Neurobiology of Aging 75 (2019) 225.e1–225.e8

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Polymorphisms in DNA methylation–related genes are linked to the phenotype of Machado-Joseph disease

Dongxue Ding [a], Chunrong Wang [a], Zhao Chen [a], Huirong Peng [a], Kai Li [a], Xin Zhou [a], Yun Peng [a], Puzhi Wang [a], Xiaocan Hou [a], Tianjiao Li [a], Rong Qiu [b], Kun Xia [c], Jorge Sequeiros [d], Tetsuo Ashizawa [e], Beisha Tang [a,c,f,g], Hong Jiang [a,c,f,h,*]

[a] Department of Neurology, Xiangya Hospital, Central South University, Changsha, Hunan, P.R. China
[b] School of Information Science and Engineering, Central South University, Changsha, P.R. China
[c] State Key Laboratory of Medical Genetics, Central South University, Changsha, Hunan, P.R. China
[d] IBMC - Institute for Molecular and Cell Biology, i3S - Instituto de Investigacao e Inovacao na Saude, ICBAS, Univ. Porto, Porto, Portugal
[e] Department of Neurology, Houston Methodist Neurological Institute & Houston Methodist Research Institute, Houston, TX, USA
[f] Key Laboratory of Hunan Province in Neurodegenerative Disorders, Central South University, Changsha, Hunan, P.R. China
[g] National Clinical Research Center for Geriatric Diseases, Changsha, Hunan, P. R. China
[h] Xinjiang Medical University, Xinjiang, P.R. China

ARTICLE INFO

Article history:
Received 26 April 2018
Received in revised form 6 September 2018
Accepted 8 November 2018
Available online 16 November 2018

Keywords:
MJD
SCA3
ATXN3
DNA methylation
Age at onset
Epigenetic regulation

ABSTRACT

DNA methylation has been reported as an important regulator of genomic structure stability, including large tandem repeats. To test the modulation effect of variants in DNA methylation–related genes on distribution of expanded $(CAG)_n$ alleles and age at onset (AO) of patients with Machado-Joseph disease (MJD), we conducted an association analysis on 23 selected SNPs in these genes in 613 patients with MJD and 581 controls. There were significant differences in the distribution of rs12957023 between patients and controls (OR = 1.296, $p = 0.007$ and OR = 1.206, $p = 0.008$, for genotype and alleles, respectively). The distribution of $(CAG)_n$ size was also different between patients carrying a CC and the other genotypes (TT and TC, $p = 0.011$ for expanded $(CAG)_n$ and $p = 0.012$ for normal size alleles), indicating that DNA methylation might modulate the $(CAG)_n$ instability. We found also that rs13420827 in *DNMT3A* and rs7354779 in *DNMT3L* contribute to AO of MJD ($p = 0.019$ and $p = 0.008$, respectively). In conclusion, our data provide the first evidence that SNPs in DNA methylation–related genes may contribute to $(CAG)_n$ instability and modulate the AO of this disease.

© 2018 Elsevier Inc. All rights reserved.

1. Introduction

As an autosomal dominantly inherited neurodegenerative disease, Machado-Joseph disease/spinocerebellar ataxia type 3 (MJD/SCA3) is caused by the abnormal expansion of a CAG repeat within the coding region of *ATXN3*, leading to an expanded polyglutamine stretch in the ATXN3 protein (Kawaguchi et al., 1994). The individuals with MJD usually carry CAG repeats longer than 56, and are clinically characterized by progressive ataxia, dystonia, dysarthria, dysphagia, peripheral signs, and oculomotor anomalies (Paulson, 2007, 2012). Average survival period after disease onset ranges 20–25 years (Klockgether et al., 1998). Unfortunately, so far, there are no therapy strategies available to prevent or stop its progression.

Serving as an important individual variation criterion of the MJD, age at onset (AO) is inversely correlated with the size of expanded CAG repeats. Modifier effects on the CAG repeats in other polyQ-related genes may contribute to the AO variation in different populations (Tezenas et al., 2014). Similarly, our team found as well that the long normal *ATXN2* allele and rs7969300 in *ATXN2* were linked to the earlier AO of patients with MJD (Chen et al., 2016a,b; Ding et al., 2016). The reported modifiers could only partially explain the AO variability (Bettencourt et al., 2011; Chen et al., 2016a,b); therefore other genetical and epigenetical factors might exist to modulate the unknown portion of AO variation.

Although the single causal gene *ATXN3* mutation alone genetically leads to the MJD, a wide variety of aberrant cell metabolic pathways mediated by the initial *ATXN3* mutation render the pathogenesis very complicated consequently (Goncalves et al.,

* Corresponding author at: Hong Jiang Department of Neurology, Xiangya Hospital, 87 of Xiangya Road, Changsha, Hunan Province, China. Tel.: +86-731-84327216; Fax: (86)731-84327332.
E-mail address: jianghong73868@126.com (H. Jiang).

0197-4580/$ – see front matter © 2018 Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.neurobiolaging.2018.11.002

2013; Koch et al., 2011; Nascimento-Ferreira et al., 2013; Simoes et al., 2012, 2014). Among the aberrant cell processes, altered transcription might serve as the pilot event although the exact pathways that contribute to gene expression alteration remain elusive (Chatterjee et al., 2015; Chou et al., 2008; Gao et al., 2015). It has been believed that epigenetic modification, particularly DNA methylation, may exert profound effects on regulation and orchestration of multiple gene expression in certain neurodegenerative diseases (Li et al., 2016; Lu et al., 2013). Polymorphisms in DNA methylation–related genes have been linked with Alzheimer's disease (Chouliaras et al., 2015), Parkinson's disease (Liao et al., 2013), and schizophrenia (Murphy et al., 2013). Recent evidences support the link between aberrant global or localized DNA methylation patterns and the trinucleotide repeat diseases, such as the fragile X syndrome (Godler et al., 2010a,b), Friedreich ataxia (Al-Mahdawi et al., 2008), SCA2 (Laffita-Mesa et al., 2012), and Huntington disease (Ng et al., 2013). Indeed, DNA methylation in the promoter region of the *ATXN3* has been proposed to contribute to the AO (Emmel et al., 2011; Wang et al., 2017) and CAG instability in MJD (Wang et al., 2017). However, there is little information available regarding the possible association between DNA polymorphisms in methylation-related genes and the AO, as well as the distribution of CAG repeats in MJD.

Here, we collected a large cohort of patients with MJD from Chinese population and tested if SNPs in DNA methylation–related genes (*DNMT1*, *DNMT2*, *DNMT3A*, *DNMT3B*, *DNMT3L*, *MBD1*, *MBD2*, *MeCP2*, *MTHFR*, and *TET1*) are associated with variation in AO and the distribution of the expanded $(CAG)_n$ alleles in MJD.

2. Materials and methods

2.1. Clinical sample collection

All patients in this study were consecutively recruited from the Department of Neurology, Xiangya Hospital and the State Key Laboratory of Medical Genetics, Central South University, from 2000 to 2017. All controls were collected from the same region of patients by the Medical Examination Centre of Xiangya Hospital, and all control individuals were independent from each other and have no relation with all MJD patients. Written informed consent was obtained from all subjects, after they read a document with detailed information about the nature and possible consequences of the study. This study was approved by the Ethics Committee of Xiangya Hospital, Central South University.

Totally, 613 patients from 499 families with both clinical and molecular diagnosis of MJDand 581 healthy controls were enrolled in this study. All the enrolled patients had detailed clinical data. AO was defined as the age at which the first ataxia symptom was reported by the patients and confirmed by their family members. About 298 patients and 281 controls from Hunan province were selected for preliminary exploration. The remaining 315 patients and 300 controls were used to verify the potential significance found in the preliminary analysis for allelic and genotypic distribution of SNPs between the patient and control groups, as well as for the distribution of $(CAG)_n$ alleles and AO among different genotypes in patients with MJD.

2.2. Isolation of genomic DNA and size determination of the CAG repeats

Genomic DNA was extracted from peripheral blood leukocytes via standard phenol-chloroform extraction methods. Size of the CAG repeat in *ATXN3* was determined in all the 613 patients by capillary electrophoresis and DNA sequencing with T-vector cloning.

2.3. Selection of SNPs and genetic analysis

SNPs were selected on the basis of their functional significance with minor allele frequency >0.01 in Chinese population by using the genome browsers of the 1000 Genomes (http://www.internationalgenome.org/home), UCSC (http://genome.ucsc.edu), and dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). All the selected SNPs in 298 patients and 281 controls were genotyped by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry via the MassARRAY system (Sequenom). To validate this method, 30 patients and 30 controls were randomly selected for Sanger sequencing. In the remaining 315 patients and 300 controls, the potentially significant SNPs found in the preliminary analysis including rs12957023, rs13420827, and rs7354779, were genotyped by Sanger sequencing. All primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/).

2.4. Statistical analysis

Pairwise linkage disequilibrium (LD) among the selected SNPs located on same chromosomes was computed for both D′ and R^2 values by using the SHEsis software. The functional effect of each SNP was identified using F-SNP database (http://compbio.cs.queensu.ca/F-SNP/). Allele and genotype frequencies of all the selected SNPs were analyzed for significance of the differences between cases and controls using a binary logistic regression. Odds ratio (OR) and 95% confidence intervals (CIs) were used as a measure of the relationship strength in the genotype and allelic frequencies between patients and controls. To further test the association between rs12957023 and the distribution of CAG units, binary logistic regressions were performed for differences in the $(CAG)_n$ distribution among the genotypes in all the 613 patients with MJD.

Because of the heterogeneity of the AO variance, a log transformation was conducted on the AO in second round of analysis. A linear regression was performed for ln(AO) on the length of expanded repeats. The constant and the partial regression coefficient in the analysis were calculated. These parameters were used to construct an expected value of AO for each individual based on their CAG repeat length followed by the residual estimation through subtracting the actual and expected AO. The extent of the AO variability explained by each SNP was tested with a linear regression of these residuals on the number of minor alleles of the SNP. Overall association of AO across the two significant SNPs (rs13420827 and rs7354779) was tested by combining the *p* values of each association using Brown's method. To visualize the effect of rs13420827 and rs7354779 on the AO, a linear regression was performed by defining ln(AO) as the dependent variable and these two SNPs as independent variables after having controlled the effect of expanded $(CAG)_n$. As mentioned previously, long normal alleles in ATXN2 might be a significant genetic modifier for MJD. Therefore, its effect on the AO of patients with MJD has also been analyzed using linear regression to exclude the possibility of false positive. A hypothesis test was conducted for regression coefficients to explore interaction between expanded $(CAG)_n$ and each genotype before the differences in AO according to genotype of each SNP were tested by covariance analysis.

All analyses were performed using SPSS v.18.0 (SPSS Inc, Chicago, IL) with nominal significance assigned if it meets $p \leq 0.05$.

3. Results

Characteristics of the 298 patients with MJD and 281 healthy controls were summarized in Supplementary Table 1. Among the 298 patients with MJD (Supplementary Table 1) with mean age

42.33 ± 11.28 years and range 16–74, the ratio of female to male was almost 1:1 (151: 147). Similarly, the healthy controls were matched for gender, ethnicity, and area of residence, and had a mean age of 41.50 ± 18.83 years with a wide range of 14–81 as well. There were neither significant differences in the gender composition ($\chi^2 = 0.006$, $p = 0.936$) nor the current age (F = 0.419, $p = 0.518$) between patients and controls. No significant differences in age were found between genders within each group with $p = 0.506$ in patients and $p = 0.614$ in controls. All the selected SNPs for 298 patients with MJD and 281 controls were successfully genotyped. Distribution of genotypes in all SNPs was in agreement with the Hardy-Weinberg equilibrium in all groups ($p > 0.05$).

3.1. LD analysis in the selected SNPs

To further analyze the nonrandom associations between DNA methylation–related polymorphisms and the MJD phenotype, the LD of the selected SNPs was statistically determined as shown in Supplementary Table 2 and Fig. 1, respectively. Clearly, strong LD ($r^2 > 0.8$) was observed in rs2228611 and rs759920 in *DNMT1*; rs2424932, rs998382, and rs1569686 in *DNMT3B*; rs2075596 and rs2239464 in *MeCP2*, in contrast to the SNPs with weak LD in other genes. More specifically, the rs2228611 was located in the coding region, whereas the rs759920 in the intron of the *DNMT1*, and from F-SNP database, their FS scores are 0.314 and 0.208, respectively. Thus, the SNP rs2228611 is predicted to confer alteration of the splicing regulation. By contrast, the rs2424932 was a 3′UTR variant, whereas the rs998382 and the rs1569686 were intronic ones of the *DNMT3B* gene, and no information was given in the F-SNP database yet for this gene. Similarly, both rs2075596 and rs2239464 were located in the intronic region of *MeCP2* gene. In addition, because the FS score is 0.176 for the rs2075596 and 0 for the rs2239464, the transcriptional regulation was predictably altered in the rs2075596 but no change for the rs2239464. Given their locations and predicted functional effects, rs759920, rs998382, rs1569686, and rs2239464 were not included in subsequent analysis.

3.2. Association of SNPs in DNA methylation–related genes with distribution of expanded CAGs

To further explore the association of the SNPs in DNA methylation–related genes with distribution of the expanded $(CAG)_n$, genotype, and allele frequencies, OR and 95% CI for the 18 SNPs mentioned previously were calculated as shown in Table 1. The lowest p value was observed for the rs12957023 (*MBD1*) for the patients and controls ($p_{allelic} = 0.024$ and $p_{genotype} = 0.030$). The power of the allelic testing was 0.706 (Power and Sample Size Calculation program, http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize, with the level of significance of 0.05 and OR of 1.394). After a Bonferroni correction for the 19 of SNPs, the statistical significance for this logistic analysis was $p = 0.0026$. Thus, we expanded the sample scale by adding 315 patients and 300 controls for the subsequent analysis of the rs12957023. With increase of sample scale, after a Bonferroni correction for the two SNPs, even more significant difference was observed in genotype of rs12957023 in *MBD1* between patients and controls ($p_{allelic} = 0.008$ and $p_{genotype} = 0.007$) and the power of allelic testing increased to 0.826 (with the level of significance of 0.05 and OR of 1.287) in this cohort. The distribution of rs12957023 genotype was 312/256/45 in patients with MJD versus 338/214/29 in the controls. Accordingly,

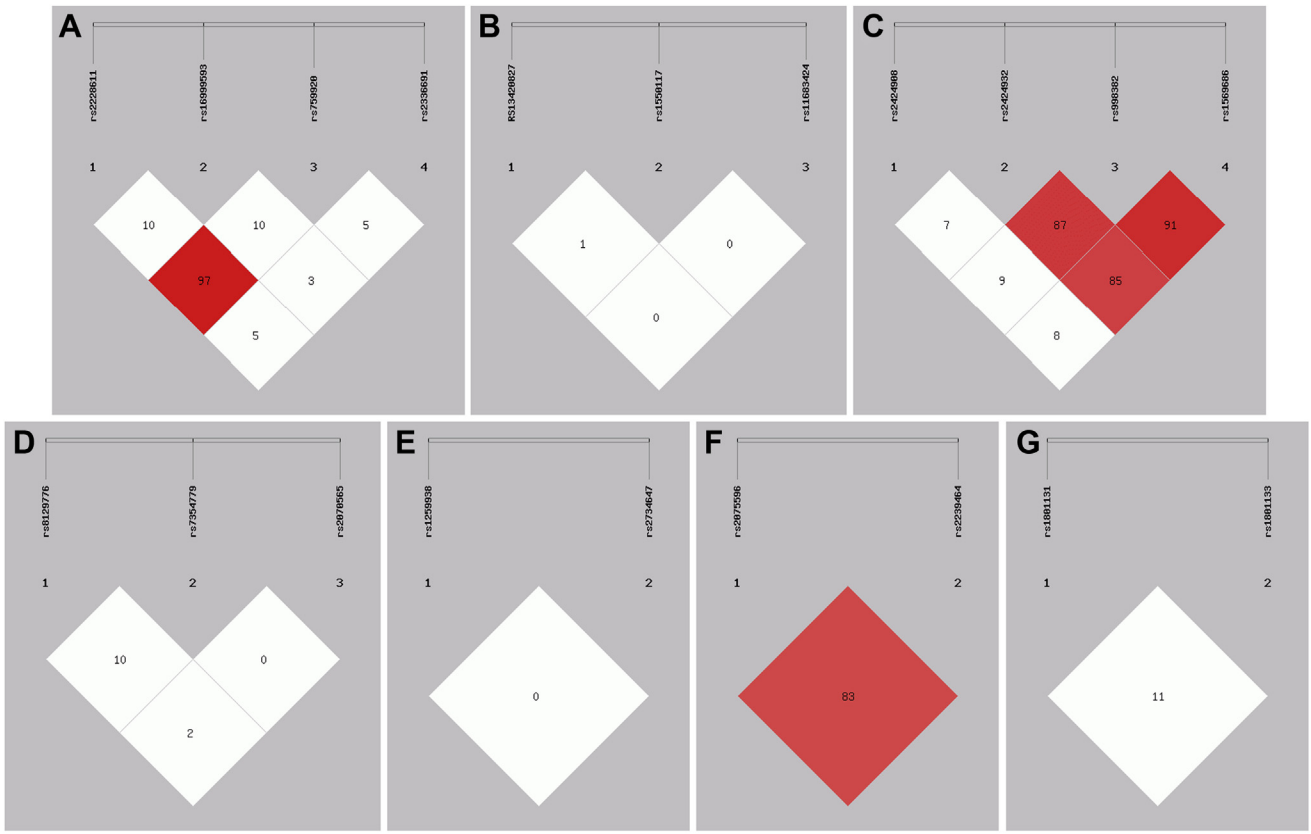

Fig. 1. Linkage disequilibrium structure of *DNMT1*(A), *DNMT3A*(B), *DNMT3B*(C), *DNMT3L*(D), *MBD2*(E), *MeCP2*(F), and *MTHFR*(G) SNPs results from the preliminary analysis cohort. Values are shown as R^2.

Table 1
Association of polymorphisms in DNA methylation–related genes and MJD

Gene	SNPs	Location	MAF (MAF in Chinese)	Minor allele	Genotype cases[a]	Genotype controls[a]	$p_{allelic}$	$p_{genotype}$	$OR_{allelic}$ (95% CI)	$OR_{genotype}$ (95 % CI)	FS score
DNMT1	rs2228611	Coding region	0.47 (0.29)	T	150/124/24	140/114/27	0.810	0.804	0.828 (0.178–3.845)	0.823 (0.177–3.825)	0.314
	rs16999593	Coding region	0.05 (0.20)	C	195/89/14	176/92/13	0.216	0.294	0.817 (0.593–1.125)	0.841 (0.608–1.163)	0.575
	rs2336691	Intron	0.02 (0.11)	A	233/60/5	221/55/5	0.963	0.989	0.991 (0.672–1.460)	0.997 (0.678–1.468)	0.208
	rs759920	Intron	0.46 (0.32)	A	149/125/24	139/116/26	0.918	0.875	1.085 (0.233–5.043)	1.132 (0.243–5.272)	0.208
DNMT2	rs11254413	Coding region	0.38 (0.25)	A	166/111/20	154/114/13	0.835	0.670	1.032 (0.767–1.389)	1.063 (0.802–1.410)	0.237
DNMT3A	rs13420827	3′UTR	0.21 (0.23)	G	192/87/19	180/89/12	0.780	0.746	1.046 (0.765–1.430)	1.049 (0.786–1.401)	0.065
	rs1550117	5'upstream	0.11 (0.25)	A	178/105/15	180/92/9	0.121	0.102	1.287 (0.935–1.772)	1.290 (0.951–1.750)	0.109
	rs11683424	Intron	0.10 (0.09)	T	277/19/2	265/13/3	0.637	0.576	1.168 (0.613–2.224)	1.184 (0.656–2.136)	0.242
DNMT3B	rs2424908	Intron	0.64 (0.46)	C	97/151/50	95/138/48	0.934	0.722	1.012 (0.769–1.332)	1.048 (0.809–1.359)	unknown
	rs2424932	3′UTR	0.20 (0.04)	A	267/31/0	251/29/1	0.333	0.302	2.223 (0.441–11.203)	2.354 (0.464–11.950)	unknown
	rs998382	Intron	0.36 (0.04)	A	264/34/0	246/34/1	0.927	0.974	0.917 (0.141–5.957)	0.969 (0.145–6.497)	unknown
	rs1569686	Intron	0.28 (0.04)	G	263/35/0	244/36/1	0.345	0.285	0.461 (0.093–2.301)	0.411 (0.081–2.097)	unknown
DNMT3L	rs8129776	Intron	0.55 (0.30)	A	142/129/27	138/116/27	0.681	0.431	1.063 (0.794–1.425)	1.119 (0.846–1.478)	0.5
	rs7354779	Coding region	0.17 (0.05)	C	277/21/0	249/32/0	0.055	0.054	0.548 (0.297–1.013)	0.539 (0.287–1.011)	0.269
	rs2070565	Splice region	0.21 (0.15)	T	210/79/8	193/82/6	0.748	0.993	0.946 (0.673–1.330)	1.001 (0.719–1.395)	1
MBD1	rs12957023	Intron	0.35 (0.27)	C	150/118/30	163/102/16	0.024	0.030	1.394 (1.045–1.859)	1.347 (1.030–1.762)	0.208
MBD2	rs1259938	3′UTR	0.18 (0.03)	T	283/15/0	262/18/1	0.175	0.193	0.610 (0.299–1.246)	0.623 (0.305–1.271)	unknown
MeCP2	rs2734647	3′UTR	0.63 (0.29)	C	204/52/42	185/57/39	0.107	0.183	0.566 (0.297–1.013)	0.687 (0.394–1.195)	0.050
	rs2239464	Intron	0.45 (0.18)	G	213/46/39	201/45/35	0.955	0.996	1.021 (0.487–2.144)	1.001 (0.546–1.837)	0
	rs2075596	Intron	0.37 (0.81)	A	46/45/207	36/52/193	0.244	0.337	1.595 (0.728–3.498)	1.354 (0.729–2.514)	0.176
MTHFR	rs1801133	Coding region	0.25 (0.28)	A	121/132/45	130/121/30	0.166	0.213	1.211 (0.924–1.587)	1.181 (0.909–1.534)	0.493
	rs1801131	Coding region	0.25 (0.33)	G	204/87/7	180/91/10	0.268	0.570	0.834 (0.605–1.150)	0.909 (0.655–1.262)	0.533
TET1	rs150689919	Coding region	0.01 (0.03)	T	276/22/0	259/22/0	0.870	0.910	0.948 (0.502–1.791)	0.963 (0.505–1.837)	unknown

Key: MAF, minimum allele frequency in Southern China.
p^* p values after the Bonferroni correction.
[a] The order of genotypes: DD/Dd/dd (d is the minor allele).

frequency of the minor allele C was significantly higher in the patients with MJD than in the controls.

For the total 613 MJD patients, significant differences were detected in the normal $(CAG)_n$ distribution ($p = 0.012$, 95% CI: 1.272 (1.053–1.536)) and in the expanded $(CAG)_n$ distribution ($p = 0.011$, 95% CI: 1.244 [1.050–1.473]) between patients carrying genotype CC and CT+TT. As more detailed in Fig. 2, the $(CAG)_n$ distribution in *ATXN3* among different genotypes indicated that the CC genotype in rs12957023 may affect the distribution of CAG allele in MJD.

3.3. Association of the SNPs in DNA methylation–related genes with AO in MJD

To assess the potential association of individual SNPs on residual AO, statistic study was conducted. As shown in Table 2, p value for rs13420827 in *DNMT3A* and rs7354779 in *DNMT3L* was 0.048 and 0.073, respectively, indicating these two SNPs may tend to be associated with AO. In the expanded samples, both rs13420827 and rs7354779 remain significant after a Bonferroni correction for 2 tests ($p = 0.019$ and $p = 0.008$, respectively). Overall, both SNPs are significantly associated with AO ($p = 0.000152$) based on Brown's method.

In the expanded sample as shown in Fig. 3 and Table 3, the value of R^2 suggested that the size of the expanded $(CAG)_n$ alone account for 60.3% of the AO variation. To study the effect of normal *ATXN3* alleles, *ATXN2* alleles, and the two SNPs on the AO, various combinations were made to exclude the possibility of false positive. As shown in Table 3, inclusion of normal *ATXN3* alleles and *ATXN2* alleles did not raise the value of R^2 significantly. However, after inclusion of rs13420827 and rs7354779 to the model, R^2 value was increased to 0.613 ($p = 0.035$ and $p = 0.003$, respectively), indicating that the two SNPs were responsible for approximately 1% of the variability in AO.

Before the covariance analysis, no significant interaction was observed between genotypes and expanded $(CAG)_n$ for the rs13420827 and the rs7354779. Further analysis of rs13420827 showed that the patients with GG genotype (38.00 ± 10.39) performed a later AO approximately +3.5 years as compared with the other genotype (34.68 ± 10.16; $F = 3.065$, $p = 0.047$). Similarly, for the rs7354779 after adjusting for size of the repeat, AO of patients with CT genotype (38.43 ± 8.76) was approximately 4-year later than those with TT genotype (34.54 ± 10.24; $F = 9.557$, $p = 0.002$). Given that only three patients carry both GG and CT genotypes, it is unnecessary statistically to perform the further analysis.

4. Discussion

Initial discoveries support that DNA methylation mainly refers to the addition of a methyl group to the 5′ position of a cytosine in a CpG dinucleotide (Bheemanaik et al., 2006; Du et al., 2015), and to the 6'position of adenosine (unpublished data). Several protein families function as modulation of the methylation pattern, including DNA methyltransferase (DNMTs) (Bestor et al., 1988; Okano et al., 1998), methyl-CpG-binding domain (MBD) proteins (Bogdanovic and Veenstra, 2009; Filion et al., 2006; Hendrich and Bird, 1998), and ten-eleven translocation (TET) protein family (Pastor et al., 2013; Smith and Meissner, 2013). In our present study, 23 SNPs detected in the genes encoding all the three protein families were linked with differences in the distribution of $(CAG)_n$ alleles or AO variation in MJD.

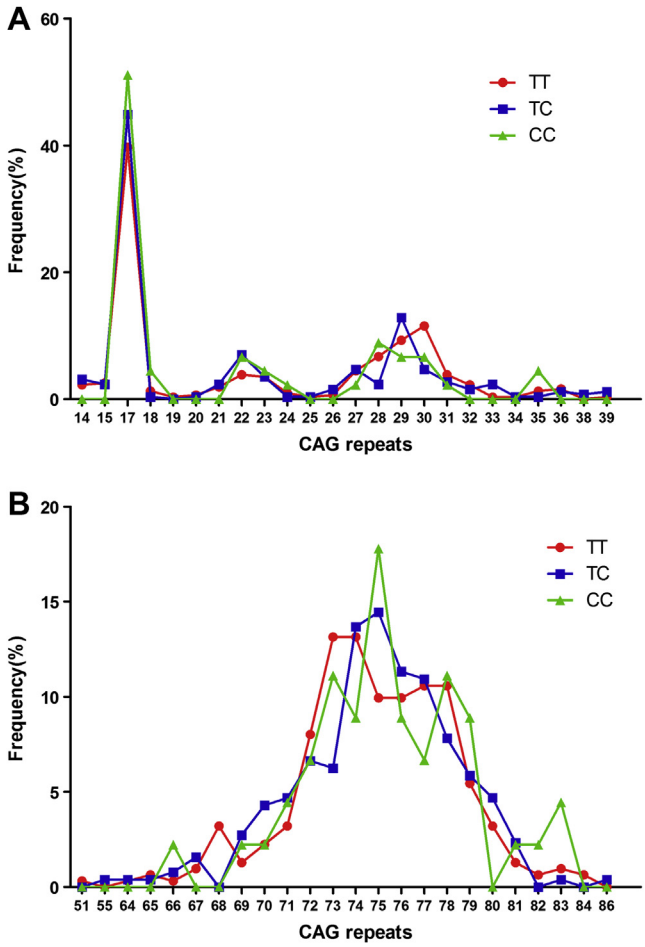

Fig. 2. CAG allele distribution in *ATXN3*, in a total of 613 patients with MJD among different genotypes of rs12957023 in *MBD1*. (A) Distribution of normal $(CAG)_n$ alleles: CC versus TC genotype ($p = 0.044$, OR = 1.218, 95% CI: 1.005–1.476); CC versus TT genotype ($p = 0.028$, OR = 1.220, 95% CI: 1.022–1.457); CC versus TT+TC genotype ($p = 0.012$, OR = 1.272, 95% CI: 1.053–1.536); (B) Distribution of normal $(CAG)_n$ alleles: CC versus TC genotype ($p = 0.013$, OR = 1.242, 95% CI: 1.047–1.472); CC versus TT genotype ($p = 0.030$, OR = 1.287, 95% CI: 1.024–1.617); CC versus TT+TC genotype ($p = 0.011$, OR = 1.244, 95% CI: 1.050–1.473). Abbreviation: MJD, Machado-Joseph disease.

rs12957023, an intronic SNP in *MBD1*, showed significant differences of genotypic and allelic distributions between patients with MJD and controls. Frequency of its minor C allele is significantly higher in patients relative to normal controls. Moreover, the distribution of normal and expanded $(CAG)_n$ in 613 patients with MJD were significantly different between who carrying genotype CC and the other two genotypes CT and TT. Thus, this SNP may affect CAG repeat distribution and be associated with CAG instability.

Structurally, DNA methylation status determines DNA conformational alteration (Landgrave-Gomez et al., 2015), whereas trinucleotidic repeats are among the most unstable regions in the genome (Simard et al., 2014). CAG instability occurs during both meiotic and mitotic stages (Souza et al., 2016), the main process of spermatogenesis that is critically regulated by epigenetic events. MBD1 can bind to aberrantly methylated promoters to dysregulate gene expression (Bogdanovic and Veenstra, 2009). Thus, it is plausible to speculate that MBD1 might modulate the CAG instability in MJD by being involved in the process of DNA methylation at meiotic or mitotic stages. In our present study, by using the FS score from F-SNP (Lee et al., 2008), function of the rs12957023 in *MBD1* is predicted to alter its regulation on transcription. However, the definite function of this SNP has not been investigated to explain exactly how the MBD1 associates with CAG repeats expansion.

Table 2
Regression of residual AAO on each SNP in the preliminary analysis

Gene	SNPs	Partial regression coefficient of constant	Partial regression coefficient of SNP	*p* Value
DNMT1	rs2228611	1.919	−0.027	0.973
	rs16999593	2.364	−1.173	0.188
	rs2336691	1.909	−0.024	0.983
DNMT2	rs11254413	1.782	0.242	0.772
DNMT3A	rs13420827	1.203	1.670	0.048[a]
	rs1550117	1.610	0.648	0.458
	rs11683424	1.791	1.458	0.410
DNMT3B	rs2424908	1.702	0.239	0.751
	rs2424932	1.797	1.024	0.544
DNMT3L	rs8129776	1.269	1.033	0.194
	rs7354779	1.650	3.593	0.073[b]
	rs2070565	1.613	0.843	0.395
MBD1	rs12957023	1.963	−0.101	0.897
MBD2	rs2734647	1.947	−0.097	0.891
MeCP2	rs2239464	1.930	−0.065	0.929
MTHFR	rs1801133	2.661	−1.018	0.165
	rs1801131	1.603	0.886	0.370
TET1	rs150689919	1.729	2.359	0.231

[a] *p* value below 0.05.
[b] *p* value close to 0.05.

Strong association was observed between the SNPs rs13420827 in *DNMT3A* and rs7354779 in *DNMT3L* with AO in MJD. The patients with the GG genotype of rs13420827 or the CT genotype of rs7354779 conferred later AO relative to those with other genotypes. And the combined effect of these two significant SNPs could explain approximately 1% of the variability in AO. Altogether suggests that DNA methylation plays important roles in the modulation of AO in patients with MJD. However, the effect of *ATXN2* allele on AO found in the previous studies was not completely replicated. This might be because of the limited number of long normal *ATXN2* CAG repeats carriers (18 subjects) in this cohort. Since the exclusion of the ever-known contributing factors, the influence of rs13420827 and rs7354779 are more reliable for us to make the conclusion.

To further understand the potential mechanisms for the association of SNPs rs13420827 in *DNMT3A* and rs7354779 in *DNMT3L* with AO in MJD, it is of importance to remember that *DNMT3A* is a de novo methyltransferase, forming the methylation patterns

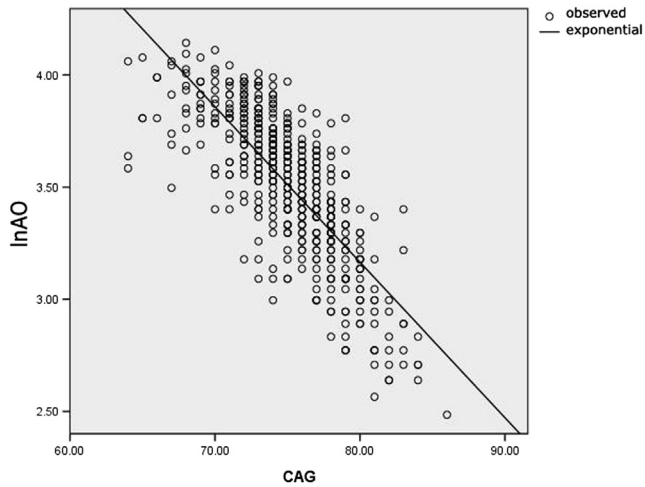

Fig. 3. Modification of AO attributed to expanded CAG repeat length in *ATXN3* of patients with MJD (linear regression). The X-axis denotes the CAG repeat size, which was regarded as an independent variable, and the Y-axis indicates the dependent variable, ln AO. Model parameters: ln(AO) = 8.710−0.069*Exp, where Exp = expanded CAG repeats in *ATXN3*. Abbreviations: AO, age at onset; MJD, Machado-Joseph disease.

Table 3
Regression analysis of normal CAG repeats in *ATXN3*, *ATXN2*, and rs13420827, rs7354779 on age at onset in MJD

Group	R^2	$\triangle R^2$	Coefficient of the equation	*p* value
①	0.603	-	8.710 (Constant)	<0.001
			−0.069 (Exp)	<0.001
① + ②	0.603	0	8.656 (Constant)	<0.001
			−0.069 (Exp)	<0.001
			0.001 (normal CAG repeats in *ATXN3*)	0.939
① + ③	0.605	0.002	8.701 (Constant)	<0.001
			−0.069 (Exp)	<0.001
			0.064 (A1 group in *ATXN2*)	0.127
① + ④	0.603	0	8.707 (Constant)	<0.001
			−0.069 (Exp)	<0.001
			0.002 (A2 group in *ATXN2*)	0.907
① + ⑤	0.604	0.001	8.677 (Constant)	<0.001
			−0.069 (Exp)	<0.001
			0.013 (A3 group in *ATXN2*)	0.502
① + ⑥	0.607	0.004	8.708 (Constant)	<0.001
			−0.069 (Exp)	<0.001
			0.032 (rs13420827)	**0.028**
①+ ⑦	0.610	0.007	8.686 (Constant)	<0.001
			−0.069 (Exp)	<0.001
			0.098 (rs7354779)	**0.002**
①+ ⑥ + ⑦	0.613	0.010	8.684 (Constant)	<0.001
			−0.069 (Exp)	<0.001
			0.031 (rs13420827)	**0.035**
			0.095 (rs7354779)	**0.003**
① + ⑤ + ⑥ + ⑦	0.613	0.010	8.656 (Constant)	<0.001
			−0.069 (Exp)	<0.001
			0.030 (rs13420827)	**0.039**
			0.095 (rs7354779)	**0.003**
			0.011 (A3 group in *ATXN2*)	0.578
①+ ② + ⑤ +⑥ + ⑦	0.613	0.010	8.657 (Constant)	<0.001
			−0.069 (Exp)	<0.001
			0.001 (normal CAG repeats in *ATXN3*)	0.933
			0.030 (rs13420827)	**0.039**
			0.095 (rs7354779)	**0.003**
			0.010 (A3 group in *ATXN2*)	0.599

The bold *p* values were below 0.05, indicating that the equation after inclusion of these SNPs were significant.
①Expanded CAG repeats in *ATXN3*; ② Normal CAG repeats in *ANXN3*; ③ A1 group of CAG repeats in *ATXN2*; ④A2 group of CAG repeats in *ATXN2*; ⑤A3 group of CAG repeats in *ATXN2*; ⑥rs13420827 in *DNMT3A*; ⑦rs7354779 in *DNMT3L*.
Exp: expanded CAG repeats in *ATXN3*.
Normal CAG repeats in *ATXN3* were divided into 3 subgroups: 19/19−25/26−40.
A1 = grouped by repeat length of shorter allele (<22/22) in *ATXN2*, A2 = grouped by repeat length of longer allele (22/23−26/>26) in *ATXN2*. A3 = The *ATXN2* genotypes were divided into four classes: 1) at least one short allele (<22), 2) homozygous medium 22 CAG alleles, 3) at least one intermediate allele (>26), and 4) at least one short intermediate allele (23−26) with or without a medium 22 CAG allele.

during early embryogenesis (Landgrave-Gomez et al., 2015). Significant abundance of DNMT3A at synapses and in mitochondria suggests its essential roles in neuronal functions (Chestnut et al., 2011). Indeed, required for neurogenesis and neuronal differentiation, DNMT3A is involved in regulation of neurogenic genes transcription (Wu et al., 2010). A functional polymorphism rs13420827 located in the 3′UTR of *DNMT3A* was found to be associated with AO in MJD in this study. The possible mechanism of this SNP could include but not limited to potential effect on DNMT3A expression and consequent DNA methylation activity.

Although lacking the amino acid residues necessary for methyltransferase activity, *DNMT3L* is critically involved in DNA methylation, as it acts as a regulator of de novo methyltransferases by simulating the activity of *DNMT3A* and *DNMT3B* (Gowher et al., 2005; Suetake et al., 2004). In this study, as mentioned previously, SNP rs7354779 in *DNMT3L* was shown to be associated with AO in MJD. To interpret the mechanism, it is necessary to look at the characterization of this SNP. rs7354779 is a nonsynonymous polymorphism that results in a G278R change and occurs at the C-terminal portion of DNMT3L that interacts with the active catalytic methyltransferase domain of DNMT3A and DNMT3B (Chen et al., 2005; Jia et al., 2007), suggesting the roles of the rs7354779 in regulation of *DNMT3A* and *DNMT3B*.

In the present study, rs13420827 (in *DNMT3A*) and rs7354779 (in *DNMT3L*) showed a combined effect on the AO in MJD, potentially indicating that these two genes might be involved in the regulation of AO through same metabolic pathway.

In conclusion, altogether this study suggest that DNA methylation is still another pathogenic mechanism in MJD and that the SNPs in some key enzyme genes involved in the methylation may regulate the expression and the activity of the enzymes responsible for the dynamic methylation patterns and levels. However, the analysis was limited by the sample size and the population composition in this study. Further study on the role of SNPs in DNA methylation regulation as well as the methylation mediated AO modulation in MJD, will open a novel avenue for understanding the pathogenesis and may provide new insight into the epigenetic therapy for the MJD by using larger sample size and population compositions in other cohorts.

Disclosure statement

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors are grateful to all the subjects for their participation in the study. They thank Dr Yujing Li, the professor of Emory University, for reviewing the article and for offering valuable suggestion.

This study was supported by the National Key Research and Development Program of China (Nos. 2016YFC0905100 and 2016YFC0901504 to Hong Jiang), the National Natural Science Foundation of China (Nos. 81471156, 81771231 to Hong Jiang), the Clinical Research Funds of Xiangya Hospital (2014L03 to Hong Jiang), the Clinical and rehabilitation fund of Peking University Weiming Biotech Group (No. xywm2015I10 to Hong Jiang) and the National Natural Science Foundation of China (No. 81430023, 81130021 to Beisha Tang), NIH (NS083564 to Tetsuo Ashizawa).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurobiolaging.2018.11.002.

References

Al-Mahdawi, S., Pinto, R.M., Ismail, O., Varshney, D., Lymperi, S., Sandi, C., Trabzuni, D., Pook, M., 2008. The Friedreich ataxia GAA repeat expansion mutation induces comparable epigenetic changes in human and transgenic mouse brain and heart tissues. Hum. Mol. Genet. 17, 735–746.

Bestor, T., Laudano, A., Mattaliano, R., Ingram, V., 1988. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. J. Mol. Biol. 203, 971–983.

Bettencourt, C., Raposo, M., Kazachkova, N., Cymbron, T., Santos, C., Kay, T., Vasconcelos, J., Maciel, P., Donis, K.C., Saraiva-Pereira, M.L., Jardim, L.B., Sequeiros, J., Lima, M., 2011. The APOE epsilon2 allele increases the risk of earlier age at onset in Machado-Joseph disease. Arch. Neurol. 68, 1580–1583.

Bheemanaik, S., Reddy, Y.V., Rao, D.N., 2006. Structure, function and mechanism of exocyclic DNA methyltransferases. Biochem. J. 399, 177–190.

Bogdanovic, O., Veenstra, G.J., 2009. DNA methylation and methyl-CpG binding proteins: developmental requirements and function. Chromosoma 118, 549–565.

Chatterjee, A., Saha, S., Chakraborty, A., Silva-Fernandes, A., Mandal, S.M., Neves-Carvalho, A., Liu, Y., Pandita, R.K., Hegde, M.L., Hegde, P.M., Boldogh, I., Ashizawa, T., Koeppen, A.H., Pandita, T.K., Maciel, P., Sarkar, P.S., Hazra, T.K., 2015. The role of the mammalian DNA end-processing enzyme polynucleotide kinase 3'-phosphatase in spinocerebellar ataxia type 3 pathogenesis. PLOS Genet. 11, e1004749.

Chen, S., Gan, S.R., Cai, P.P., Ni, W., Zhou, Q., Dong, Y., Wang, N., Wu, Z.Y., 2016a. Mitochondrial NADH Dehydrogenase Subunit 3 polymorphism associated with an earlier age at onset in male Machado-Joseph disease patients. CNS Neurosci. Ther. 22, 38–42.

Chen, Z., Zheng, C., Long, Z., Cao, L., Li, X., Shang, H., Yin, X., Zhang, B., Liu, J., Ding, D., Peng, Y., Wang, C., Peng, H., Ye, W., Qiu, R., Pan, Q., Xia, K., Chen, S., Sequeiros, J., Ashizawa, T., Klockgether, T., Tang, B., Jiang, H., 2016b. (CAG)n loci as genetic modifiers of age-at-onset in patients with Machado-Joseph disease from mainland China. Brain 139 (Pt 8), e41.

Chen, Z.X., Mann, J.R., Hsieh, C.L., Riggs, A.D., Chedin, F., 2005. Physical and functional interactions between the human DNMT3L protein and members of the de novo methyltransferase family. J. Cell Biochem 95, 902–917.

Chestnut, B.A., Chang, Q., Price, A., Lesuisse, C., Wong, M., Martin, L.J., 2011. Epigenetic regulation of motor neuron cell death through DNA methylation. J. Neurosci. 31, 16619–16636.

Chou, A.H., Yeh, T.H., Ouyang, P., Chen, Y.L., Chen, S.Y., Wang, H.L., 2008. Polyglutamine-expanded ataxin-3 causes cerebellar dysfunction of SCA3 transgenic mice by inducing transcriptional dysregulation. Neurobiol. Dis. 31, 89–101.

Chouliaras, L., Kenis, G., Visser, P.J., Scheltens, P., Tsolaki, M., Jones, R.W., Kehoe, P.G., Graff, C., Girtler, N.G., Wallin, A.K., Rikkert, M.O., Spiru, L., Elias-Sonnenschein, L.S., Ramakers, I.H., Pishva, E., van Os, J., Steinbusch, H.W., Verhey, F.R., van den Hove, D.L., Rutten, B.P., 2015. DNMT3A moderates cognitive decline in subjects with mild cognitive impairment: replicated evidence from two mild cognitive impairment cohorts. Epigenomics 7, 533–537.

Ding, D., Li, K., Wang, C., Chen, Z., Long, Z., Peng, Y., Zhou, X., Peng, H., Qiu, R., Xia, K., Tang, B., Jiang, H., 2016. ATXN2 polymorphism modulates age at onset in Machado-Joseph disease. Brain 139, e59.

Du, Q., Luu, P.L., Stirzaker, C., Clark, S.J., 2015. Methyl-CpG-binding domain proteins: readers of the epigenome. Epigenomics 7, 1051–1073.

Emmel, V.E., Alonso, I., Jardim, L.B., Saraiva-Pereira, M.L., Sequeiros, J., 2011. Does DNA methylation in the promoter region of the ATXN3 gene modify age at onset in MJD (SCA3) patients? Clin. Genet. 79, 100–102.

Filion, G.J., Zhenilo, S., Salozhin, S., Yamada, D., Prokhortchouk, E., Defossez, P.A., 2006. A family of human zinc finger proteins that bind methylated DNA and repress transcription. Mol. Cell Biol. 26, 169–181.

Gao, R., Liu, Y., Silva-Fernandes, A., Fang, X., Paulucci-Holthauzen, A., Chatterjee, A., Zhang, H.L., Matsuura, T., Choudhary, S., Ashizawa, T., Koeppen, A.H., Maciel, P., Hazra, T.K., Sarkar, P.S., 2015. Inactivation of PNKP by mutant ATXN3 triggers apoptosis by activating the DNA damage-response pathway in SCA3. PLOS Genet. 11, e1004834.

Godler, D.E., Slater, H.R., Amor, D., Loesch, D.Z., 2010a. Methylation analysis of fragile X-related epigenetic elements may provide a suitable newborn screening test for fragile X syndrome. Genet. Med. 12, 595.

Godler, D.E., Tassone, F., Loesch, D.Z., Taylor, A.K., Gehling, F., Hagerman, R.J., Burgess, T., Ganesamoorthy, D., Hennerich, D., Gordon, L., Evans, A., Choo, K.H., Slater, H.R., 2010b. Methylation of novel markers of fragile X alleles is inversely correlated with FMRP expression and FMR1 activation ratio. Hum. Mol. Genet. 19, 1618–1632.

Goncalves, N., Simoes, A.T., Cunha, R.A., de Almeida, L.P., 2013. Caffeine and adenosine A(2A) receptor inactivation decrease striatal neuropathology in a lentiviral-based model of Machado-Joseph disease. Ann. Neurol. 73, 655–666.

Gowher, H., Liebert, K., Hermann, A., Xu, G., Jeltsch, A., 2005. Mechanism of stimulation of catalytic activity of Dnmt3A and Dnmt3B DNA-(cytosine-C5)-methyltransferases by Dnmt3L. J. Biol. Chem. 280, 13341–13348.

Hendrich, B., Bird, A., 1998. Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol. Cell Biol. 18, 6538–6547.

Jia, D., Jurkowska, R.Z., Zhang, X., Jeltsch, A., Cheng, X., 2007. Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. Nature 449, 248–251.

Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I., Kimura, J., Narumiya, S., Kakizuka, A., et al., 1994. CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. Nat. Genet. 8, 221–228.

Klockgether, T., Ludtke, R., Kramer, B., Abele, M., Burk, K., Schols, L., Riess, O., Laccone, F., Boesch, S., Lopes-Cendes, I., Brice, A., Inzelberg, R., Zilber, N., Dichgans, J., 1998. The natural history of degenerative ataxia: a retrospective study in 466 patients. Brain 121 (Pt 4), 589–600.

Koch, P., Breuer, P., Peitz, M., Jungverdorben, J., Kesavan, J., Poppe, D., Doerr, J., Ladewig, J., Mertens, J., Tuting, T., Hoffmann, P., Klockgether, T., Evert, B.O., Wullner, U., Brustle, O., 2011. Excitation-induced ataxin-3 aggregation in neurons from patients with Machado-Joseph disease. Nature 480, 543–546.

Laffita-Mesa, J.M., Bauer, P.O., Kouri, V., Pena, S.L., Roskams, J., Almaguer, G.D., Montes, B.J., Martinez, R.P., Gonzalez-Zaldivar, Y., Almaguer, M.L., Cuello-Almarales, D., Aguiar, S.J., 2012. Epigenetics DNA methylation in the core ataxin-2 gene promoter: novel physiological and pathological implications. Hum. Genet. 131, 625–638.

Landgrave-Gomez, J., Mercado-Gomez, O., Guevara-Guzman, R., 2015. Epigenetic mechanisms in neurological and neurodegenerative diseases. Front. Cell Nerrosci. 9, 58.

Lee, P.H., Shatkay, H., 2008. F-SNP: Computationally predicted functional SNPs for disease association studies. Nucleic Acids Res. 36, D820–D824.

Li, H., Guo, Z., Guo, Y., Li, M., Yan, H., Cheng, J., Wang, C., Hong, G., 2016. Common DNA methylation alterations of Alzheimer's disease and aging in peripheral whole blood. Oncotarget 7, 19089–19098.

Liao, X.X., Zhan, Z.X., Luo, Y.Y., Li, K., Wang, J.L., Guo, J.F., Yan, X.X., Xia, K., Tang, B.S., Shen, L., 2013. Association study between SNP rs150689919 in the DNA demethylation gene, TET1, and Parkinson's disease in Chinese Han population. BMC Neurol. 13, 196.

Lu, H., Liu, X., Deng, Y., Qing, H., 2013. DNA methylation, a hand behind neurodegenerative diseases. Front. Aging Neurosci. 5, 85.

Murphy, T.M., Mullins, N., Ryan, M., Foster, T., Kelly, C., McClelland, R., O'Grady, J., Corcoran, E., Brady, J., Reilly, M., Jeffers, A., Brown, K., Maher, A., Bannan, N., Casement A, Lynch, D., Bolger, S., Buckley, A., Quinlivan, L., Daly, L., Kelleher, C., Malone, K.M., 2013. Genetic variation in DNMT3B and increased global DNA methylation is associated with suicide attempts in psychiatric patients. Genes Brain Behav. 12, 125–132.

Nascimento-Ferreira, I., Nobrega, C., Vasconcelos-Ferreira, A., Onofre, I., Albuquerque, D., Aveleira, C., Hirai, H., Deglon, N., Pereira, D.A.L., 2013. Beclin 1 mitigates motor and neuropathological deficits in genetic mouse models of Machado-Joseph disease. Brain 136 (Pt 7), 2173–2188.

Ng, C.W., Yildirim, F., Yap, Y.S., Dalin, S., Matthews, B.J., Velez, P.J., Labadorf, A., Housman, D.E., Fraenkel, E., 2013. Extensive changes in DNA methylation are associated with expression of mutant huntingtin. Proc. Natl. Acad. Sci. U S A. 110, 2354–2359.

Okano, M., Xie, S., Li, E., 1998. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat. Genet. 19, 219–220.

Pastor, W.A., Aravind, L., Rao, A., 2013. TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. Nat. Rev. Mol. Cell Biol. 14, 341–356.

Paulson, H., 2012. Machado-Joseph disease/spinocerebellar ataxia type 3. Handb Clin. Neurol. 103, 437–449.

Paulson, H.L., 2007. Dominantly inherited ataxias: lessons learned from Machado-Joseph disease/spinocerebellar ataxia type 3. Semin. Neurol. 27, 133–142.

Simard, O., Gregoire, M.C., Arguin, M., Brazeau, M.A., Leduc, F., Marois, I., Richter, M.V., Boissonneault, G., 2014. Instability of trinucleotidic repeats during chromatin remodeling in spermatids. Hum. Mutat. 35, 1280–1284.

Simoes, A.T., Goncalves, N., Koeppen, A., Deglon, N., Kugler, S., Duarte, C.B., Pereira, D.A.L., 2012. Calpastatin-mediated inhibition of calpains in the mouse brain prevents mutant ataxin 3 proteolysis, nuclear localization and aggregation, relieving Machado-Joseph disease. Brain 135 (Pt 8), 2428–2439.

Simoes, A.T., Goncalves, N., Nobre, R.J., Duarte, C.B., Pereira, D.A.L., 2014. Calpain inhibition reduces ataxin-3 cleavage alleviating neuropathology and motor impairments in mouse models of Machado-Joseph disease. Hum. Mol. Genet. 23, 4932–4944.

Smith, Z.D., Meissner, A., 2013. DNA methylation: roles in mammalian development. Nat. Rev. Genet. 14, 204–220.

Souza, G.N., Kersting, N., Krum-Santos, A.C., Santos, A.S., Furtado, G.V., Pacheco, D., Goncalves, T.A., Saute, J.A., Schuler-Faccini, L., Mattos, E.P., Saraiva-Pereira, M.L., Jardim, L.B., 2016. Spinocerebellar ataxia type 3/Machado-Joseph disease: segregation patterns and factors influencing instability of expanded CAG transmissions. Clin. Genet. 90, 134–140.

Suetake, I., Shinozaki, F., Miyagawa, J., Takeshima, H., Tajima, S., 2004. DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. J. Biol. Chem. 279, 27816–27823.

Tezenas, D.M.S., Durr, A., Bauer, P., Figueroa, K.P., Ichikawa, Y., Brussino, A., Forlani, S., Rakowicz, M., Schols, L., Mariotti, C., van de Warrenburg, B.P., Orsi, L., Giunti, P., Filla, A., Szymanski, S., Klockgether, T., Berciano, J., Pandolfo, M., Boesch, S., Melegh, B., Timmann, D., Mandich, P., Camuzat, A., Goto, J., Ashizawa, T., Cazeneuve, C., Tsuji, S., Pulst, S.M., Brusco, A., Riess, O., Brice, A., Stevanin, G., 2014. Modulation of the age at onset in spinocerebellar ataxia by CAG tracts in various genes. Brain 137 (Pt 9), 2444–2455.

Wang, C., Peng, H., Li, J., Ding, D., Chen, Z., Long, Z., Peng, Y., Zhou, X., Ye, W., Li, K., Xu, Q., Ai, S., Song, C., Weng, L., Qiu, R., Xia, K., Tang, B., Jiang, H., 2017. Alteration of methylation status in the ATXN3 gene promoter region is linked to the SCA3/MJD. Neurobio Aging 53, 192–195.

Wu, H., Coskun, V., Tao, J., Xie, W., Ge, W., Yoshikawa, K., Li, E., Zhang, Y., Sun, Y.E., 2010. Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science 329, 444–448.