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Research paper

Global methylation patterns in primary plasma cell leukemia

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ABSTRACT

Primary plasma cell leukemia (pPCL) is a rare and very aggressive variant of multiple myeloma (MM). Specific clinical, biological and molecular patterns distinguish pPCL from MM. Here, we performed a genome-wide methylation analysis by high-density array in 14 newly diagnosed pPCL patients along with 60 MMs, and 5 patients affected by monoclonal gammopathy of uncertain significance (MGUS). Our analysis revealed a global hypomethylation profile associated with pPCL. Additionally, differential methylation patterns were found related to distinct chromosomal aberrations and *DIS3* mutations, affecting genes with roles in bone metabolism, cell migration, transcription regulation or DNA damage response. When compared with MM patients, pPCL showed a distinct methylation profile mostly characterized by hypomethylated probes specific for genes involved in several processes like cell adhesion and migration. Furthermore, decreasing methylation levels were evidenced for genes significantly modulated in the progressive phases of plasma cell dyscrasias, from MGUS to MM and pPCL. Overall, our data provide new insights into the molecular characterization of pPCL, thus being potentially useful in the prognostic stratification or identification of novel molecular targets.

1. Introduction

Plasma cell leukemia (PCL) is a rare form of PC dyscrasia diagnosed by the presence of more than 20% of PCs in peripheral blood and/or an absolute PC count $\geq 2 \times 10^9$ /L [1–4]. It may occur *de novo*, as primary PCL (pPCL), or as secondary PCL (sPCL) from the leukemic transformation of relapsed/refractory multiple myeloma (MM) [1–4]. Main chromosomal aberrations observed in MM, with the exception of hyperdiploidy, are also identified in pPCL, albeit with peculiar frequencies [5–7]. Additionally, specific gene and miRNA expression profiles, and mutational patterns distinguish pPCL from MM [5,8–10].

A number of complex and interdependent epigenetic modifications are known to cooperate with genetic alterations in cancer development and progression [11,12]. DNA methylation interacts with other components involved in epigenetic control, such as nucleosome remodeling machinery, histone modifications or non-coding RNAs (ncRNA). This interaction plays a pivotal role in establishing chromatin architecture and in the control of DNA accessibility to regulatory protein complexes, which in turn modulate transcription processes [12]. Early methylation studies have been focused on CpG islands (CGIs) at Transcription Starting Sites (TSSs). More recent genome-wide methylation profiling approaches have underlined the importance of the position of methylation sites within the entire transcriptional unit. In particular, DNA methylation in the proximity of the TSS was evidenced to block transcription initiation, whereas gene body methylation might activate transcription elongation and possibly even influence splicing [13,14]. Interestingly, methylation of CpG-rich sites in gene body has been associated with highly expressed genes in human B cells [15].

Concerning PC dyscrasias, early studies investigating CpG methylation patterns, mainly in promoter regions, reported relevant DNA methylation changes compared to healthy controls, and their association with disease progression and specific cytogenetic subgroups

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[16,17]. A global DNA hypomethylation was evidenced in the transition from pre-malignant conditions to overt MM [16–18] and associated with poor prognosis [19]. However, distinct DNA hypermethylation events were also found in CGI, which may be associated with main molecular classes [17] and poor clinical outcome in MM [20]. Finally, a more recent high-throughput methylation study in MM using a high-density array [21] revealed the presence of hypermethylated sites even outside CGI and particularly associated with intronic enhancers, indicating a new type of epigenetic alteration [21].

In the present study, we used high-density arrays to obtain a comprehensive DNA methylation profiling of pPCL. The integrative analysis with global gene expression data revealed a number of genes with a significant correlation in methylation-expression levels, which may contribute to the definition of peculiar altered molecular pathways in pPCL.

2. Materials and methods

2.1. Samples and clinical data

Highly purified (≥ 90%) CD138+ PCs were obtained from bone marrow (BM) aspirates of fourteen pPCL patients at onset, as previously reported [22]. Main genomic aberrations, such as 14q32 IGH chromosomal translocations, del(13q), del(17p), 1q gain and 1p loss, were investigated by fluorescence in situ hybridization (FISH), as previously described [23]. The mutational status of *DIS3*, *BRAF*, *N-, K-RAS* and *TP53* genes was previously investigated [24–26]. The molecular features of PCL cases are reported and summarized in Supplementary Table S1. Nine of the 14pPCL patients were enrolled in an Italian multicenter prospective GIMEMA phase II clinical trial [27].

Purified PCs from BM samples of 5 monoclonal gammopathies of undetermined significance (MGUS) and 60 newly diagnosed MM cases [25] were also included in the study. The Institutional Review Board approved the design of this study. Written informed consent was obtained in accordance with the Declaration of Helsinki.

2.2. Array-based DNA methylation profiling

Total genomic DNA (500 ng) was treated with sodium bisulfite using the Zymo EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the Infinium HD Methylation Assay protocol. The bisulfite converted genomic DNA was processed, and hybridized on Infinium HumanMethylation450 BeadChip array (Illumina Inc., San Diego, CA, USA), following manifacturer's procedure. After washing and staining procedures, chips scanning was performed by the Illumina HiScanSQ system.

2.3. Processing and analysis of methylation data

Illumina Infinium Human Methylation 450 K raw data were imported to R/Bioconductor environment (https://www.r-project.org/; https://www.bioconductor.org/) for quality control and processing. The measurement success rate per sample resulted > 99.9% (Supplementary Table 1), by considering for each sample the methylation probes with a detection p-value < 0.05. Subsequently, a global filter was applied to remove all the methylation probes with a detection p-value ≥ 0.05 in at least one sample of the entire dataset (1,345 probes). Then, methylation data were normalized by SWAN method [28], using the minfi package [29]. Finally, the probes not associated to CpGs, measuring single nucleotide polymorphisms (SNPs) or localized on X or Y chromosomes were discarded, thus resulting in 469, 874 probes for the further studies.

Methylation levels were expressed as β-value [30]. The Illumina Methylation Analyzer (IMA) and Annotation for Illumina 450 K methylation arrays (IlluminaHumanMethylation450kanno.ilmn12. hg19) R packages were used for the annotation of the methylation probes to the UCSC RefGene Name, in relation to the genomic context (including enhancer prediction) and the CGI proximity. They were annotated up to 1.5 kb or 200 bp upstream of the TSS (TSS1500 or TSS200, respectively) of RefSeq and miRBase-annotated genes (GRCh37/hg19); and in intragenic regions, namely 5′UTR, exon 1, gene body and 3′UTR [31]. Annotation was also performed in relation to the location in CGIs and the progressively upstream or downstream regions, such as CGI shores and shelves.

Global DNA Methylation data from eight tonsillar normal PCs (NPCs) profiled on the same array were obtained from the BLUEPRINT Epigenome Consortium (http://www.blueprint-epigenome.eu) [21,32]. Genome-wide methylation levels were calculated in NPCs, by using the described procedure as for the pPCL dataset.

Processing and visualization of the images was performed by ggplot2 and ComplexHeatmap packages, and some customizations were obtained by means of graphics editor tools (http://inkscape.org; http://gimp.org).

Hierarchical clustering (centered Pearson's correlation, average linkage) was applied on the most variable probes in methylation levels (249,658 probes with standard deviation/mean > 0.4) across the 14 pPCL dataset, by means of lumi [33], WGCNA and amap packages. M-values were computed as $\log_2$ (Methylated/Unmethylated signals) and used for differential methylation analysis [29]. Samples stratified according to the occurrence of the main molecular lesions were compared to select significant (q-value < 0.05) differentially methylated probes (dmps), with a mean β-value difference of at least 0.2.

In order to take into account the different number of methylation probes per gene on the array, gene ontology (GO) and KEGG pathway analyses were performed on the lists of significant CpG probes, by means of the gometh function from the missMethyl package, setting all CpG sites on 450 K array as the appropriate background [34]. The topGO and topKEGG functions in limma were then called to obtained the top significant GO categories or KEGG pathways, ranked by p-value [34].

To infer genome-wide copy number alterations (CNAs) by global methylation data, intensities from Illumina Infinium HumanMethylation450 BeadChip array were processed using the Chip Analysis Methylation Pipeline (ChAMP) Bioconductor package, using NPCs as control [35]. Each sample's CNAs and their frequency in pPCL group were calculated by using 0.3 or -0.3 cutoff value as the threshold for calling a gain or loss. The association between global DNA methylation and copy number levels was investigated by the chi-square test and subsequent standardized residual analysis.

Global methylation profiling data have been deposited at NCBI Gene Expression Omnibus repository (http://www.ncbi.nlm.mih.gov/geo) and are accessible through GEO Series accession number GSE104770.

2.4. Gene expression profiling data

Gene expression profiling (GEP) array data were previously obtained using GeneChip® Gene 1.0 ST array (Affymetrix Inc., Santa Clara, CA) [9] and were available under GEO Series accession number GSE66293, for 13 out of 14 pPCL samples included in the present study. Raw intensity expression values were processed by Robust Multi-array Average procedure [36] with the re-annotated Chip Definition Files from BrainArray libraries version 20.0.0 [37], available at http://brainarray.mbni.med.umich.edu/. Pearson's correlation between gene methylation and expression levels was calculated. Differential gene expression levels were assessed by Wilcoxon or Kruskal-Wallis tests.

3. Results

3.1. Global methylation patterns and copy number alterations in pPCL dataset

Global methylation profiles were investigated in 14 newly