News Release

Title

A simple and robust methylation test for risk stratification of patients with juvenile myelomonocytic leukemia

Key Points

- A total of 137 JMML patients have been analyzed using the Digital Restriction Enzyme Analysis of Methylation (DREAM).
- A robust DNA methylation test that is highly consistent with the array-based international consensus definition for JMML could be developed.

Summary

A research group led by Professor Yoshiyuki Takahashi and Lecturer Hideki Muramatsu of the Department of Pediatrics, Nagoya University Graduate School of Medicine, Professor Yusuke Okuno of the Department of Virology, Nagoya City University Graduate School of Medicine, and Dr. Hironobu Kitazawa, Chief of the Department of Pediatrics, Nagoya Daiichi Hospital, Japanese Red Cross Aichi Medical Center and their colleagues have developed a simple and robust methylation test for risk stratification of juvenile myelomonocytic leukemia (JMML) patients.

JMML is a rare myelodysplastic/myeloproliferative neoplasm that develops during infancy and early childhood. The array-based international consensus definition of DNA methylation has recently classified patients with JMML into the following three groups: high methylation (HM), intermediate methylation (IM), and low methylation (LM). To develop a simple and robust methylation clinical test, 137 patients with JMML have been analyzed using the Digital Restriction Enzyme Analysis of Methylation (DREAM), which is a next-generation sequencing based methylation analysis. Unsupervised consensus clustering of the discovery cohort (n=99) using the DREAM data has identified HM and LM subgroups (HM_DREAM, n=35; LM_DREAM; n=64). Of the 98 cases that could be compared with the international consensus classification, 90 cases of HM (n=30) and LM (n=60) had 100% concordance with the DREAM clustering results. For the remaining eight cases classified as the IM group, four cases were classified into the HM_DREAM group and four cases into the LM_DREAM group. A machine-learning classifier has been successfully constructed using a Support Vector Machine (SVM), which divided the validation cohort (n=38) into HM (HM_SVM; n=18) and LM (LM_SVM; n=20) groups. Patients with the HM_SVM profile had a significantly poorer 5-year overall survival rate than those with the LM_SVM profile. In conclusion, a robust methylation test has been developed using the DREAM analysis for patients with JMML. This simple and straightforward test can be easily incorporated in diagnosis to generate a methylation classification for patients so that they can receive risk-adapted treatment in the context of future clinical trials.

Research Background

Disruption of the epigenome is a common finding in cancer cells, often resulting in altered DNA methylation

patterns that can be accurately assessed using DNA methylation analysis. Some aberrant DNA methylation patterns have been associated with specific genetic mutations that promote neoplasia, and these patterns can be used as biomarkers for disease progression.

Juvenile myelomonocytic leukemia (JMML) is a rare myelodysplastic/myeloproliferative neoplasm that develops during infancy and early childhood. JMML is characterized by excessive myelomonocytic cell proliferation and hypersensitivity to granulocyte–macrophage colony–stimulating factor. We and other groups conducted a genome–wide methylation profiling of promoter–associated CpG sites using the Infinium Human Methylation 450K BeadChip (450K; Illumina, San Diego, CA), which permitted for the ident JMML patients with high–methylation (HM) profile. The HM profile correlated significantly with genetic markers predicting poor outcome, including the *PTPN11/NF1* gene mutations, two or more genetic mutations, an acute myeloid leukemia–type gene expression profile, and *LIN28B* overexpression. Furthermore, the array–based international consensus definition of DNA methylation has been recently reported categorizing JMML patients into three groups: HM, intermediate methylation (IM), and low methylation (LM), in an international collaborative study. In order to incorporate methylation profiling into the clinical decision–making process and future clinical trials, a robust but simple and less labor–intensive method for evaluating methylation patterns in patients with JMML is needed.

The Digital Restriction Enzyme Analysis of Methylation (DREAM) is a method for quantitative mapping of DNA methylation at tens of thousands of CpG sites on a genome using a next–generation sequencing technology. Methylation levels at each of the target CpG sites are calculated by high–throughput sequencing of DNA fragments with specific signatures for unmethylated and methylated CpG sites obtained by sequential digestion of genomic DNA using restriction enzymes *Sma*I and *Xma*I, which have the same CCCGGG recognition site but different sensitivity to CpG methylation and cleavage patterns (**Fig 1**).

In this study, the DREAM was performed on 137 patients [juvenile myelomonocytic leukemia (JMML), n = 124; Noonan syndrome–associated myeloproliferative disorder (NS/MPD), n = 13] using genomic DNA from the peripheral blood or bone marrow mononuclear cells. The patients were divided into two groups: a discovery cohort (n = 99) and a validation cohort (n = 38).

Research Results

Clustering analysis

An unsupervised hierarchical clustering of the discovery cohort has been performed using DREAM data for 7,360 promoter–associated CpG sites, and high–methylation (HM_DREAM, n = 35) and low–methylation subgroups (LM_DREAM, n = 64) with a 95% concordance (94 of 99 samples) with the previously reported 450K clustering results have been identified. Of the 98 cases that could be compared with the international consensus classification, all 90 cases of HM (n = 30) and LM (n = 60) had 100% concordance with the DREAM clustering results. For the eight cases classified as the IM group by the international consensus classification, four cases were classified into the HM_DREAM group and four cases into the LM_DREAM group (**Fig 2**).

Overall survival (OS) for both cohorts were estimated using the Kaplan–Meier method. JMML patients with HM_DREAM profile in the discovery cohort (excluding NS/MPD) had a significantly poorer OS than those with the LM_DREAM profile, with five–year OS for the HM_DREAM being 41.9% (95% CI: 25.3–57.6%) *vs.* 71.4%

(95% CI: 56.2%–82.1%) for LM_DREAM ($P = 3.45 \times 10^{-3}$).

An unsupervised clustering of the validation cohort (n = 38) has also been performed using the DREAM data obtained from the aforementioned 7,360 promoter-associated CpG sites. The patients in the validation cohort were also classified into the HM_DREAM (n = 18) and LM_DREAM subgroups (n = 20; **Fig 2**).

Support Vector Machine classifier construction for DREAM clustering

In order to develop a machine-learning classifier model using a Support Vector Machine (SVM), 84 CpG sites that were among those that exhibited a distinct difference in the average methylation levels (>0.3) between JMML-associated HM_DREAM and LM_DREAM profiles have been selected. The samples from the discovery cohort were randomly assigned into training (n = 59) and test datasets (n = 40) and were used along with the tune.svm function of the e1071 package in R to optimize parameters. The best gamma parameter and the best cost parameter were 0.015 and 0.14, respectively. A classifier with the lowest mean square error (MSE = 0) was created.

Using the SVM, patients assigned to the validation cohort were classified as either high methylation (HM_SVM; n = 18) or low methylation (LM_SVM; n = 20) (**Fig 2**). Discrepancies in profiling results between the clustering analysis and the SVM were observed in 2 out of 38 cases (5%) in the validation cohort. Patients with the HM_SVM profile had a significantly poorer five-year OS [26.3%; 95% CI: 1.9%–64.0%] than those with the LM_SVM profile [80.5%; 95% CI: 49.1%–93.6%], with a *P*-value of 0.024 (**Fig 3**).

Fig.1 Schematic outline of the principles of DNA methylation analysis. In the Digital Restriction Enzyme Analysis of Methylation (DREAM), DNA is sequentially cut using two restriction enzymes, *Sma*I and *Xma*I.

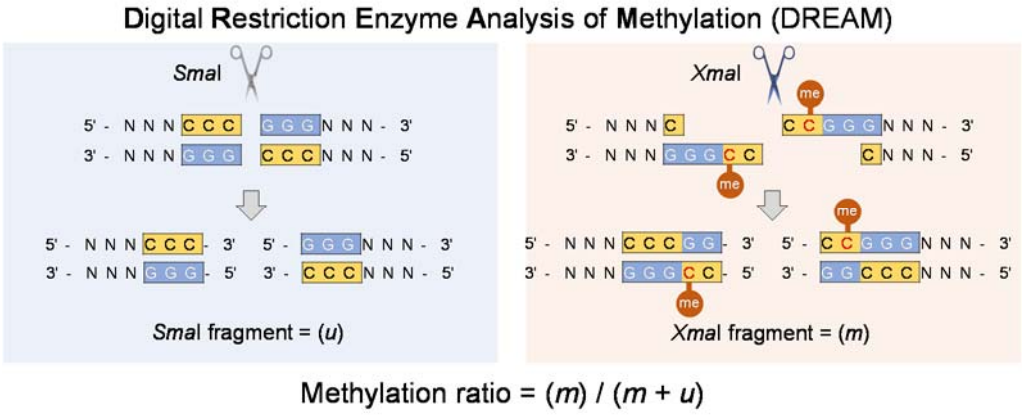


Fig.2 Classifier construction for risk stratification of patients with juvenile myelomonocytic leukemia using the Digital Restriction Enzyme Analysis of Methylation data. Juvenile myelomonocytic leukemia (JMML) and Noonan syndrome-associated myeloproliferative disorder (NS/MPD) patients (discovery cohort n = 99; validation cohort n = 38) were subjected to unsupervised hierarchical clustering using the Digital Restriction Enzyme Analysis of Methylation (DREAM) data. The heat map displays the methylation ratios calculated for a selected subset of 1,000 CpG sites with high differential average methylation levels between the high-methylation (HM_DREAM) and low-methylation (LM_DREAM) subgroups. The methylation ratios were color coded with a gradual change

from blue (0% methylation) to red (100% methylation). Known clinical and biological features were annotated for each patient. Each column indicates one patient.

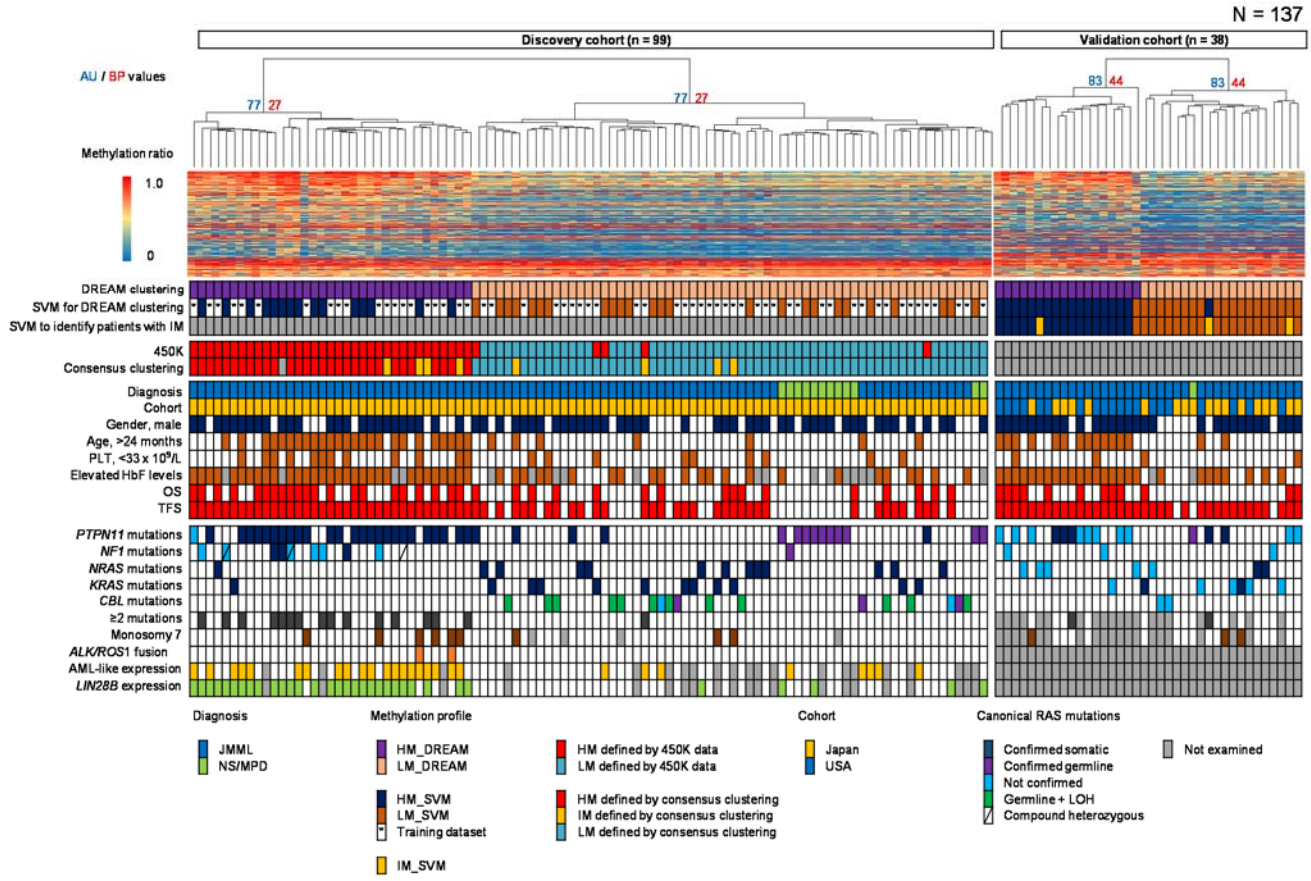
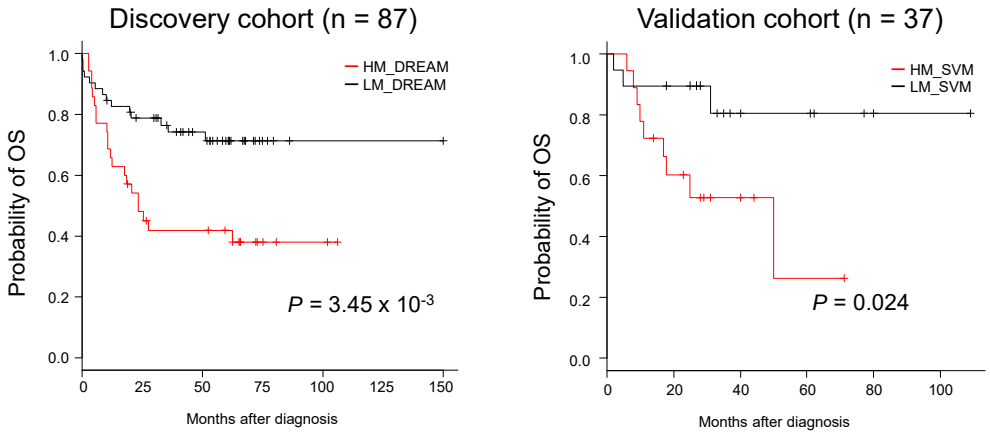


Fig.3 Overall survival (OS) of JMML patients in the discovery cohort using clustering with DREAM data. OS of JMML patients in the validation cohort using SVM with DREAM data.



Research Summary and Future Perspective

Outcomes of JMML patients were successfully predicted by classifying JMML patients into the HM_DREAM and

LM_DREAM subgroups using the DREAM analysis, and a clear reproducibility to the international consensus classification using 450K platform has been documented, despite the fact that the majority of CpG sites used for each classification were not shared. The findings have been validated in an independent cohort of patients which again revealed a poor prognosis for JMML patients with an HM subgroup profile. Furthermore, a prediction model using SVM as a clinical test was also constructed.

The DREAM analysis is quantitative and highly reproducible, and performing tests on each sample to obtain results in a timely manner is possible. Methylation testing using the DREAM analysis in newly diagnosed patients could be used for patient stratification in prospective clinical trials and could be integrated into the clinical decision-making process.

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