Identification of a CpG Island Methylator Phenotype that Defines a Distinct Subgroup of Glioma

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SUMMARY

We have profiled promoter DNA methylation alterations in 272 glioblastoma tumors in the context of The Cancer Genome Atlas (TCGA). We found that a distinct subset of samples displays concerted hypermethylation at a large number of loci, indicating the existence of a glioma CpG island methylator phenotype (G-CIMP). We validated G-CIMP in a set of non-TCGA glioblastomas and low-grade gliomas. G-CIMP tumors belong to the proneural subgroup, are more prevalent among lower-grade gliomas, display distinct copy-number alterations, and are tightly associated with IDH1 somatic mutations. Patients with G-CIMP tumors are younger at the time of diagnosis and experience significantly improved outcome. These findings identify G-CIMP as a distinct subset of human gliomas on molecular and clinical grounds.

INTRODUCTION

Human gliomas present as heterogeneous disease, primarily defined by the histologic appearance of the tumor cells. Astrocytoma and oligodendroglioma constitute the infiltrating gliomas (Adamson et al., 2009). The cell or cells of origin have not definitively been identified, however, the identification of tumorigenic, stem-cell like precursor cells in advanced stage gliomas suggests that human gliomas may have a neural stem cell origin (Canoll and Goldman, 2008; Dirks, 2006; Galli et al., 2004). Gliomas are subdivided by the World Health Organization (WHO) by histological grade, which is an indication of

Significance

Glioblastoma (GBM) is a highly aggressive form of brain tumor, with a patient median survival of just over one year. The Cancer Genome Atlas (TCGA) project aims to characterize cancer genomes to identify means of improving cancer prevention, detection, and therapy. Using TCGA data, we identified a subset of GBM tumors with characteristic promoter DNA methylation alterations, referred to as a glioma CpG island methylator phenotype (G-CIMP). G-CIMP tumors have distinct molecular features, including a high frequency of IDH1 mutation and characteristic copy-number alterations. Patients with G-CIMP tumors are younger at diagnosis and display improved survival times. The molecular alterations in G-CIMP tumors define a distinct subset of human gliomas with specific clinical features.
differentiation status, malignant potential, response to treatment, and survival. Glioblastoma (GBM), also described as grade IV glioma, accounts for more than 50% of all gliomas (Adamson et al., 2009). Patients with GBM have an overall median survival time of only 15 months (Brandes et al., 2001; Martinez et al., 2010; Parsons et al., 2008). Most GBMs are diagnosed as de novo or primary tumors and are more common in males. A subset of ~5% of GBM tumors, termed secondary GBM, progress from lower-grade tumors (grade II/III), are seen in younger patients, are more evenly distributed among the sexes, and exhibit longer survival times (reviewed in Adamson et al., 2009; Furnari et al., 2007).

There is currently great interest in characterizing and compiling the genome and transcriptome changes in human GBM tumors to identify aberrantly functioning molecular pathways and tumor subtypes. The Cancer Genome Atlas (TCGA) pilot project identified genetic changes of primary DNA sequence and copy number, DNA methylation, gene expression, and patient clinical information for a set of GBM tumors (The Cancer Genome Atlas Research Network, 2008). TCGA also reaffirmed genetic alterations in TP53, PTEN, EGFR, RB1, NF1, ERBB2, PIK3R1, and PIK3CA mutations and detected an increased frequency of NF1 mutations in GBM patients (The Cancer Genome Atlas Research Network, 2008). Recent DNA sequencing analyses of primary GBM tumors with a more comprehensive approach (Parsons et al., 2008) also identified somatic mutations in isocitrate dehydrogenase 1 (IDH1) that occur in 12% of all GBM patients. IDH1 mutations have only been detected at the arginine residue in codon 132, with the most common change being the R132H mutation (Parsons et al., 2008; Yan et al., 2009), which results in a gain of enzyme function, resulting in increased patient survival (Bals et al., 2008; Hartmann et al., 2009; Yan et al., 2009). Higher IDH1 mutation rates are seen in grade II and III astrocytomas and oligodendrogliomas (Bals et al., 2008; Bleeker et al., 2009; Hartmann et al., 2009; Yan et al., 2009), suggesting that IDH1 mutations generally occur in the progressive form of glioma, rather than in de novo GBM. Mutations in the related IDH2 gene are of lower frequency and generally nonoverlapping with tumors containing IDH1 mutations (Hartmann et al., 2009; Yan et al., 2009).

Cancer-specific DNA methylation changes are hallmarks of human cancers, in which global DNA hypomethylation is often seen concomitantly with hypermethylation of CpG islands (reviewed in Jones and Baylin, 2007). Promoter CpG island hypermethylation generally results in transcriptional silencing of the associated gene (Jones and Baylin, 2007). CpG island hypermethylation events have also been shown to serve as biomarkers in human cancers, for early detection in blood and other bodily fluids, for prognosis or prediction of response to therapy, and to monitor cancer recurrence (Laird, 2003).

A CpG island methylator phenotype (CIMP) was first characterized in human colorectal cancer by Toyota and colleagues (Toyota et al., 1999) as cancer-specific CpG island hypermethylation of a subset of genes in a subset of tumors. We confirmed and further characterized colorectal CIMP using MethylLight technology (Weisenberger et al., 2006). Colorectal CIMP is characterized by tumors in the proximal colon, a tight association with BRAF mutations, and microsatellite instability caused by MLH1 promoter hypermethylation and transcriptional silencing (Weisenberger et al., 2006).

DNA methylation alterations have been widely reported in human gliomas, and there have been several reports of promoter-associated CpG island hypermethylation in human GBM and other glioma subtypes (Kim et al., 2006; Martinez et al., 2009; Martinez et al., 2007; Nagarajan and Costello, 2009; Stone et al., 2004; Tepel et al., 2008; Uhleman et al., 2003). Several studies have noted differences between primary and secondary GBMs with respect to epigenetic changes. Overall, secondary GBMs have a higher frequency of promoter methylation than primary GBM (Oghi and Kleihues, 2007). In particular, promoter methylation of RB1 was found to be approximately three times more common in secondary GBM (Nakamura et al., 2001).

Hypermethylation of the MGMT promoter-associated CpG island has been shown in a large percentage of GBM patients (Esteller et al., 2000; Esteller et al., 1999; Hegi et al., 2005; Hegi et al., 2008; Herman and Baylin, 2003). MGMT encodes for an O6-methylguanine methyltransferase that removes alkyl groups from the O-6 position of guanine. GBM patients with MGMT hypermethylation showed sensitivity to alkylating agents such as temozolomide, with an accompanying improved outcome (Esteller et al., 2000; Esteller et al., 1999; Hegi et al., 2005; Hegi et al., 2008; Herman and Baylin, 2003). However, initial promoter methylation of MGMT, in conjunction with temozolomide treatment, may result in selective pressure to lose mismatch repair function, resulting in aggressive recurrent tumors with a hypermutator phenotype (Cahill et al., 2007; Hegi et al., 2005; Silber et al., 1999; The Cancer Genome Atlas Research Network, 2008).

Here, we report the DNA methylation analysis of 272 GBM tumors collected for TCGA, extend this to lower-grade tumors, and characterize a distinct subgroup of human gliomas exhibiting CIMP.

RESULTS
Identification of a Distinct DNA Methylation Subgroup within GBM Patients

We determined DNA methylation profiles in a discovery set of 272 TCGA GBM samples. At the start of this study, we relied on the Illumina GoldenGate platform, using both the standard Cancer Panel I, and a custom-designed array (The Cancer Genome Atlas Research Network, 2008) (Figures S1A–S1C available online and Experimental Procedures), but migrated to the more comprehensive Infinium platform (Figure 1 and Figure S1C), as it became available. DNA methylation measurements were highly correlated for CpG dinucleotides shared between the two platforms (Pearson’s r = 0.94, Figure S1D). Both platforms interrogate a sampling of about two CpG dinucleotides per gene. Although this implies that nonrepresentative CpGs may be assessed for some promoters, it is likely that representative results will be obtained for most gene promoters, given the very high degree of locally correlated DNA methylation behavior (Eckhardt et al., 2006).

We selected the most variant probes on each platform and performed consensus clustering to identify GBM subgroups
We identified three DNA methylation clusters using either the GoldenGate or Infinium data, with 97% concordance (61/63) in cluster membership calls for samples run on both platforms (Table S1). Cluster 1 formed a particularly tight cluster on both platforms with a highly characteristic DNA methylation profile (Figure 1 and Figure S1), reminiscent of the CpG island methylator phenotype described in colorectal cancer (Toyota et al., 1999; Weisenberger et al., 2006). CIMP in colorectal cancer is characterized by correlated cancer-specific CpG island hypermethylation of a subset of genes in a subset of tumors and not just a stochastic increase in the frequency of generic CpG island methylation across the genome (Toyota et al., 1999; Weisenberger et al., 2006). CIMP in colorectal cancer is characterized by correlated cancer-specific CpG island hypermethylation of a subset of genes in a subset of tumors and not just a stochastic increase in the frequency of generic CpG island methylation across the genome (Toyota et al., 1999; Weisenberger et al., 2006). CIMP in colorectal cancer is characterized by correlated cancer-specific CpG island hypermethylation of a subset of genes in a subset of tumors and not just a stochastic increase in the frequency of generic CpG island methylation across the genome (Toyota et al., 1999; Weisenberger et al., 2006). Cluster 1 GBM samples show similar concerted methylation changes at a subset of loci. We therefore designated cluster 1 tumors as having a glioma CpG island methylator phenotype (G-CIMP). Combining Infinium and GoldenGate data, we identified 24 of 272 TCGA GBM samples (8.8%) as the G-CIMP subtype (Table S1).

Characterization of G-CIMP Tumors within Gene Expression Clusters

Four gene expression subtypes (proneural, neural, classical, and mesenchymal) have been previously identified and characterized with TCGA GBM samples (Verhaak et al., 2010). We compared the DNA methylation consensus cluster assignments for each sample to their gene expression cluster assignments (Figure 1, Figure 2A, Figures S1A and S1B, and Table S1). The G-CIMP sample cluster is highly enriched for proneural GBM tumors, whereas the DNA methylation clusters 2 and 3 are moderately enriched for classical and mesenchymal expression groups, respectively. Of the 24 G-CIMP tumors, 21 (87.5%) were classified within the proneural expression group. These G-CIMP tumors represent 30% (21/71) of all proneural GBM tumors, suggesting that G-CIMP tumors represent a distinct subset of proneural GBM tumors (Figure 2A, Figure S2A, and Table S1). The few nonproneural G-CIMP tumors belong to neural (2/24 tumors, 8.3%), and mesenchymal (1/24 tumors, 4.2%) gene expression groups.

In order to obtain an integrated view of the relationships of G-CIMP status and gene expression differences, we performed pairwise comparisons between members of different molecular subgroups (Figure 2B). We calculated the mean Euclidean distance in both DNA methylation and expression for each possible pairwise combination of the five different subtypes: G-CIMP-positive proneural, G-CIMP-negative proneural, classical, mesenchymal, and neural tumors. We observed the high
dissimilarity of the GP, GN, GC, and GM pairs (Figure 2B), supporting the hypothesis that G-CIMP-positive tumors are a unique molecular subgroup of GBM tumors and more specifically that G-CIMP status provides further refinement of the proneural subset. Indeed, among the proneural tumors, the G-CIMP-positive tumors are distinctly dissimilar to the mesenchymal tumors (GM pair), whereas the G-CIMP-negative proneural tumors are relatively similar to mesenchymal tumors (PM pair). We focused downstream analyses on comparisons between G-CIMP-positive versus G-CIMP-negative tumors within the proneural subset, to avoid misidentifying proneural features as G-CIMP-associated features.

Clinical Characterization of G-CIMP Tumors

We further characterized G-CIMP tumors by reviewing the available clinical covariates for each patient. Although patients with proneural GBM tumors are slightly younger (median age, 56 years) than all other nonproneural GBM patients (median age, 57.5 years), this was not statistically significant (p = 0.07). However, patients with G-CIMP tumors were significantly younger at the time of diagnosis compared with patients diagnosed with non-G-CIMP proneural tumors (median ages of 36 and 59 years, respectively; p < 0.0001; Figure 2C).

The overall survival for patients of the proneural subtype was not significantly improved compared to other gene expression
subtypes (Figure 2D), but significant survival differences were seen for groups defined by DNA methylation status (Figures 2E and 2F and Figure S2B). We observed significantly better survival for proneural G-CIMP-positive patients (median survival of 150 weeks) than proneural G-CIMP-negative patients (median survival of 42 weeks) or all other nonproneural GBM patients (median survivals of 54 weeks). G-CIMP status remained a significant predictor of improved patient survival ($p = 0.0165$) in Cox multivariate analysis after adjusting for patient age, recurrent versus non-recurrent tumor status and secondary GBM versus primary GBM status.

**IDH1 Sequence Alterations in G-CIMP Tumors**

Nine genes were found to have somatic mutations that were significantly associated with proneural G-CIMP-positive tumors (Figure S3A and Table S2). *IDH1* somatic mutations, recently identified primarily in secondary GBM tumors (Balss et al., 2008; Parsons et al., 2008; Yan et al., 2009), were found to be very tightly associated with G-CIMP in our data set (Table 1), with 18 *IDH1* mutations primarily observed in 23 (78%) G-CIMP-positive tumors, and 184 G-CIMP-negative tumors were *IDH1*-wild-type ($p < 2.2 \times 10^{-16}$). The five discordant cases of G-CIMP-positive, *IDH1*-wild-type tumors are not significantly different in age compared to G-CIMP-positive, *IDH1* mutant tumors (median ages of 34 and 37 years respectively; $p = 0.873$). However, the five discordant cases of G-CIMP-positive, *IDH1*-wild-type tumors are significantly younger at the time of diagnosis compared to patients with G-CIMP-negative, *IDH1*-wild-type tumors (median ages of 37 and 59 years respectively; $p < 0.008$). Interestingly, two of the five patients each survived more than 5 years after diagnosis. We did not observe any *IDH2* mutations in the TCGA data set. Tumors displaying both G-CIMP-positive and *IDH1* mutation occurred at low frequency in primary GBM, but were enriched in the set of 16 recurrent (treated) tumors and to an even greater degree in the set of four secondary GBM (Table 1). We also identified examples of germline mutations (nine genes) and loss of heterozygosity (six genes) that were significantly associated with proneural G-CIMP-positive tumors (Figures S3B and S3C and Table S2).

**DNA Copy-Number Variation in Proneural G-CIMP Tumors**

In order to elucidate critical alterations within proneural G-CIMP-positive tumors, we analyzed gene-centric copy-number variation data (see Supplemental Information). We identified significant copy-number differences in 2875 genes between proneural G-CIMP-positive and G-CIMP-negative tumors (Figure 3A and Table S3). Although chromosome 7 amplifications are a hallmark of aggressive GBM tumors (The Cancer Genome Atlas Research Network, 2008), copy-number variation along chromosome 7 was reduced in proneural G-CIMP-positive tumors. Gains in chromosomes 8q23.1-q24.3 and 10p15.3-p11.21 were identified (Figures 3B and 3C). The 8q24 region contains the MYC oncogene, is rich in sequence variants, and was previously shown as a risk factor for several human cancers (Amundadottir et al., 2006; Freedman et al., 2006; Haiman et al., 2007a; Haiman et al., 2007b; Schumacher et al., 2007; Shete et al., 2009; Visakorpi et al., 1995; Yeager et al., 2007). Accompanying the gains at chromosome 10p in proneural G-CIMP-positive tumors, we also detected deletions of the same chromosome arm in G-CIMP-negative tumors (Figure 3C). Similar copy-number variation results were obtained when comparing all G-CIMP-positive to G-CIMP-negative samples (Figure S4). These findings point to G-CIMP-positive tumors as having a distinct profile of copy-number variation when compared to G-CIMP-negative tumors.

**Identification of DNA Methylation and Transcriptome Expression Changes in Proneural G-CIMP Tumors**

To better understand CpG island hypermethylation in glioblastoma, we investigated the differentially methylated CpG sites of these samples (Figure 1B). Among 3153 CpG sites that were differentially methylated between proneural G-CIMP positive and proneural G-CIMP-negative tumors, 3098 (98%) were hypermethylated (Figure 4A). In total, there were 1550 unique genes, of which 1520 were hypermethylated and 30 were hypomethylated within their promoter regions. We ranked our probe list by decreasing adjusted p values and increasing beta-value difference in order to identify the top most differentially hypermethylated CpG probes within proneural G-CIMP-positive tumors (Table S3). The Agilent transcriptome data were used to detect genes showing both differential expression and G-CIMP DNA methylation, in G-CIMP-positive and negative proneural samples. Gene expression values were adjusted for regional copy number changes, as described in Supplemental Information. A total of

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CpG Island Methylation Phenotype in Gliomas

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**Table 1. G-CIMP and IDH1 Mutation Status in Primary, Secondary, and Recurrent GBMs**

<table>
<thead>
<tr>
<th>GBMs</th>
<th>G-CIMP</th>
<th>+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDH1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>184</td>
<td>5</td>
<td>189</td>
</tr>
<tr>
<td>Mutant</td>
<td>0</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>184</td>
<td>23</td>
<td>207</td>
</tr>
<tr>
<td>Primary Tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDH1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
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<td>4</td>
<td>175</td>
</tr>
<tr>
<td>Mutant</td>
<td>0</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>171</td>
<td>16</td>
<td>187</td>
</tr>
<tr>
<td>Recurrent Tumors</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IDH1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Mutant</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>4</td>
<td>16</td>
</tr>
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<td>Secondary Tumors</td>
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<td>IDH1</td>
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<td>2</td>
</tr>
<tr>
<td>Mutant</td>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

G-CIMP and IDH1 mutation status are compared for All analyzed GBM tumors ($p < 2.2 \times 10^{-16}$), Primary tumors, Recurrent tumors, and Secondary tumors. P value is calculated from Fisher’s exact test. See also Figure S3 and Table S2.
1,030 genes were significantly downregulated and 654 genes were significantly upregulated among proneural G-CIMP-positive tumors (Figure 4B and Table S3). The differentially downregulated gene set was highly enriched for polysaccharide, heparin and glycosaminoglycan binding, collagen, thrombospondin, and cell morphogenesis (p < 2.2E-04, Table S3). In addition, the significantly upregulated gene set was highly enriched among functional categories involved in regulation of transcription, nucleic acid synthesis, metabolic processes, and cadherin-based cell adhesion (p < 6.2E-04). Zinc finger transcription factors were also found to be highly enriched in genes significantly upregulated in expression (p = 3.1E-08). Similar findings were obtained when a permutation analysis was performed and when Affymetrix gene expression data were used (data not shown). We also identified 20 miRNAs that showed significant differences in their gene expression between proneural G-CIMP-positive and proneural G-CIMP-negative tumors (Figure S5 and Table S3).

Integration of the normalized gene expression and DNA methylation gene lists identified a total of 300 genes with both significant DNA hypermethylation and gene expression changes in G-CIMP-positive tumors compared to G-CIMP-negative tumors within the proneural subset. Of these, 263 were significantly downregulated and hypermethylated within proneural G-CIMP-positive tumors (Figure 4C, lower-right quadrant). To validate these differentially expressed and methylated genes, we replicated the analysis using an alternate expression platform (Affymetrix) and derived consistent results (Figure S5). Among the top ranked genes were FABP5, PDPN, CHI3L1, and LGALS3 (Table 2), which were identified in an independent analysis to be highly prognostic in GBM with higher expression associated with worse outcome (Colman et al., 2010). Gene ontology analyses showed G-CIMP-specific downregulation of genes associated with the mesenchyme subtype, tumor invasion, and the extracellular matrix as the most significant terms (Table S3). Genes with roles in transcriptional silencing, chromatin structure
modifications, and activation of cellular metabolic processes showed increased gene expression in proneural G-CIMP-positive tumors. Additional genes differentially expressed in proneural G-CIMP-positive samples are provided in Table S3.

To extend these findings, we subjected the differentially silenced genes to a NextBio (www.nextbio.com) meta-analysis to identify data sets that were significantly associated with our list of 263 hypermethylated and downregulated genes. There was an overlap with downregulated genes in low- and intermediate-grade glioma compared to GBM in a variety of previously published data sets (Ducray et al., 2008; Liang et al., 2005; Sun et al., 2006) (Figure S5 and Table S3). The overlap of the 263-gene set with each of these additional datasets was unlikely to be due to chance (all analyses p < 0.00001). To further characterize this gene set, we tested the survival association of these gene expression values in a collection of Affymetrix profiling data from published and publicly available sources on which clinical annotation was available. This data set included cohorts from the Rembrandt set (Madhavan et al., 2009) as well as other sources and did not include TCGA data (given that TCGA data were used to derive the gene list). In this combined data set, the expression of the 263 gene set was significantly associated with patient outcome (Figure S5G). Together, these findings suggest that G-CIMP-positive GBMs tumors have epigenetically related gene expression differences, which are more consistent with low-grade gliomas as well as high-grade tumors with favorable prognosis.

Validation of G-CIMP in GBM and Incidence in Lower-Grade Gliomas

To validate the existence of G-CIMP loci and better characterize the frequency of G-CIMP in gliomas, we used MethyLight to assay the DNA methylation levels in eight G-CIMP gene regions in seven hypermethylated loci (ANKRD43, HFE, MAL, LGALS3, FAS-1, FAS-2, and RHO-F) and one hypomethylated locus,

Figure 4. Comparison of Transcriptome versus Epigenetic Differences between Proneural G-CIMP-Positive and G-CIMP-Negative Tumors

(A) Volcano plots of all CpG loci analyzed for G-CIMP association. The beta value difference in DNA methylation between the proneural G-CIMP-positive and proneural G-CIMP-negative tumors is plotted on the x axis, and the p value for a FDR-corrected Wilcoxon signed-rank test of differences between the proneural G-CIMP-positive and proneural G-CIMP-negative tumors (−1·log10 scale) is plotted on the y axis. Probes that are significantly different between the two subtypes are shown in red.

(B) Volcano plot for all genes analyzed on the Agilent gene expression platform.

(C) Starburst plot for comparison of TCGA Infinium DNA methylation and Agilent gene expression data normalized by copy-number information for 11,984 unique genes. Log10(FDR-adjusted p value) is plotted for DNA methylation (x axis) and gene expression (y axis) for each gene. If a mean DNA methylation β-value or mean gene expression value is higher (greater than zero) in G-CIMP-positive tumors, −1 is multiplied to log10(FDR-adjusted p value), providing positive values. The dashed black lines indicate the FDR-adjusted p value at 0.05. Data points in red indicate those that are significantly up- and downregulated in their gene expression levels and significantly hypo- or hypermethylated in proneural G-CIMP-positive tumors. Data points in green indicate genes that are significantly downregulated in their gene expression levels and hypermethylated in proneural G-CIMP-positive tumors compared to proneural G-CIMP-negative tumors. See also Figure S5 and Table S3.
TABLE 2. The Top-50 Most Differentially Hypermethylated and Downregulated Genes in Proneural G-CIMP-Positive Tumors

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>DNA Methylation Wilcoxon Rank p Value</th>
<th>DNA Methylation Beta Value Difference</th>
<th>Gene Expression Wilcoxon Rank p Value</th>
<th>Gene Expression Fold Change</th>
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<tr>
<td>G0S2</td>
<td>2.37E-07 0.76</td>
<td>2.12E-13</td>
<td>-3.92</td>
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<tr>
<td>RBPP1</td>
<td>2.37E-07 0.84</td>
<td>1.07E-14</td>
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</tr>
<tr>
<td>FABP5</td>
<td>2.30E-05 0.3</td>
<td>1.39E-12</td>
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<td></td>
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<tr>
<td>CA3</td>
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<td>RARRES2</td>
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<td>PDNP</td>
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<td>1.47E-07</td>
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DOCK5, in the tumor samples. These eight markers were evaluated in paraffin-embedded tissues from 20 TCGA samples of known G-CIMP status (ten G-CIMP-positive and ten G-CIMP-negative). We observed perfect concordance between G-CIMP calls on the array platforms and those with the MethyLight markers, providing validation of the technical performance of the platforms and of the diagnostic marker panel. These 20 samples were excluded from the validation set described below. A sample was considered G-CIMP positive if at least six genes displayed a combination of DOCK5 DNA hypomethylation and/or hypermethylation of the remaining genes in the panel. Using these criteria, we tested an independent set of non-TCGA GBM samples for G-CIMP status. Sixteen of 208 tumors (7.6%) were found to be G-CIMP positive (Figure 5A), very similar to the findings in TCGA data.

To further expand these observations, we determined the IDH1 mutation status for an independent set 100 gliomas (WHO grades II, III, and IV). Among 48 IDH1 mutant tumors, 35 (72.9%) were positive for G-CIMP. However, only 3/52 cases (5.8%) without an IDH1 mutation were G-CIMP positive (odds ratio = 42; 95% confidence interval (CI), 11-244; Figure S3D), validating the tight association of G-CIMP and IDH1 mutation.

On the basis of the association of G-CIMP status with features of the progressive, rather than the de novo GBM pathway, we hypothesized that G-CIMP status was more common in the low- and intermediate-grade gliomas. We extended this analysis by evaluating 60 grade II and 92 grade III gliomas for G-CIMP DNA methylation using the eight gene MethyLight panel. Compared to GBM, grade II tumors showed a ~10-fold increase in G-CIMP-positive tumors, whereas grade III tumors had an intermediate proportion of tumors that were G-CIMP positive (Figure 5A and Figure S3E). When low- and intermediate-grade gliomas were separated by histologic type, G-CIMP positivity appeared to be approximately twice as common in oligodendroglomas (52/56, 93%) as compared to astrocytomas (43/95, 45%). G-CIMP positive status correlated with improved patient survival within each WHO-recognized grade of diffuse glioma, indicating that the G-CIMP status was prognostic for glioma patient survival (p < 0.032, Figure 5B). G-CIMP status was an independent predictor (p < 0.01) of survival after adjustment for patient age and tumor grade (Figure S3F). Together, these
findings show that G-CIMP is a prevalent molecular signature in low-grade gliomas and confers improved survival in these tumors.

**Stability of G-CIMP at Recurrence**

Because epigenetic events can be dynamic processes, we examined whether G-CIMP status was a stable event in glioma or whether it was subject to change over the course of the disease. To test this, we obtained a set of samples from 15 patients who received a second surgical procedure after tumor recurrence, with time intervals of up to 8 years between initial and second surgical procedures. We used the eight-gene MethyLight panel to determine their G-CIMP status and found that eight samples were G-CIMP positive, whereas seven were G-CIMP negative. Interestingly, among the G-CIMP-positive cases, 8/8 (100%) recurrent samples retained their G-CIMP-positive status. Similarly, among seven G-CIMP-negative cases, all seven remained G-CIMP negative at recurrence, indicating stability of the G-CIMP phenotype over time (Figure 5C).

**DISCUSSION**

In this report, we have identified and characterized a distinct molecular subgroup in human gliomas. Analysis of epigenetic changes from TCGA samples identified the existence of a proportion of GBM tumors with highly concordant DNA methylation of a subset of loci, indicative of a CpG island methylator phenotype (G-CIMP). G-CIMP-positive samples were associated with secondary or recurrent (treated) tumors and tightly associated with IDH1 mutation. G-CIMP tumors also showed a relative lack of copy-number variation commonly observed in GBM, including EGFR amplification, chromosome 7 gain, and chromosome 10 loss. Interestingly, G-CIMP tumors displayed copy-number alterations that were also shown in gliomas with IDH1 mutations in a recent report (Sanson et al., 2009). Integration of the DNA methylation data with gene expression data showed that G-CIMP-positive tumors represent a subset of proneural tumors. G-CIMP-positive tumors showed a favorable prognosis within GBMs as a whole and also within the proneural subset, consistent with prior reports for IDH1 mutant tumors (Parsons et al., 2008; Yan et al., 2009). Interestingly, of the five discordant cases of G-CIMP-positive, IDH1-wild-type tumors, two patients survived more than 5 years after diagnosis, suggesting that G-CIMP-positive status may confer favorable outcome independent of IDH1 mutation status. However, studies with many more discordant cases will be needed to carefully dissect the effects of G-CIMP status versus IDH1 mutation on survival. To a large extent, the improved prognosis conferred by proneural tumors (Phillips et al., 2006) can be accounted for by the G-CIMP-positive subset. These findings indicate that G-CIMP could be used to further refine the expression-defined groups into an additional subtype with clinical implications.

G-CIMP is highly associated with IDH1 mutation across all glioma tumor grades, and the prevalence of both decreases with increasing tumor grade. Tumor grade is defined by morphology only and therefore can be heterogeneous with respect to molecular subtypes. Within grade IV glioblastoma tumors are a subset of patients who tend to be younger and have a relatively favorable prognosis. It is only through molecular characterization with markers such as IDH1 and G-CIMP status that one could prospectively identify such patients. Conversely, these markers could also be used to identify patients with...
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low- and intermediate-grade gliomas who may exhibit unfavorable outcome relative to tumor grade.

In the non-TCGA independent validation set examined in this study, an IDH1 mutation was detected in 40/43 (93%) low- and intermediate-grade gliomas, but only 7/57 (13%) of primary GBMs. Similarly, we detected nearly 10-fold more G-CIMP-positive gliomas in grade II tumors as compared to grade IV GBMs. The improved survival of G-CIMP gliomas at all tumor grades suggests that there are molecular features within G-CIMP gliomas that encourage a less aggressive tumor phenotype. Consistent with this, we identified G-CIMP-specific DNA methylation changes within a broad panel of genes whose expression was significantly associated with patient outcome. We observed that this large subset of differentially silenced genes was involved in specific functional categories, including markers of mesenchyme, tumor invasion, and extracellular matrix. This concept builds upon our prior finding of a mesenchymal subgroup of glioma which shows poor prognosis (Phillips et al., 2006). According to this model, a lack of methylation of these genes in G-CIMP-negative tumors would result in a relative increase in expression of these genes, which in turn would promote tumor progression and/or lack of response to currently available treatment modalities. A comparison of the G-CIMP gene list with prior gene expression analyses (meta-analyses) suggests that G-CIMP positive tumors may be less aggressive because of silencing of key mesenchymal genes.

We found that a minority of genes with significant promoter hypermethylation showed a concomitant significant decrease in associated gene expression (263/1520, 17%). This is consistent with previous reports, in which we found similarly low frequencies of inversely correlated promoter hypermethylation and gene expression (Houshdaran et al., 2010; Pike et al., 2008). The lack of an inverse relationship between promoter hypermethylation and gene expression for most genes may be attributed to several scenarios, including the lack of appropriate transcription factors for some unmethylated genes and the use of alternative promoters for some genes with methylated promoters. Epigenetics controls expression potential, rather than expression state.

RB1 and G0S2 are the two genes showing the strongest evidence for epigenetic silencing in G-CIMP tumors. RB1 has been previously reported to be epigenetically silenced in cancer cell lines and primary tumors, and the association of its encoded protein with retinoic acid receptors (RARs) has been well characterized (Esteller et al., 2002). G0S2 gene expression is regulated by retinoic acid (RA) and encodes a protein that promotes apoptosis in primary cells, suggesting a tumor-suppressor role (Kitareewan et al., 2008; Welch et al., 2009). The vitamin A metabolite RA is important for both embryonic and adult growth. RA has diverse roles involving neuronal development and differentiation mediated by RARs (reviewed in Malik et al., 2000). Interestingly, studies in breast cancer cells have shown that silencing of RB1 plays an important role in RA signaling by lowering all-trans-retinoic acid production and loss of RAR levels and activity mediated by derepression of PI3K/Akt signaling pathway, leading to loss of cell differentiation and tumor progression (Farias et al., 2005a; Farias et al., 2005b). This mechanism may help describe the molecular features of tumorigenesis in G-CIMP tumors. Thus, dissecting the gene expression and DNA methylation alterations of G-CIMP tumors among lower-grade gliomas will be helpful for better understanding of the roles of a mutant IDH1 and G-CIMP DNA methylation on tumor grade and patient survival.

The highly concerted nature of G-CIMP methylation suggests that this phenomenon may be caused by a defect in a trans-acting factor normally involved in the protection of a defined subset of CpG island promoters from encroaching DNA methylation. Loss of function of this factor would result in widespread concerted DNA methylation changes. We propose that transcriptional silencing of some CIMP genes may provide a favorable context for the acquisition of specific genetic lesions. Indeed, we have recently found that IGFBP7 is silenced by promoter hypermethylation in BRAF mutant CIMP+ colorectal tumors (Hinoue et al., 2009). Oncogene-induced senescence by mutant BRAF is known to be mediated by IGFBP7 (Wajapeyee et al., 2008). Hence, CIMP-mediated inactivation of IGFBP7 provides a suitable environment for the acquisition of BRAF mutation. The tight concordance of G-CIMP status with IDH1 mutation in GBM tumors is very reminiscent of colorectal CIMP, in which DNA hypermethylation is strongly associated with BRAF mutation (Weisenberger et al., 2006). We hypothesize that the transcriptional silencing of as yet unknown G-CIMP targets may provide an advantageous environment for the acquisition of IDH1 mutation.

In our integrative analysis of G-CIMP tumors, we observed upregulation of genes functionally related to cellular metabolic processes and positive regulation of macromolecules. This expression profile may reflect a metabolic adjustment to the proliferative state of the tumor, in conjunction with the gain-of-function IDH1 mutation (Dang et al., 2009). Such a metabolic adjustment may be consistent with Warburg’s observation that proliferating normal and tumor cells require both biomass and energy production and convert glucose primarily to lactate, regardless of oxygen levels, whereas nonproliferating differentiated cells emphasize efficient energy production (reviewed in Vander Heiden et al., 2009).

In summary, our data indicate that G-CIMP status stratifies gliomas into two distinct subgroups with different molecular and clinical phenotypes. These molecular classifications have implications for differential therapeutic strategies for glioma patients. Further observation and characterization of molecular subsets will probably provide additional information enabling insights into the development and progression of glioma, and may lead to targeted drug treatment for patients with these tumors.

EXPERIMENTAL PROCEDURES

Samples and DNA Methylation Assays

Genomic DNAs from TCGA GBM tumors were isolated by the TCGA Biospecimen Core Resource (BCR) and delivered to USIC as previously described (The Cancer Genome Atlas Research Network, 2008). One sample (TCGA-06-0178) with a confirmed IDH1 mutation was removed from our analyses, given that it became clear that an incorrect tissue type had been shipped for the DNA methylation analysis. Four brain genomic DNA samples from apparently healthy individuals were included as controls. All tissue samples (patients and healthy individuals) were obtained with institutional review board-approved protocols from University of Southern California (TCGA GBM samples), Johns Hopkins School of Medicine (normal brain samples), and
The University of Texas MD Anderson Cancer Center (glioma validation samples). Tissue samples were deidentified so that patient confidentiality was ensured. Genomic DNA methylated in vitro with M.SssI methylase or whole-genome amplified (WGA) as positive and negative controls for DNA methylation, respectively, were also included. Details are in the Supplemental Information.

The GoldenGate assays were performed according to the manufacturer’s instructions and as described previously (Bibikova et al., 2006). The GoldenGate methylation assays survey the DNA methylation of up to 1536 CpG sites—a total of 1505 CpGs spanning 807 unique gene loci are interrogated in the OMA-002 probe set (Bibikova et al., 2006), and 1498 CpGs spanning the same number of unique gene regions are investigated in the OMA-003 probe set (The Cancer Genome Atlas Research Network, 2008).

The Infinium methylation assays were performed in accordance with the manufacturer’s instructions. The assay generates DNA methylation data for 27,578 CpG dinucleotides spanning 14,473 well-annotated, unique gene promoter and/or 5’ gene regions (from –1,500 to +1,500 from the transcription start site). The assay information is available at www.illumina.com and the probe information is available on the TCGA Data Portal website. Data from 91 TCGA GBM samples (batches 1, 2, 3, and 10) were included in this analysis. Batches 1–3 (63 samples) were run on both Infinum and GoldenGate, whereas batches 4–8 (182 samples) were analyzed exclusively on GoldenGate, and batch 10 (28 samples) was analyzed exclusively on Infinum. All data were packaged and deposited onto the TCGA Data Portal website (http://tcga.cancer.gov/dataportal). Figure S1C illustrates a Venn diagram with the overlapping samples between different DNA methylation platforms. Additional details on DNA methylation detection protocols and TCGA GBM data archived versions are in the Supplemental Information.

**Integrative TCGA Data Platforms**

Although ancillary data (expression, mutation, and copy number) were available for additional tumor samples, we only included those samples for which there were DNA methylation profiling (either GoldenGate or Infinium). Given that the Agilent gene expression platform contained a greater number of genes for which DNA methylation data were available, we limited our primary analysis to only the Agilent gene expression data set. Where appropriate, we confirmed results using the Affymetrix gene expression data. Additional details are in the Supplemental Information.

**Clustering Analysis and Measurement of Differential DNA Methylation and Differential Gene Expression**

Probes for each platform were filtered by removal of those targeting the X and Y chromosomes, those containing a single-nucleotide polymorphism (SNP) within five base pairs of the targeted CpG site, and probes containing repeat element sequences ≥10 base pairs. We next retained the most variably methylated probes (standard deviation > 0.20) across the tumor set in each DNA methylation platform. These final data matrices were used for unsupervised Consensus/Hierarchical clustering analyses. A nonparametric approach (Wilcoxon rank-sum test) was used for determining probes/genes that are differentially DNA methylated or differentially expressed between the two groups of interest. Additional information is described in the Supplemental Information.

**G-CIMP Validation with MethyLight Technology**

Tumor samples were reviewed by a neuropathologist (K.A.) to ensure accuracy of diagnosis as well as quality control to minimize normal tissue contamination. MethyLight real-time PCR strategy was performed as described previously (Eads et al., 2000; Eads et al., 1999). Additional details are in the Supplemental Information.

**Pathway and Meta-analyses and Statistical Analyses**

Additional tools included the Molecular Signatures Database (MSigDB database v2.5), Database for Annotation, Visualization, and Integrated Discovery (DAVID) and NextBio. All statistical tests were done with R software (R version 2.9.2, 2009-08-24, (R Development Core Team, 2009) and packages in Bioconductor (Gentleman et al., 2004), except as noted. Additional details are in the Supplemental Information.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures, three tables, Supplemental Experimental Procedures, and a list of authors and affiliations of the TCGA Research Network and can be found with this article online at doi:10.1016/j.ccr.2010.03.017.

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