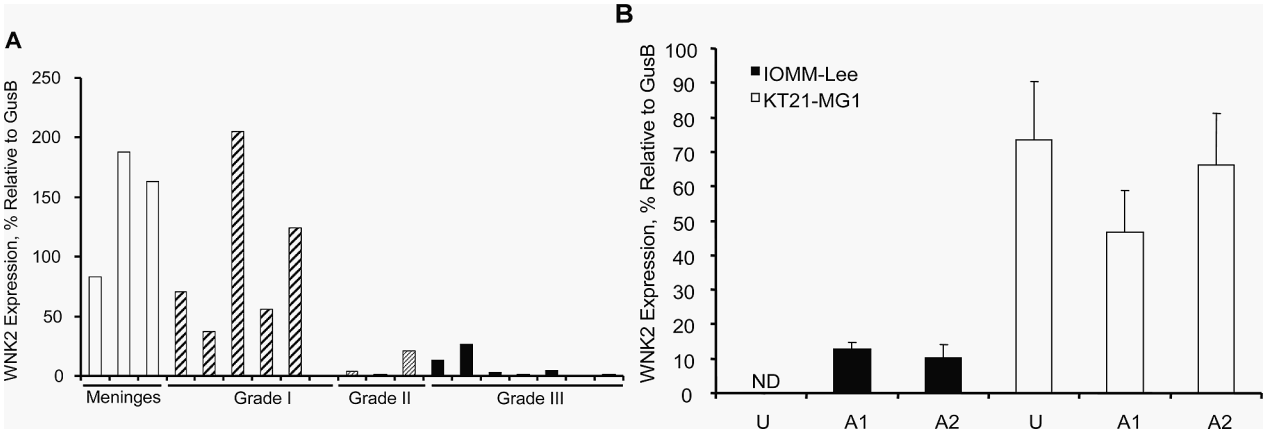

Fig. 4. WNK2 expression is significantly down-regulated in primary high-grade but not low-grade meningiomas, and demethylating treatment reactivates expression in a silenced meningioma cell line. (A) Expression of WNK2 in normal leptomeninges and primary meningioma samples was determined by quantitative RT-PCR analysis and is expressed relative to the control gene, *GusB*. Gene expression values were derived from the equation $\Delta Ct = (Ct_{WNK2} - Ct_{GusB})$ followed by $(2^{-\Delta Ct}) \times 100$. Columns indicate average of triplicates. An analysis of variance was performed among normal and different grades of tumors: $p = 0.013$ between normal and grade II; $p = 0.0001$ between normal and grade III; $p = 0.019$ between grade I and grade III. Comparisons of grade I or grade III tumors with grade II tumors were not statistically significant, likely due to the small number of grade II samples. Two independent experiments yielded similar results. (B) Expression of WNK2 in meningioma cell lines before and after treatment with 5-aza-2′-deoxycytidine as assessed by quantitative RT-PCR analysis. Abbreviations: U, untreated cells; ND, not detectable; A1 and A2, independent flasks of cells treated with 5 μM 5-aza-2′-deoxycytidine for 3 days. Columns indicate average of triplicates; error bars, standard deviation (SD).

activity, while plasmids 2 and 4 had low activity in both IOMM-Lee and KT21-MG1 cell lines. HEK293T, a human embryonic kidney cell line, was used as a positive control. These findings indicate that the 5'-end of the *WNK2* CpG island acts as a promoter element capable of initiating transcription in meningioma cells in vitro, and that cells with epigenetic silencing of endogenous *WNK2* retain the ability to transcribe from a transfected, unmethylated *WNK2* promoter. Because the aberrant methylation observed in the primary tumors extended into the 3' edge of the active promoter area but not farther, and the aberrant methylation correlated with *WNK2* silencing, the promoter analyses suggest that the aberrant methylation has an indirect effect on *WNK2* promoter activity, and/or combines with other epigenetic mechanisms such as histone deacetylation to silence *WNK2*.

Expression of Exogenous WNK2 Inhibits Colony Formation by Malignant Meningioma Cell Lines

The frequent biallelic methylation and transcriptional silencing of WNK2 in grade II and III meningiomas suggest that inactivation of this gene may confer a selective advantage. To determine whether WNK2 has an effect on proliferation, we transfected two malignant meningioma cell lines with *WNK2* and performed colony formation assays. To control for the size difference between the *WNK2* expression vector and the empty plasmid control, equimolar amounts of control and *WNK2*-containing plasmids were used for transfection. Compared with the control vector, the colony number was dramatically decreased in cells transfected with *WNK2* (Fig. 6), consistent with results in gliomas.[27] Expression of WNK2 caused a decrease in colony formation in IOMM-Lee and somewhat unexpectedly in KT21-MG1, as well. WNK2 expression was confirmed by immunocytochemical staining with antibody against the V5 epitope tag (see supplementary data, Fig. 2S).

Discussion

In this study, we integrated aCGH and RLGS findings from primary meningiomas to determine the relative contributions of genetic and epigenetic mechanisms on a defined set of loci. We found that genetic mechanisms affected progressively more loci with increasing tumor grade, which is consistent with other published reports.[5,6] The frequency of epigenetic mechanisms also increased with tumor grade, with grade I and II meningiomas having relatively more aberrantly methylated loci than genetically altered loci. Although not all aberrantly methylated CpG islands are expected to have an effect on gene expression, it appears that epigenetic mechanisms play a greater role in meningioma development than previously recognized.

Our integrative analysis also found that aberrant methylation occurs mostly at loci independent of genetic alterations, suggesting that genes are preferentially inactivated by either genetic or epigenetic mechanisms but less commonly by both. WNK2 is an example of a gene that is aberrantly methylated in a large proportion of grade II and III meningiomas. Since this locus was presumably retained genetically in all of our tumor samples, *WNK2* would have been overlooked as a potential candidate gene by traditional genetic approaches. Given that our integrated method surveys only a small fraction of the human genome, our findings raise the possibility that

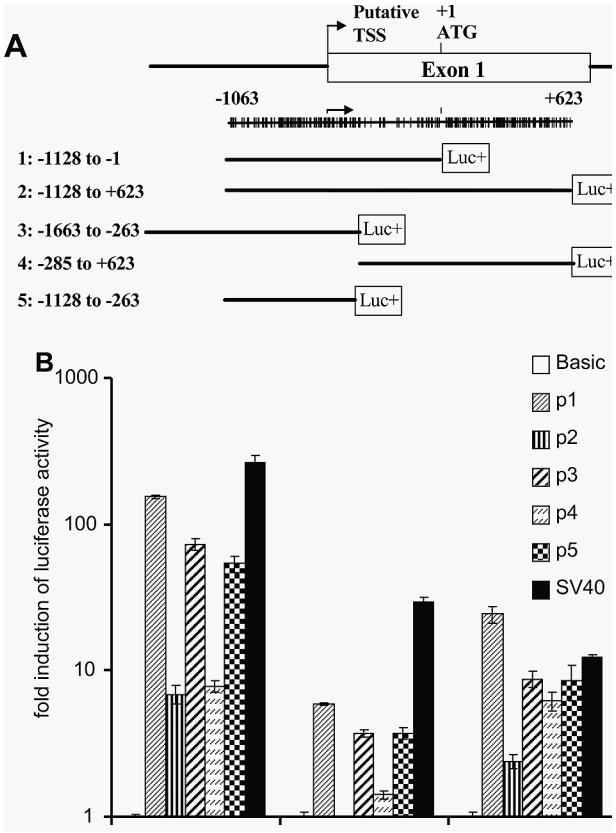

Fig. 5. The 5′-end of the *WNK2* CpG island functions as a promoter. (A) Locations of the *WNK2* transcription start site, CpG island, and five different DNA fragments cloned into pGL3-Basic luciferase vector. (B) HEK293T, IOMM-Lee, and KT21-MG1 cells were each transiently cotransfected with the resultant WNK2-promoter plasmids and pRL-TK. After 48 h, luciferase activity was measured and plotted after normalization with the *Renilla* control. pGL3-Basic vector was used as a basal level for luciferase activity and is plotted as 1, and "pGL3 promoter" containing an SV40 (simian virus 40) promoter was used as a positive control. Abbreviations: p1–p5, plasmids 1–5.

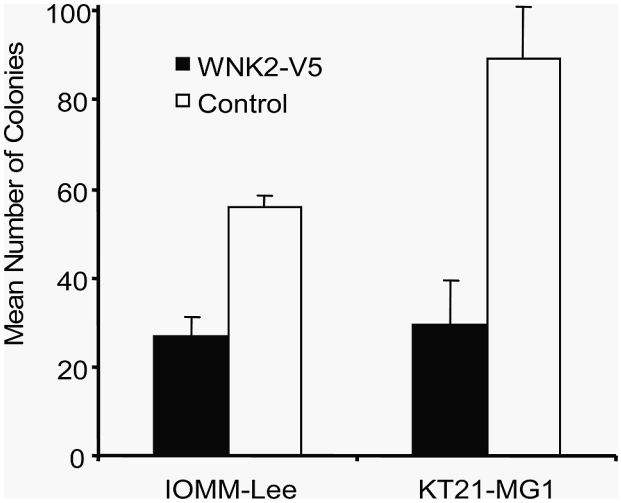

Fig. 6. Exogenous WNK2 expression inhibits colony formation by malignant meningioma cell lines. IOMM-Lee and KT21-MG1 cells were each transfected with equimolar amounts of *WNK2* expression vector or vector only. Cells were selected with G418 (an antibiotic called Geneticin, used to select cells positive for WNK2 constructs) for 10–14 days before staining with crystal violet. Colonies of >50 cells were counted. Quantitation of total colony numbers from each cell line is shown; error bars, standard deviation (SD).

many more tumor-related genes are inactivated solely by epigenetic mechanisms.

Aberrant methylation is thought to silence genes by blocking transcriptional machinery access to DNA.[8] In our grade II and III meningiomas, dense aberrant methylation was associated with decreased WNK2 expression. Conversely, the unmethylated normal leptomeninges and grade I tumors showed significantly higher levels of expression, suggesting that the methylation confined to the 3' half of the CpG island and infringing on, but not encompassing, the active promoter area contributes to *WNK2* silencing. Demethylation treatment reactivated WNK2 expression in the meningioma cell line, IOMM-Lee, which was aberrantly methylated across the entire island. Our promoter assay confirmed that much of the promoter activity resides at the 5'-end of the CpG island. There are an abundance of predicted transcription factor binding sites at the 5'-end of the promoter and two narrow clusters of predicted transcription factor binding sites in the 3'-end (see supplementary data, Fig. 3S). Similar to a subset of gliomas, meningiomas with decreased WNK2 expression exhibited aberrant DNA methylation across approximately 60 CpGs in the 3' part of the island, but very little methylation in the 5' region. Thus, additional epigenetic mechanisms, such as histone deacetylation, may syngerize with or be promoted by DNA methylation in *WNK2* silencing, as we reported previously.[27] Consistent with this hypothesis, all primary tumors with *WNK2* silencing exhibited aberrant DNA methylation, whereas most low-grade meningiomas expressed higher levels of WNK2 and did not exhibit significant DNA methylation.

The functional consequence of aberrant methylation and silencing of *WNK2* in grade II and III meningiomas is not entirely clear. WNK2 is a member of the with-no-lysine (WNK) kinase family, so named because they lack a lysine within the canonical location of the catalytic domain,[28] though the critical lysine was later discovered elsewhere in the kinase domain. Furthermore, we demonstrated that WNK2 has kinase activity on generic protein substrates and also exhibits autophosphorylation. Of the four members, WNK1 is the best characterized and is involved in trafficking NaCl transporters to the plasma membrane.[29] WNK1 is also a downstream target in the AKT/protein kinase B pathway, and its depletion in 3T3-L1 preadipocytes leads to cell proliferation.[30] Recently, in vitro studies showed a connection between WNK1 and WNK2. WNK1 phosphorylates WNK2 and WNK4 in vitro, while the WNK1 autoinhibitory domain suppresses the catalytic activity of WNKs.[31] It is also noteworthy that WNK1, independent of its kinase domain, is a negative regulator of insulin-stimulated cell proliferation, possibly through activation of its

downstream target, SGK1 (serum- and glucocorticoid-induced protein kinase), mediated by AKT (also called protein kinase B [PKB]).[32] Therefore, these proteins may interact intracellularly to traffic ion transporters, inhibit cell growth, or partake in other roles.

Point mutations of *WNK1* in breast and colon cancer and *WNK2* in lung cancer were discovered by large-scale cancer genome sequencing.[33,34] Point mutations of *WNK1*, *WNK2*, *WNK3*, and *WNK4* were also discovered in a spectrum of solid tumor types.[35] These findings suggest a potential role of the WNK family in tumorigenesis. We recently found that overexpression of WNK2 inhibits glioma cell growth in vitro.[27] Ectopic expression of WNK2 in our malignant meningioma cell lines suppressed colony formation, as well, suggesting that it functions as a growth suppressor in vitro. Interestingly, this effect was observed in both cell lines. This may be due to several factors. One possibility is that WNK2 overexpression compromises the growth-promoting effects of WNK1, which is generally expressed at high levels.[29] In our WNK2-expressing cell line, the exogenous WNK2 may have tipped the balance between WNK2 and WNK1. In HeLa cells, WNK2 inhibits cell proliferation by negatively modulating the activation of MEK1/ERK1/2 and epidermal growth factor receptor (EGFR) signaling.[36] *EGFR* is thought to be an important oncogene in meningiomas, and *WNK2* silencing could potentially enhance EGFR signaling. In fact, we have observed *WNK2* silencing in a mutant EGFR-driven mouse model of glioma. Careful titering of the levels of WNK1 and WNK2 in the meningiomas would be required to address this proposed mechanism. Alternatively, differences in the level or phosphorylation status of ERK1 or ERK5 (a WNK1 target), and/or EGFR signaling may influence the cellular response to exogenous WNK2. Finally, as yet unknown targets or modulators of WNK2, either through kinase-dependent or -independent WNK2 activities, may contribute to the observed effects on colony-forming ability.

It is also possible that WNK2 may alter intracellular homeostasis. In addition to transcriptional regulation, the WNK family members are also regulated at the posttranslational level. For instance, WNK1 promotes trafficking of the NaCl transporter to the plasma membrane by inhibiting WNK4.[37] As mentioned above, the autoinhibitory domain of WNK1 can suppress WNK2 kinase activity.[31] Thus, an additional possibility is that endogenous WNK2 in KT21-MG1 may be functionally inactive due to posttranslational regulatory mechanisms. By ectopically expressing WNK2, this balance may be altered to reduce colony formation. Further studies and reagents to assess endogenous WNK2 are required to tease apart the mechanism(s) of growth suppression.

In summary, by using an integrative approach for measuring copy number and methylation changes genomewide, we found that epigenetic mechanisms are common across meningiomas of all grades and that for specific genes, such as *WNK2*, epigenetic mechanisms including DNA methylation may be the dominant mechanism of gene inactivation. Exogenous expression of WNK2 inhibited colony formation by meningiomas, similar to what we previously reported for gliomas, implicating it as a potential cell growth suppressor. Taken together, the epigenetic silencing and functional assessment suggest that aberrations of *WNK2* may contribute to unregulated tumor cell growth, potentially through relieving inhibitory effects on EGFR signaling, though this requires further investigation.

It is important to note that Moniz and colleagues recently showed that WNK2 modulates MEK1 activity through the Rho GTPase pathway, rather than having a direct effect on MEK1 itself. This addition does not change any conclusion here, it only updates the mechanism of WNK2 modulating MEK1 activity.[38]

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