DNA Hypermethylation and 1p Loss Silence *NHE-1* in Oligodendroglioma

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Oligodendroglioma is characterized by mutations of *IDH* and *CIC*, 1p/19q loss, and slow growth. We found that *NHE-1* on 1p is silenced in oligodendrogliomas secondary to *IDH*-associated hypermethylation and 1p allelic loss. Silencing lowers intracellular pH and attenuates acid load recovery in oligodendroglioma cells. Others have shown that rapid tumor growth cannot occur without *NHE-1*–mediated neutralization of the acidosis generated by the Warburg glycolytic shift. Our findings show for the first time that the pH regulator *NHE-1* can be silenced in a human cancer and also suggest that pH deregulation may contribute to the distinctive biology of human oligodendroglioma.

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Oligodendroglioma is a brain cancer with a distinctive histopathology and propensity to grow slowly even when high grade. Hemizygous mutations of *IDH-1* or *IDH-2* are thought to be the initiating events, which then combine with subsequent codeletions of chromosomes 1p and 19q to cause this cancer.[1] Furthermore, certain clinical features of oligodendroglioma, such as sensitivity to chemotherapy and affinity for ^{11}C-methionine, have long hinted at an unusual biology. Because 1p and 19q losses persist as oligodendrogliomas evolve, these chromosomal regions likely contain genes that contribute to their causation or phenotype. Among these genes is *CIC* at 19q13.2. Mutations of *CIC* occur in up to 70% of oligodendrogliomas with 1p/19q loss.[2,3] Despite intense sequencing, similarly frequent mutations of genes on chromosome 1p have not yet been found.[2–4]

Because *IDH* mutations lead to DNA hypermethylation,[5] we reasoned that epigenetic silencing of 1p genes together with 1p haploinsufficiency might contribute to the behavior or pathogenesis of oligodendrogliomas, and we developed a strategy to identify such alterations. Our approach relied on brain tumor-initiating cells (BTICs), including oligodendroglioma lines established by our group.[6,7]

We compared gene expression profiles from oligodendroglioma, glioblastoma (GBM), and human fetal neural stem cells and found *NHE-1* at 1p36.1-1p35 to be highly underexpressed in oligodendroglioma cells. *NHE-1* is a ubiquitous pH regulator that increases intracellular pH (pHi) by electroneutral exchange of intracellular H^+ for extracellular Na^+.[8] In the setting of increasing $[H^+]$ production, a byproduct of intermediary metabolism that lowers pHi, *NHE-1* is upregulated to maintain intracellular homeostasis.[9] *NHE-1* was of interest because its promoter is methylated in primary human astrocytes engineered to express mutant *IDH-1*[5] and because 1p36 is a known site of microdeletions on the retained 1p allele in some oligodendrogliomas.[10] We now

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report silencing of *NHE-1* in oligodendroglioma cells and tumors by combined DNA hypermethylation and 1p loss.

MATERIALS AND METHODS

BTIC Cultures

BTICs from oligodendrogliomas (BT054; BT088) and GBMs (n = 18) were described previously.[6,11] Whole-arm losses of 1p/19q and translocations of 1q/19p were preserved in BT054 and BT088.[7] Both were derived from tumors with *IDH-1* and *CIC* mutations,[3,6] and *MGMT* was methylated. BT054 retains the R132H mutation of *IDH-1*[6] and produces 2-hydroxyglutarate.[7] In GBM BTICs, retention of 1p/19q alleles was confirmed by single nucleotide polymorphism (SNP) analysis and wild-type *IDH* by sequencing.

NHE-1 Re-expression Studies

Cells were plated in 3μm 5-azacytidine (Sigma, St Louis, MO). Fresh media and 5-azacytidine were added daily for 72 hours. Adenovirus was used to re-express *NHE-1* in BTICs as described elsewhere.[8]

NHE-1 Immunohistochemistry and IDH Status

NHE-1 immunohistochemistry (IHC) was performed on tissue microarrays with an antibody from Santa Cruz Biotechnology (Santa Cruz, CA; sc-28758). Triplicate cores were evaluated for each tumor sample. A neuropathologist blinded to diagnosis and genotype scored the intensity of staining as none, weak, moderate, or strong. The percentage of cases in each intensity category was then plotted by histological type. The status of codon 172 *(IDH-2)* in tissues was assessed by Sanger sequencing; codon 132 *(IDH-1)* was evaluated by IHC using an anti-human R132H antibody from Dianova (Hamburg, Germany) and by sequencing.

Other Experimental Methods

Primers and antibodies for real time polymerase chain reaction (PCR) and Western blotting were purchased from Applied Biosystems (Foster City, CA; Hs00300047_m1) and Abcam (Cambridge, MA; ab58304). Basal pHi and acid load recovery capability were measured in BTICs using the pH-dependent fluorophore BCECF as described elsewhere.[12] Pyrosequencing of DNA was performed by EpigenDx (Orlando, FL) using protocol ADS1552.

Results

Real time PCR and Western blotting confirmed the absence of *NHE-1* expression in BT054 and BT088 cells (Fig 1A, B). Genes adjacent to *NHE-1* were expressed similarly in oligodendroglioma and GBM cells, indicating selective underexpression of *NHE-1* in BT054 and BT088, while excluding gene–dose effects due to 1p allelic loss as the sole cause of silencing (Supplementary Fig). SNP analysis and whole genome sequencing of

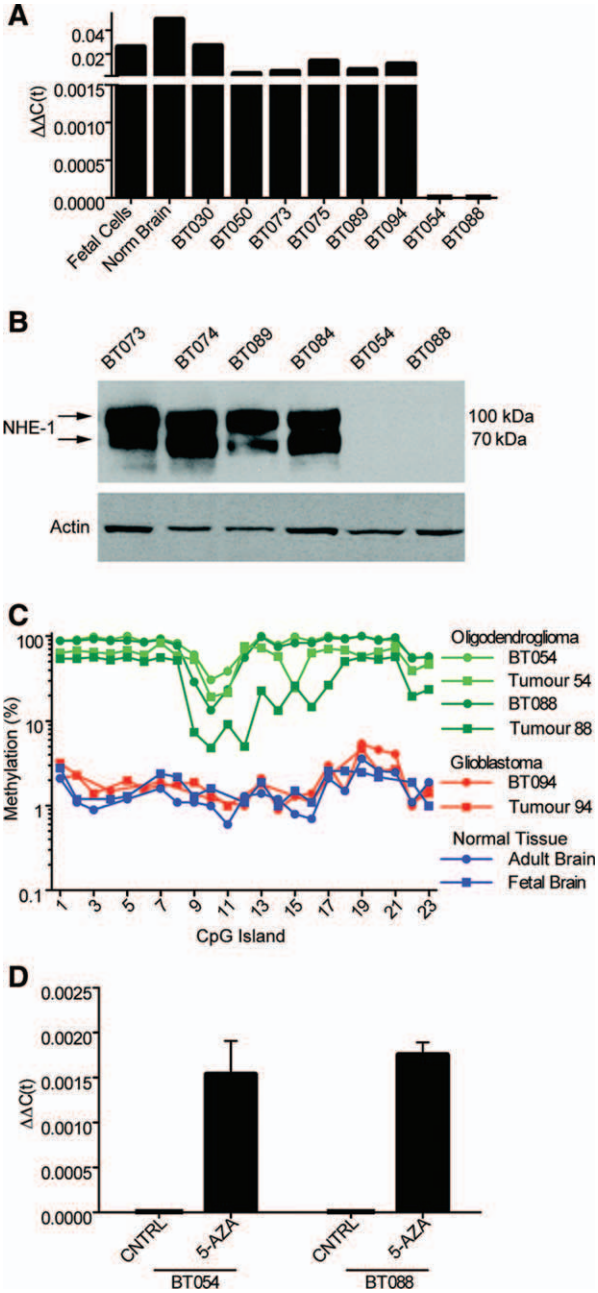

FIGURE 1: Absence of *NHE-1* in oligodendroglioma brain tumor-initiating cells is shown. (A) Real time polymerase chain reaction for *NHE-1* was performed on glioblastoma (GBM) and fetal neural stem cells, normal adult brain and oligodendroglioma cells, BT054, and BT088. Transcript was present in all cells and tissues except BT054 and BT088. (B) NHE-1 protein was absent in BT054 and BT088 but present in GBM cells. (C) Pyrosequencing of 23 CpG islands in the *NHE-1* promoter revealed methylation in BT054, BT088, and parent tumors, but not in BT094, its parent GBM, or normal brain. (D) Treatment with 3μm 5-azacytidine (5-AZA) for 3 days restored *NHE-1* expression. Error bars = standard error of the mean.

BT054 and BT088 and exome sequencing of codeleted oligodendrogliomas revealed no deletions or mutations of *NHE-1*.[3] However, methylation of the *NHE-1* promoter

was seen in BT054 and BT088 cells and parent tumors (see Fig 1C). Promoter methylation was not detected in BT094, its parent GBM, or normal brain. Moreover, exposure of BT054 and BT088 cells to 5-azacytidine restored expression of *NHE-1* (see Fig 1D), confirming that promoter methylation in cells from *IDH*-mutant oligodendrogliomas contributed to its silencing.

To determine whether *NHE-1* was commonly silenced in oligodendrogliomas—not simply a feature of our 2 lines and their parent tumors—we evaluated methylation of the *NHE-1* promoter in oligodendrogliomas, GBMs, and normal brain tissues. We found methylation in oligodendrogliomas (n = 19), but not GBMs (n = 32) or normal brain (n = 3). The average level of methylation was 14.91% in oligodendrogliomas (standard deviation [SD] = 18.53) versus 1.79% in GBMs (SD = 0.574; $p = 0.0002$, unpaired t test; Fig 2A). GBMs and normal brain had equally low levels. IHC performed on an expanded panel of oligodendrogliomas (n = 27) and GBMs (n = 26) confirmed silencing of *NHE-1* at the protein level. The former were *IDH* mutant with 1p/19q codeletion, and the latter were *IDH* wild type with intact 1p/19q. *NHE-1* expression was lower in oligodendrogliomas than GBMs (see Fig 2B; $p < 0.0001$, Mann–Whitney test) and undetectable in 12 of 27 (44%) versus 1 of 26 (4%). Low levels were observed in both low-grade and high-grade oligodendrogliomas ($p = 0.4980$, Mann–Whitney test). Expression and methylation varied in oligodendrogliomas, perhaps reflecting degrees of admixed normal tissue. These data show that methylation and low expression of *NHE-1* are features of oligodendrogliomas.

To explore the contributions of 1p loss and *IDH* mutation to silencing, we examined *NHE-1* methylation and expression in *IDH*-mutant, 1p-intact oligoastrocytomas and astrocytomas. *NHE-1* promoter methylation was not specific to oligodendrogliomas; methylation was also detected in astrocytic gliomas (n = 15) in association with mutations of *IDH* ($p = 0.0009$, unpaired t test; see Fig 2A). (In data not shown, we also detected methylation of the NHE-1 promoter in 4 secondary GBMs.) However, *NHE-1* staining intensity was lower in oligodendrogliomas than 1p-intact astrocytic tumors (n = 32, $p = 0.0001$, Mann–Whitney test; see Fig 2B–D). Moreover, *NHE-1* expression in astrocytic gliomas increased with tumor grade ($p = 0.0092$, Mann–Whitney test) and was absent in only 3 of 32 cases (9%). *NHE-1* promoter methylation was a feature of *IDH*-mutant tumors, but low expression was characteristic of oligodendrogliomas, implying that 1p loss contributed to silencing.

We then inquired whether *NHE-1*–deficient BT054 and BT088 cells had a pH disturbance. First, we

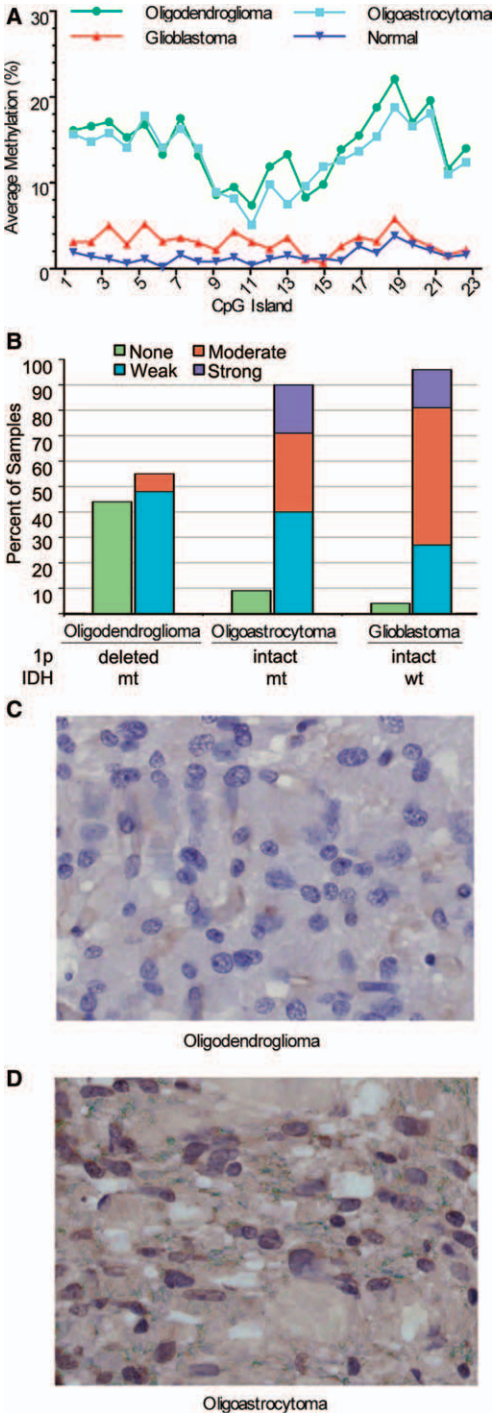

FIGURE 2: Low *NHE-1* expression in oligodendrogliomas is shown. (A) Pyrosequencing of the *NHE1* promoter shows average percentage methylation in oligodendrogliomas (n = 19), astrocytic gliomas (n = 15), glioblastomas (GBMs) (n = 32), and normal brain (n = 3). (B) *NHE-1* immunohistochemistry (IHC) on microarrays containing oligodendrogliomas (n = 27), astrocytic gliomas (n = 32), and GBMs (n = 26) is shown. Expression for each tumor sample was scored as none, weak, moderate, or strong. The percentage of samples in each of these categories was then plotted by tumor type as shown. (C, D) *NHE-1* IHC shows absence of staining of an anaplastic oligodendroglioma compared to moderate staining of an anaplastic oligoastrocytoma (capillary staining is visible in the oligodendroglioma).

observed that BT054 and BT088 had an acidic pHi; their basal pHi was 6.8 and 6.9 versus 7.3 and 7.8 for GBM lines (Fig 3) and 7.4 for mouse neural stem cells (data not shown). We then showed that pH regulation was disturbed in BT054 and BT088. After nigericin-induced acid loading, a maneuver that mimics physiological acidic stress, the pHi of oligodendroglioma cells recovered slowly and partially, whereas that of glioblastoma BTICs and mouse neural stem cells (data not shown) rapidly returned to basal levels. When *NHE-1* was re-expressed in BT054 and BT088, their pHi increased, a change accompanied by full acid load recovery. These results are consistent with the known role of *NHE-1* in restoring physiological pHi after acid stress,[13] and affirm that loss of *NHE-1* has important effects on oligodendroglioma cell physiology.

Discussion

NHE-1 encodes a transmembrane protein that plays a central role in the regulation of pHi. In the context of metabolically active cancers, silencing of *NHE-1* is unprecedented because *NHE-1* is required to maintain a physiological pHi in the face of the high acid load generated by aerobic glycolysis, the preferred metabolic state of cancer cells (ie, the Warburg effect). Yet, we found that *NHE-1* was expressed at low levels in codeleted oligodendroglioma tumor tissues and silenced in oligodendroglioma cell lines. Silencing disrupted pH regulation in cells and was only observed in tumors with 1p allelic loss. To date, no normal adult or cancer tissue has been identified in which *NHE-1* is silenced, but our findings are consistent with the recent observation that expression of mutant *IDH-1* in astrocytes causes selective hypermethylation, including the *NHE-1* promoter.[5] Could silencing of *NHE-1* on 1p contribute to the behavior or pathogenesis of oligodendrogliomas?

Absence of *NHE-1* has the potential to alter the metabolism of cancer cells. Transformed cells that rely on glycolysis to generate adenosine triphosphate require *NHE-1* for survival,[14] whereas those that use oxidative phosphorylation for energy production can survive without *NHE-1*. A cancer in which *NHE-1* is silenced would grow slowly because neutralization of cellular acidosis is necessary to support the rapid growth that glycolysis facilitates.[15] Silencing of *NHE-1* has recently been shown to blunt in vivo tumor growth.[16] The indolent behavior of oligodendrogliomas is consistent with these observations. Paradoxically, silencing could also be tumorigenic; in experimental models, inhibition of *NHE-1* is associated with DNA damage[17] and low pHi with sister chromatid exchange.[18] The bizarre karyotypes of BT054 and

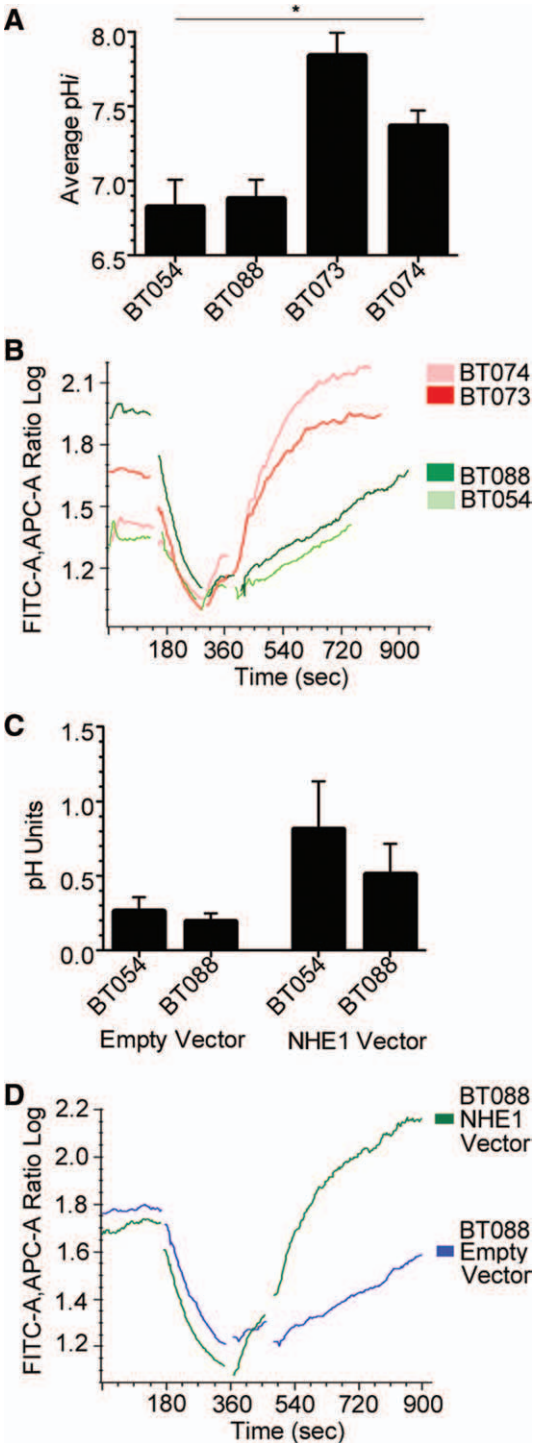

FIGURE 3: *NHE-1* functional studies in oligodendroglioma brain tumor-initiating cells are shown. (A) Basal intracellular pH (pHi) was measured in BT054 and BT088 and GBM lines BT073 and BT074. The pHi of BT054 and BT088 was 6.8 to 6.9 versus 7.3 to 7.8 for BT073 and BT074 ($p = 0.0025$, 2-way analysis of variance). (B) Ability to recover from an acid load was measured. BT073 and BT074 recovered rapidly and completely; BT054 and BT088 recovered slowly and partially (representative curves from 3 independent experiments). (C) After restoration of *NHE-1*, pHi increased to alkaline levels, and (D) acid load recovery normalized. *$p = 0.01$ to 0.05; error bars = standard error of the mean.

BT088 cells are consistent with such effects.[6] Furthermore, *NHE-1* inhibition in the presence of growth factors induces mitogen-activated protein kinase, causing persistent cell proliferation.[19] Through sustained proliferation and genomic instability, loss of *NHE-1* could enhance cancer risk.

Oligodendrogliomas with 1p/19q codeletion display the usual hallmarks of cancer, but many grow slowly, even when anaplastic, or relapsing after treatment. Those that recur quickly frequently have 1p polysomy.[20] Such paradoxes might be explained by a genetic mechanism such as silencing of *NHE-1* that promotes tumorigenesis while simultaneously restricting the rapid cellular growth typical of cancer.

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Authorship

J.A.C. and J.G.C. contributed equally to this work.

Potential Conflicts of Interest

Nothing to report.

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