DNA methylation and targeted sequencing of methyltransferases family genes in canine acute myeloid leukaemia, modelling human myeloid leukaemia

I. Bronzini[1], L. Aresu[2], M. Paganin[1], L. Marchioretto[7], S. Comazzi3, F. Cian[4],

F. Riondato[5], L. Marconato[6], V. Martini[3] and G. te Kronnie[1]

1 Oncohematology Laboratory, Department of Women's and Children's Health, University of Padova, Padova, Italy

2 Department of Comparative Biomedicine and Food Science, University of Padova, Padova, Italy

3 Department of Veterinary Science and Public Health, University of Milan, Milano, Italy

4 Department of Veterinary Medicine, University of Cambridge, Cambridge, UK

5 Department of Veterinary Sciences, University of Turin, Turin, Italy

6 Centro Oncologico Veterinario, Sasso Marconi, Bologna, Italy

7 Department of Cellular Biotechnology and Hematology, Sapienza University of Rome, Rome, Italy

Abstract

Tumours shows aberrant DNA methylation patterns, being hypermethylated or hypomethylated compared with normal tissues. In human acute myeloid leukaemia (hAML) mutations in DNA methyltransferase (DNMT3A) are associated to a more aggressive tumour behaviour. As AML is lethal in dogs, we defined global DNA methylation content, and screened the C-terminal domain of DNMT3 family of genes for sequence variants in 39 canine acute myeloid leukaemia (cAML) cases. A heterogeneous pattern of DNA methylation was found among cAML samples, with subsets of cases being hypermethylated or hypomethylated compared with healthy controls; four recurrent single nucleotide variations (SNVs) were found in DNMT3L gene. Although SNVs were not directly correlated to whole genome DNA methylation levels, all hypomethylated cAML cases were homozygous for the deleterious mutation at p.Arg222Trp. This study contributes to understand genetic modifications of cAML, leading up to studies that will elucidate the role of methylome alterations in the pathogenesis of AML in dogs.

Introduction

Acute myeloid leukaemia (AML) is a malignant haematopoietic disease characterized by uncon- trolled clonal cell proliferation of the myeloid lineage that affects both humans and dogs.1 In human acute myeloid leukaemia (hAML), somatic acquired chromosomal abnormalities and mutations lead to clonal overgrowth and overt disease. Recent whole genome sequence analysis of hAML identified mutations affecting genes that are involved in DNA methylation. In particular, a mutation hotspot in DNMT3A at R882H was found in 22% of adult AML patients2 and it is thought to have a role in the epigenetic dysregulation, which is a common feature of cancer.

Myeloid disorders are rare in dogs, being 10 times less frequent than lymphoprolifera- tive neoplasms, and are generally characterized by an aggressive and rapid disease progres- sion and a poor prognosis.3 The aetiology of spontaneously occurring leukaemias in dogs is far from being fully elucidated and it is likely that both genetic and environmental factors are implicated. Although extensive studies on kary- otype and somato-genomic aberrations of canine acute myeloid leukaemia (cAML) are lacking, advances in molecular genetics, including chro- mosome painting and genomic hybridization led to the identification of missense mutations in FLT3, C-KIT and RAS sequences in cAML.4 The latter aberrations have also been described in hAML hinting to common mechanisms of the two diseases.

A recent study comparing leukaemia in human and dog highlighted how diagnosis and classi- fication of AML remain challenging. The most prominent characteristic of the disease in both species is the lack of recurrent genomic aberration if compared with other leukaemias.5

Recent evidence suggests that the epigenetic layer of gene expression regulation may be impli- cated in AML pathogenesis. In mammals DNA methylation plays an important role in organ devel- opment, gene regulation, genomic imprinting, and cellular differentiation. Aberrant DNA methylation profiles are reported in several cancer types, and are considered a hallmark of cancer, being reported in several cancer types where genome-wide loss of DNA methylation commonly co-exist with aberrant promoter hypermethylation.6 On the one hand, DNA hypermethylation that leads to inappropriate silencing of tumour suppressor genes has emerged as an important mechanism in development of tumours, such as AML.7,8 On the other hand global genome hypomethylation, espe- cially occurring on repetitive elements can lead to genomic instability, re-expression of silenced trans- posable elements, increased DNA mutation rate, aberrant activation of proto-oncogenes, and altered boundaries between methylated and unmethylated genomic regions.9 – 11 In mammals, a family of DNA methyltransferases (DNMT3s) establishes and maintains DNA methylation patterns dur- ing adult life. These methyltransferases catalyse the transfer of a methyl group from S-adenosyl methionine (AdoMet) onto the 5′ position of cytosine at CpG rich sites. Members of this gene family DNMT3A and its homologue DNMT3B are both de novo methyltransferases as they generate new DNA methylation patterns throughout the genome; in addition, a third member (DNMT3L) is a catalytic inactive co-factor, that directly interacts with DNMT3A and DNMT3B stimulating their DNA methylation activity.8,12

As different hAML subtypes show heterogeneous DNA methylation profiles, both hypermethylated and hypomethylated13,14 the primary aim of this study was to investigate the global DNA methyla- tion level in cAML compared with healthy controls, in order to establish if cAML pathogenesis might be associated with globally altered DNA methylation levels. The second aim was to analyse the mutational status of the principal members of the DNMT3 fam- ily genes (DNMT3A, DNMT3B and DNMT3L) and to determine their role in cAML.

Materials and methods

Specimens

Thirty-nine blood samples were obtained from dogs with AML (see also Novacco et al. 2015)15 as part of normal diagnostic procedures and 20 blood samples were obtained from healthy dogs presented for periodical examination between 2009 and March 2014 (including general health check and/or regular vaccination). Written con- sent from owners was obtained for utilization of residual blood specimens for scientific purposes. Samples were not collected specifically for the purpose of this study. All samples were used to detect the global 5-methylcytosine (5-mC) content in genomic DNA and to assess the screening of mutations in DNMT3 family of genes. The diagno- sis of AML was achieved by careful morphological assessment of blood smears, coupled with cell blood count and immunophenotyping by flow cytometry.

Genomic DNA extraction

Genomic DNA extraction of AML and controls samples was performed with Gentra Puregene Blood Kit (QIAGEN Science, Germantown, MD, USA) according to manufacturer's instructions. Lysis of red blood cells was previously performed for specimens derived from whole peripheral blood. DNA was then quantified and quality con- trolled with NanodropTM (ThermoFisher Scientific, Waltham, MA, USA). For precise DNA quantifi- cation and in order to perform global 5-mC detection, Qubit Fluorimetric Quantitation (Life Technologies, Carlsbad, CA, USA) was used.

Flow cytometry analysis

Flow cytometric data were obtained as previously described,10 including a wide panel of antibodies (Table S1) with a multicolour approach to detect surface and cytoplasmic antigens. All antibodies were previously tittered to define correct working dilutions. Furthermore, isotype-matched controls were included for each labelling. Samples were pro- cessed either with a BD FACScalibur or a BD Accuri C6 flow cytometer (Becton Dickinson, San Jose, CA, USA) and analysed with the specific software CellQuest or Cflow Plus (Becton Dickinson).

Measurement of global 5-mC content

To measure the global content of 5-mC, a 5-mC DNA ELISA Kit (Zymo Research, Irvine, CA, USA) was used according to manufacturer's instruc- tion; 50 ng of purified genomic DNA in total was used both for AML and healthy control samples. The 5-mC content was quantified comparing the absorbance to a reference standard curve (gen- erated by mixing negative and positive control from 0% methylation to 100% methylation). Each analysis was repeated twice. Absorbances were quantified using a spectrophotometer (Victor X4 Multilabel Plate Reader, PerkinElmer, Bridgeville, PA, USA) at 450 and 405 nm.

Amplicon library preparation

The C-terminal domains of three DNMT3 family genes were screened to verify the presence of muta- tions through 454 deep sequencing approaches (Roche Applied Science®, Basel, Switzerland) in 16 high quality DNA cAML samples and 12 healthy controls. Four specific primer pairs were designed to amplify exons from 19 to 22 both for DNMT3A and its homologous DNMT3B; for DNMT3L, five specific primer pairs were designed in order to cover exons from 4 to 8. All the primer pairs were further elongated with a M13 sequence (Table S2); 20 ng μL^{-1} of genomic DNA were used in

order to obtain amplicons in a first step of poly- merase chain reaction (PCR) amplification. These amplicons were purified with Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany) and further elongated with specific key adaptors and multiplex identifiers (MIDs) specifically tag- ging each different sample through another PCR amplification step. Purification of the second PCR was performed with Agencourt AMPure XP beads (Beckman Coulter) according to manufacturer's instructions.

454 Next-generation pyrosequencing

After amplicons purification, library was quantified with Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. Amplicons were then pooled in equimolar ratios in order to allow clonal amplifi- cation using an emPCR Amplification Kit (Lib-A; Roche Applied Science®). Breaking of the emulsion and DNA library bead enrichment was performed following manufacturer's instructions. Enriched beads obtained were loaded on the Pico Titer Plate (PTP) plate of the Genome Sequencer Junior instrument (Roche Applied Science®).

Data and statistical analysis

For the analysis of the 454 sequencing output data were generated using GS Sequencer Instrument software version 2.3 (Roche Applied Science®). For each run, raw images and internal control analysis were performed using settings of the GS Run Browser Software version 2.3 (Roche Applied Science®). GS Amplicon Variant Analyzer software version 2.3 (Roche Applied Science®) was used for sequence analyses and variants detection. Only variants above 5% both in forward and in reverse reads were retained; further filters were applied to exclude mutations within introns and silent variants. Variants were annotated using COSMIC database. Continuous variables were compared by two-tailed Mann– Whitney test, one-way analysis of variance (ANOVA) test or unpaired t-test using Graphpad software 6.0f (GraphPad Software, San Diego, CA, USA).

Results

Characterization of diagnostic features of the canine AML specimens

Thirty-nine cAMLs were included in this study. The majority of dogs were in their middle age (7.5 years, SD ± 3.2) and 54% (21/39) were of large breeds, including eight Golden Retriever, six Labrador, three German Shepherd, two Irish Setter, one Doberman and one Weimaraner; 47% (18/39) were medium or small size breeds including 12 mixed breeds, 2 Cavalier King Charles Spaniel, 2 American Staffordshire Terrier, 1 Cocker Spaniel and 1 French Bulldog. The ratio between male (16/39) and female (19/39) cAML cases was 0.8; sex was not available for four dogs affected by AML. The mean leuko- cyte count derived from complete blood counts (CBC) was 144.45 ± 146.64 × 103 µL−1 (median: 86.04 × 103 µL−1; range: 6.5 – 572 × 103µL−1). Based on flow cytometry results, lymphoid markers were negative in all samples, myeloperoxidase (MPO) stained positive in 28.2% of cases, CD4 was positive in 7.7%, CD14 in 48.7% and CD11b in 53.8%.

Methylation levels of genomic DNA in cAML

Quantification of 5-mC content of genomic DNA was successfully performed in 36 out of 39 cAMLs and in all the controls. In general, the controls showed homogeneous global methylation levels (mean methylation

4.45%, SD 0.7), whereas methy- lation levels in cAMLs were heterogeneous (mean methylation 4.21%, SD 1.74). One dog (case 4) was excluded from further analysis as an outlier (Fig. 1). Altogether, there was no statistical difference in 5-mC content between healthy controls and cAML samples ($P \geq 0.05$). Considering the heterogeneity of the 5-mC content in cAMLs, a further analysis was performed to investigate the observed hetero- geneous methylation profiles among the cAML samples. Global cAMLs methylation levels ±2 × SD of control samples were fixed as thresholds. In this case, when comparing canine AML samples with global 5-mC content ≥5.85% (above the mean +2 × SD of control samples) and ≤3.05% (below the mean −2 × SD of control samples) a significant difference in methylation content was found with respect to healthy controls ($P = 0.0001$; Fig. 2). Interestingly, a positive correlation was found between global methylation content and white blood cell (WBC) count at diagnosis (Fig. S1A, Supporting information).

No correlation was observed for 5-mC content and age or sex among cAML samples (Fig. S1B,C).

Single nucleotide variants in DNMT3 family genes of canine blood samples

In order to investigate if the difference in methyla- tion levels in cAMLs was associated to alterations in DNMT3 family genes, analysis of single nucleotide variants (SNVs) was performed in 28 high quality DNA samples (16 cAMLs and 12 healthy controls).

Only SNVs with a mutation allele frequency (MAF)

≥5% in forward and reverse reads was considered. In addition, intronic variants and single nucleotide polymorphisms (SNPs) were excluded as well as silent mutations. Finally, 46 SNVs were detected corresponding to four unique mutations (Table 1). All 46 SNVs were found across DNMT3L, whereas no SNVs were identified in the C-terminal domain of DNMT3A and DNMT3B. Two mutations were identified in exon 7 of DNMT3L, Arg241Gln and Arg222Trp. The other two mutations were respectively in exon 6 (Ala200Thr) and in exon

8 (Ala269Thr). These two mutations localize in the C-terminal domain of the DNMT3L protein, which is the one that interacts with the active cat- alytic methyltransferase domain of DNMT3A and DNMT3B. Prediction analyses of the functional impact of these mutations were performed using Polyphen2 software16 and Arg222Trp resulted to predict damaging mutation (Table 2).

DNMT3L and not DNMT3A and DNMT3B is mutated in canine samples

By Next Generation Sequencing (NGS), four SNVs in DNMT3L were identified in 15 of 16 cAML sam- ples (94%) and in 9 of 12 healthy controls (75%). In particular, among cAMLs, 9 of 16 (56%) had two or three variants, and 6 of 16 (37%) had exclusively one variant. On the other hand, five healthy con- trols (42%) had two or three variants and four (33%) showed only one variant. Considering the MAF,

67% (10/15) of cAMLs had at least one homozygous mutation (MAF ≥ 70%), conversely only 2 of 12 (16%) healthy controls were homozygous for at least one variant (Table 1). In particular, the predicted deleterious mutation p.Arg222Trp was homozy- gous in 4 of 16 (25%) cAMLs and in 2 of 12 (16%) healthy controls. By comparing cAML cases and healthy controls, or cAMLs with high methylation level and cAMLs with low methylation content, no association was found between the global methy- lation level and the presence of one or more SNV (Fig. S2). Notably, all hypomethylated cAML cases were homozygous for the deleterious mutation at p.Arg222Trp and not mutated for the other variants.

Discussion

In this study we determined if some of the epi- genetic changes seen in hAML were also present in cAML. Whole genome methylation levels were analysed in 36 dogs affected by AML and the mutational status of the three principal methyl- transferase genes was investigated. Then, we have compared these results to the data generated from 20 healthy control dogs analysed with the same approach.

Recent studies highlighted the role of methy- lation deregulation in the pathogenesis of hAML, in particular the mutations affecting DNMT3A (R882H), a methyltransferase involved in de novo DNA methylation during cell maturation processes.17 Some studies have also investigated the functional role of mutated methyltransferases in haematological malignancies using in vitro or murine models.12,18 However, due to the absence of natural occurring malignancies in the mice19 and lack of DNMT3 mutations these models have serious limitations regarding the validity of their results and their ability to faithfully recapitulate the diseases.

Here we investigated AML in dogs, which is a natural spontaneous occurring malignancy with phenotypical and biological similarities to human AML.20 Studying dogs with cancer is likely to pro- vide a valuable perspective on the aetiology of the disease different from that generated by the study of human or rodent cancers alone.21

The first goal of this study was to measure the global methylation content in the genomic DNA of cAML compared with healthy control dogs. We found that the global 5-mC content of cAML was heterogeneous, with the majority of the dogs showing either hypermethylation or hypomethy- lation. Conversely, in healthy dogs methylation levels were homogeneous, with a limited standard deviation. These results prompted us to infer that cAML is characterized by varying levels of whole genome methylation, and that specific methylation patterns and regulation could be implicated in the pathogenesis of cAML. This observation is further corroborated by the absence of any correlation between global methylation content in cAML and biological characteristics; methylation level differ- ences could not be attributed to sex or age. However, we found that cAMLs with high numbers of WBC were hypomethylated and vice versa; an inverse correlation between global methylation content and WBC count was previously observed also in B-ALL in human.22 Partly this can be attributed to the observation that a decrease of global methy- lation drives differentiation of haematopoietic progenitors towards the myeloid lineage, which is in fact characterized by hypomethylation when compared with lymphoid precursors.23,24 Alter- ations of epigenetic regulation of genes involved in tumourigenesis have been previously described in canine diffuse large B-cell lymphoma (DLBCL),25,26 but so far no data regarding the alteration of global methylation levels in tumours have been reported in dogs. To further investigate DNA methylation of cAML we questioned if alterations of methyla- tion family enzymes occurred in dogs in a similar way to what has been described in adult human AML. In line, we investigated the mutational status of DNMT3A, DNMT3B and DNMT3L in cAML using a deep sequencing approach (NGS) thereby obtaining qualitative as well as quantitative information on the mutational status. Our results revealed that cAMLs do not carry mutations in DNMT3A and DNMT3B neither in major nor in minor sub-clones. Interestingly, absence of mutations in DNMT3A was also shown in a large cohort of paediatric AML patients,27 in contrast to hot spot mutations (R883) present in adult AML patients. However, in dogs, four specific mutations were identified in DNMT3L. Recently reported experimental studies clarified the important role of DNMT3L as stimulatory factor for de novo methylation. In particular, several studies showed that DNMT3L directly interacts with DNMT3A and DNMT3B by stimulating DNA methylation activity of both DNMT3s under in vitro and in vivo conditions.28 – 32 Moreover DNMT3L enhances the catalytic activity of DNMT3A to produce an enhancement of catalysis by

stabilizing the DNMT3A-DNA complex. Mutations in DNMT3L suggest a potential role of these mutations in dog. Specifically, (1) mutations were detected more frequently in cAMLs than in healthy controls; (2) homozygous mutations were more frequent in cAMLs compared with the healthy controls where mutations were mostly heterozygous; (3) cAML affected dogs frequently carried two or more mutations compared with healthy controls. DNMT3s are considered to function as tumour suppressors and homozygous DNMT3A mutations have been reported in adult T-ALL.33,34 Inherited mutations in DNMT's with a secondary somatic hit in the bone marrow needs to be considered as a possible incentive of leukemogenesis: in fact, control dogs carry the same specific mutations in DNMT3L. Notably all hypomethylated cAML were homozygous for the mutation p.Arg222Trp. The latter mutation is known to have a deleterious effect on the protein function, thereby affecting the ability of DNMT3L to sustain correct methylation patterns. The observation that the same muta- tion in homozygosis was also found in healthy dogs together with the absence of a correlation between DNMT3L mutation status and global methylation point to additional compensatory mechanisms of epigenetic regulation that need further investigations.

In conclusion, this study provides a first under- standing of epigenetic changes in cAML. Moreover, this is the first study reporting the global methyla- tion profiles and the occurrence of new mutations in a gene of the methyltransferase family in a large number of cAML. Our data suggest that cAML similar to human AML is at least in part a methy- lome disorder with perturbed global methylation patterns and deleterious mutations in exon 7 of DNMT3L. Our data justify comparative approaches in oncology where cAML may be a relevant disease to model human AML and lead up to further studies on the epigenetic changes in both human and canine AML.

Acknowledgements

The authors wish to thank all the colleagues who provided samples and follow-up data for this study. In particular authors would like to thank Roche Italy for the technical support; a special thanks to Dr Bresolin S., Lana T., Borga C. and Trentin L., for their support on the interpretation of next-generation sequencing data and Dr Poggi A., for her support to the flow data analysis.

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Figure 1. Graph of global 5-mC content of 56 samples. [Colour figure can be viewed at wileyonlinelibrary.com].

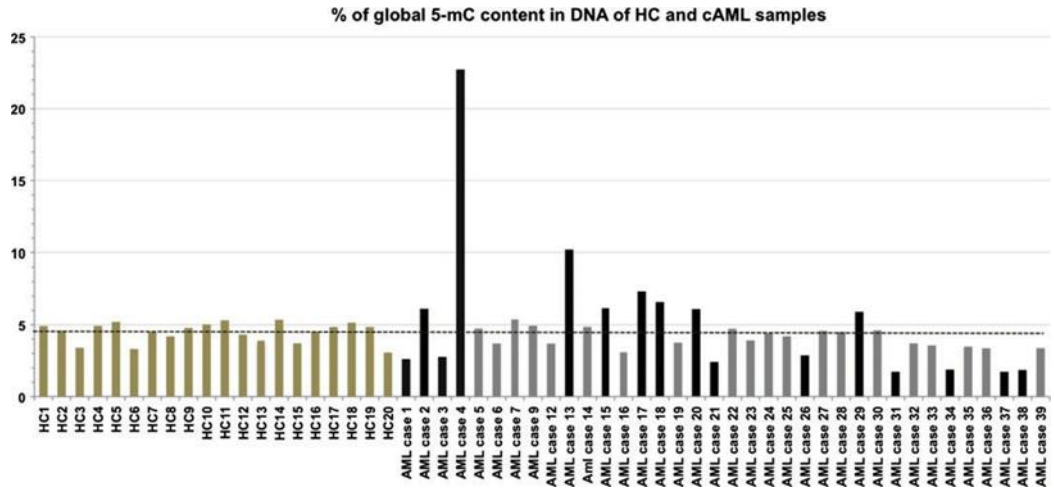

Figure 2. Global level of 5-mC comparing cAML and healthy dogs. [Colour figure can be viewed at wileyonlinelibrary.com].

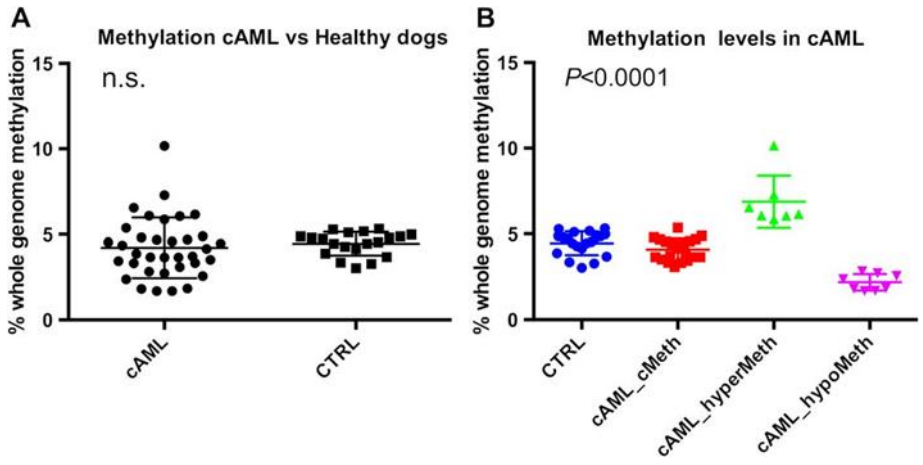

Table 1. Mutation status of 4 recurrent single nucleotide variants (SNVs) of DNMT3L and the total number of SNVs for each AML case and Healthy control.

	Number of SNV	p.Arg222Trp MAF (%)	p.Arg241Gln MAF (%)	p.Ala200Thr MAF (%)	p.Ala269Thr MAF (%)
	0	wt	wt	wt	wt
AML case 1	1	**99**	wt	wt	wt
AML case 2	1	56	wt	wt	wt
AML case 3	1	**99**	wt	wt	wt
AML case 8	1	**100**	wt	wt	wt
AML case 9	1	**100**	wt	wt	wt
AML case 15	1	55	wt	wt	wt
AML case 4	2	wt	47	36	wt
AML case 10	2	wt	**70**	wt	58
AML case 12	2	wt	**100**	wt	**100**
AML case 13	2	wt	wt	36	43
AML case 14	2	wt	wt	**100**	**98**
AML case 6	3	48	52	30	wt
AML case 7	3	43	61	30	wt
AML case 11	3	52	wt	36	36
AML case 16	3	50	wt	31	34
HC 1	0	wt	wt	wt	wt
HC 2	0	wt	wt	wt	wt
HC 3	0	wt	wt	wt	wt
HC 4	1	**99**	wt	wt	wt
HC 5	1	53	wt	wt	wt
HC 6	1	**98**	wt	wt	wt
HC 7	1	55	wt	wt	wt
HC 9	2	56	wt	27	wt
HC 10	2	wt	45	28	wt
HC 11	3	60	wt	32	47
HC 12	3	58	wt	17	51
HC 8	3	58	38	22	wt

HC, healthy control; wt, wild type.

Table 2. Description of the mutations identified by NGS. Four unique mutations were identified and their functional impact was scored using Polyphen2 software (0.000 = benign; 1 = deleterious)

Type of mutation	Database description	Exon	Mutation nucleotide position	Mutation codon position	Polyphen prediction
Missense	Described	7	c.722G>A	p.Arg241Gln	Tolerated (0.000)
Missense	Described	7	c.664C>T	p.Arg222Trp	Deleterious (0.970)
Missense	Not described	6	c.558G>A	p.Ala200Thr	Tolerated (0.001)
Missense	Not described	8	c.804G>A	p.Ala269Thr	Tolerated (0.067)

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Antibody panel used for flow cytome- try analysis of CD markers in cAML

Table S2. Primers used to amplify exons 19 to 22 of DNMT3A and DNMT3B and exons 5 to 8 of DNMT3L. Nucleotide in red represents the M13 specific sequence

Figure S1. Correlation between methylation level, WBC, age and sex. (A) Comparing the methylation percentages among cAML with low versus high WBC using a paired t-test a significant difference was found between the two groups. (B) Methylation levels comparing three age groups were not significantly different in a one-way ANOVA test. (C) Methylation levels comparing male and female cAML were not significantly different using an unpaired t-test.

Figure S2. Correlation between 5-mC con- tent and SNV in DNMT3L. Relation of wild type (wt), heterozygous and homozygous status of four variants in DNMT3L and distribution of wt, heterozygous and homozygous status per SNV and groups of cAML's specimens: no significant correlation was noted between hypermethylated or hypomethylated AML and mutation in DNMT3L. HC = healthy control.