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Identification and Functional Relevance of *de novo* DNA Methylation in Cancerous B-Cell Populations

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Abstract

Epigenetic remodeling is a hallmark of cancer, with the frequent acquisition of *de novo* DNA methylation in CpG islands. However, the functional relevance of *de novo* DNA methylation in cancer is less well-defined. To begin to address this issue in B-cells, we used BeadArray assays to survey the methylation status of 1,500 cancer-related CpG loci in two molecular subtypes of diffuse large B-cell lymphoma (ABC-DLBCL and GCB-DLBCL) and cognate normal B-cell populations. We identified 81 loci that showed frequent *de novo* DNA methylation in GCB-DLBCL and 67 loci that showed frequent *de novo* DNA methylation in ABC-DLBCL. These *de novo* methylated CpG loci included reported targets of polycomb repressive complexes (PRC) in stem cells. All candidate loci in GCB-DLBCL are proximal to genes that are poorly expressed or silent in purified normal germinal center (GC) B-cells. This is consistent with the hypothesis that *de novo* DNA methylation in cancer is more frequently involved in the maintenance rather than the initiation of gene silencing (*de novo* repression). This suggests that epigenetic switching occurs during tumorigenesis with *de novo* DNA methylation locking in gene silencing normally mediated by transcriptional repressors. Furthermore, we propose that similar to *de novo* genetic mutations, the majority of *de novo* DNA methylation events observed in tumors are passengers not causally involved in tumorigenesis.

Keywords

Epigenetic; DNA methylation; Gene expression; Lymphoma; B-cell

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of cancers arising from normal B-cell populations that have acquired characteristic groups of genetic and epigenetic changes [Coiffier, 2001; De Paepe and De Wolf-Peeters, 2007]. Gene expression profiling of tumor and normal B-cells have identified two major subgroups of DLBCL, germinal center B-cell-like DLBCL (GCB-DLBCL) and activated B-cell-like DLBCL (ABC-DLBCL), which have distinct clinical outcome [Alizadeh et al., 2000; Lossos and Morgensztern, 2006; Rosenwald et al., 2002; Wright et al., 2003]. In addition, these subgroups show differences in chromosomal abnormalities [Bea et al., 2005; Chen et al., 2006; Lenz et al., 2008; Rosenwald et al., 2002; Tagawa et al., 2005] and miRNA expression [Lawrie et al., 2007; Malumbres et al., 2009]. Recent evidence that ABC-DLBCL and GCB-DLBCL cases also show different responses to chemotherapeutic regimens raises the possibility that genetic testing could inform more personalized medical interventions in the future [Dunleavy et al., 2009].

In addition to these extensive genetic characterizations, DNA methylation profiling studies of different B-cell lymphomas are now being conducted [Guo et al., 2005; Killian et al., 2009; Martin-Subero et al., 2009; Pike et al., 2008; Shi et al., 2007; Yang et al., 2003]. Previously, we used qualitative CpG island microarray and PCR-based assays to DNA methylation in DLBCL [Pike et al., 2008]. In these studies, we identified CpG islands that were frequently methylated in DLBCL and found that the bulk of these CpG islands were proximal to genes that were poorly expressed in all tumors, regardless of methylation status [Pike et al., 2008]. However, we could not determine if the observed DNA methylation was acquired during tumorigenesis or if it was associated with the silencing of genes normally expressed in B-cell populations thought to give rise to these tumors.

In this study, we use quantitative BeadArray assays to evaluate the DNA methylation status of a novel expanded set of CpG loci proximal to carefully chosen cancer-related genes in a larger cohort of DLBCL and purified normal B-cell populations of origin. This now allows us to identify DNA methylation acquired *de novo* during B-cell tumorigenesis and begin to investigate the functional relevance DNA methylation has on gene expression in B-cells. This focused epigenetic evaluation provides an important counterpart to studies aimed at determining the functional significance of *de novo* mutations acquired during B-cell tumorigenesis.

Materials and Methods

Tissue Specimens

Frozen tissue samples from diagnostic tumor biopsies are obtained from patients before anthracycline-based chemotherapy at the University of Nebraska Medical Center. DNA and RNA samples are from 46 specimens, 21 from patients with ABC-DLBCL, 25 from patients with GCB-DLBCL. There was consensus central pathology re-review of the specimens to confirm the diagnosis of DLBCL and the samples had >75% tumor cells. Clinical information has been obtained from all patients according to a protocols approved by the University of Nebraska Medical Center Institutional Review Board.

Isolation of Normal Human B-Cell Populations

Germinal center B-cells were purified from healthy lymph node tissues obtained from adult human donors. Lymph node tissues were minced and passed through 30 micrometer nylon mesh to obtain single cell suspensions. B-cells were enriched from the cell suspensions by positive selection with CD19 multisort kit (Miltenyi Biotec, Auburn, CA). Germinal center B-cells were isolated from the total B-cells by sorting $IgD^{-}CD20^{+}CD38^{+}$ B-cells using

direct fluorescence-activated cell sorting (FACS). In keeping with previous studies [Liu and Banchereau, 1997], analytical FACS analyses confirmed that a greater than 85% purity. Human B-cells were purified from peripheral blood obtained from healthy adult donors. Monocytes were isolated using Ficoll density gradients. $CD19^+$ B-cells were isolated by positive selection using the CD19 multisort kit (Miltenyi Biotec, Auburn, CA). The purity was greater than 85% [Allman et al., 1996].

B-Cell Activation

The isolated CD19+ B-cells were cultured at 2×10^6 cells/ml in RPMI containing 10% fetal calf serum (FCS), 1μg/ml glutamine, 1μg/ml each of penicillin and streptomycin, and 5×10^5 mol/L 2-mercaptoethanol. Activated peripheral B-cells were obtained by exposing purified cultured CD19+ B-cells to one of three types of mitogenic agents, 25μg/ml LPS, 1μg/ml IL4 plus 1μg/ml CD40 ligand, or 20μg/ml anti-IGM-Ab, for 44 hours. These treatments were used in the prior studies to obtain an activated B-cell gene expression signature [Alizadeh et al., 2000].

DNA Methylation Profiling Using the GoldenGate BeadArray Platform

The DNA methylation levels of 1536 specific CpG sites in 371 genes (1~9 CpG sites per gene) were measured on the Illumina (San Diego, CA) GoldenGate BeadArray® platform, as previously described [Bibikova et al., 2006]. Briefly, this primer extension-ligation assay monitors the extension of oligonucleotide primers designed to bind to methylated or unmethylated CpG sites in bisulfite-converted templates. Extended templates are PCR amplified, labeled with fluorescent dyes, and hybridized to the BeadArrays. The relative hybridization signals from PCR products derived from primers specific for methylated and unmethylated templates are used to calculate relative fractions of DNA methylation in the original templates.

The GoldenGate DNA methylation assays measure the DNA methylation levels of a given locus as β-values ranging from 0 (no DNA methylation detected) to 1 (complete DNA methylation). Following the nomenclature established in prior Illumina GoldenGate BeadArray DNA methylation studies, we defined a hypermethylated locus as having $\beta>0.8$ and a hypomethylated locus as having $\beta<0.2$ [Killian et al., 2009]. We deemed loci with a $\beta>0.4$ (i.e. loci with any measureable level of DNA methylation in our assay system) as being “methylated”. We chose $\beta>0.4$ since it is 0.2 units above the upper threshold of the hypomethylated category. Since the GoldenGate assays are reported to be able to distinguish 0.15 β-units of DNA methylation [Bibikova et al., 2006], this provides a reasonable definition of the lower limits of β-values that reflect the presence of DNA methylation.

We applied multiple quality control metrics to analyze high quality DNA methylation data. Assays showing $\beta>0.6$ in either of two whole genome amplification (WGA) fully hypomethylated controls or $\beta<0.4$ in either of two M.*Sss*I-treated fully methylated controls were excluded from further analysis. Likewise, CpGs associated with imprinted genes (based on the publicly available databases http://igc.otago.ac.nz/home.html and http://www.geneimprint.com/site/genes-by-species and [Murphy and Jirtle, 2003], as described in [Martin-Subero et al., 2009]), X-chromosomal genes, and single-nucleotide polymorphism known to affect the performance of the Illumina GoldenGate BeadArray DNA methylation platform [Byun et al., 2009] were excluded from the analysis.

Bisulfite Sequencing

Bisulfite conversion was performed on 0.5–1μg tumor DNA samples using EZ DNA methylation kit (Zymo Research, Orange, CA). The converted DNA samples served as templates for PCR reactions using primers were designed using Methprimer software [Li,

2007; Li and Dahiya, 2002] (Supplemental Table 1). The resultant amplicons were sub-cloned and at least 12 colonies were sequenced for each selected CpG site. Sequence analysis and visualization was completed using BiQ Analyzer Software [Bock et al., 2005].

Gene Expression Profiling

Total RNA from normal GC B-cells was isolated and subjected to gene expression profiling analysis on Human Genome U133Plus2 Arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's recommended protocols. We report normalized log2-transformed gene expression data for probe tilings with minimal cross-hybridization potential (i.e. disregarding `_x_at' probe sets designated by Affymetrix, Santa Clara, CA) that interrogate NCBI-designated Reference Sequence (RefSeq) transcripts associated with genes proximal to or containing candidate *de novo* DNA methylation in GCB-DLBCL (Supplemental Table 2). All scaled fluorescent intensity values and .cel files from normal B-cells are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/) under Series Accession Number GSE16712.

RESULTS

Our initial goal was to identify *de novo* DNA methylation in DLBCL that are acquired during B-cell tumorigenesis. To address this issue, we conducted a wide-spread survey of DNA methylation in cancer-related genes in a cohort of GCB-DLBCL (N=25), ABC-DLBCL (N=21), purified normal germinal center B-cells (N=6, DNA obtained from five donors with two lymph nodes obtained from one donor), and activated peripheral B-cells (N=6, cultured under 3 different stimulation conditions each - see Methods). We used Illumina GoldenGate BeadArray® assays to measure the DNA methylation levels of over 1,500 carefully selected cancer-related CpG loci as β-values ranging from 0 (no DNA methylation detected) to 1 (complete DNA methylation) (see Methods).

Although we have acquired DNA methylation data on all of 1,500 loci, we focused our attention on 690 autosomal loci that passed rigorous quality control metrics and are not subject to the confounding effects of imprinting (see Methods). These allowed us to conduct for a focused and rigorous analysis DNA methylation and gene expression profiles. Due to technological improvements and the focused nature of the current study, we profile a different group of CpG loci from our previous studies that investigated a discovery set of only 14 DLBCL tumors [Pike et al., 2008]. All β-values for these 690 loci are available in Supplemental Table 3, with complete annotation provided in Supplemental Table 4.

Overview of DNA Methylation in DLBCL and Normal B-Cells

In Fig. 1, we present an unsupervised hierarchical clustering analysis of DNA methylation data from 690 autosomal loci proximal to (defined hereafter as being located within 1,500-bp of the transcription start site, as detailed in Supplemental Table 3) or within 238 unique cancer-related genes (see Methods). With only four exceptions (i.e. GCB-DLBCL tumors 4, 8, 10, and 16), 91.3% (42/46) of the tumors clustered separate from the normal B-cells (N=24, including all activated and germinal center B-cells). However, the ABC-DLBCL and GCB-DLBCL tumors could not be distinguished based on this unsupervised clustering method. As discussed in our previous study [Pike et al., 2008], this could be due to the non-concordance of genes whose expression levels can be used to differentiate the ABC-DLBCL and GCB-DLBCL subgroups and the CpG loci interrogated by the BeadArrays. Likewise, GC B-cells could not be distinguished from the activated peripheral blood B-cell cultures, generated using three different established protocols, in our DNA methylation-based hierarchical clustering analyses (Fig. 1).

From these hierarchical clustering analyses, it was apparent that methylated loci in DLBCL were either frequently methylated in normal B-cell populations or acquired *de novo* during tumorigenesis (Fig. 1 and Supplemental Fig. 1). Below, we will discuss our efforts to identify methylated loci that fall into these two categories and relate them to existing gene expression and copy number data.

DNA Methylation in DLBCL that Derive from Normal B-Cell Populations

To identify robust DNA methylation in GCB-DLBCL tumors that derive from the epigenetic state of normal GC B-cell populations, we focused on candidate loci in which every allele present was methylated (β>0.8) in >95% of GCB-DLBCL tumors and in >95% of normal B-cell preparations. In the latter case, we combined data from all purified germinal center (GC) B-cells and activated B-cells (N=24) since they could not be distinguished from one another by hierarchical clustering analyses (Fig. 1). This observation is especially striking since these samples were obtained from different donors. This suggests that the cancer-related CpG loci we examined have robust B-cell methylation signatures and that B-cell subtype-specific DNA methylation profiles, at least for the most abundant B-cell species, are far less frequent.

Overall, we identified 16 CpG loci that our criteria for being methylated in both normal and cancerous B-cells. These were proximal to or within 13 genes (ASCL2, BRCA2, C4B, COL1A2, CTBP1, DDX17, HFE, MC2R, PPP2R1B, SMARCA3 (aka *HLTF*), *TGFB1*, *UBB*, and *VHL*) (Supplemental Table 5). These genes did not appear to have highly related specific functions as GeneOntology (GO) analyses did not identify any enriched functional categories (≥2 genes, P<0.01).

We note that the GoldenGate BeadArray platform evaluates the relative levels of DNA methylation of all alleles present in a sample. In normal diploid B-cells, we can assume that a β-value of 1 reflects DNA methylation of both autosomal alleles. However, in a tumor sample, a β-value of 1 could reflect DNA methylation of all copies of a haploid locus (due to a deletion), polyploid locus (due to an amplification), or diploid locus (no copy number change). Nevertheless, based on array-CGH (comparative genomic hybridization) analyses of 17 GCB-DLBCL in our cohort, two of the CpG loci (*MC2R* and *VHL*) that were methylated in GCB-DLBCL and normal B-cells showed both a deletion and DNA methylation (β>0.8) in one tumor sample each (Supplemental Table 5). Intriguingly, loci proximal to or within *ASCL2, BRCA2, C4B, COL1A2, HEF, MCR2, PPP2R1B*, and *UBB* (all methylated in GCB-DLBCL tumors and normal B-cells) showed DNA methylation (β>0.8) and an increase in gene copy number in one or two GCB-DLBCL tumors profiled by array-CGH (Supplemental Table 5). This suggests that DNA methylation present in normal B-cells was retained after amplification that occurred during tumorigenesis.

Next, we focused on the differences between ABC-DLBCL and normal B-cells (N=24, including all activated B-cells and germinal center B-cells). These differences were less well-defined since activated B-cell cultures may not necessarily represent the epigenetic state of normal B-cell populations that result in ABC-DLBCL (which are currently unknown), even though their gene expression profiles show similarities [Alizadeh et al., 2000; Rosenwald et al., 2002; Wright et al., 2003]. A total of seven loci proximal to or within six genes were hypermethylated (β>0.8) in >95% of ABC-DLBCL and >95% of all normal B-cell samples (Supplemental Table 6). These included *ASCL2*, *C4B*, *COL1A2*, *DDX17*, *MC2R*, and *RARRES1*. Interestingly, all genes but *RARRES1* were also methylated in ≥95% of the GCB-DLBCL tumors. Again, the specific functions of this subset of genes were not highly related given that GO analyses did not identify any enriched functional categories (≥2 genes, P<0.01).

Identification of Common *de novo* DNA Methylation in DLBCL

To identify *de novo* DNA methylation frequently acquired during GCB-DLBCL tumorigenesis, we screened for loci that were methylated (β>0.4) in ≥20% of GCB-DLBCL tumors (i.e. at least 5) and hypomethylated (β<0.2) in >95% of all normal B-cell samples. We chose less stringent criteria for DNA methylation in tumors (β>0.4) since we wanted to include all loci in which *de novo* DNA methylation occurred in a single allele in the absence of amplification. Overall, we detected 81 such candidate loci proximal to or within the following 42 genes: ABO, ADAMTS12, APBA1, APP, ASCL2, CAV1, CCNA1, CDH3, COL1A2, CSF1, CSPG2 (aka *VCAN*), CYP1A1, EPHA3, EPO, EYA4, F2R, FN1, HRASLS, IGFBP7, IGSF4 (aka *CADM1*), IL17RB, IMPACT, LAT, LOX, LRP2, MT1A, MYCN, MYOD1, NEFL, NPY, PAX6, POMC, PROK2, PTPRO, RAB32, RARB, RASGRF1, RET, SEZ6L, TMEFF1, TNFRSF10D, and *ZMYND10* (Supplemental Table 7). As above, the specific functions of these genes were not highly related given that GO analyses did not identify any enriched functional categories (≥2 genes, P<0.01).

Based on previous array-CGH analyses of 17 GCB-DLBCL tumors in our cohort, four of the loci (*EYA4*, *NEFL*, *RAB32* and *ZMYND10*) with common *de novo* DNA methylation in GCB-DLBCL tumors showed both a deletion and DNA methylation (β>0.4) in one or two GCB-DLBCL tumors (Supplemental Table 7). Intriguingly, loci with *de novo* DNA methylation proximal to or within ADAMTS12, ASCL2, CAV1, CCNA1, CDH3, COL1A2, EPO, FN1, HRASLS, IGSF4 (aka *CADM1*), *IMPACT*, *LRP2*, *MT1A*, *MYCN*, *NEFL*, *NPY*, *PAX6*, *POMC*, and *PTPRO* showed DNA methylation (β>0.4) and an increase in gene copy number in one or two of the GCB-DLBCL tumors previously profiled by array-CGH (Supplemental Table 7). However, based on this data, we cannot determine if *de novo* DNA methylation occurred prior to amplification and was maintained or if *de novo* DNA methylation occurred independently at all loci after amplification. The former hypothesis provides a more parsimonious explanation since it requires the fewest *de novo* epigenetic changes in each tumor.

As in our above analysis of *de novo* DNA methylation in GCB-DLBCL tumors, we screened for loci that were methylated (β>0.4) in ≥20% of ABC-DLBCL tumors and hypomethylated (β<0.2) in >95% of all normal B-cells (N=24, including all activated and GC B-cells). Overall, we found 67 loci meeting the *de novo* DNA methylation criteria either proximal to or within 32 unique genes (ABO, ADAMTS12, APBA1, APP, ASCL2, CCNA1, COL1A2, CSF1, CSPG2 (aka *VCAN*), *CYP1A1*, *EPHA3*, *EPO*, *EYA4*, *HRASLS*, *IGFBP7*, *IGSF4* (aka *CADM1*), IMPACT, LOX, LRP2, MT1A, MYCN, MYOD1, NEFL, NPY, PAX6, PROK2, PTPRO, RAB32, RARB, RASGRF1, RET, and *SEZ6L*) (Supplemental Table 8). A total of 64 loci proximal to or within 32 unique genes showed *de novo* DNA methylation in both GCB-DLBCL and ABC-DLBCL. All 32 genes proximal to or encompassing loci showing common *de novo* DNA methylation in ABC-DLBCL also showed common *de novo* DNA methylation in GCB-DLBCL.

There is literature precedent for DNA methylation occurring proximal to a subset of the above-stated genes in lymphoma. For example, *de novo* methylation of loci proximal to or within *CCNA1*, *EYA4*, *IGFBP7*, *LOX*, *MT1A*, *MYOD1*, *NEFL*, *PROK2*, and *RAB32* was also observed in mature aggressive B-cell NHLs (maB-NHLs), including molecular Burkitt's lymphoma and DLBCL [Martin-Subero et al., 2009]. These loci comprised part of the evidence for the proposal that *de novo* methylation in maB-NHLs are enriched for polycomb repressive complex (PRC) targets and suggestion that maB-NHLs originate from cells with stem cell features or stemness acquired during lymphomagenesis by epigenetic remodeling [Martin-Subero et al., 2009]. We also note agreement with our prior observation of frequent DNA methylation of a locus proximal to *MYOD1* in both ABC-DLBCL and GCB-DLBCL, based on Methylight assays [Pike et al., 2008].

Furthermore, we previously proposed that epigenetic processes affect *IGSF4* expression in DLBCL due to the observed proportional reductions of *IGSF4* expression with increasing DNA methylation levels of a nearby locus [Pike et al., 2008]. DNA methylation of loci proximal to *IGSF4* in DLBCL was also previously reported by others [Martin-Subero et al., 2009]. Thus, we propose that further functional analyses into the epigenetic regulation of *IGFS4* expression in DLBCL are warranted.

Intriguingly, loci proximal to *ADAMTS12*, *ASCL2*, *CCNA1*, *EYA4*, *IGFS4* (aka *CADM1*), *IMPACT*, *LOX*, *MYOD1*, *NEFL*, *NPY*, *PEX6*, and *RAB32* have been reported to show robust increases in DNA methylation in follicular lymphoma (FL) relative to normal follicular hyperplasia (FH) [Killian et al., 2009]. We propose that the shared common *de novo* methylation events in DLBCL and FL are related to their B-cell origins. In fact FL commonly transforms into DLBCL (t-FL) [Davies et al., 2007]. However, it should be noted that t-FL has a gene expression phenotype similar to GCB-DLBCL, but not ABC-DLBCL [Davies et al., 2007]. This makes the interpretation of the epigenetic profiles amongst these diseases challenging.

Based on array-CGH analyses of seven ABC-DLBCL tumors in our cohort, eight of the loci (*ABO*, *CSF1*, *EYA4*, *IGFBP7*, *LRP2*, *MYCN*, *RAB32*, and *RASGRF1*) that showed *de novo* DNA methylation in ABC-DLBCL tumors also showed both a deletion and DNA methylation ($\beta>0.4$) in one or more ABC-DLBCL tumors (Supplemental Table 8). Intriguingly, loci with *de novo* DNA methylation proximal to or within *ADAMTS12*, *COL1A2*, *EPHA3*, *HRASLS*, *IMPACT*, *NPY*, *PROK2*, *PTPRO*, *RARB*, and *RET* also showed DNA methylation ($\beta>0.4$) and an increase in gene copy number in one or two of the ABC-DLBCL tumors previously profiled by array CGH (Supplemental Table 8).

Comparisons of DNA Methylation in ABC-DLBCL and GCB-DLBCL

Although ABC-DLBCL and GCB-DLBCL tumors could not be distinguished by our hierarchical clustering analysis (Fig. 1), we uncovered a subset of loci showing robust differences in DNA methylation levels across these two DLBCL subtypes (Table 1). We found 19 loci showing differential DNA methylation (Benjamini and Hochberg corrected Wilcoxon $P<0.05$ and >0.4 units difference in geometric means of ABC-DLBCL and GCB-DLBCL β-values). These loci are proximal to or within 16 genes (*ABCC5*, *APBA2*, *CAPG*, *CD44*, *DAD1*, *DMP1*, *EPM2A*, *GP1BB*, *IL13*, *IRF5*, *LTB4R*, *RUNX3*, *SEZ6L*, *SMARCA3* (aka *HLTF*), *TMEFF1*, and *TSP50*). Based on BeadArray probe identifiers, none of these loci showed common *de novo* DNA methylation for either ABC-DLBCL or GCB-DLBCL.

Confirmatory Bisulfite Sequencing Analyses of BeadArray DNA Methylation Data

Although the BeadArray DNA methylation platform has been extensively characterized [Bibikova and Fan, 2009; Bibikova et al., 2006; Killian et al., 2009; Martin-Subero et al., 2009], we sought to evaluate independently its performance in our study. We performed bisulfite sequencing analysis of nine loci with differential DNA methylation between ABC-DLBCL and GCB-DLBCL or *de novo* DNA methylation in tumors (Table 2 and Supplemental Fig. 2). These included CpG islands proximal to or within *CCNA1*, *DBC1*, *IGFBP1*, *IRF5*, *MYOD1*, *RAB32*, *RUNX3*, and *TSP50* (Table 2). Overall, the results from the bisulfite sequencing and BeadArray analyses were in excellent agreement (Table 2 and Supplemental Fig. 2). Furthermore, they demonstrate that the DNA methylation status of CpG dinucleotide(s) interrogated by the BeadArray assays largely reflects the overall DNA methylation status of flanking CpG sites.

Common *de novo* DNA Methylation in GCB-DLBCL Occur Proximal to or Within Genes that are Poorly Expressed in Normal GC B-Cell Populations

Next, we sought to determine the relationships between common *de novo* DNA methylation in GCB-DLBCL and the expression levels of proximal or encompassing genes in normal GC B-cells. Through Affymetrix GeneChip analyses, we were able to obtain expression data for 39 of the 42 genes proximal to or containing loci showing common *de novo* DNA methylation in GCB-DLBCL. We found that >87% (34/39) of the *de novo* DNA methylation in GCB-DLBCL occurred proximal to or within genes that were poorly expressed in normal GC B-cells (i.e. below the upper 50th percentile of gene expression scores – see Methods).

In fact, the five exceptions (*APBA1*, *EPO*, *IGFBP7*, *IGSF4* (aka *CADM1*), and *RAB32*) showed only modest expression in normal GC B-cells. None of the gene expression scores ranked within the upper 25th percentile for any normal GC B-cell sample (Additional File 9). However, we have previously noted that *IGSF4* gene expression levels in DLBCL tumors were inversely proportional to the DNA methylation levels of a proximal CpG island [Pike et al., 2008]. *IGSF4* is a tumor suppressor gene that encodes an immunoglobulin superfamily member protein important for cell adhesion and motility. It is inactivated in multiple cancers including non-small cell lung cancer (NSCLC) [Murakami, 2005] and nasal NK/T-cell lymphoma [Fu et al., 2009]. To our knowledge, *APBA1*, *EPO*, *IGFBP7*, or *RAB32* methylation has not yet been highlighted in lymphoma. However, the DNA methylation status of a locus proximal to *IGFBP7* is inversely associated with *IGFBP7* gene expression in cultured human colon cancer cell lines [Lin et al., 2007]. Likewise, it has been reported that *RAB32* expression is down-regulated in response to DNA methylation in a group of primary colon cancer and colon cancer cell lines [Mori et al., 2004].

DISCUSSION

The combined analysis of DNA methylation profiles in DLBCL and normal B-cell populations provides an opportunity to discriminate *de novo* DNA methylation arising during tumorigenesis from DNA methylation occurring in normal B-cells. A subset of *de novo* DNA methylation events could be of functional significance in the development and/or progression of disease. However, the identification of functional *de novo* DNA methylation will be challenging, even when both tumor and normal progenitor cell DNAs can be obtained from the same individual to help control for age, gender, and/or environmental exposures [Foley et al., 2009; Fraga et al., 2005; Woo et al., 2009].

In our attempts to identify epigenetic changes of functional significance, we observed that *de novo* DNA methylation occurred most frequently in CpG islands proximal to or within genes that are already poorly expressed in normal B-cell populations. This supports a model in which *de novo* DNA methylation is most frequently involved in the maintenance, as opposed to the initiation, of gene silencing [Weber and Schubeler, 2007]. Overall, our data is consistent with similar observations of pervasive epigenetic remodeling in FL, wherein genes that are poorly expressed in normal B-cells were proximal to loci that were methylated in tumors [Killian et al., 2009]. Analyses in aggressive B-cell non-Hodgkin lymphomas have also demonstrated that genes proximal to or encompassing *de novo* methylated loci are expressed at low levels in lymphomas and normal hematopoietic tissues [Martin-Subero et al., 2009].

Furthermore, there is increasing evidence that the above observations can be generalized to other cancers. For example, genes proximal to or containing methylated loci in colon tumor samples have been shown to be expressed at low levels in normal colon as well as in colorectal adenocarcinomas [Keshet et al., 2006]. Functional studies in cultured human colon cancer [Bachman et al., 2003] and prostate cancer cells [Gal-Yam et al., 2008] and

mouse erythroleukemia (MEL) cells [Feng et al., 2006] reached a similar conclusion that *de novo* DNA methylation locks in rather than initiates gene silencing (*de novo* repression). Gel-Yam and colleagues provide compelling evidence of frequent epigenetic switching in PC3 prostate cancer cell cultures by which DNA methylation replaces Polycomb repressive complex (PRC) marks found in normal prostate epithelial cells [Gal-Yam et al., 2008]. Given the overlap in PRC targets and the *de novo* methylation found in our studies and in a different survey of maB-NHLs, we agree with the speculation that epigenetic switching can frequently occur in DLBCL [Martin-Subero et al., 2009]. However, rigorous functional studies in primary tumor cells are needed to validate these speculations.

Nevertheless, it has been recently reported that the methylation status of CpG island shore sequences, located on the edges of CpG islands, is strongly associated with the transcription of associated genes (within 2-kb) in normal tissues and colon cancer [Irizarry et al., 2009]. The BeadArray assays used in our study are not optimal for addressing the methylation status of CpG island shore sequences. Thus, large-scale, comprehensive analyses of DNA methylation and gene expression profiles are needed to elucidate the likely complex nature of their relationships. Although this will require further technology development, continued advances in sequencing technologies could make this more feasible in the future.

We note that there are multiple scenarios by which maintaining the silence of an otherwise normal gene (i.e. one not carrying genetic mutations that compromise its activity) would have functional significance in tumorigenesis. For example, the epigenetic silencing of genes whose primary function is to prevent the division or promote the death of cells with genetic instability could have a profound impact in cancer development. In principle, such genes could be poorly expressed in normal cells under non-stressful physiological conditions.

In our attempts to identify epigenetic changes of functional significance, we observed a limited numbers of cases in which *de novo* DNA methylation was associated with a chromosomal deletion. For tumor suppressor genes, the association of gene deletions and epigenetic silencing would be consistent with Knudson's two-hit hypothesis of cancer [Knudson, 1971]. It is possible that some loci showing *de novo* DNA methylation are proximal to functionally significant genes that are haploinsufficient or have inactivating point mutations or deletions not detected by array CGH analyses in their second copy.

Even taking into consideration the caveats discussed above, the relationships among *de novo* DNA methylation, gene expression, and locus copy number call into question the functional significance of *de novo* DNA methylation uncovered in this study and others [Martin-Subero et al., 2009]. This would be in keeping with genetic studies suggesting that `driver' mutations that are causally involved in cancer development and progression can be significantly less numerous than `passenger' mutations that have little to no functional significance [Greenman et al., 2007; Sjoblom et al., 2006]. The discrimination between epigenetic `driver' and `passenger' changes in tumors will require functional analyses. Although challenging, the development of novel technologies to introduce specific epigenetic changes into cancer and normal cells would provide a powerful tool to facilitate such studies.

Regardless of their functional significance, *de novo* DNA methylation could represent epigenetic biomarkers that are useful for monitoring residual disease levels in body fluids, especially during remission periods. The decreasing costs of sequencing technologies bode well for the development of personalized genetic and epigenetic biomarkers of this type based on data acquired from individual tumors. We believe the combined analysis and application of genetic and epigenetic data will lead to a more sophisticated understanding of

the molecular etiology of cancer and the development of more sensitive and specific clinical biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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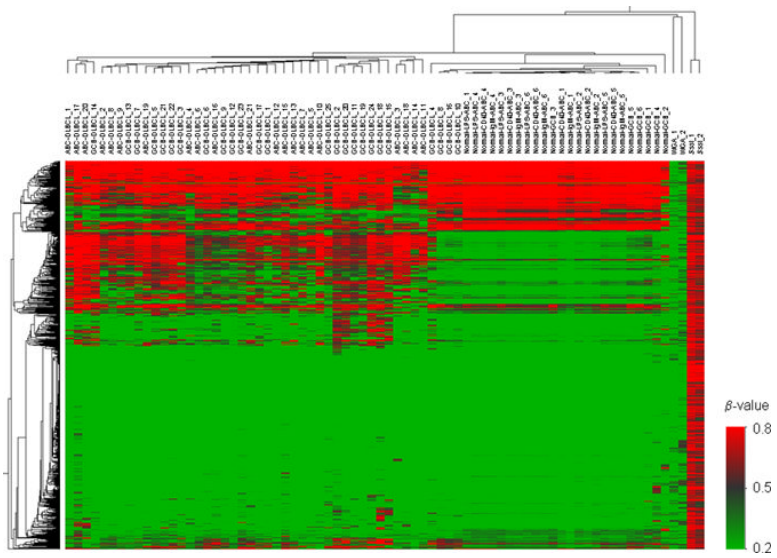

FIG. 1.

Hierarchical clustering of DNA methylation profiles in diffuse large B-cell lymphoma (DLBCL) and normal B-cells. Analyses were based on 690 autosomal loci proximal to or within 238 unique genes with no strong evidence of imprinting and having the fully methylated (*Sss*I: M.*Sss*I-treated whole genome amplification materials generated from human genomic DNA) and fully unmethylated (WGA: whole genome amplification from human genomic DNA) controls pass quality control metrics (see Methods). Hierarchical clustering was conducted using Pearson absolute distance and average-linkage.

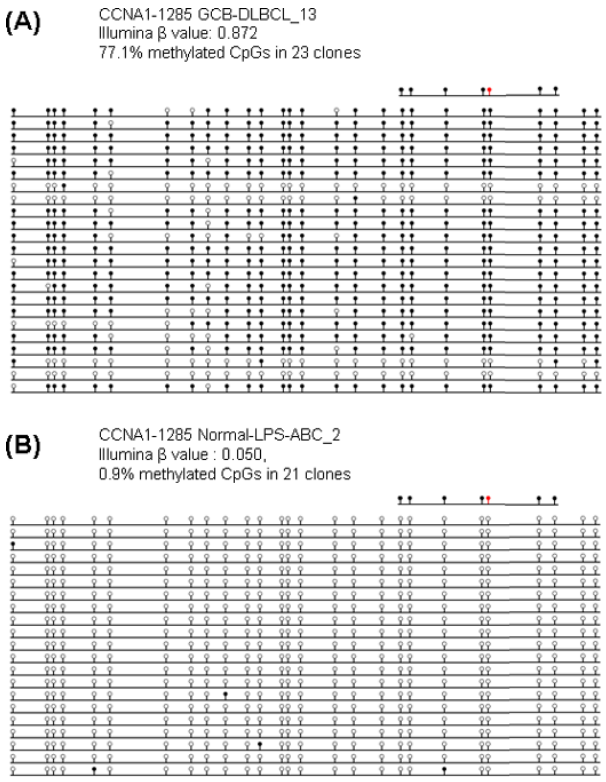

FIG. 2.

Bisulfite sequencing of CpG island proximal to *CCNA1*. Blackened and empty circles denote methylated and unmethylated CpG dinucleotides, respectively. The regions encompassing Illumina reaction CCNA1-1285 (*CCNA1*) was subject to bisulfite sequencing analysis in (**A**) GCB-DLBCL_13 and (**B**) peripheral B-cells activated by LPS treatment. Light gray and blackened circles denote unmethylated and methylated CpG dinucleotides, respectively. The *CCNA1* amplicon spans nucleotide positions 35904243 – 35904473 of chromosome 13, based on the May 2004 human genome assembly provided at http://genome.ucsc.edu/.

Table I

Summary of Candidate DLBCL Subtype-Specific Methylation

Gene	TargetID	ABC-DLBCL[a]	GCB-DLBCL[a]	P-value[b]
ABCC5	ABCC5-154	0.56	0.82	0.0395
APBA2	APBA2-1274	0.37	0.89	0.0021
CAPG	CAPG-337	0.61	0.88	0.0329
CD44	CD44-985	0.54	0.79	0.0402
DAD1	DAD1-655	0.44	0.86	0.0021
DMP1	DMP1-1367	0.29	0.60	0.0029
EPM2A	EPM2A-666	0.33	0.61	0.0021
GP1BB	GP1BB-348	0.47	0.95	0.0127
IL13	IL13-55	0.17	0.59	0.0450
	IL13-298	0.20	0.58	0.0090
IRF5	IRF5-897	0.53	0.91	0.0037
LTB4R	LTB4R-1441	0.24	0.55	0.0297
RUNX3	RUNX3-516	0.28	0.50	0.0395
SEZ6L	SEZ6L-592	0.14	0.42	0.0063
	SEZ6L-856	0.57	0.84	0.0317
SMARCA3	SMARCA3-1167	0.10	0.34	0.0342
TMEFF1	TMEFF1-876	0.19	0.40	0.0188
TSP50	TSP50-1320	0.38	0.64	0.0395
	TSP50-1231	0.22	0.47	0.0192

[a] Median β-value that reflects the level of methylation for each locus.

[b] Benjamini and Hochberg corrected Wilcoxon t-test.

Table II

Confirmatory Bisulfite Sequencing Analysis

Target ID	Sample ID	β-value	Fraction of methylated CpGs[a]	Assays within 0.2 units?
CCNA1-1285	GCB-DLBCL_13	0.87	0.77	Yes
	GCB-DLBCL_10	0.24	0.08	Yes
	LPS-ABC_2[b]	0.05	0.01	Yes
DBC1-1053	GCB-DLBCL_13	0.56	0.43	Yes
	GCB-DLBCL_15	0.71	0.92	No
IGFBP1-1484	GCB-DLBCL_8	0.07	0.10	Yes
	GCB-DLBCL_13	0.85	0.88	Yes
	CD40-ABC_6[c]	0.15	0.16	Yes
IRF5-897	ABC-DLBCL_4	0.37	0.45	Yes
	GCB-DLBCL_2	0.94	0.94	Yes
MYOD1-1193	GCB-DLBCL_1	0.26	0.23	Yes
	ABC-DLBCL_1	0.95	0.94	Yes
RAB32-1026	GCB-DLBCL_2	0.56	0.86	No
	GCB-DLBCL_4	0.12	0.37	No
RAB32-1080	GCB-DLBCL_2	0.79	0.86	Yes
	GCB-DLBCL_4	0.15	0.37	No
RUNX3-516	ABC-DLBCL_7	0.09	0.08	Yes
	GCB-DLBCL_6	0.67	0.51	Yes
TSP50-1231	ABC-DLBCL_9	0.12	0.25	Yes
	GCB-DLBCL_8	0.67	0.63	Yes

[a] Based on the sequenced regions in all the analyzed subclones

[b] Normal peripheral B-cell cultures activated with lipopolysaccharide (Methods)

[c] Normal peripheral B-cell cultures activated with CD40 (Methods)