5-Hydroxymethylcytosine Is Strongly Depleted in Human Cancers but Its Levels Do Not Correlate with IDH1 Mutations

Seung-Gi Jin\textsuperscript{1}, Yong Jiang\textsuperscript{1}, Runxiang Qiu\textsuperscript{2}, Tibor A. Rauch\textsuperscript{4}, Yinsheng Wang\textsuperscript{3}, Gabriele Schackert\textsuperscript{5}, Dietmar Krex\textsuperscript{5}, Qiang Lu\textsuperscript{5}, and Gerd P. Pfeifer\textsuperscript{1}

Abstract
The base 5-hydroxymethylcytosine (\textit{5hmC}) was recently identified as an oxidation product of 5-methylcytosine in mammalian DNA. Here, using sensitive and quantitative methods to assess levels of 5-hydroxymethyl-2'-deoxycytidine (\textit{5hmCdC}) and 5-methyl-2'-deoxycytidine (\textit{5mCdC}) in genomic DNA, we investigated whether levels of \textit{5hmC} can distinguish normal tissue from tumor tissue. In squamous cell lung cancers, levels of \textit{5hmCdC} were depleted substantially with up to 5-fold reduction compared with normal lung tissue. In brain tumors, \textit{5mCdC} showed an even more drastic reduction with levels up to more than 30-fold lower than in normal brain, but \textit{5hmCdC} levels were independent of mutations in isocitrate dehydrogenase-1. Furthermore, immunohistochemical analysis indicated that \textit{5hmC} is remarkably depleted in many types of human cancer. Importantly, an inverse relationship between \textit{5hmC} levels and cell proliferation was observed with lack of \textit{5hmC} in proliferating cells. The data therefore suggest that \textit{5hmCdC} is strongly depleted in human malignant tumors, a finding that adds another layer of complexity to the aberrant epigenome found in cancer tissue. In addition, a lack of \textit{5hmC} may become a useful biomarker for cancer diagnosis. Cancer Res; 71(24); 7360–5. ©2011 AACR.

Introduction
Recently, 5-hydroxymethylcytosine (\textit{5hmC}) has been identified as an oxidation product of 5-methylcytosine in mammalian DNA (1, 2) generated by the \textalpha-ketoglutarate-dependent Tet dioxygenases (2, 3). Levels of \textit{5hmC} are tissue dependent and the highest levels have been found in the central nervous system (4, 5). The biological function of \textit{5hmC} is currently unknown. \textit{5hmC} may be an intermediate in DNA demethylation processes that accomplishes the conversion of \textit{5mC} to cytosine (6). The distribution of \textit{5hmC} in embryonic stem cells (7–12), mouse cerebellum (13), and human prefrontal cortex (14) has been mapped by array- or sequencing-based assays. \textit{5hmC} was enriched at promoters and within gene bodies.

Several hematologic malignancies carry mutations in one of the \textit{TET} genes, \textit{TET2} (15). \textit{TET2} mutations were linked to aberrant levels of \textit{5hmC} and \textit{5mC} in these cancer genomes (16, 17). Also, mutations in isocitrate dehydrogenase-1 (\textit{IDH1}) have been linked to abnormal DNA methylation patterns (18). One attractive proposal is that mutated \textit{IDH1} produces a new metabolite, 2-hydroxyglutarate (2HG; ref. 19), which can inhibit TET proteins potentially leading to altered levels of \textit{5hmC} and \textit{5mC} in tumors (20).

Systematic studies on levels of \textit{5hmC} in human cancers are lacking. Here, we have used liquid chromatography/tandem mass spectrometry (LC/MS-MS), to assess the levels of 5-hydroxymethyl-2'-deoxycytidine (\textit{5hmCdC}) and 5-methyl-2'-deoxycytidine (\textit{5mCdC}) in human lung carcinomas and in brain tumor DNA. We also used immunofluorescence staining to assess \textit{5hmC} in a series of normal and malignant tissue sections.

Materials and Methods

\textbf{DNA samples}
Stage-I lung squamous cell carcinoma (SCC) and adenocarcinoma samples and matched normal tissues were obtained from the frozen tumor bank of the City of Hope Medical Center under an Institutional Review Board approved protocol. Samples were obtained from tumors without laser-capture microdissection. DNA from primary small cell lung cancers and matched normal lung was obtained from Asterand, BioChain, and Cureline. Normal human brain tissue DNAs of the prefrontal cortex were obtained from Capital Biosciences and BioChain. DNA from neurons and astrocytes of fetal (24 weeks of gestation) human brain was obtained from ScienCell.
Twenty-seven astrocytomas (World Health Organization, grade II–III) were obtained on Institutional Review Board approved protocols at the Department of Neurosurgery at the University Hospital in Dresden. DNA was isolated by standard procedures with phenol-chloroform extraction and ethanol precipitation. Eight additional brain tumor DNAs were obtained from Asterand. Genomic DNAs from tissues and cell lines were isolated by the help of the DNeasy Tissue Kit (QIAGEN).

**IDH mutations**

For sequencing of IDH1 and IDH2 exon 4, 40 ng of genomic DNA was used for PCR amplification using the following primers: for IDH1, forward 5'-TGCCACCAACGCACAGTC and reverse 5'-CATGCAAATCACATATTTG; for IDH2, forward 5'-TGAAAGATGGCGGCTGCAGT and reverse 5'-GGGGTGAAGACCATTTTGAA.

**Simultaneous quantification of 5mC and 5hmC by LC/MS-MS**

Genomic DNA (1–2 μg) was incubated with 5 units of DNA Degradase Plus (Zymo Research) at 37°C for at least 2 hours. The stable isotope labeled 5hmC (21, 22) and labeled 2'-deoxyguanosine (Cambridge Isotope Laboratories) were added as internal standards. Aliquots of the mixture were subjected directly to LC/MS-MS analysis. LC/MS-MS was carried out with a Thermo Accela 600 HPLC pump interfaced with a TSQ Vantage triple stage quadruple mass spectrometer (Thermo Fisher Scientific). A 2.1 x 50 mm Kinetex XB-C18 column (2.6 μm in particle size and 100 Å in pore size; Phenomenex) was used for separation at a flow rate of 400 μL/min. The TSQ mass spectrometer was optimized and set up in selected reaction monitoring scan mode for monitoring the [M + H]^+ ions of 5hmC (m/z 258.1 → 142.1), 5mC (m/z 242.1 → 126.1), dG (m/z 268.1 → 152.1), labeled 5hmC (m/z 261.1 → 144.1) and labeled dG (m/z 273.1 → 157.1). Thermo Xcalibur software (version 2.1) was used to conduct data analysis. Immunodot blot analysis for 5hmC was conducted as described previously (14).

**Immunohistochemistry**

Frozen tissue arrays were from Biochain (catalog no. T6235700-5 and lot no. B403109). They contain normal brain tissue and craniopharyngioma, normal breast and invasive ductal carcinoma, normal colon and adenocarcinoma, normal skeletal muscle and rhabdomyosarcoma, normal kidney and renal cell carcinoma, normal liver and hepatocellular carcinoma, normal lung and SCC, normal pancreas and adenocarcinoma, normal prostate and adenocarcinoma, normal skin and malignant melanoma, normal small intestine and malignant mesenchymoma, normal stomach and adenocarcinoma, normal uterus and adenocarcinoma, and normal ovary and cystadenocarcinoma. The tissue sections were boiled in 10 mmol/L sodium citrate for antigen retrieval followed by blocking with 10% goat serum, 0.1% Triton X-100 in PBS for 1 hour at room temperature (RT). Sections were incubated with primary anti-5hmC polyclonal antibody (dilution 1:1000; Active Motif) in 5% goat serum, 0.01% Triton X-100 in PBS at 4°C, overnight. After washing with PBS at RT, sections were incubated with Rhod Red-X-AffiniPure conjugated goat anti-rabbit secondary antibody (dilution 1:200; Jackson ImmunoResearch) for 1 hour at RT, then washed with PBS and water, and mounted with fluoromount-G solution (SouthernBiotech). Ki67 staining was carried out with Ki67 antibody (BD Pharmingen; catalog...
The anti-5mC antibody was from Eurogentec (catalog no. BI-MECY-0100; dilution 1:200). Slides were counterstained with Hoechst 33258 dye. All fluorescent images were taken with an inverted Olympus IX 81 fluorescence microscope.

**Reverse transcriptase PCR**

Quantitative reverse transcriptase PCR was carried out as described (23).

**Results and Discussion**

To determine the levels of 5hmC and 5mC in normal and tumor tissues, we developed a sensitive LC/MS-MS assay with isotope-labeled internal standards (Supplementary Fig. S1A). 5mC was quantitated with reference to the dG standard. Supplementary Fig. S1B shows examples of LC separation and how mass spectrometric analysis of 5hmC and 5mC was achieved. The method is strictly quantitative as shown by standard curves (Supplementary Fig. S2). We initially tested its performance by measuring 5hmC and 5mC in several cell and tissue DNA samples (Supplementary Fig. S3). The data obtained were consistent with values reported in the literature (2, 4) and were also generally in agreement with a less quantitative immunodot blot assay (Supplementary Fig. S4).

Using the LC/MS-MS assay, we measured 5hmC in 24 stage-I lung SCC DNAs and in matched normal lung DNA (Fig. 1A). The levels of 5hmC, expressed as percentage of dG, were between 0.078% and 0.182% in normal lung. In every SCC tumor except one (LT2), we saw a significant reduction of 5hmC level compared with the paired normal lung sample ($P<0.05$ for each sample pair; $t$ test; Fig. 1A). 5hmC levels were generally 2- to 5-fold lower in the tumors than in normal lung ($P = 8.88 \times 10^{-7}$; paired $t$ test). We also quantitated 5mC (Fig. 1B). 5mC was depleted in most tumor samples with a few exceptions (tumors 1, 2, 6, 7, 15, and 16). In many cases, 5mC levels were lower by only approximately 5% to 20% ($P = 0.023$; paired $t$ test). IDH1 or IDH2 mutations were not found in these lung tumors. We also analyzed 5mC in lung adenocarcinomas and primary small cell lung cancers (Supplementary Fig. S5). As with SCC, 5mC was depleted in most of these tumors relative to matched normal tissue.

Next, we analyzed the 2 modified 2'-deoxynucleosides in 6 normal brain DNA samples and in 33 stage II and III astrocytomas (astrocytic gliomas) and in 2 glioblastomas. We found high levels of 5hmC in normal human brain prefrontal cortex DNA (Fig. 2A), in which 5hmC was between 0.82% and 1.18% of dG. We also measured levels of 5hmC and 5mC in astrocytes and in neurons from human fetal brain. Levels of 5hmC were higher (1.45% 5hmC/dG) in neurons than in astrocytes (0.23% 5hmC/dG; Supplementary Fig. S6). In brain tumors, 5mC was strongly depleted relative to normal brain (Fig. 2A). Some astrocytomas contained only 0.03% to 0.04% of 5hmC, a reduction of more than 30-fold ($P = 1.55 \times 10^{-11}$; unpaired $t$ test). Because astrocytomas initiate in neural stem cells or glial progenitor cells, their decreased level of 5hmC may be due to either the malignant state or to the cell of origin of these tumors. The varying levels of 5hmC in tumors did not...
correlate with patient age or whether the tumor was stage II or III or with patient survival (data not shown). Levels of 5mC showed only a small reduction in some brain tumors (Fig. 2B; \( P = 0.3; \) unpaired \( t \) test). There was no correlation between levels of 5hmC and levels of 5mC. A substantial fraction of stages II and III gliomas contain mutations in IDH1 and much more rarely, in IDH2 (24). We determined the mutation status of IDH1 at codon 132 (Supplementary Fig. S7). We identified 16 stage II/III tumors with the typical codon R132H mutation, and 17 stage II/III tumors without any IDH1 mutation. The R132H IDH1 mutation produces a neomorphic enzyme with the capacity to generate 2HG (19). We expected that IDH1-mutant tumors would have lower levels of 5mC according to the presumed role of 2HG as an inhibitor of TET oxidases (see Supplementary Fig. S8). Surprisingly, however, the levels of 5hmC were evenly distributed between the low and high ranges, both in IDH1 wild type and in IDH1-mutant tumors (Fig. 2A; \( P = 0.53; \) \( t \) test, nonpaired). This finding is in contrast to a previous report, which observed a significant reduction of 5hmC in IDH1-mutant gliomas by immunohistochemistry (20). Similarly, IDH1-mutant and wild-type cases did not show differences in levels of 5mC (Fig. 2B).

To investigate whether loss of 5hmC is a feature of human cancers in general, we conducted immunohistochemical staining with an anti-5hmC antibody (Fig. 3 and Supplementary Fig. S9). This antibody was verified previously by us and used for detecting 5hmC in early embryos (14, 23). Normal tissue sections and corresponding tumor were stained with this antibody. We observed substantial 5hmC staining of almost all cells in most normal tissues. However, staining in corresponding tumors was universally decreased with only a few cells (<10%) staining positive for 5hmC. The only exception was a tumor originating in the colon (Supplementary Fig. S9). Additional lung tumor slides were also analyzed (Supplementary Fig. S10) including tumor and adjacent normal lung (Supplementary Fig. S10A).

We also did parallel staining of several normal and tumor sections for 5mC using an anti-5mC antibody (Supplementary Fig. S11) and did not observe a substantial decrease of 5mC staining in the tumors. This means that the loss of 5hmC is not simply due to loss of 5mC in tumors. We then determined whether reduced staining of 5hmC in tumors is due to increased cell proliferation. We used an anti-Ki67 antibody to stain proliferating cells. Sections of normal brain were almost

Figure 3. Immunohistochemical analysis of 5hmC on human tissue arrays. Human tissue arrays containing samples of malignant tumor and corresponding normal tissue were stained with anti-5hmC antibody. Staining with Hoechst 33258 is shown as a control. The magnification of all panels is 10-fold.
completely devoid of Ki67 antigen but brain tumors contained many Ki67-positive cells that lacked 5hmC staining (Supplementary Fig. S12). Similarly, there was little Ki67 staining in normal lung, but in adjacent carcinoma tissue, we saw mutually exclusive staining of Ki67 and 5hmC (Fig. 4 and Supplementary Fig. S10). The same was found for sections of breast, pancreatic tumors, and uterus tumors (Supplementary Fig. S13). Tissue sections of normal small intestine showed strong Ki67 staining for proliferating cells at the bottom of crypts with lack of 5hmC staining, whereas the more differentiated cells contained high levels of 5hmC and lacked Ki67 staining (Fig. 4). Thus, one straightforward explanation for the loss of 5hmC in tumors is the enhanced rate of cell proliferation in tumors that could lead to a passive loss of 5hmC, which is not a substrate for DNA methyltransferase 1 (25). We noticed that not all cells that lack 5hmC staining in the tumors are Ki67 positive perhaps due to past history of proliferation leading to permanent loss of 5hmC.

The inverse link between 5hmC levels and cell proliferation may also reflect intrinsic differences in cell type, as we are comparing differentiated cells with undifferentiated cancer cells that carry stem cell–like properties or originate from somatic stem cells. Another alternative is the possible existence of aberrations in 5hmC production or elimination pathways in tumors. Mutations in TET genes have not been reported in solid tumors. With the exception of TET3 in brain, there was no substantial reduction of TET gene expression in lung and brain tumors relative to normal tissue as confirmed by reverse transcriptase PCR (Supplementary Fig. S14).

The loss of 5hmC in tumors may have profound effects on DNA methylation patterns. For example, if 5hmC is an intermediate in DNA demethylation, its loss at specific genomic locations may make these sequences more prone to acquire methylation. It will be essential to understand the mechanisms of how 5hmC is lost in tumors. Finally, loss of 5hmC could become a useful molecular biomarker for cancer detection and diagnosis, perhaps in conjunction with Ki67 staining.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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