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Array-based DNA methylation profiling in follicular lymphoma

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Abstract

Quantitative methylation profiling was performed using the Illumina GoldenGate Assay in untreated Follicular Lymphoma (FL) (164), paired pre- and post-transformation FL (20), benign haematopoietic (24) samples and purified B & T cells from two FL cases. Methylation values allowed separation of untreated FL samples from controls with one exception based primarily on tumour-specific gains of methylation typically occurring within CpG islands. Genes which are targets for epigenetic repression in stem cells by Polycomb Repressor Complex 2 were significantly overrepresented among hypermethylated genes. Methylation profiles were conserved in sequential FL and t-FL biopsies suggesting that widespread methylation represents an early

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event in lymphomagenesis and may not contribute substantially to transformation. Significant ($p<0.05$) correlation between FL methylation values and reduced gene expression was demonstrated for up to 28% of loci. Methylation changes occurred predominantly in B cells with variability in the amount of non-malignant tissue between samples preventing conclusive correlation with survival. This represents an important caveat in attributing prognostic relevance to methylation and future studies in cancer will optimally require purified tumour populations to address the impact of methylation on clinical outcome.

Keywords

Methylation; follicular lymphoma; gene expression; polycomb; transformation

Introduction

Aberrant cytosine methylation occurring at CpG rich areas known as CpG islands has conventionally been associated with transcriptional silencing in neoplasia (1-3). Unlike gene mutation which selectively targets a number of discrete loci, methylation is less discriminatory and frequently affects numerous genes with diverse functions (3, 4). Such epigenetic changes are potentially reversible and represent therapeutic targets using demethylating agents, approved for use in the treatment of Myelodysplastic Syndrome (5-7). These agents are currently under evaluation in Follicular Lymphoma (FL) (8), a neoplasm of germinal centre B lymphocytes that accounts for 20-25% of all Non-Hodgkin's lymphomas (9).

In this study, we undertook methylation profiling in a large series of FL cases. Previous studies have suggested that FL may be more susceptible to methylation than other B cell lymphomas (10-12). FL has an heterogenous clinical course with a relatively long median survival of 8-10 years. For as many as half of patients, transformation to a more aggressive lymphoma (t-FL), usually diffuse large B-cell lymphoma, occurs and this event is often associated with particularly poor outcomes (13-15). Cytogenetically, up to 95% of FL cases are characterised by the t(14;18)(q32;q21) translocation (16, 17). However, this alone is not sufficient for lymphomagenesis and a complex heterogenous accumulation of secondary genetic events in the tumour and alterations within the tumour microenvironment frequently occur (18).

Investigation of methylation in FL has been predominantly restricted to studies on small patient cohorts or focused on individual candidate tumour suppressor genes, using locus-specific non-quantitative methods (reviewed by Hayslip and Montero (19)). With the introduction of newer technologies, the focus is moving away from studies of single genes to a global analysis of the lymphoma "methylome". In this study, we generated methylation profiles on a large clinically and molecularly well characterised patient cohort including previously untreated FL lymph node biopsy samples (u-FL), paired pre-transformation (pre-t-FL) and post-transformation (t-FL) samples and non-tumour tissue using the GoldenGate Methylation Cancer Panel 1 (Illumina, San Diego, California, USA). These results were correlated with previously established gene expression profiles to provide a comprehensive epigenetic analysis of FL.

Materials & Methods

Samples

DNA from whole lymph node biopsies from 164 patients with FL at diagnosis with accompanying clinical, gene expression (20) and genomic Single Nucleotide Polymorphism

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(SNP) array data (21) was obtained through the Lymphoma / Leukemia Molecular Profiling Project (LLMPP). DNA extracted from 10 paired pre- and post-transformation lymph node biopsies was also available (22). In addition, 27 non-tumour samples were examined (Table 1) including 19 lymph nodes from a variety of anatomical locations with multiple non-malignant histological diagnoses. Characteristics are summarised in Table 1. Two FL samples were separated into T and B cell components using CD3 or CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the CD3 and CD19 positive fractions was found to be >90% in all cases. The SNP data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (23) and are accessible through GEO Series accession number GSE14582 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14582).

Illumina GoldenGate Methylation Cancer Panel 1

DNA was bisulfite modified using the EZ DNA Methylation kit (Zymo Research, California, USA) and 250ng of starting bisulfite-modified gDNA was analysed using the Illumina GoldenGate Methylation Cancer Panel 1 (24). Methylation values are expressed as a beta (ß) value (between 0 and 1) for each CpG site representing a continuous measurement from 0 (completely unmethylated) to 1 (completely methylated). This value is calculated by subtracting background hybridisation levels obtained from negative control probes on the array and calculating the ratio of the fluorescent signal from the methylated allele (M) to the sum of the fluorescent signals from both unmethylated (U) and methylated alleles (max(M, 0))/(|U| + |M| + 100). Samples were analysed on 96 well arrays (x 3); replicate samples (n=12) were included to assess inter- and intra-array reproducibility.

Data analysis

Data was analysed using the methylation module in Illumina BeadStudio software. 84 CpG loci on the X chromosome were excluded from analysis to eliminate any gender bias as were 157 loci showing a mean ß value of <0.5 in enzymatically methylated control DNA samples (CpGenome™ Universal Methylated DNA; Chemicon) (24) (excluded genes listed in Supplementary Table S1). Analysis was subsequently restricted to the remaining 1264 loci (739 genes), 865 located within CpG islands and 399 outside of these regions. For each sample, a ß value between 0 (unmethylated) and 1 (fully methylated) was assigned to each of these 1264 CpG loci.

Differential methylation

Differential methylation was assessed by comparing the mean methylation level (ß value) of FL samples to the mean ß value of the reference group (19 non-tumour lymph node samples in Table 1) using BeadStudio software. Selection of the most significantly differentially methylated loci was based on (1) a ß value difference (Delta Beta ($\Delta ß$)) of at least 0.34 between the reference and tumour groups and (2) a P value of < .00001 as determined by two-sided t-test including a False Discovery Rate correction factor (25). These criteria were selected as they represent the most stringent criteria applied in previous studies which used the Illumina GoldenGate Methylation array (24, 26, 27).

Validation by pyrosequencing and methylation-specific PCR (MSP)

Pyrosequencing assays were designed using PSQ AssayDesign Software to analyse quantitative methylation values at 5 CpG loci corresponding to 5 GoldenGate probes from 4 genes (*CDH1, DAPK1, FAT, SLIT2)*. Pyrosequencing was performed on 25 samples (FL (n=20), Benign lymph nodes (n=4), enzymatically methylated human genomic DNA (n=1)) according to published protocols with minor modifications (28). Primer sequences and annealing temperatures are listed in Supplementary Table S2. MSP was performed on

bisulfite-modified DNA from 30 patients for 2 genes (*IGFBP7, MGMT*). Primer sequences and PCR conditions are listed in Supplementary Table S3.

Gene expression profiling

Gene expression profiling was previously performed on all FL samples studied using the Affymetrix U133 A and U133B arrays (20). In comparing methylation with gene expression, only those Affymetrix probesets with a mean $\log_2$ expression greater than 7.0 were considered well measured. If multiple probesets were available for the same gene, the one with the highest average log-expression was used. A one sided Fisher Z-score transformation was used to test for anti-correlation between methylation score and gene expression. The false discovery rates for the number of probes for which methylation was positively or negatively correlated with gene expression was calculated by comparing the observed results with those of 10,000 data sets, generated by randomly permuting the gene expression profiles among the samples.

Results

Methylation profiling distinguishes tumour from non-tumour

DNA methylation levels were measured using the Illumina GoldenGate Methylation Cancer Panel 1 in 164 diagnostic FL samples, 10 pairs of FL / t-FL samples, 19 benign lymph nodes, 4 tonsils, pooled peripheral blood mononuclear cells and 3 commercially available controls (Table 1). Unsupervised hierarchical cluster analysis of ß values of the untreated FL and non-tumour material separated samples into two distinct groups, one consisting of 163 tumour samples (blue colour bar in Figure 1) and a group that included all benign haematopoietic samples (yellow). The human male genomic DNA and the human fetal cell line DNA (CpGenome™ Universal Unmethylated DNA Vials A & B respectively; Chemicon) and a single tumour sample also clustered with these benign samples. The benign haematopoietic samples showed little variation in methylation profiles with a mean standard deviation of ß value of 0.04. The enzymatically methylated genomic DNA control (brown) clustered separately from all other samples.

Distinct clusters were identified based on their change in ß value relative to controls (Δß); we have focused on two of these clusters, referred to as low Δß (n=34, green branches of dendrogram, Figure 1) and high Δß (n=29, red branches of dendrogram) groups. These groups demonstrated a mean Δß relative to the control group of 0.1 and 0.23 respectively. There was no significant difference in the demographics or clinical outcome of patients with regard to age, grade of malignancy, stage, progression-free or overall survival between these groups. No association between methylation values of all samples and survival outcomes was identified.

We did note, however, a strong association between a higher Δß and an abnormal SNP profile using the Affymetrix 10K 2.0 GeneChip (HighΔß group: 25/29 samples with abnormal SNP; LowΔß group: 11/34 (Fisher's exact test $P<0.0001$); colour bar in Figure 1). Since detection of abnormalities by SNP profiling is dependent on the level of malignant cells (29), we reasoned that the differences in methylation may be related to amount of non-tumour tissue in the sample. Therefore, we performed methylation analysis on purified B and T cell populations and whole lymph node DNA obtained from 2 patients with FL. Both fractions showed distinct methylation patterns. The two purified B cell samples showed a mean Δß across all CpG loci of 0.29 and 0.27 and clustered with the high Δß group. In contrast the corresponding T cell samples showed a lower mean Δß of 0.11 and 0.14 and clustered within the low Δß group while the corresponding whole tumour samples showed mean Δß values of 0.24 and 0.23, intermediate between the values for purified B and T cell

samples. This demonstrated that the changes in methylation are derived predominantly from the tumour B cell population and that non-tumour cells within the tumour sample affect the methylation value.

Analysis of individual CpG loci

To identify the CpGs demonstrating the most significant tumour-specific changes in methylation relative to controls, a mean ß value for each CpG was determined for the 164 FL samples and compared to the corresponding mean ß value in our reference non-tumour lymph node group (n=19). Using the criteria of $p<0.00001$ and $\Delta ß$ of ≥ 0.34, 199 loci (corresponding to 133 genes) were identified with significant increase in methylation in the u-FL group (1-50 listed in Table 2 - all loci in Supplementary Table S4). These loci preferentially located within a CpG island with only one of the 199 CpG loci located outside of a CpG island ($P<0.0001$, Fisher's exact test). No loci located within imprinted regions (30) were included in the hypermethylated gene set. Using the same criteria, only 6 CpG loci showed significant loss of methylation in FL relative to controls. Using a less stringent $\Delta ß$ of ≥ 0.17, 70 CpG loci demonstrated hypomethylation in FL (Supplementary Table S4). CpG loci outside of CpG islands were significantly overrepresented in this group (61 of 70 (87%); $p<0.0001$, Fisher's exact test).

Reproducibility and validation of array results

There was excellent inter- and intra- array correlation between replicate DNA samples obtained from FL and benign tissue, and the human genomic DNA sample (mean $r^2 = 0.974$; range 0.909 - 0.995). Correlation between replicate human fetal cell line DNA and the enzymatically methylated control DNA samples were more variable (mean $r^2 = 0.728$; range 0.59 - 0.8). Pearson and Spearman correlation coefficients for all replicate samples are listed in Supplementary Table S5. Comparison of quantitative methylation values at 5 CpG sites studied by both the GoldenGate array and pyrosequencing assays in 25 samples confirms the accuracy of the array, particularly for identifying differential methylation (mean $r^2 = 0.852$; range 0.787 - 0.886; Supplementary Table S6, Supplementary Figure S1). MSP results demonstrated a mean beta value of 0.05 for those samples with a negative methylation-specific PCR reaction with a mean beta value of 0.35 for those samples with a positive methylation-specific PCR reaction (Supplementary Figure S2).

Identification of genes targeted by Polycomb Repressor Complex 2

As the Polycomb Repressor Complex 2 (PRC2) has been implicated in reversible transcriptional repression of genes by epigenetic mechanisms in embryonic stem cells, it was of interest to analyse whether the hypermethylated gene set included targets of the PRC2 complex. Genes examined in our study were identified as epigenetic repressive targets of PRC2 in embryonic stem cells (ESCs) based on previously published data from Lee and colleagues (31). The authors examined for the presence of three Polycomb group (PcG) marks in over 16,000 genes - occupancy by Polycomb group proteins SUZ12 and EED and association with trimethylation of Lysine 27 of Histone H3. Information on these three PcG marks was available for 674 of the genes examined on our methylation array. Comparison of frequency of the repressive PcG marks among the hypermethylated gene set, the full array and in the initial genome-wide mapping studies is shown in Figure 2. Polycomb repressor target genes were significantly overrepresented in the hypermethylated group compared to the entire set of genes analysed on the array (Fisher's exact test $p<0.0001$): genes demonstrating all three PcG marks were overrepresented (30% (36/121) in the hypermethylated group compared to 9.5% (64/674) of all genes included for analysis) as were genes showing at least one of the three PcG marks (55% (66/121) in hypermethylated group compared to 22% (145/674) of all genes included for analysis). This enrichment within the hypermethylated gene set was also present when separate analyses for the

presence of each individual PcG mark were performed. The same analyses of PcG marks also confirmed enrichment within the hypermethylated gene set compared to the reference gene list examined in the genome-wide studies by Lee et al. (chi-square test $p<0.0001$).

Analysis of methylation in sequential pre-t-FL and t-FL biopsies

Since previous studies of methylation in t-FL had been limited to non-quantitative methylation-specific PCR in a small series of patients (32), we analysed paired pre-t-FL and t-FL biopsies to determine whether there was dynamic alteration in methylation status over time with disease treatment and transformation. Ten pairs of samples which previously demonstrated chromosomal abnormalities by SNP analysis (33) were selected to ensure a minimum of contaminating non-malignant cells. Of the pre-t-FL samples 2 were obtained at diagnosis, 1 at progression after "watch and wait" management, 3 at first relapse, 3 at second relapse and 1 at seventh relapse. Significant increases in methylation relative to controls ($p<0.00001$; $\Delta ß \geq 0.34$) were demonstrated in 213 loci (corresponding to 142 genes) in the pre-t-FL group and 256 loci (163 genes) in the t-FL group. The number of hypermethylated genes in these groups is marginally higher than the u-FL group, which may reflect our efforts to limit the effects of contaminating non-tumour tissue by restricting analysis to samples with an abnormal SNP profile. There was extensive overlap between the hypermethylated CpG loci in all three groups (Figure 3). Cluster analysis of the paired samples and non-tumour samples demonstrated discrete separation of the 20 lymphoma samples from the non-tumour samples, validating the discriminative ability of the assay (Figure 4). Methylation patterns were more strongly related to the individual than to transformation status with predominantly similar profiles in patients pre- and post-transformation. Pre-t-FL and t-FL samples from 6 pairs clustered adjacent to each other (Figure 4). Analysis of variation (ANOVA) confirmed that the 'patient effect' was larger than the 'transformation status effect': 876 loci were differentially methylated between individuals across transformation status with 413 loci differentially methylated between transformation status groups across individuals (False Discovery Rate = 0.01).

Correlation with gene expression

In order to identify genes where methylation resulted in transcriptional silencing and reduction in gene expression, we correlated methylation results with previously established gene expression profiles (20). Of the 1264 probe sets measuring methylation, 819 had well measured gene expression available across the FL dataset. Of these, 226 loci (FDR 0.30) showed significant ($p<0.05$) inverse correlation between gene expression and methylation, with 77/819 (FDR 0.03) being highly significant ($p<0.001$) (Supplementary Table S7, Figure 5). Loci within CpG islands were overrepresented in this group compared with the remaining loci ($p=0.016$). Of the 226 loci showing significant inverse correlation between methylation and gene expression, 47 (corresponding to 32 genes) were present in our list of 199 significantly differentially methylated probes (Supplementary Table S8). A significant ($p<0.05$) positive correlation with methylation value was observed for 114 of the 819 (FDR 0.36) loci while 30/819 (FDR 0.05) had a strong positive correlation ($p<0.001$). CpG loci outside of CpG islands were significantly overrepresented in this group ($p<0.0001$).

Discussion

The study of methylation in FL to date has been predominantly restricted to analysis of small patient cohorts or focused on individual candidate tumour suppressor genes, using locus-specific non-quantitative methods. In our study we use array technology to quantitatively assess methylation profiles in a large, well-characterised cohort of newly diagnosed FL patients and sequential biopsies from patients who underwent transformation to DLBCL.

This approach generated a methylation profile which demonstrated extensive methylation changes between FL and benign lymph node allowing separation of tumour samples from non-tumour samples with a single exception (Figure 1). This discriminatory ability was based primarily on tumour-specific gains of methylation within CpG islands. Haematopoietic control samples demonstrated remarkably consistent methylation profiles across a wide range of ages and pathological diagnoses. While many CpG loci were noted to have similar methylation levels across FL samples, the pattern of methylation at a number of loci was heterogeneous within the u-FL group. From our comparison with SNP profiles and analysis of purified B and T cell populations, we believe that the degree of change in methylation relative to the control group predominantly reflects the relative proportions of neoplastic B cells and non-neoplastic infiltrating immune cells contained within the biopsies rather than heterogeneity of methylation within tumour cells.

This potential confounding effect of non-neoplastic tissue on quantitative methylation analysis is often overlooked as the majority of methylation studies use whole tumour samples. This emphasises the importance of purified tumour populations in future attempts to define the epigenome of various cancers, which will undoubtedly gain impetus as technology to allow high-resolution large-scale methylation mapping becomes available.

Despite this, our study was able to discriminate all but 1 of 164 tumours from non-tumour tissue, indicating that changes in methylation in FL are sufficient to distinguish from benign samples regardless of any potential effect of tumour content in the vast majority of cases. This has important practical applications as it suggests the potential for methylation-based discriminators in cancer e.g. as a less invasive method of confirming disease recurrence (e.g. by fine needle aspiration rather than core needle or open biopsies) or as an adjunct to diagnosis when material is limited. Moreover, the recent findings by Killian et al. (34) showing excellent reproducibility between matched frozen tissue and formalin-fixed, paraffin embedded tissue from individuals with FL confirms the potential clinical application of this methylation technology.

While the majority of loci examined showed similar methylation levels in both benign and malignant samples, the discriminative ability of the array was predominantly based on relative hypermethylation at selected loci in tumour samples relative to controls (Figure 1; Table 2; Supplementary Table S4). Hypermethylated and hypomethylated groups were significantly associated with CpG location, within and outside of CpG islands, respectively. This finding supports the basic methylation theory that CpGs located within CpG islands in non-tumour tissue are unmethylated while CpGs outside CpG islands are methylated with the inverse pattern occuring in tumour tissue (1).

The significant overrepresentation in the hypermethylated gene set of genes targeted for repression by Polycomb group proteins is in keeping with recent studies in carcinomas (35-37). It has been proposed that methylation of these genes are early events which "lock in stem cell phenotypes" and lead to abnormal clonal expansion (35, 38). Indeed, similar findings have recently been reported in mature aggressive B cell lymphomas by Martin-Subero and colleagues who discussed the potential implications of this finding for our understanding of lymphomagenesis (39). Recent observations suggesting that methylation of polycomb target genes is mediated by inflammation (40) and that transcriptional activity of polycomb target genes may be regulated by enzymes expressed by macrophages (41) are also intriguing, particularly given the role of the microenvironment in determining prognosis in FL (20). Sequential analysis of biopsy material from 10 paired samples also suggests that changes in methylation are acquired early in the process of lymphomagenesis and conserved with treatment and transformation.

The hypermethylated CpG loci identified in this study include both previously reported and novel methylated genes with a wide range of functions. While numerous studies have identified methylated genes in a range of human tumours, it is unclear if these changes are causal events in tumourigenesis or are merely a consequence of tumour development (42) - what have been referred to as 'epipolymorphisms' (43). In this series it seems likely that many of the 199 CpG loci hypermethylated in u-FL will represent part of widespread epigenetic change brought about through instructive mechanisms rather than individual random methylation events leading to gene silencing (44, 45), particularly given the relatively low inverse correlation between gene expression and methylation. Previous studies have shown that many genes methylated in lymphoma are expressed at low level in benign haematopoietic tissue (34, 39, 46). There are a number of other potential reasons for this low correlation. There may be a "threshold effect" in regulating gene expression whereby gene expression may not be unduly altered until a certain level of methylation has arisen(47, 48). In addition, the effect of the microenvironment on gene expression is unknown while other factors involved in methylation-associated gene silencing such as histone modifications could also contribute to these results.

In summary, this study confirms frequent aberrant tumour-specific methylation in FL in support of ongoing clinical trials of demethylating agents in FL. The similarities in the methylation profiles observed in sequential FL and t-FL biopsies and the overrepresentation within the hypermethylated gene set of genes that are targets for epigenetic repression by PRC2 in stem cells suggests that the widespread methylation may represent an early event in lymphomagenesis. It is likely that future studies will focus on elucidating the links between Polycomb group proteins and widespread tumour-specific methylation with a view to gaining insight into the timing and significance of these changes in lymphomagenesis. Methylation profiles provide a robust discriminator between benign and malignant tissue, irrespective of the confounding effect of non-neoplastic tissue, which prevented conclusive correlation with clinical outcome in this study. This is an important caveat in analysing studies attributing prognostic relevance to methylation of individual genes and future work will optimally require purified tumour populations using quantitative methylation studies to identify the true impact of methylation on tumour biology and outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. Nov 20; 2003 349(21):2042–2054. [PubMed: 14627790]
2. Feinberg AP, Tycko B. The history of cancer epigenetics. Nat Rev Cancer. Feb; 2004 4(2):143–153. [PubMed: 14732866]
3. Jones PA, Baylin SB. The epigenomics of cancer. Cell. Feb 23; 2007 128(4):683–692. [PubMed: 17320506]
4. Frigola J, Song J, Stirzaker C, Hinshelwood RA, Peinado MA, Clark SJ. Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. Nat Genet. May; 2006 38(5):540–549. [PubMed: 16642018]
5. Mack GS. Epigenetic cancer therapy makes headway. J Natl Cancer Inst. Oct 18; 2006 98(20): 1443–1444. [PubMed: 17047192]
6. Muller CI, Ruter B, Koeffler HP, Lubbert M. DNA hypermethylation of myeloid cells, a novel therapeutic target in MDS and AML. Curr Pharm Biotechnol. Oct; 2006 7(5):315–321. [PubMed: 17076647]
7. Oki Y, Aoki E, Issa JP. Decitabine--bedside to bench. Crit Rev Oncol Hematol. Feb; 2007 61(2): 140–152. [PubMed: 17023173]
8. U.S. National Institutes of Health clinical trials database. Accessed February 26th,2009http://clinicaltrials.gov
9. The Non-Hodgkin's Lymphoma Classification Project. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. Blood. Jun 1; 1997 89(11): 3909–3918. [PubMed: 9166827]
10. Guo J, Burger M, Nimmrich I, Maier S, Becker E, Genc B, et al. Differential DNA methylation of gene promoters in small B-cell lymphomas. Am J Clin Pathol. Sep; 2005 124(3):430–439. [PubMed: 16191512]
11. Rahmatpanah FB, Carstens S, Guo J, Sjahputera O, Taylor KH, Duff D, et al. Differential DNA methylation patterns of small B-cell lymphoma subclasses with different clinical behavior. Leukemia. Oct; 2006 20(10):1855–1862. [PubMed: 16900213]
12. Taylor KH, Kramer RS, Davis JW, Guo J, Duff DJ, Xu D, et al. Ultradeep bisulfite sequencing analysis of DNA methylation patterns in multiple gene promoters by 454 sequencing. Cancer Res. Sep 15; 2007 67(18):8511–8518. [PubMed: 17875690]
13. Bastion Y, Sebban C, Berger F, Felman P, Salles G, Dumontet C, et al. Incidence, predictive factors, and outcome of lymphoma transformation in follicular lymphoma patients. J Clin Oncol. Apr; 1997 15(4):1587–1594. [PubMed: 9193357]
14. Horning SJ, Rosenberg SA. The natural history of initially untreated low-grade non-Hodgkin's lymphomas. N Engl J Med. Dec 6; 1984 311(23):1471–1475. [PubMed: 6548796]
15. Montoto S, Davies AJ, Matthews J, Calaminici M, Norton AJ, Amess J, et al. Risk and clinical implications of transformation of follicular lymphoma to diffuse large B-cell lymphoma. J Clin Oncol. Jun 10; 2007 25(17):2426–2433. [PubMed: 17485708]
16. Rowley JD. Chromosome studies in the non-Hodgkin's lymphomas: the role of the 14;18 translocation. J Clin Oncol. May; 1988 6(5):919–925. [PubMed: 3284977]
17. Horsman DE, Gascoyne RD, Coupland RW, Coldman AJ, Adomat SA. Comparison of cytogenetic analysis, southern analysis, and polymerase chain reaction for the detection of t(14; 18) in follicular lymphoma. Am J Clin Pathol. Apr; 1995 103(4):472–478. [PubMed: 7726146]
18. Bende RJ, Smit LA, van Noesel CJ. Molecular pathways in follicular lymphoma. Leukemia. Jan; 2007 21(1):18–29. [PubMed: 17039231]
19. Hayslip J, Montero A. Tumor suppressor gene methylation in follicular lymphoma: a comprehensive review. Mol Cancer. 2006; 5:44. [PubMed: 17026765]
20. Dave SS, Wright G, Tan B, Rosenwald A, Gascoyne RD, Chan WC, et al. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. N Engl J Med. Nov 18; 2004 351(21):2159–2169. [PubMed: 15548776]

21. O'Shea D, O'Riain C, Gupta M, Waters R, Yang Y, Wrench D, et al. Regions of acquired uniparental disomy at diagnosis of follicular lymphoma are associated with both overall survival and risk of transformation. Blood. Mar 5; 2009 113(10):2298–2301. [PubMed: 19141865]
22. Davies AJ, Lee AM, Taylor C, Clear AJ, Goff LK, Iqbal S, et al. A limited role for TP53 mutation in the transformation of follicular lymphoma to diffuse large B-cell lymphoma. Leukemia. Aug; 2005 19(8):1459–1465. [PubMed: 15902285]
23. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. Jan 1; 2002 30(1):207–210. [PubMed: 11752295]
24. Bibikova M, Lin Z, Zhou L, Chudin E, Garcia EW, Wu B, et al. High-throughput DNA methylation profiling using universal bead arrays. Genome Res. Mar; 2006 16(3):383–393. [PubMed: 16449502]
25. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Statist Soc. 1995; B57:289–300.
26. Bibikova M, Chudin E, Wu B, Zhou L, Garcia EW, Liu Y, et al. Human embryonic stem cells have a unique epigenetic signature. Genome Res. Sep; 2006 16(9):1075–1083. [PubMed: 16899657]
27. Ladd-Acosta C, Pevsner J, Sabunciyan S, Yolken RH, Webster MJ, Dinkins T, et al. DNA methylation signatures within the human brain. Am J Hum Genet. Dec; 2007 81(6):1304–1315. [PubMed: 17999367]
28. Tost J, Gut IG. DNA methylation analysis by pyrosequencing. Nat Protoc. 2007; 2(9):2265–2275. [PubMed: 17853883]
29. Zhao X, Li C, Paez JG, Chin K, Janne PA, Chen TH, et al. An integrated view of copy number and allelic alterations in the cancer genome using single nucleotide polymorphism arrays. Cancer Res. May 1; 2004 64(9):3060–3071. [PubMed: 15126342]
30. Houshdaran S, Cortessis VK, Siegmund K, Yang A, Laird PW, Sokol RZ. Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. PLoS ONE. 2007; 2(12):e1289. [PubMed: 18074014]
31. Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, et al. Control of developmental regulators by Polycomb in human embryonic stem cells. Cell. Apr 21; 2006 125(2): 301–313. [PubMed: 16630818]
32. Rossi D, Capello D, Gloghini A, Franceschetti S, Paulli M, Bhatia K, et al. Aberrant promoter methylation of multiple genes throughout the clinico-pathologic spectrum of B-cell neoplasia. Haematologica. Feb; 2004 89(2):154–164. [PubMed: 15003890]
33. Fitzgibbon J, Iqbal S, Davies A, O'Shea D, Carlotti E, Chaplin T, et al. Genome-wide detection of recurring sites of uniparental disomy in follicular and transformed follicular lymphoma. Leukemia. Jul; 2007 21(7):1514–1520. [PubMed: 17495976]
34. Killian JK, Bilke S, Davis S, Walker RL, Killian MS, Jaeger EB, et al. Large-scale profiling of archival lymph nodes reveals pervasive remodeling of the follicular lymphoma methylome. Cancer Res. Feb 1; 2009 69(3):758–764. [PubMed: 19155300]
35. Widschwendter M, Fiegl H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, et al. Epigenetic stem cell signature in cancer. Nat Genet. Feb; 2007 39(2):157–158. [PubMed: 17200673]
36. Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet. Feb; 2007 39(2):237–242. [PubMed: 17211412]
37. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet. Feb; 2007 39(2):232–236. [PubMed: 17200670]
38. Schuebel K, Chen W, Baylin SB. CIMPle origin for promoter hypermethylation in colorectal cancer? Nat Genet. Jul; 2006 38(7):738–740. [PubMed: 16804535]
39. Martin-Subero JI, Kreuz M, Bibikova M, Bentink S, Ammerpohl O, Wickham-Garcia E, et al. New insights into the biology and origin of mature aggressive B-cell lymphomas by combined epigenomic, genomic, and transcriptional profiling. Blood. Mar 12; 2009 113(11):2488–2497. [PubMed: 19075189]

40. Hahn MA, Hahn T, Lee DH, Esworthy RS, Kim BW, Riggs AD, et al. Methylation of polycomb target genes in intestinal cancer is mediated by inflammation. Cancer Res. Dec 15; 2008 68(24): 10280–10289. [PubMed: 19074896]

41. De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. Cell. Sep 21; 2007 130(6):1083–1094. [PubMed: 17825402]

42. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, et al. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet. Jun; 2002 31(2):141–149. [PubMed: 11992124]

43. Martin-Subero JI, Ballestar E, Esteller M, Siebert R. Towards defining the lymphoma methylome. Leukemia. Oct; 2006 20(10):1658–1660. [PubMed: 17041636]

44. Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, et al. Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat Genet. Feb; 2006 38(2):149–153. [PubMed: 16444255]

45. Ohm JE, Baylin SB. Stem cell chromatin patterns: an instructive mechanism for DNA hypermethylation? Cell Cycle. May 2; 2007 6(9):1040–1043. [PubMed: 17457052]

46. Pike BL, Greiner TC, Wang X, Weisenburger DD, Hsu YH, Renaud G, et al. DNA methylation profiles in diffuse large B-cell lymphoma and their relationship to gene expression status. Leukemia. Feb 21.2008 22:1035–1043. [PubMed: 18288132]

47. Irizarry RA, Ladd-Acosta C, Carvalho B, Wu H, Brandenburg SA, Wen B, et al. Comprehensive high-throughput arrays for relative methylation (CHARM). Genome Res. Mar 3.2008 18:780–790. [PubMed: 18316654]

48. Reinhold WC, Reimers MA, Maunakea AK, Kim S, Lababidi S, Scherf U, et al. Detailed DNA methylation profiles of the E-cadherin promoter in the NCI-60 cancer cells. Mol Cancer Ther. Feb; 2007 6(2):391–403. [PubMed: 17272646]

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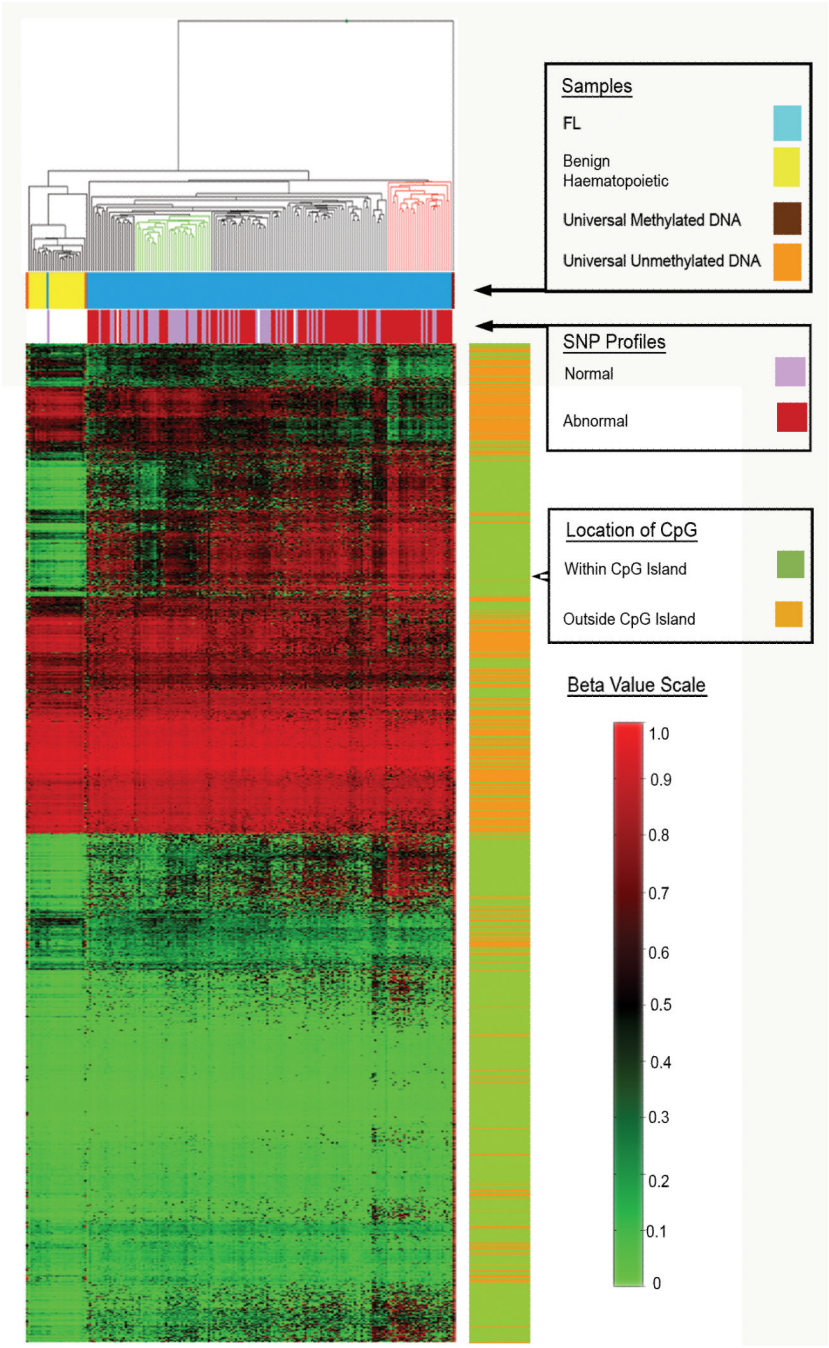

Figure 1. Hierarchical clustering of methylation values (ß) from 1264 CpG loci from diagnostic Follicular Lymphoma samples and control non-tumour samples

Columns represent samples; rows represent CpG loci. Colour represents methylation level ß from 0 - 1 as per colour bar (Red = high methylation level; Green= Low methylation level). Analysis restricted to autosomal loci and those with ß value of ≥0.5 for enzymatically methylated control DNA. Vertical colour bar indicates location of CpG locus within CpG island (Green) or outside of CpG island (Red). Top horizontal colour bar indicates tumour samples (Blue; n=164), benign haematopoietic samples (Yellow; n=24), human genomic DNA and human fetal cell line DNA (Orange; n=2), enzymatically methylated human DNA (Brown; n=1). The enzymatically methylated DNA clusters separately from all other

samples. The remaining samples separate into two distinct groups; a group consisting of 163 tumour samples (n=163) and a group including all benign / control tissue and a single tumour sample. Lower horizontal colour bars indicate presence (red) or absence (purple) of abnormality on 10K 2.0 SNP array. White indicates unavailable / not tested. Two sub-clusters representing the extremes of methylation change (Δß) relative to control group are highlighted on the dendrogram- a low Δß group (green coloured dendrogram bars) and a high Δß group (red coloured dendrogram bars).

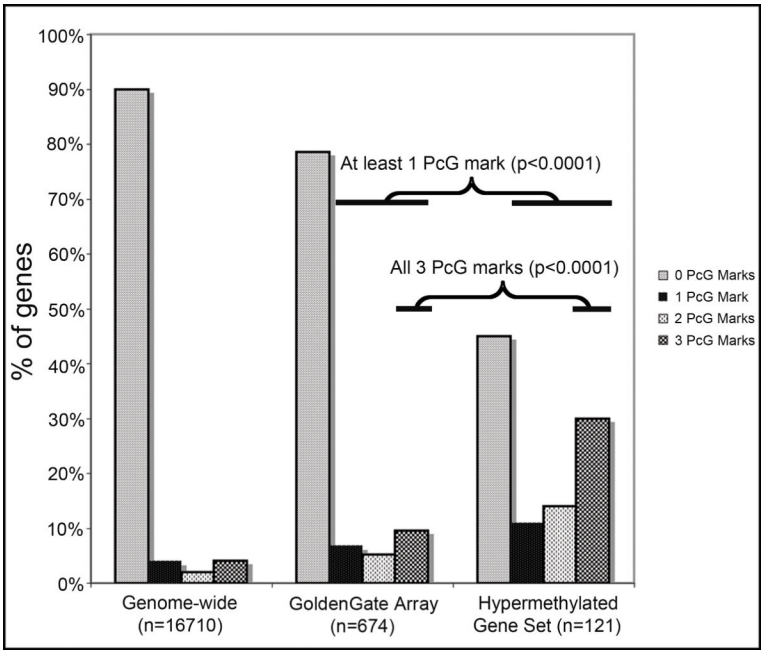

Figure 2. Overrepresentation of Polycomb Repressor Complex 2 target genes within the hypermethylated gene set

Polycomb group (PcG) marks are based on the study of Lee and colleagues (31) who examined for 3 PcG marks (occupancy by Polycomb group proteins SUZ12 and EED and association with trimethylation of Lysine 27 of Histone H3) in 16710 genes in embryonic stem cells. The hypermethylated gene set is significantly ($p<0.0001$; Fisher's exact test) enriched for genes showing PcG marks compared to the entire GoldenGate array gene set. This enrichment applies to both genes showing all 3 PcG marks and those showing at least 1 of 3 PcG marks.

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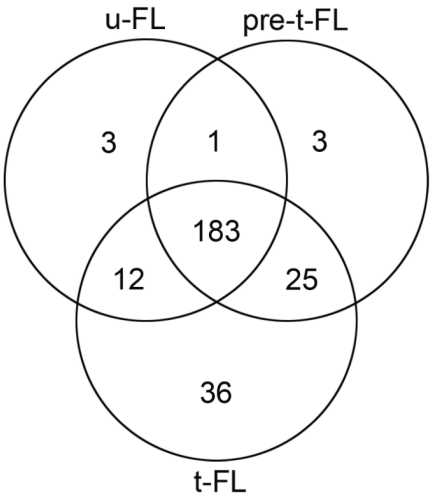

Figure 3. Venn diagram showing overlap of hypermethylated CpG loci among u-FL samples(n=199 CpG loci) and the pre-t-FL (n=212) and t-FL (n=256) paired samples

Hypermethylation defined as (1) a ß value difference (Delta Beta ($\Delta ß$)) of at least 0.34 between the reference and tumour groups and (2) a *P* value of < .00001 as determined by two-sided t-test. Of the 36 loci unique to the t-FL group, 36/36 (100%) in the pre-t-FL group and 33/36 (92%) in the u-FL group showed hypermethylation at less stringent criteria of (1) a $\Delta ß$ of at least 0.17 between the reference and tumour groups and (2) a *P* value of < .004 as determined by two-sided t-test.

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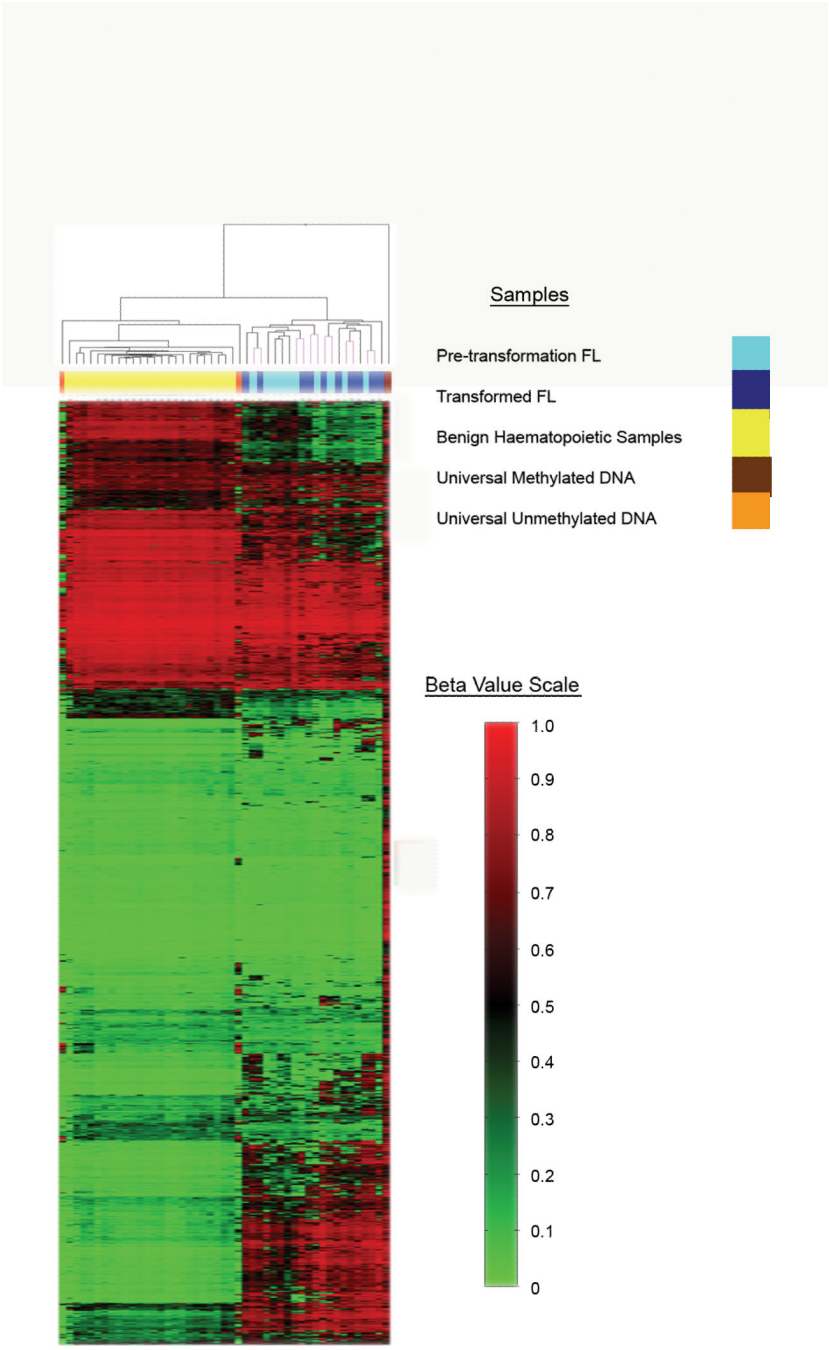

Figure 4. Hierarchical clustering of methylation values (ß) from 1264 CpG loci from 10 pairs of pre- and post-transformation Follicular Lymphoma samples and control non-tumour samples

Columns represent samples; rows represent CpG loci. Colour represents methylation level ß from 0 - 1 as per colour bar (Red = high methylation level; Green= Low methylation level). Analysis restricted to autosomal loci and those with ß value of ≥0.5 for enzymatically methylated control DNA. Horizontal colour bar indicates pre-transformation follicular lymphoma samples (Light blue; n=10), transformed FL samples (Dark blue; n=10), benign haematopoietic samples (Yellow; n=24), human genomic DNA and human fetal cell line DNA (Orange; n=2), enzymatically methylated human DNA (Brown; n=1). The enzymatically methylated DNA clusters separately from all other samples. The remaining

samples separate into two distinct groups consisting of malignant samples and benign controls. Individual samples from six pairs cluster adjacent to each other (purple bars of dendrogram).

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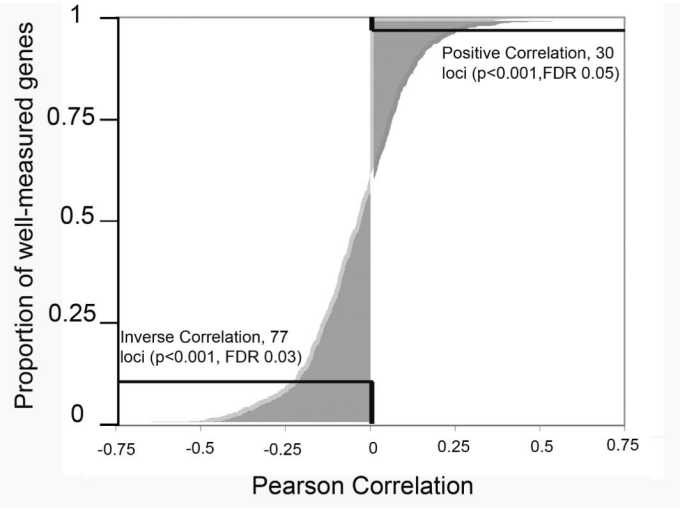

Figure 5. Analysis of correlation between methylation level and gene expression

Gene expression profiling was previously performed on all FL samples studied using the Affymetrix U133 A and U133B arrays (20). 819 CpG loci (y axis) had well measured corresponding gene expression data available across the FL dataset. Pearson correlation values between gene expression and methylation are shown on the x axis with negative values representing inverse correlation and positive values representing positive correlation. Highly significant ($p<0.001$) inverse correlation was identified for 77/819 loci (FDR 0.03) while 30/819 loci (FDR 0.05) had a strong positive correlation ($p<0.001$). FDR = False Discovery Rate.

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Table 1
List of samples examined using Illumina GoldenGate Methylation Cancer Panel 1

Sample	n=	Mean Age, years (Range)
Malignant Lymph Node DNA	184	
Previously untreated Follicular Lymphoma (FL)	164	52 (23-90)
Paired FL and transformed FL:		
Pre-transformation FL	10	
Transformed FL	10	
Benign Lymph Node DNA *	19	27 (4-55)
Hyperplastic	14	
Granulomatous	3	
PTGC	3	
Dermatopathic lymphadenitis	2	
Granulation Tissue	1	
Tonsils / Adenoids	4	13 (7-27)
Hyperplastic	4	
Pooled Peripheral Blood MNCs	1	
CpGenome Universal Unmethylated DNA (Chemicon)		
Vial A - Human genomic DNA	1	
Vial B - Human fetal cell line DNA	1	
CpGenome Universal Methylated DNA (Chemicon)	1	
FL Cell Suspension DNA		
Whole tumour	2	
Purified CD19+ve B cells	2	
Purified CD3+ve T cells	2	

MNC indicates mononuclear cells; PTGC, Progressive transformation of Germinal Centres.

* Some benign lymph nodes included multiple diagnostic categories.

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Table 2

List of 50 CpG loci showing most significant increases in methylation in Follicular Lymphoma (n=164) relative to lymph node controls (n=19). All p values <1 × 10^{-14}

	Lymph Node(ß)		Follicular Lymphoma(ß)		Delta Beta (Δß)
TargetID	**Mean**	**Standard Deviation**	**Mean**	**Standard Deviation**	
IGF2_E134_R	0.10	0.05	0.82	0.18	0.72
DLK1_E227_R	0.08	0.05	0.79	0.13	0.71
SLC22A3_E122_R	0.07	0.04	0.77	0.14	0.70
IGSF4_P86_R	0.09	0.02	0.78	0.14	0.69
TUSC3_E29_R	0.10	0.06	0.77	0.13	0.67
HOXA9_E252_R	0.08	0.03	0.75	0.18	0.67
DAPK1_P10_F	0.04	0.02	0.70	0.20	0.66
ASCL2_E76_R	0.09	0.04	0.74	0.13	0.65
GAS7_P622_R	0.20	0.10	0.85	0.16	0.65
NTSR1_P318_F	0.05	0.02	0.70	0.20	0.65
HTR1B_P222_F	0.08	0.06	0.73	0.18	0.65
TFAP2C_E260_F	0.03	0.02	0.67	0.20	0.64
SEMA3C_E49_R	0.06	0.03	0.70	0.19	0.64
ISL1_P379_F	0.06	0.05	0.70	0.17	0.64
HOXA9_P1141_R	0.10	0.03	0.74	0.16	0.64
ETV1_P235_F	0.09	0.09	0.73	0.21	0.64
TJP1_P390_F	0.03	0.02	0.67	0.22	0.64
SFRP1_E398_R	0.04	0.02	0.67	0.19	0.64
SCGB3A1_E55_R	0.06	0.05	0.69	0.19	0.64
FAT_P279_R	0.12	0.05	0.76	0.13	0.64
SLIT2_P208_F	0.10	0.05	0.73	0.16	0.63
CDH13_E102_F	0.08	0.05	0.71	0.18	0.63
HS3ST2_E145_R	0.16	0.07	0.79	0.13	0.63
NEFL_P209_R	0.11	0.07	0.74	0.13	0.62
IGFBP3_P423_R	0.07	0.04	0.69	0.15	0.62
CDH1_P52_R	0.05	0.03	0.67	0.21	0.62

	Lymph Node(fl)		Follicular Lymphoma(fl)		Delta Beta (Δfl)
TargetID	**Mean**	**Standard Deviation**	**Mean**	**Standard Deviation**	
MAF_P826_R	0.04	0.02	0.66	0.25	0.62
FRZB_E186_R	0.09	0.06	0.71	0.20	0.62
CHGA_E52_F	0.06	0.04	0.66	0.18	0.61
SFRP1_P157_F	0.04	0.02	0.65	0.21	0.61
MYOD1_E156_F	0.12	0.06	0.73	0.15	0.61
ASCL2_P360_F	0.08	0.04	0.69	0.14	0.61
FAT_P973_R	0.10	0.04	0.71	0.16	0.61
SOX1_P294_F	0.10	0.04	0.70	0.16	0.61
GATA6_P726_F	0.06	0.02	0.66	0.20	0.61
CDH13_P88_F	0.22	0.08	0.82	0.12	0.60
WT1_E32_F	0.04	0.02	0.65	0.25	0.60
DAPK1_P345_R	0.04	0.02	0.64	0.26	0.60
ONECUT2_E96_F	0.08	0.04	0.68	0.19	0.59
TIMP3_seq_7_S38_F	0.04	0.03	0.64	0.21	0.59
PLXDC2_E337_F	0.03	0.02	0.62	0.27	0.59
ISL1_E87_R	0.07	0.04	0.65	0.19	0.59
PALM2-AKAP2_P420_R	0.08	0.03	0.66	0.17	0.58
NGFB_E353_F	0.05	0.03	0.63	0.24	0.58
FGF3_P171_R	0.06	0.03	0.63	0.23	0.57
NGFB_P13_F	0.12	0.03	0.69	0.17	0.57
DBC1_P351_R	0.10	0.06	0.67	0.15	0.57
GRB10_E85_R	0.03	0.02	0.60	0.23	0.57
ONECUT2_P315_R	0.04	0.02	0.60	0.24	0.57
NOTCH3_P198_R	0.16	0.07	0.73	0.19	0.57