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EBV transformation and cell culturing destabilizes DNA methylation in human lymphoblastoid cell lines

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ABSTRACT

Recent research suggests that epigenetic alterations involving DNA methylation can be causative for neurodevelopmental, growth and metabolic disorders. Although lymphoblastoid cell lines have been an invaluable resource for the study of both genetic and epigenetic disorders, the impact of EBV transformation, cell culturing and freezing on epigenetic patterns is unknown. We compared genome-wide DNA methylation patterns of four white blood cell samples, four low-passage lymphoblastoid cell lines pre and post freezing and four high-passage lymphobastoid cell lines, using two microarray platforms: Illumina HumanMethylation27 platform containing 27,578 CpG sites and Agilent Human CpG island Array containing 27,800 CpG islands. Comparison of genome-wide methylation profiles between white blood cells and lymphoblastoid cell lines demonstrated methylation alterations in lymphoblastoid cell lines occurring at random genomic locations. These changes were more profound in high-passage cells. Freezing at low-passages did not have a significant effect on DNA methylation. Methylation changes were observed in several imprinted differentially methylated regions, including DIRAS3, NNAT, H19, MEG3, NDN and MKRN3, but not in known imprinting centers. Our results suggest that lymphoblastoid cell lines should be used with caution for the identification of disease-associated DNA methylation changes or for discovery of new imprinted genes, as the methylation patterns seen in these cell lines may not always be representative of DNA methylation present in the original B-lymphocytes of the patient.

Introduction

Immortalized lymphoblastoid cell lines (LCLs) are of great practical value for human genetics, because they provide a virtually unlimited source of DNA. Many publicly available research repositories store LCLs derived from both healthy individuals and individuals with various disorders. These cell lines have been particularly useful for genetic studies despite rare cases of genomic instability [1].They have also been successfully used for gene expression analyses in neuropsychiatric disorders [2,3]. Given the attention focused now on discovery of epigenetic determinants in human disorders, we studied the stability of epigenetic marks in LCLs, easily accessible cell lines for such research.

DNA methylation is an important epigenetic mechanism regulating gene expression. It occurs predominantly at cytosines of CpG dinucleotides. CpGs are distributed non-randomly in the genome. For 98% of the genome, CpG sites occur at low frequency once per 80 dinucleotides. CpG islands range in size from 200 bp to several kb, comprise 1–2% of the genome, and have a five-fold higher concentration of CpGs than the rest of the genome [4]. The CpG islands commonly associated with gene promoters are usually not methylated. Important exceptions are the imprinted genes and genes on the inactive X chromosome [4]. Normally methylation of promoters containing CpG islands causes gene silencing. However, for CpG poor promoters there are conflicting reports about the effect of methylation on gene expression [5,6].

Aberrant DNA methylation can result in various disorders including cancer [7] and imprinting disorders [8,9]. Whole genome based approaches such as microarrays and deep genome sequencing have presented new opportunities to identify epigenetic defects associated with human diseases. In many cases, DNA from LCLs might be considered the best available material for such experiments. However, the essential question is: normal methylation marks retained in LCLs? There are several factors that could potentially change methylation patterns in LCLs as compared to B-lymphocytes, from which they originate. These include Epstein Barr virus infection and subsequent immortalization, cell culture conditions and freezing cycles. Two types of altered methylation patterns could theoretically occur. *De novo* methylation of normally unmethylated sequences could occur via DNA methyltransferase activity. Alternatively, loss of methylation could occur either passively through failure of DNA

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methylation maintenance following DNA replication cycle, or actively by demethylating activity; however no enzyme with such activity has been identified to date. If DNA methylation changes were to occur in LCLs at specific genomic locations, this would not be a problem for epigenetic research, as the probes for these genomic regions could be excluded from the analysis. However, if methylation changes occur at random genomic locations in LCLs, this could invalidate experiments targeting the identification of candidate DNA methylation changes associated with a specific disease. This is because it would not be clear whether the altered DNA methylation pattern represents the original patient's DNA profile or whether it reflects in vitro changes that occur during cell line establishment and maintenance.

Our experiments were designed to explore the scope of epigenetic alterations occurring in LCLs and how that might impact the use of these lines for epigenetic discoveries. We compared genome-wide DNA methylation profiles from LCLs and white blood cells (WBCs) using two technologies: methylated DNA immunoprecipitation followed by Agilent CpG island array (27,800 CpG islands) and Illumina's Infinium HumanMethylation27 BeadChip (27,578 individual CpG dinucleotides spanning 14,495 genes). Our goal was to determine the utility of these two platforms to identify methylation changes in comparisons of methylation profiles of WBCs and LCLs. We compared four WBC samples, four low passage LCLs (LP_LCLs) and four low passage LCLs after freezing (FR_LCLs) from the same individuals and four unrelated high passage previously frozen LCLs (HP_LCLs). We used both Illumina and Agilent platforms for genome-wide correlation analysis and analysis of DNA methylation changes in imprinted genes. The Agilent platform was used for differential methylation analysis of one WBC and LP_LCL pair in order to select regions of several hundred bps with methylation changes, as except for imprinted genes Illumina coverage was limited to two CpG sites for most genes. The Illumina array and pyrosequencing were used to validate differences detected by Agilent in a larger number of LP_LCL pairs.

Our results show that the various technologies we used validate each other in identifying DNA methylation changes. We found random methylation changes in LP_LCLs, FR_LCLs and HP_LCL; the largest frequency was in HP_LCLs. The methylation profiles in FR_LCLs were similar to LP_LCLs, indicating that one cycle of freezing does not induce significant methylation changes.

We also checked DNA methylation patterns at differentially methylated regions of imprinted genes. Imprinted genes are expressed from only one of the parental alleles. There are approximately 60 imprinted genes found in the human genome (http://www.geneimprint.com). There are a number of single imprinted genes (e.g. DIRAS3, NAP1L5, NNAT) but the majority identified to date are clustered in the genome [10]. The parent-of-origin specific expression of imprinted genes within the cluster is usually regulated by allelic methylation at a single imprinting centre (IC), and often additional differentially methylated regions (DMRs) are present within the cluster, usually within the promoters of the imprinted genes. These parent of origin specific allelic methylation patterns are maintained in somatic cell lineages, with few exceptions [8]. However, it is not known whether this differential allelic methylation is consistently maintained in immortalized LCLs. Our results showed that several imprinted DMRs, but none of the known ICs, have methylation changes. These occur in all type of LCLs, with more changes occurring in HP_LCLs.

Results

We compared DNA methylation between WBCs and LCLs at two levels of resolution: CpG islands and single CpG sites, using two microarray platforms, the Illumina HumanMethylation27 BeadChip and the Agilent CpG island array. For the Agilent array, the methylated DNA immunoprecipitation (MeDIP) was carried out prior to hybridization.

MeDIP is a recently developed technique [11] used to capture methylated DNA sequences. We labeled the immunoprecipitated DNA and input DNA with two different fluorescent dyes and co-hybridized to the Agilent microarrays. Log2 ratios of immunoprecipitated DNA versus input (MeDIP/Input) were used to assess the levels of methylation with higher log2 ratios representing higher levels of methylation. The log2 (MeDIP/Input) is not an absolute methylation value, but can be used to compare methylation levels between samples at the same genomic locations (i.e. microarray probes), but not to compare methylation levels among different genomic locations, as MeDIP enrichment depends on the density of the methylated CpG sites [11,12]. The Illumina platform utilizes bisulphite converted DNA and thus measures absolute methylation levels at specific CpG sites. The methylation level of each site is measured as $C/(C+T)$ and is represented as a β value ranging continuously from 0 (0% methylated) to 1 (100% methylated).This method provides more accurate methylation level measurements than MeDIP, but less genomic coverage as only one CpG site is measured by each array probe.

We implemented both of the above described techniques in order to study DNA methylation stability in LCLs. We assessed changes in DNA methylation for DNA fragments 500 bp and longer by MeDIP and Agilent CpG island microarray and the DNA methylation changes occurring at specific CpG sites by Illumina methylation platform.

To assess the methylation differences between DNA from WBCs and LCLs, we analyzed methylation profiles for four sets of WBCs, low passage LCLs, and frozen LCLs from two males and two females (WM1/LM1_LP/LM1_FR, WM2/LM2_LP/LM2_FR, WF1/LF1_LP/LF1_FR, WF2/LF2_LP/LF2_FR) using the Illumina platform, and one pair of WBC and low passage LCL (WM1/LM1_LP) using MeDIP and the Agilent platform (Table 1). DNA was extracted from the low passage LCLs directly after EBV transformation prior to freezing the cells, and then DNA was extracted a second time after one cycle of freezing (frozen LCLs). Four high passage LCLs (LM3_HP, LF3_HP, LM4_HP, LPID11_HP) had undergone an unknown number of freezing cycles and cell divisions, and were studied using the Illumina array; and two of them (LF3_HP and LPID11_HP) using the Agilent array. As well, one un-paired WBC sample (WF4) was studied with the Agilent array (Table 1).

Comparison of genome-wide DNA methylation levels between LCLs and WBCs

DNA methylation across the genome was assessed to determine consistency among WBCs versus LCLs. We first assessed cell type specific differences between WBC and three types of LCLs by performing Manhattan hierarchical clustering analyses of Illumina methylation data. Clustering showed that WBC samples and LCLs cluster in separate groups, whereas there was no clustering of LCLs according to the type (low passage, frozen, high passage) (Fig. 1). This suggests that DNA methylation changes in each group could be either specific for the group or random.

To further assess cell type specific differences, we compared genome-wide DNA methylation profiles between individual samples by calculating correlation coefficients (R) for linear regressions of pair-wise sample comparisons. Sex chromosomes (X and Y) were excluded from these analyses, in order to pick up only cell type specific differences, but not sex specific differences.

Illumina methylation correlations

Correlation coefficients (R) for Illumina methylation are shown in Table 1A. R values among WBC samples ranged from 0.985 to 0.99 (Table 2A), showing very small inter-individual variation in the DNA methylation of WBCs. However, for pairs of WBCs and LCLs from the same individuals, R values were in range of 0.933–0.947 for WBC/