Letter to the Editor

Characterization of Serous Cell-Free DNA in Myelodysplastic Syndromes

Hongbo Zhu¹*, Guangjia Feng²*, Na Zhao², Lei Wu², and Zhiguo Long²

Abstract
Myelodysplastic syndromes (MDS) are a group of malignant clonal diseases presenting abnormal development of acquired hematopoietic progenitor/stem cell myeloid differentiation. MDS have been clinically divided into different types. There is a lack of clear gold standard, which makes the diagnosis of MDS with clinical signs and laboratory examination difficult. Cell-free DNA (cfDNA) is a resource of DNA fragments from apoptotic or necrotic cells, and has been considered as a measurement with ample sensitive, specific, and effective traits for auxiliary diagnosis. In this study, we collected 25 cases of relatively high-risk MDS (HRM), 22 cases of low-risk MDS (LRM), and 15 cases of benign blood diseases (control) and conducted reduced representation bisulfite sequencing (RRBS) to investigate the variants and DNA methylation of cfDNA in serum of three cases of each group. We observed increased single-nucleotide polymorphisms (SNPs) particularly distributed in intergenic and intronic regions in HRM compared with LRM and control. Moreover, HRM presented more nonsynonymous and harmful variants that would affect amino acid sequence. Meanwhile, we also observed that global DNA methylation on non-CpG sites (CHG and CHH) in HRM was obviously higher than that in LRM and control. Finally, we picked up the candidate genes with specific variants and abnormal methylation at the promoter in HRM and LRM, and combined to examine the specificity and sensitivity of HRM and LRM diagnosis in our collection. We found that FANCM with T49G mutation at first exon and promoter hypermethylation (−835 to transcription start site [TSS]) was indicated as the most confident factor with the highest area under curve (AUC) value (0.9271) for HRM. Similarly, ICAM1 with C1211T mutation at sixth exon and promoter hypermethylation (−282 to TSS) was suggested to identify LRM (AUC = 0.9338). Taken together, our study characterized the variants and methylation pattern of cfDNA in MDS, and provided the potential biomarkers for HRM and LRM identification.

Keywords
myelodysplastic syndromes, cell-free DNA, DNA methylation, FANCM, ICAM1

Dear editor,

Myelodysplastic syndromes (MDS) are a group of malignant clonal diseases caused by the abnormal development of acquired hematopoietic progenitor/stem cell myeloid differentiation¹. MDS have been clinically divided into different types. There is a lack of clear gold standard, which makes the diagnosis of MDS with clinical signs and laboratory examination difficult. Cell-free DNA (cfDNA) is a resource of DNA fragments from apoptotic or necrotic cells, and has been considered as a measurement with ample sensitive, specific, and effective traits for auxiliary diagnosis. In this study, we collected 25 cases of relatively high-risk MDS (HRM), 22 cases of low-risk MDS (LRM), and 15 cases of benign blood diseases (control) and conducted reduced representation bisulfite sequencing (RRBS) to investigate the variants and DNA methylation of cfDNA in serum of three cases of each group. We observed increased single-nucleotide polymorphisms (SNPs) particularly distributed in intergenic and intronic regions in HRM compared with LRM and control. Moreover, HRM presented more nonsynonymous and harmful variants that would affect amino acid sequence. Meanwhile, we also observed that global DNA methylation on non-CpG sites (CHG and CHH) in HRM was obviously higher than that in LRM and control. Finally, we picked up the candidate genes with specific variants and abnormal methylation at the promoter in HRM and LRM, and combined to examine the specificity and sensitivity of HRM and LRM diagnosis in our collection. We found that FANCM with T49G mutation at first exon and promoter hypermethylation (−835 to transcription start site [TSS]) was indicated as the most confident factor with the highest area under curve (AUC) value (0.9271) for HRM. Similarly, ICAM1 with C1211T mutation at sixth exon and promoter hypermethylation (−282 to TSS) was suggested to identify LRM (AUC = 0.9338). Taken together, our study characterized the variants and methylation pattern of cfDNA in MDS, and provided the potential biomarkers for HRM and LRM identification.

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and double-stranded DNA, with a resolution of about 0.18–21 kB². CfDNA has become one of the most popular research hotspots in translational medicine with the following advantages: (1) sensitivity. Previous studies have shown that the content of cfDNA in peripheral blood in tumor patients is usually dozens of times larger than in normal population³,⁴; (2) convenience. Sufficient cfDNA can be already achieved from as low as 1 ml of serum for polymerase chain reaction (PCR) or sequencing by the present technical conditions⁵. CfDNA with its simple and minimally invasive sampling method and high correlation with diseases can be used to detect diseases hidden in the body as early as possible. Compared with other imaging methods, cfDNA has a broader clinical application prospect in the early detection of diseases⁶; (3) multidimension. CfDNA has been proved to be closely correlated with the occurrence and development of diseases from multiple aspects, such as mutation, single-nucleotide polymorphism (SNP), abundance frequency of a gene, and epigenetic modifications. Although cfDNA, as a measurement with ample sensitive, specific, minimally invasive, simple, and effective traits, has been proved in a variety of diseases⁶–⁹, it has still not been involved in MDS to distinguish from different phenotypes.

Here, we collected 25 cases of relatively high-risk MDS (HRM), 22 cases of relatively low-risk MDS (LRM), and 15 cases of benign blood diseases (control) and conducted reduced representation bisulfite sequencing (RRBS) to investigate the variants and DNA methylation of cfDNA in serum of three cases of each group. We observed a total of 343,547 SNPs particularly distributed in intergenic and intronic regions in HRM much higher than LRM (n = 282,170, F = 6.595, P = 0.031) and control (161,364, F = 56.76, P = 0.002) (Fig. 1A), as well as increased nonsynonymous variants in HRM (n = 3,048) compared with LRM (n = 2,684, F = 5.827, P = 0.039) and control (n = 2,693, F = 30.073, P = 0.005) (Fig. 1B). Meanwhile, we also observed 23,245 hypermethylated and 20,376 hypomethylated differential methylated regions (DMRs) in HRM compared with LRM (Fig. 1C), as well as 13,571 hypermethylated and 57,124 hypomethylated DMRs in LRM compared with control (meth.diff > 25 or < −25, P < 0.05) (Fig. 1D).

Furthermore, we also found that CCAR2, CHRNB4, CIAO3, DYM, FANCM, GRN, MON2, PDC6I, PHLD3, PRICKLE1, RPUSD1, TG, TMEM132E, TRPM3, WWTR1, ZFPM1, and ZFVE1 could be considered as a series of biomarkers for HRM on variants and methylation. On the contrary, HAL, ICAM1, INPP4A, KCNJ15, MAT1A, MED4, PLXNB2, SERPINA5, SPIDR, TIRAP, TPO, WDR38, and ZNF81 might be used to distinguish LRM from other types of MDS. We traced back all collected specimens (25 HRM, 22 LRM, and 15 benign controls) to examine cfDNA using PCR and majorly calculated the specificity and sensitivity of mutations and methylated promoter (before transcription start site [TSS]) by receiver operating characteristic (ROC) curve. We determined that FANCM and ICAM1 were the potential biomarkers for the identification

**Figure 1.** Characterization of cfDNA variants and methylation in MDS. Total counts of SNP in tested samples of HRM (n = 3), LRM (n = 3), and control groups (n = 3) (A). The counts of SNPs on cfDNA that cause nonsynonymous variants (B). DMRs between HRM and LRM (C) as well as between LRM and control (D) by |meth.diff| > 25 and P < 0.05. The oncoplot shows 24 genes containing the specific deleterious missense mutations in HRM and 17 genes in LRM. "Multiple hits" means the multiple missense mutations (E). MDS: myelodysplastic syndromes; HRM: high-risk MDS; LRM: low-risk MDS; control: benign blood diseases; DMRs: differential methylated regions; multiple hits: multiple missense mutations; cfDNA: cell-free DNA; SNP: single-nucleotide polymorphism.
of MDS in our system. FANCM with T49G mutation at first exon and promoter hypermethylation (−835 to TSS) was indicated as the most confident factor with the highest area under curve (AUC) value (0.9271) for HRM. Similarly, ICAM1 with C1211T mutation at sixth exon and promoter hypermethylation (−282 to TSS) was suggested to identify LRM (AUC = 0.9338).

DNA methylation seemingly has little effect on the traditional methylated CG loci, while a remarkable distinction in CHG and CHH. Methylation on non-CpG context has extensively been reported in genomes of plant, microbes, reproductive stem cells, and mitochondrion\textsuperscript{10–12}, but the role has not been clearly connected to any biological function and arouses controversy in current scientific literature\textsuperscript{13}. Methylation at non-CpG sites may be a substitute for CpG methylation in some stress condition\textsuperscript{14}. The epigenetic mechanism behind the elevated methylation of CHG and CHH in HRM needs to be further addressed in future study.

In addition, this limitation of this study is the insufficient sample size that may cause us to overlook more informative signals. More specimens will be enrolled in this study in future to optimize the specificity and sensitivity for MDS identification based on our initial analysis.

In summary, our study characterized the variants and methylation pattern of cfDNA in MDS, and provided the potential biomarkers for HRM and LRM identification.

**Ethical Approval**

This study was approved by the Fudan University Ethics Committee (2016/6768/I), Shanghai, China.

**Statement of Human and Animal Rights**

All of the experimental procedures involving human specimens were conducted in accordance with biomedical research guidelines of the Declaration of Helsinki and approved by the Fudan University Ethics Committee, Shanghai, China.

**Statement of Informed Consent**

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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