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Variants of the Mitochondrial Displacement Loop in Patients with Myelodysplastic Syndromes

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OBJECTIVE Some mtDNA mutations have been detected in patients with myelodysplastic syndromes (MDSs). As the non-coding region of mitochondria, the displacement loop (D-loop) region of mtDNA contains important elements for mtDNA replication and transcription. Variants of the D-loop region were found to be related to the cause of many diseases. The aim of our study was to investigate mutations and single nucleotide polymorphisms in the D-loop region of MDS patients.
METHODS The mutations and SNPs in the hypervariable regions of the D-loop were detected by direct sequencing in MDS patients and normal controls.
RESULTS Sixty-four SNPs were found in the D-loop region in MDS cases and control group. Among the SNPs, the 16,189 variant (T > C transition) was found to have an increased frequency in the MDS group (P = 0.044). However, no mutations were detected in neither group.
CONCLUSION Our data provide evidence for a highly polymorphic D-loop region in patients with MDS, but do not support the presence of mutations in the mitochondrial D-loop region in MDS cases. The mtDNA T16,189C variant, which may be a functional variant, is associated with increased susceptibility to a MDS.

KEY WORDS: myelodysplastic syndrome, mitochondrial DNA, single nucleotide polymorphism, mutation.

Introduction

Mitochondrial DNA (mtDNA) is a 16,569 bp, closed circular, double-stranded DNA, which codes for small (12 s) and large (16 s) rRNA, 22 transfer RNAs, and 13 polypeptides of the mitochondrial respiratory chain. The displacement loop (D-loop), which contains 1,124 bp (positions 16,024-576), is a non-coding region of mitochondia, containing crucial elements for mtDNA replication and transcription, and acting as the promoter for both the heavy and light strands of mtDNA. It includes two hypervariable regions, HV1 at positions 16,024-16,383 and HV2 at positions 57-372[1].

As mtDNA lacks protective histones and efficient repair mechanisms, the mutation rate of mtDNA is approximately 10 times higher than that of the nuclear genome[2]. Recently, many mtDNA variations including substitutions, insertions and deletions have been identified in a broad spectrum of human diseases. Myelodysplastic syndromes (MDSs) are a heterogeneous group of acquired clonal hematologic disorders, characterized by ineffective hematopoiesis and an in-

creased risk of developing acute myelogenous leukemia. Cytogenetic abnormalities have been implicated in MDSs, just as in other malignant tumors. In MDSs, mitochondria often show ultrastructural abnormalities, including a bizarre-shape, iron deposits and swollen cristae[3,4]. mtDNA is also susceptible to mutagenesis in MDS patients, and mtDNA variants play an important role in MDS pathopoiesis[5,6].

The D-loop region is a control region for mtDNA with alterations in this region possibly modifying the essential replication and transcription process of mitochondria. In previous studies, the frequency of mtDNA variants was shown to be especially higher in the D-loop region. A great deal of mutations in this region were found in leukemia[7] and in many other kinds of tumors[8-11], while a single nucleotide polymorphism (SNP) in this region was found to be correlated with some diseases such as diabetes and cardiomyopathy[12,13]. It suggested that the variants in the D-loop region might be related to the pathopoiesis of these diseases. But few studies concerning variants in the mitochondrial D-loop region in MDSs have been published.

The 2 hypervariable regions (HV1 and HV2) are the more variable regions in the mtDNA D-loop. In the present study, we examined the mutations and SNPs in the hypervariable regions of the D-loop in MDS patients and normal controls, in order to explain the relationship between genetic variants in the D-loop region and the pathophysiology of MDSs.

Materials and Methods

Patients and healthy control subjects

Bone marrow specimens were obtained form 44 patients with a MDS (23 male, 21 female, mean age = 54 years), and peripheral blood specimens were obtained from 43 normal, sex- and age-matched controls (21 males, 22 females, mean age =52 years). The MDSs were diagnosed by peripheral blood and bone marrow findings according to the criteria of the WHO classification system [14], including 8 cases of refractory anemia (RA), 3 cases of refractory anemia with ringed sideroblasts (RARS), 9 cases of refractory cytopenia with multilineage dysplasia (RCMD), 12 cases of refractory anemia with excess blasts-1 (RAEB-1), and 12 cases of refractory anemia with excess blasts-2 (RAEB-2). The normal control subjects were healthy volunteers. Patients with a MDS and healthy controls are described in Table 1. This study followed ethical standards formulated in the Helsinki Declaration of 1975 (revised 1983). It was approved by the Ethics Committee of the Qilu Hospital, Shandong University, and informed consent was obtained from every individual.

Samples

Bone marrow and peripheral blood specimens (3~4 ml) were collected in tubes containing EDTA as an anticoagulant. Mononuclear cells were separated by density gradient centrifugation and washed twice in phosphate-buffered saline.

Table 1. Characteristics of MDS patients and healthy controls.

Characteristic	MDS (n = 44)	Healthy controls (n = 43)
Median age, years (range)	54 (32~74)	52 (30~71)
Sex, n (%)		
Male	23 (52.3%)	21 (48.8%)
Female	21 (47.7%)	22 (51.2%)
WHO classification		
RA	8 (18.2%)	-
RARS	3 (6.8%)	-
RCMD	9 (20.4%)	-
RAEB-1	12 (27.3%)	-
RABE-2	12 (27.3%)	-

MDS, myelodysplastic syndrome; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RABE-1, refractory anemia with excess blasts-1; RAEB-2, refractory anemia with excess blasts-2.

DNA Extraction

DNA was extracted from the mononuclear cells by standard proteinase K treatment followed by phenol/chlorophorm/isoamyl alcohol extraction. Extracted DNA was resuspended in TE buffer (pH 8.0) containing 10 mM Tris and 1mM EDTA.

PCR amplification of the hypervariable regions in the D-loop region

HV1 and HV2 regions were amplified using 2 pairs of primers respectively. Primers were as follows: HV1: F 15,972 5'-TAA CTC CAC CAT TAG CAC C-3', R 16,422 5'-ATT GAT TTC ACG GAG GAT G-3'; HV2: F 16,495 5'-CGA CAT CTG GTT CCT ACT TC-3', R 394 5'-GAA ATC TGG TTA GGC TGG TG-3'. 100 ng sample DNA was added to each polymerase chain reaction (PCR). The reaction mixture contained 0.2 μM of each primer, 0.1 μM of each deoxynucleotide triphosphate (dNTP), 1.0 U *Taq* polymerase (TaKaRa), 2.0 mM $MgCl_2$, and 5 μl 10 × buffer. The PCR reaction was performed in a DNA thermal cycler (Eppendorf) and conducted under the following cycling conditions: the initial denaturation of 50 μl reaction at 95ºC for 5 min, followed by 94ºC for 45 s, 56ºC for 45 s, and 72ºC for 45 s for 35 cycles, and a final extension step at 72ºC for 7 min. The PCR product was analyzed in an ethidium bromide-stained, 2.0% agarose gel to verify the amplification product and assess the purity.

DNA Sequencing Analysis

PCR products were purified using a Qiaquick gel extraction kit (Qiagen). Direct sequencing was carried out on an ABI 3730 automated DNA sequencer (Applied Biosystems) using the DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences). The results of DNA sequencing were compared with the revised human mitochondrial Cambridge reference sequence deposited in the mitochondrial genome database (http://www.mitomap.org/, GenBank accession no. AC_000021), using the BLAST2 Sequence program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). We then checked each of the DNA sequence differences with the reported polymorphisms in the mitochondrial genome database (http://www.mitomap.org/). Variants recorded in the database were categorized as reported SNPs. Among the detected SNPs in the MDSs, SNPs with high frequencies (with the frequency > 10 %) were selected. Variants not recorded in the database were categorized as mutations, and, if simultaneously found in both patients and healthy controls, categorized as novel mtDNA polymorphisms.

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Science program (SPSS for Windows, version 11.5). The chi-square test was used to compare the frequencies of selected SNPs (with the frequency > 10 %) between the patients and healthy controls. Logistic regression analysis was used to analyze the association of the variant with the MDSs. $P < 0.05$ was considered statistically significant.

Results

MtDNA sequence variants in HV1 and HV2 of the D-loop region

The 451 bp PCR products for HV1 and 461 bp PCR products for HV2 were amplified followed by direct sequencing. A total of 64 variants were found in MDSs and healthy controls. There were 39 mtDNA sequencing variants in HV1 (Fig.1A), and 12 in HV2 (Fig.2A) detected among 44 patients with a MDS. Among 43 healthy control subjects, 36 variants in HV1 (Fig.1B) and 12 in HV2 (Fig.2B) were noted. We checked each variant with the reported polymorphisms in the mitochondrial genome database (http://www.mitomap.org/). All of the variants were single nucleotide polymorphisms reported in the database. Neither new polymorphisms nor mutations were found in the HV1 or HV2 regions among patients or healthy controls.

SNPs in the D-loop region

Among the detected SNPs, there were 16 SNPs with a frequency > 10% (Table 2). The variant at bp 16,189 (T > C transition) in HV1 was present in 18 patients with a MDS (40.9%) compared with 9 healthy controls (20.9%). The difference in the frequency of occurrence of this variant between the MDS group and control group was statistically significant ($P = 0.044$). The odds ratio (OR) for the association between the 16,189 variant and the MDS was 2.615 (95% confidence interval, 1.012~6.757, $P = 0.047$). No relation between a subtype of MDS and a 16,189 variant could be demonstrated

Table 2. Frequency of D-loop single nucleotide polymorphisms in MDS patients and healthy controls.

Nucleotide position	Nucleotide variant	Hypervariable region	MDS ($n = 44$)	Healthy controls ($n = 43$)
16129	G→A	HV1	13 (29.5%)	8 (18.6%)
16172	T→C	HV1	6(13.6%)	9 (20.9%)
16183	A→C	HV1	11 (25.0%)	6 (14.0%)
16189	T→C	HV1	18 (40.9%)*	9 (20.9 %)*
16223	C→T	HV1	32 (72.7%)	36 (83.7 %)
16298	T→C	HV1	6 (13.6%)	5 (11.6%)
16304	T→C	HV1	7 (15.9%)	4 (9.3%)
16319	G→A	HV1	8 (18.2%)	6 (14.0%)
16362	T→C	HV1	15 (34.1%)	18 (41.9%)
73	A→G	HV2	44 (100%)	43 (100%)
146	T→C	HV2	8 (18.2%)	5 (11.6%)
150	C→T	HV2	10 (22.7%)	9 (20.9%)
152	T→C	HV2	5 (11.4%)	7 (16.3%)
263	A→G	HV2	44 (100%)	43 (100%)
303	T insertion	HV2	31 (70.5%)	27 (62.8%)
315	T insertion	HV2	38 (86.4%)	36(83.7%)

MDS, myelodysplastic syndrome; *, $P < 0.05$

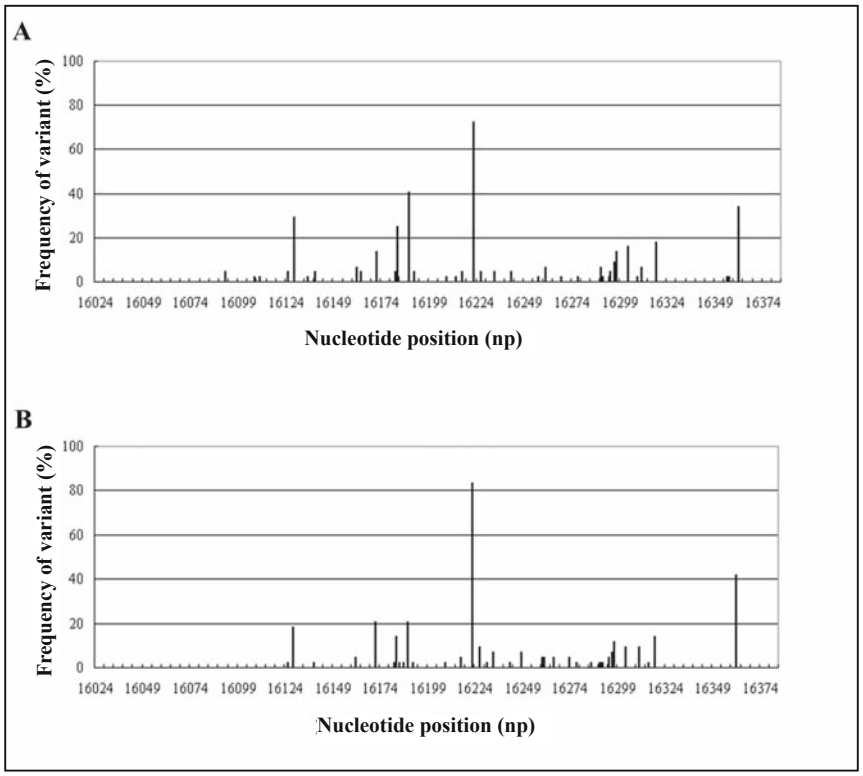

Fig.1. The frequency of mtDNA D-loop variants in the hypervariable region 1. X axis, nucleotide position in mtDNA. (A) The frequency of variants in the MDS group. (B) The frequency of variants in the healthy group.

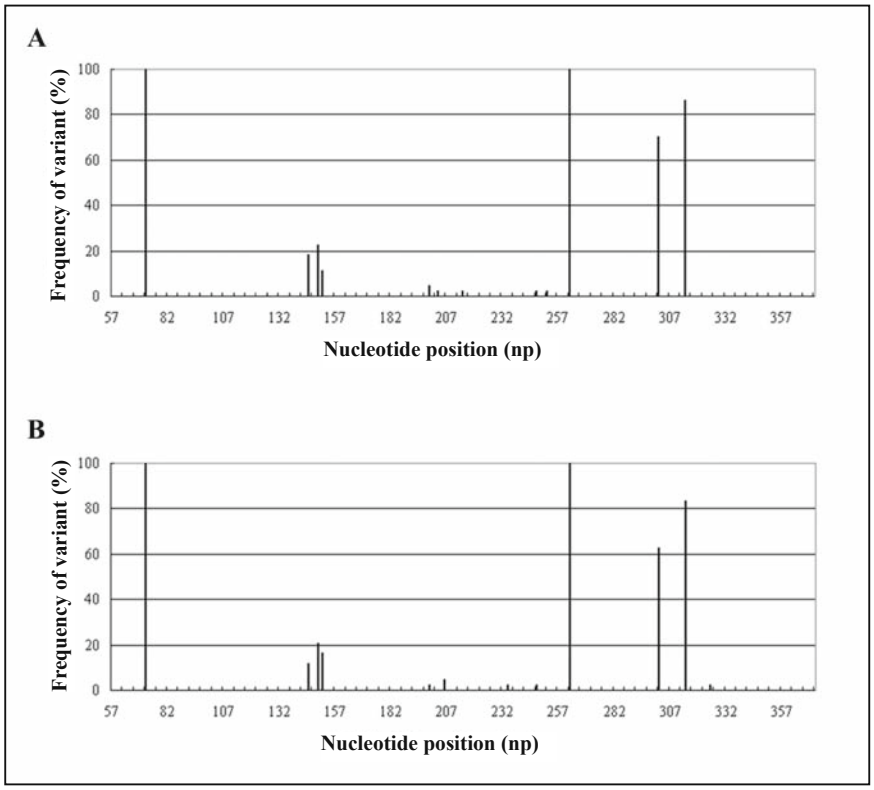

Fig.2. The frequency of mtDNA D-loop variants in the hypervariable region 2. X axis, nucleotide position in mtDNA. (A) The frequency of variants in the MDS group. (B) The frequency of variants in the healthy group.

(not shown). The 16,189 variant generated an uninterrupted homopolymeric C-stretch, which caused a heteroplasmic length variation in the mtDNA (Fig.3B). The length of the C-stretch was between 9 and 12 poly cytosine. In one healthy control, the C-stretch was not detected for another C to T transition occurring simultaneously in this region (Fig.3C). Other SNPs with higher frequencies did not reach significant differences between the 2 groups.

Discussion

Recently, some mtDNA mutations have been reported in MDSs [5,6]. As the promoter for both the heavy and light strands of mtDNA, the D-loop region is highly polymorphic and subject to mutations, especially in the 2 hypervariable regions, the so-called hot spots[15]. In our study, we directly sequenced the HV1 and HV2 regions to compare the variants in the D-loop region in MDSs with those in sex- and age-matched healthy volunteers. However, neither group displayed mutations.

Our results concerning D-loop mutations in this study differed from the investigations of other cancers. Many mutations in the D-loop region have been reported in gastric[8], hepatocellular[9], breast[10], and uterine[11] cancers, as well as other hematologic malignancies. By sequencing, Grist et al.[7] detected mutations in the D-loop in 8 of 22 patients (36%) with AML, and in 15 of 26 patients (58%) with ALL. This implies that the mutations in the D-loop region in these cancers might play an important role in tumorigenesis. But interestingly, our study failed to detect mutations in the D-loop region of MDS patients, a malignant disease of the hematologic system. It suggests that mutations have no definite role in the D-loop region of MDSs.

At present, the Cambridge Reference Sequence is thought to be the standard for mtDNA sequences. A number of polymorphisms can be found to distribute in the whole mitochondrial genome, especially in the D-loop region. These data are deposited in the mitochondrial genome database (http://www.mitomap.org/). The polymorphisms vary among different races. In our study, the detected variants reflected a highly polymorphic area in the D-loop region of MDS cases. Although no mutation was detected, the frequency of SNP (T16,189C variant) increased in the MDS group (P = 0.044). Other detected SNPs just showed higher frequencies in the MDS group and healthy group simultaneously, suggesting that there are increased frequencies in the population studied in China, but not reflecting a statistical relationship with MDS.

The 16,189 variant has been shown to be related to type 2 diabetes and cardiomyopathy[12,13]. Our study also suggests that there is a relationship between this variant and MDSs (odds ratio 2.615, 95% CI 1.012~6.757, P = 0.047). The 16,189 variant is a T to C transition at bp 16,189 in the HV1 of the mitochondrial genome. It generates an uninterrupted homopolymeric C-tract. The poly C causes a heteroplasmic length variation of the D-loop, presumably as a result of DNA polymerase slipping during mtDNA replication. The variation in length perhaps combines with some other defects in the fidelity of the mtDNA replication machinery to influence mtDNA replication and transcription. In our study, the number of

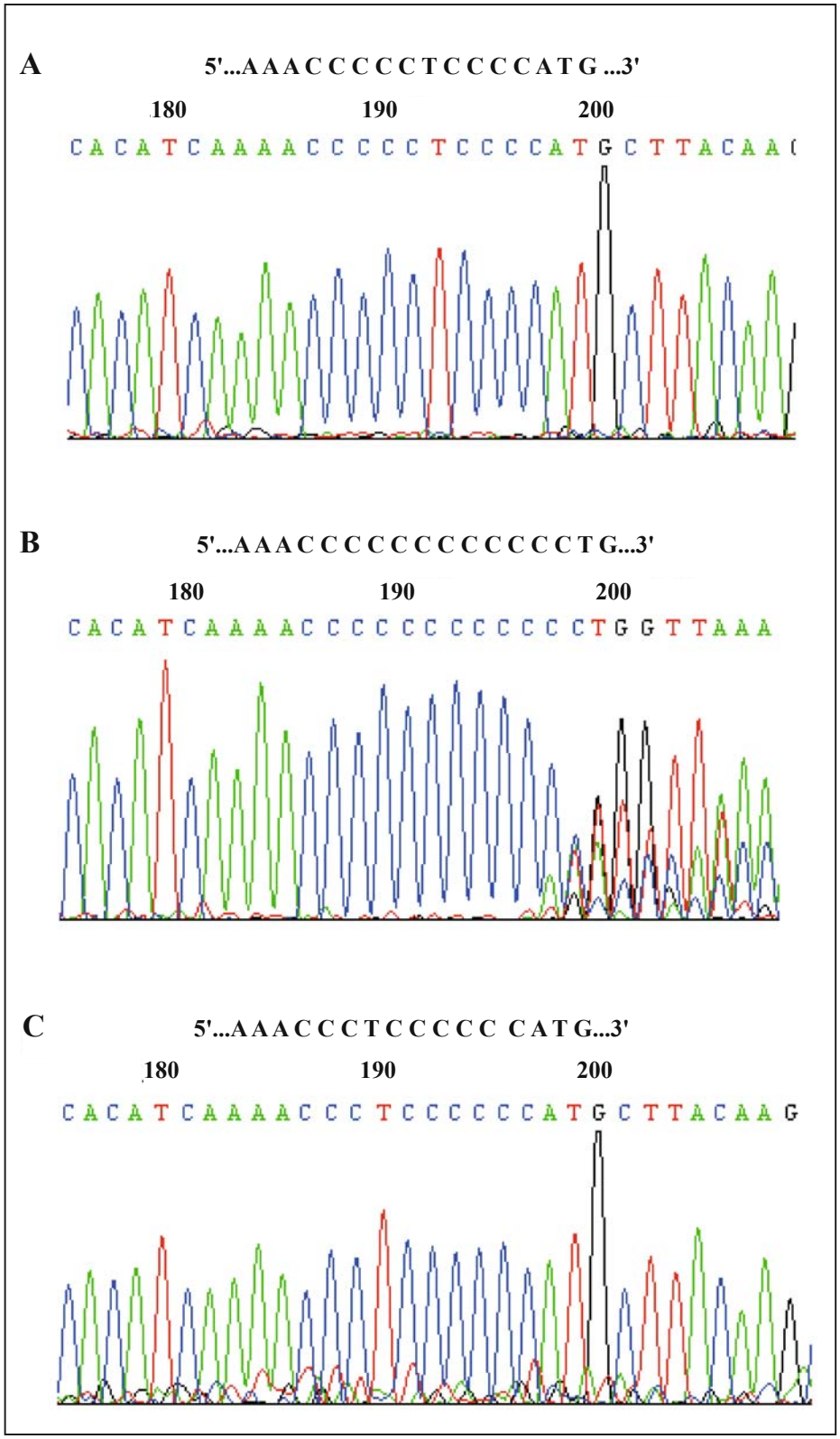

Fig.3. Sequence of the T16,189C mtDNA variant. (A) Wild type. (B) The T16,189C variant caused an uninterrupted homopolymeric C-stretch and heteroplasmic length variation of mtDNA. (C) T16,189C variant and another C > T transition simultaneously in this region did not cause an uninterrupted homopolymeric C-stretch.

poly Cs ranged form 9 to 12, and the poly Cs caused an unreadable sequence beyond the C-stretch. But it did not occur if anther C to T transition occurred in this region. In one healthy control, a C to T transition was seen at bp 16,187 simultaneously, which stopped the C-stretch and thus did not cause the heteroplasmic length variation. Because the 16,189 variant lies within the control region for mtDNA, the presence of the 16,189 variant might influence either interactions of binding proteins or interactions between binding proteins and mtDNA, which are important for mtDNA replication.

Mitochondria play a fundamental role in ATP synthesis by oxidative phosphorylation. They also have important contributions to cellular homeostasis, heme synthesis and initiation of cellular apoptosis. In previous studies of mtDNA in MDSs, mutations were detected in cytochrome b, ATPase 8, the cytochrome c-oxidase gene and so on[5,6]. The 16,189 variant might combine with other mutations of mtDNA to influence the level of oxidative phosphorylation and other important functions in mitochondria that may contribute in part to the pathophysiology of MDSs.

In conclusion, our study showed 64 variants in HV1 and HV2 of the mtDNA D-loop region in MDSs and healthy controls, but no mutations were detected. Our results, however, display an association between MDSs and a common polymorphism, T16,189C, suggesting that it might be a functional variant.

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