

## Neuropathology does not Correlate with Regional Differences in the Extent of Expansion of CTG Repeats in the Brain with Myotonic Dystrophy Type 1

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Myotonic dystrophy (DM1) is known to be an adult-onset muscular dystrophy caused by the expansion of CTG repeats within the 3' untranslated region of the dystrophin myotonin protein kinase (*DMPK*) gene. The clinical features of DM1 include CNS symptoms, such as cognitive impairment and personality changes, the pathogenesis of which remains to be elucidated. We hypothesized that the distribution of neuropathological changes might be correlated with the extent of the length of the CTG repeats in the *DMPK* genes in DM1 patients. We studied the neuropathological changes in the brains of subjects with DM1 and investigated the extent of somatic instability in terms of CTG repeat expansion in the different brain regions of the same individuals by Southern blot analysis. The neuropathological changes included état criblé in the cerebral deep white matter and neurofibrillary tangles immunoreactive for phosphorylated tau in the hippocampus and entorhinal cortex, both of which were compatible with the subcortical dementia in DM1 patients. However, the length of the CTG repeats did not correlate with the regional differences in the extent of neuropathological changes. Our data suggested that pathomechanisms of dementia in DM1 might be more multifactorial rather than a toxic gain-of-function due to mutant RNA.

**Key words:** myotonic dystrophy (DM1), dementia, dystrophin myotonin protein kinase, CTG repeats, neuropathology

### I. Introduction

Myotonic dystrophy (DM1) is the most common, autosomal dominant form of adult muscular dystrophy, affecting around 1 in 8000 individuals. DM1 is caused by the expansion of CTG repeats within the 3' untranslated region (UTR) of the dystrophin myotonin protein kinase (*DMPK*) gene [3, 4]. Normal individuals have less than 50 CTG repeats whereas DM1 patients have hundreds or even thousands. The severity and age of onset of DM1 correlate well with

the length of the repeats. In addition, somatic mosaicism of the expansion of CTG repeats has been observed in different organs of the same individuals with DM1.

The clinical features of DM1 include muscle weakness, myotonia, cardiac arrhythmia, endocrinopathy, such as frontal baldness and insulin resistance, cataracts, and CNS symptoms. CNS symptoms include cognitive impairment and particular personality changes [2, 21]. Although no detailed studies have as yet been reported, these CNS symptoms should be based upon specific neuropathological changes.

Recent molecular genetic studies suggested that the underlying pathological mechanism of both the myopathy and the CNS symptoms associated with DM1 is a toxic gain-of-function due to mutant RNA [5, 6, 11, 17, 22, 31–33]. The CUG-expanded transcripts of the mutant allele are

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retained in the cell nuclei to form intranuclear inclusions, which then compromise the regulation of alternative splicing for a subset of neuronal transcripts [7, 10, 14, 15, 18, 19].

We hypothesized that the distribution of neuropathological changes might be correlated with the extent of the length of the CTG repeats in *DMPK* genes in DM1 patients. In order to verify our hypothesis, we studied the neuropathological changes found in the brains of subjects with DM1 (M/F=5/6) and investigated the extent of somatic instability in terms of the expansion of CTG repeats in the different brain regions of the same individuals, employing the Southern blot analysis method. We found *état criblé* in the cerebral deep white matter, lesions that should be responsible for the subcortical dementia in subjects with DM1. However, no correlation was demonstrated between the extent of the length of the CTG repeats and the pathological changes.

## II. Materials and Methods

### Clinical information

Eleven patients were enrolled in this study. Age (mean: 60.4 (50–69) years old) and gender (M/F=5/6) are described in Table 1. All of the patients studied had a clinical history of dementia for several years before death. None of them had a history of hypertension.

### Neuropathological examinations

Ten autopsied brains with DM1, each with a post-mortem time within 3 hr, were studied (Table 1). After weighing, the brains were cut at the median plane and the left hemisphere was fixed with buffered formalin for histological analyses and the right side was frozen for molecular analyses. This study was approved by the Ethics Committee both at Kyoto Prefectural University of Medicine and the National Hospital Organization, Hyogo-Chuo National Hospital, and all the procedures for tissue sampling and DNA analyses were in accord with the guidelines for Genome Research issued by the Japanese Government.

After fixation, paraffin-embedded brain sections were processed for staining with hematoxylin-eosin (HE), Klüver-Barrera (KB), silver impregnation (Gallyas-Braak, Bodian), and for immunohistochemistry using the PAP method. The primary antibodies used were: Neurofilament, phosphorylated (1:100, United States Biological, Swampscott, MA, USA), MAP2 (1:1000, Chemicon/Millipore, Billerica, MA, USA), GFAP (1:1000, Chemicon/Millipore), A $\beta$ 1-40/42 (1:100, Chemicon/Millipore), Human PHF-Tau (clone AT8, 1:200, Innogenetics N.V./S.A., Gent, Belgium), ubiquitin (1:1000, Abcam, Cambridge, UK),  $\alpha$ -synuclein (1:3000, Chemicon/Millipore), CD34 (clone QBEND-10, 1:1000, Abcam) and  $\alpha$ -SMA (clone 1A4, 1:2000, Sigma Chemical Co. St Louis, MO, USA). The areas of the brain studied were the frontal, orbital, parietal, temporal, and occipital cortices and white matter, hippocampus, entorhinal cortex, basal ganglia such as nucleus caudatus, putamen, globus pallidus, nucleus lateralis and medialis of the thalamus, tegmentum, nucleus raphe dorsalis, substantia nigra of the midbrain, locus coeruleus, reticular formation and nuclei pontis of the pons, reticular formation and nucleus olivaris inferior of the medulla oblongata, cerebellar cortex and white matter, nucleus dentatus and pedunculus cerebellaris and the entire length of the spinal cord. Semiquantitative study was performed on the number or the extent of pathological changes and evaluated as follows: +: moderate or frequent (localized in the entorhinal cortex for NFT, or < approximately 2/mm<sup>2</sup> for Lewy bodies), ++: severe or very frequent (spread to the amygdala and presubiculum, subiculum and CA1 of the hippocampus for NFT, > approximately 2/mm<sup>2</sup> for Lewy bodies).

### DNA analyses

Genomic DNA was prepared from the brain tissues (n=5) according to a standard protocol as previously reported [4, 16]. The length of the CTG repeats was analyzed in the tissue specimens obtained from the contralateral side of the brain, but all of the areas studied corresponded to those used for the neuropathological examinations. In each case,

**Table 1.** Summary of clinical and neuropathological findings from analysed cases

CASE	1*	2	3	4*	5*	6*	7	8	9*	10	11	Frequency/Average
Age (years old)	50	55	58	59	61	61	62	62	63	64	69	60±5
Gender	M	M	F	M	F	M	M	F	F	F	F	M/F=5/6
Dementia	+	+	+	+	+	+	+	+	+	+	+	100%
Hypertension	–	–	–	–	–	–	–	–	–	–	–	0%
Brain weight (g)	1460	1230	900	1180	900	1200	1080	910	1060	1140	960	1092±174
Neuronal loss (cortex)	–	–	+	–	+	–	–	+	+	+	+	55%
État criblé	+	+	+	+	+	+	+	+	+	+	+	100%
NFTs	+	++	+	++	+	+	+	++	++	+	++	100%
Senile plaques	–	–	–	–	–	–	–	–	–	–	–	0%
Lewy bodies	–	–	++	–	–	–	–	–	–	–	+	18%
NFT-like in RF	–	–	+	–	+	–	–	+	+	–	+	45%

NFT: Neurofibrillary tangle; RF: Reticular formation.

\*: DNA analysed.

+: moderate/frequent; ++: severe/very frequent.

the size of the expansion of the CTG repeats at the DMPK locus was determined by Southern blot analysis of *EcoRI*- and *BglII*-digested DNA obtained from the tissue, using cDNA25 as a probe. Pulse-field gel electrophoresis (PFGE) was performed on the CHEF-DR-II system (Bio-Rad, Hercules, CA, USA) as reported elsewhere [4, 16]. In brief, eight  $\mu\text{g}$  of digested DNA fragments in buffer was added to each sample well. SeaKem GTG agarose gel (FMC BioProducts, Rockland, ME) of 0.7% was set in a chamber containing standard Tris buffer kept at 14°C. The driving conditions of the PFGE were: 120 V, 70 mA, pulse time of 1 sec ramped up to 5 sec, and a run time of 20 hr, according to the manufacturer's instructions.

### III. Results

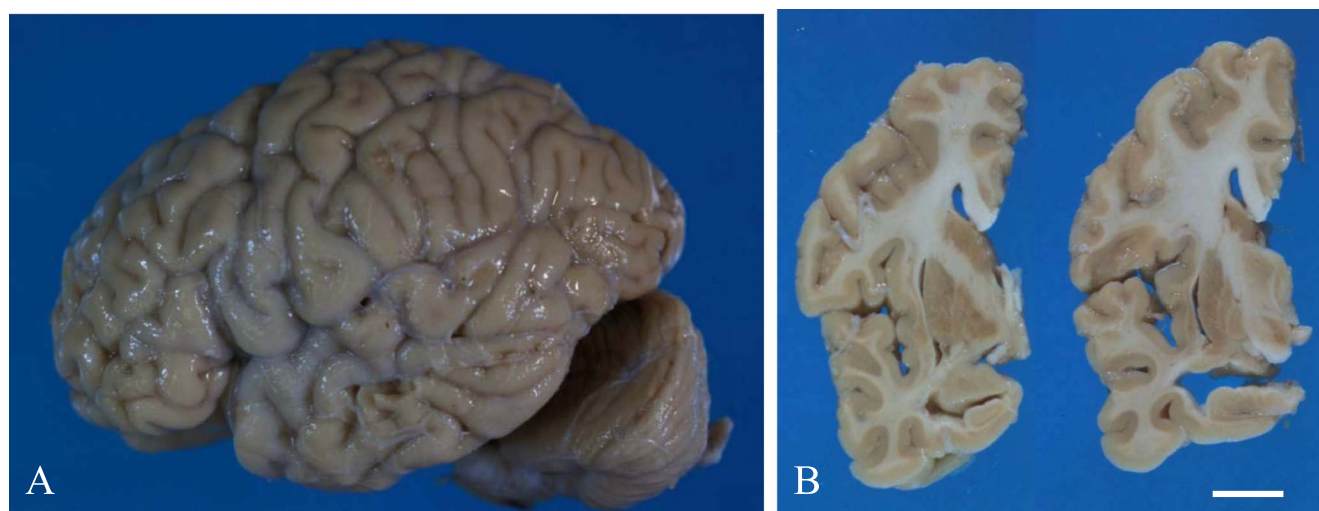
#### *Neuropathological examinations*

Macroscopically, the brains showed diffuse atrophy with a normal gyral configuration except for case 1 (10/11 cases, Fig. 1A). The proportion of the brain, cerebellum and brainstem was normal. Mild to moderate symmetrical ventricular dilatation with a decreased volume of the white matter was identified on the cut sections (10/11 cases, Fig. 1B). The basal forebrain showed dilatation of the perivascular space. Microscopically, the deep white matter in the frontal, orbital, temporal, parietal, and occipital lobes showed *état criblé*, namely dilated perivascular spaces (type III), loss of adjacent axons and myelin, capillary hyalinization and fibrillary gliosis (11/11 cases, Fig. 2A–D). Small to medium-sized arteries showed a loss of smooth muscles in the tunica media associated with a collection of perivascular CD34-immunoreactive pericytes or microglia and numerous corpora amylacea (Fig. 2E, F). The cerebral cortices showed a normal cytoarchitecture with mild neuronal loss (6/11 cases) or without neuronal loss (5/11 cases). No apparent neuronal loss was found in the CA1 of the hippocampus, subiculum and presubiculum, but there was a mild neuronal

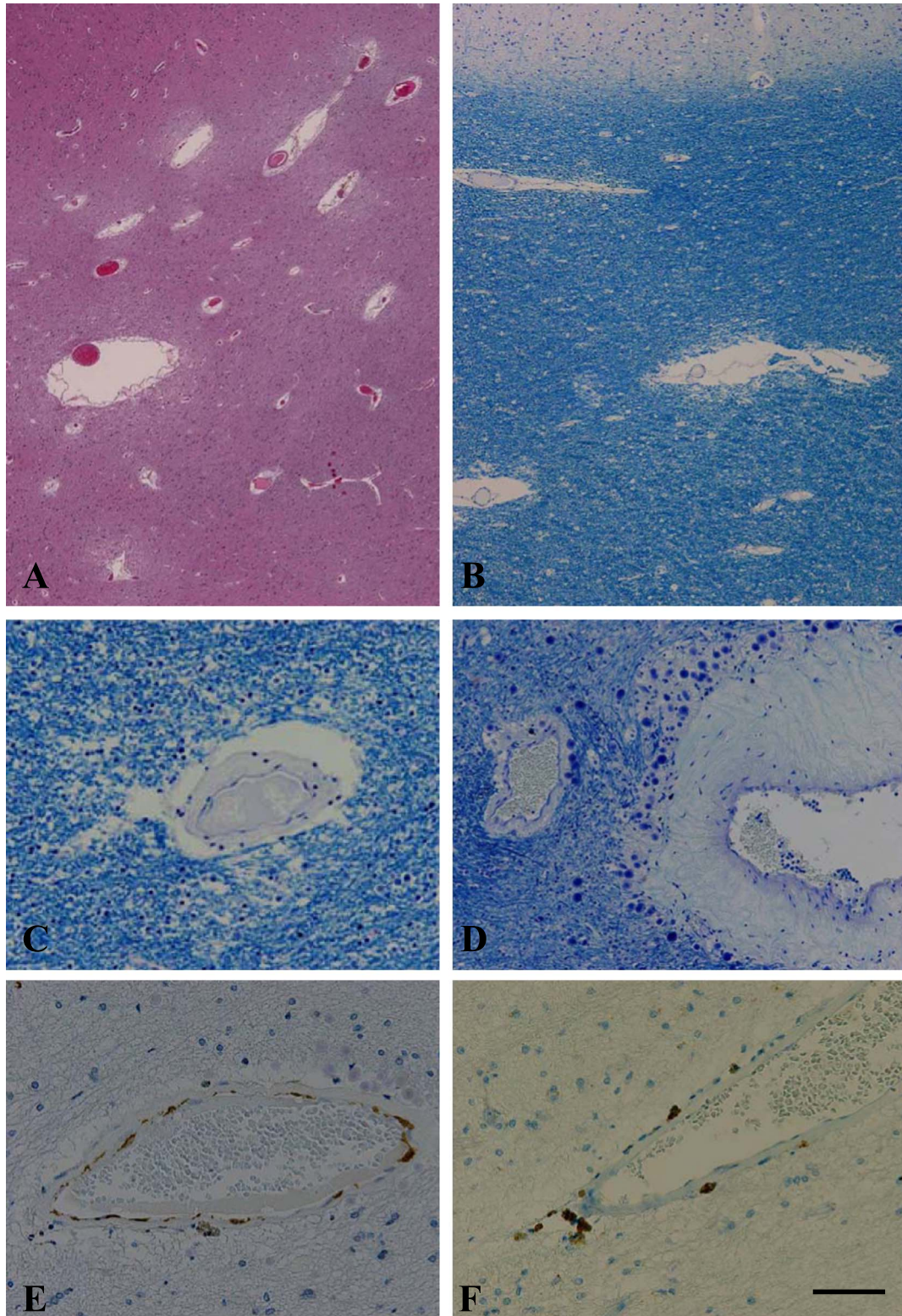
loss in the entorhinal cortex. A moderate number of neurofibrillary tangles, showing immunoreactivity for phosphorylated tau protein, were observed in the amygdala, CA1, presubiculum and subiculum of the hippocampus, and in the entorhinal cortex (11/11 cases, Fig. 3A, B). Many ghost tangles were observed in the superficial layer of the entorhinal cortex. Enlarged perivascular spaces and abundant corpora amylacea were often observed in the substantia innominata. Senile plaques, immunoreactive for  $A\beta$ , were not observed in all cases. No apparent neuronal loss was observed in the nucleus caudatus and putamen. Apparent calcification in the vessel walls was sometimes observed in the lateral and medial segments of the globus pallidus (6/11 cases). Cytoplasmic eosinophilic inclusions were seen in the thalamic nuclei in 2/11 cases (Fig. 3E), mild neuronal loss, gliosis and Lewy bodies were observed in the substantia nigra and locus coeruleus in 2/11 cases (Fig. 3C, D), mild neuronal loss and tangled masses of fibrils were observed in the reticular formation of the brainstem in 5/11 cases (Fig. 3F, G). In the pigmented neurons of the substantia nigra, an increased number of intranuclear Marinesco bodies, round to oval, and immunoreactive for ubiquitin were found (11/11 cases). No apparent neuronal loss was detected in the nuclei pontis, nucleus olivaris inferior, Purkinje cells and granule cells of the cerebellum, and the nucleus dentatus. The spinal cord showed no loss of neurons, axons, or myelin.

#### *DNA analyses (Table 2)*

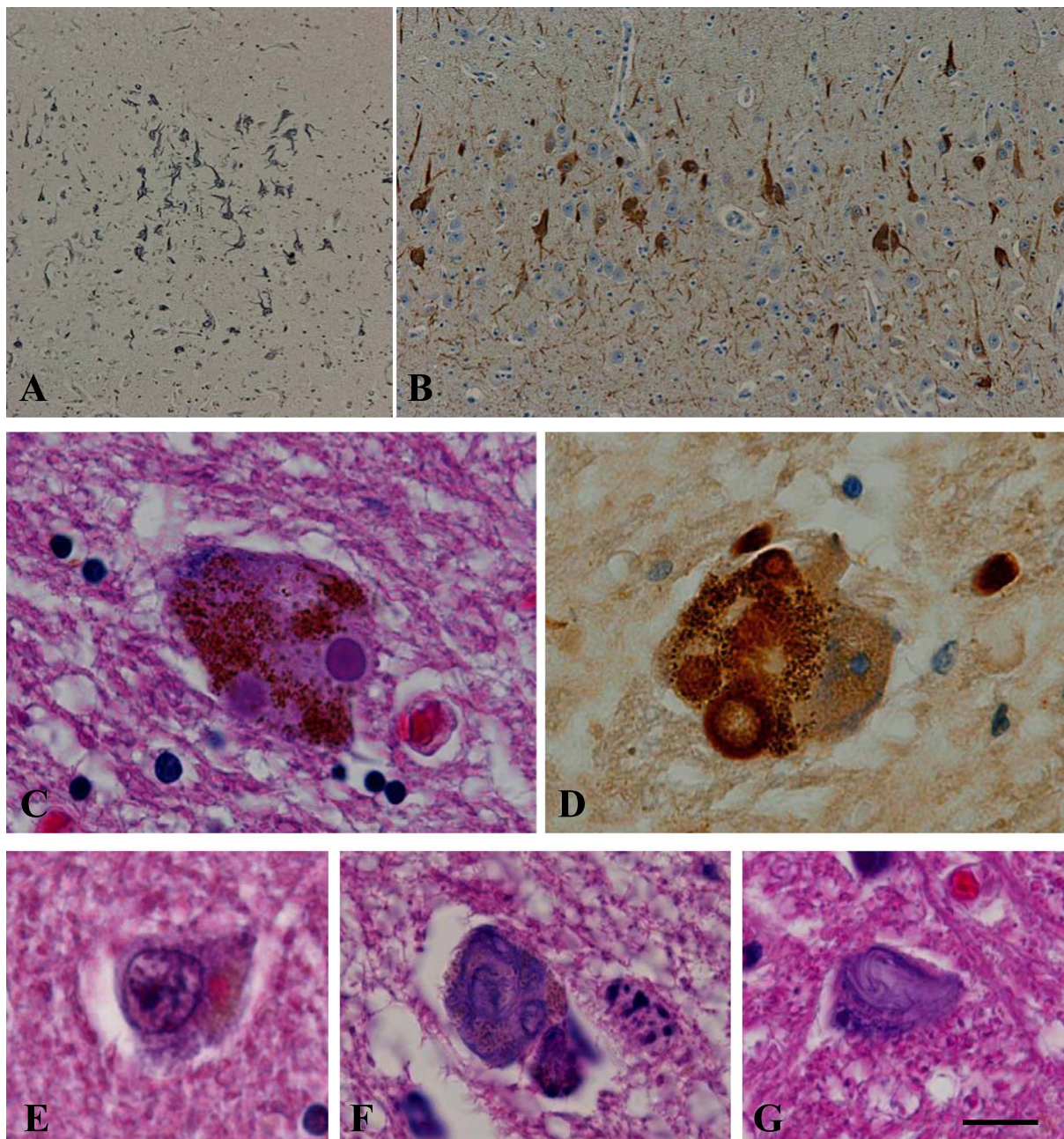
The normal allele showed the same size in all the analyzed brain regions in the DM1 cases, with a single strong band at 8.6 and 9.8 kilobases (kb) after *EcoRI* and 3.4 kb after *BglII* digestion. The expanded allele showed smear-like broad bands at around 18.0–23.9 kb after *EcoRI* and 10–14.5 kb after *BglII* digestion in all of the brain regions analyzed except for the cerebellar cortex and nucleus dentatus, where the expanded repeat was larger than that of the normal allele, but much smaller than the



**Fig. 1.** Macroscopic findings of the brain from myotonic dystrophy patients. **A:** lateral view. **B:** coronal sections at the level of basal ganglia and thalamus. Bar=2 cm.



**Fig. 2.** Deep white matter showing état criblé and hyalinized vessels. Marked dilatation of perivascular spaces (**A**, **B**), myelin pallor of adjacent perivascular areas (**C**), hyalinized vessels (**D**), showing loss of smooth muscle in the tunica media (**E**) and extravasation of CD34-immunoreactive cells (**F**). **A**: HE stain, **B**, **C**, **D**: Klüver-Barrera stain, **E**: immunohistochemistry for  $\alpha$ -SMA, **F**: immunohistochemistry for CD34. Bar=500  $\mu$ m (**A**), 250  $\mu$ m (**B**), 65  $\mu$ m (**C**, **D**), 35  $\mu$ m (**E**, **F**).



**Fig. 3.** Miscellaneous neuropathological findings in brains with myotonic dystrophy. Abundant neurofibrillary tangles immunoreactive for phosphorylated tau in the entorhinal cortex (**A**, **B**), brain-stem type Lewy bodies in the substantia nigra and locus coeruleus immunoreactive for  $\alpha$ -synuclein (**C**, **D**), eosinophilic cytoplasmic inclusion in the thalamic nuclei (**E**) and tangle-like inclusions in the nucleus raphe dorsalis (**F**) and in the reticular formation of the medulla oblongata (**G**). **A**: Bodian stain, **C**, **E**, **F**, **G**: HE stain, **B**: immunohistochemistry for phosphorylated tau, **D**: immunohistochemistry for  $\alpha$ -synuclein. Bar=130  $\mu$ m (**A**), 50  $\mu$ m (**B**), 15  $\mu$ m (**C**–**G**).

expanded repeats in other brain regions. In addition, the bands of the expanded allele in the cerebellar cortex and nucleus dentatus were intense and broad, not smeared, which indicated that less somatic mosaicism is present in these areas. Table 2 shows the ratios of expanded allele size to normal allele size (as expanded ratios). The expanded ratio of the cerebellum was significantly lower than that of the other 22 various brain regions ( $p < 0.01$ , one-way ANOVA). There were no significant differences in ex-

panded ratios among all brain regions analysed except for the cerebellum.

The size of the expanded allele showed a similar smear-like band at around 20.0–26.4 kb after *EcoRI* and 10–14.5 kb after *BglI* digestion in the skeletal muscle, heart, liver, spleen, pancreas, and testis. The largest expanded repeat was observed in the spleen where a larger somatic mosaicism was present.

**Table 2.** Expanded ratios: expanded allele size to normal allele one of DMPK locus

	case 1	case 4	case 5	case 6	case 9	mean	SD
Normal allele	1.0	1.0	1.0	1.0	1.0		
WBC	1.3	1.5	1.3	1.7	1.3	1.4±0.2	
FG	1.9	2.5	1.8	2.3	1.9	2.1±0.3	
FW	1.7	2.5	1.6	2.2	2.0	2.0±0.4	
OrG	1.8	2.5	1.7	2.1	2.0	2.0±0.3	
OrW	1.8	2.6	1.6	2.2	1.9	2.0±0.4	
PG	2.1	2.6	1.7	2.3	2.1	2.2±0.3	
PW	1.8	2.5	1.7	2.1	1.9	2.0±0.3	
OcG	1.9	2.7	1.7	2.3	2.1	2.1±0.4	
OcW	1.8	2.6	1.6	2.2	1.9	2.0±0.4	
TG	1.9	2.5	1.7	2.3	2.0	2.1±0.3	
TW	1.8	2.5	1.6	2.2	2.0	2.0±0.3	
Hip	2.0	ND	1.9	2.3	1.7	2.0±0.3	
NC	2.0	2.5	1.8	2.2	1.8	2.1±0.3	
Put	1.8	2.4	1.8	2.2	1.7	2.0±0.3	
GP	1.8	2.5	1.8	2.2	1.7	2.0±0.3	
Thal	2.0	2.5	1.8	2.3	1.7	2.1±0.3	
SN	2.1	2.4	1.8	2.1	1.7	2.0±0.3	
NR	1.8	ND	1.7	2.2	1.8	1.9±0.2	
NP	2.4	2.8	2.4	2.8	1.7	2.4±0.4	
VP	1.8	2.8	1.7	2.1	1.8	2.0±0.5	
NOI	1.7	2.4	1.8	2.3	1.9	2.0±0.3	
Cer	1.1	1.2	1.1	1.2	1.1	1.1±0.1*	
DN	1.1	1.3	ND	1.2	1.5	1.3±0.2	
Sp	1.9	ND	1.7	2.1	1.7	1.9±0.2	

$p < 0.01$ , one-way ANOVA.

WBC, white blood cell; FG, frontal grey matter; FW, frontal white matter; OrG, orbital grey matter; OrW, orbital white matter; PG, parietal grey matter; PW, parietal white matter; OcG, occipital grey matter; OcW, occipital white matter; TG, temporal grey matter; TW, temporal white matter; Hip, hippocampus; NC, nucleus caudatus; Put, putamen; GP, globus pallidus; Thal, thalamus; SN, substantia nigra; NR, nucleus ruber; NP, nuclei pontis; VP, ventral pons; NOI, nucleus olivaris inferior; Cer, cerebellum; DN, nucleus dentatus; Sp, spinal cord; ND, not done.

#### IV. Discussion

The present study demonstrated detailed neuropathological changes in DM1 brains, including neurofibrillary tangles (NFT) immunoreactive for phosphorylated tau protein localized in the CA regions of the hippocampus, amygdala and the entorhinal cortex, and état criblé (indicative of perivascular ischemic changes) in the cerebral deep white matter. The other pathological changes observed frequently, but regarded as nonspecific, were as follows: Lewy bodies and increased Marinesco bodies in the pigmented neurons of the substantia nigra, NFT-like neuronal inclusions in the reticular formation, apparent calcification of vessel walls in the globus pallidus, and cytoplasmic eosinophilic inclusions in the thalamic nuclei.

On the other hand, the length of the CTG repeats of the expanded allele did not show any considerable variations

depending on the brain region, with almost full length expansions and apparent somatic mosaicism even in the same regions. In the cerebellar cortex and white matter of all of the analyzed individuals, the expansion of the CTG repeats and the somatic mosaicism were constantly the smallest among all the brain regions studied. No correlation was demonstrated between the region-specific CTG instability and the severity of neuropathological changes throughout the brain.

The features of the neuropathological changes associated with DM1 are not well established. NFTs were often observed, mostly in the mesolimbic areas [28, 34], characterized by a reduced expression of tau isoforms, including the encoded sequences of exons 2, 3, 6 and 10 [18, 19, 30, 34]. In our study, NFTs were constantly observed in the hippocampus and entorhinal cortex in all of the analyzed DM1 cases. Other authors have reported intracytoplasmic inclusion bodies in the thalamus and substantia nigra [25], increased Marinesco bodies, and neuronal loss of the medullary arcuate nucleus and reticular formation [26, 27]. However, it is unlikely that these contribute to the cognitive impairment seen in DM1. The neuropathological changes seen in autopsy cases of DM1 have included neuronal loss in the superficial layer of the frontal and parietal cortices and extensive neuronal loss in the occipital cortex, intracytoplasmic inclusion bodies in the medial thalamic nuclei, neuronal loss and presence of Lewy bodies in the substantia nigra, and locus coeruleus and abnormalities of myelin (i.e. tigroid pattern) in the white matter [23, 29]. Lewy bodies in the substantia nigra and locus coeruleus were frequently but inconsistently seen in DM1. These changes, therefore, were considered not to be specific for DM1. Previous reports on cranial magnetic resonance imaging (MRI) findings in DM1 described a wide variety of abnormalities ranging from white matter hyperintense lesions (WML) to cerebral atrophy [9, 13, 24]. Some authors reported that large convexity Virchow-Robin spaces were significantly associated with DM1 and negatively correlated with the duration of disease, which might precede the appearance of WMLs and brain atrophy as one of the initial features of white matter involvement in the disease [8]. It might be argued that WMLs detected by MRI coincide with diffuse or perivascular loss of myelin associated with vascular hyalinization and enlarged perivascular spaces, which we observed in this study. In addition to MRI findings, cerebral hypoperfusion detected by single photon emission computed tomography and cerebral hypometabolism detected by positron emission tomography have also been reported in DM1 patients [1, 12, 20]. Taken together, it is considered that instances of état criblé in the cerebral white matter are a morphological change specific to DM1 brains, and likely responsible for the subcortical dementia associated with DM1. The pathogenetic mechanisms of WML are uncertain, and further studies on the vascular changes are necessary.

Recent studies have suggested that both the myopathy and CNS symptoms seen in DM1 reflect a toxic gain-of-function due to mutant RNA [5–7, 10, 11, 14, 15, 17–19, 22,

31–33]. In those studies, the CUG repeats in the mutant transcript were highly expanded but the portion of the mRNA encoding DMPK remained intact. CUG-expanded transcripts were retained in cell nuclei as focal inclusions. The accumulation of mutant RNA in the neuronal nucleus compromises the regulation of alternative splicing for a subset of neuronal transcripts and may also disrupt the regulation of transcription in general. Dhaenens *et al.* showed that adult DM1 brain exhibits overexpression of MBNL1 fetal isoforms and modified splicing of tau pre mRNA, resulting in the aggregation of tau isoforms that lack exon 2/3 encoded sequences [7]. Examination of post-mortem DM1 brain tissue by fluorescence *in situ* hybridization indicated that the mutant DMPK mRNA, with its expanded CUG repeats in the 3'-untranslated region, was widely expressed in cortical and subcortical neurons [15]. The mutant transcripts accumulated in discrete foci within the neuronal nuclei. Proteins in the muscleblind family (MBL1, MBL2) were recruited into the RNA foci and depleted elsewhere in the nucleoplasm. In parallel, a subset of neuronal pre-mRNAs, including tau, NMDA NR1 receptor, and amyloid precursor protein, showed abnormal regulation of alternative splicing. This report showed widely distributed RNA foci in the neurons from cerebral cortices, hippocampus, dentate gyrus, thalamus, substantia nigra and brainstem tegmentum, and less intensely in oligodendrocytes of the subcortical white matter and corpus callosum. The exception was the cerebellar cortex, where small foci were detected in some Purkinje cells, but not in neurons in the granular layer. This might be compatible with the findings of present study showing that CTG repeats were smaller in the cerebellar cortex in DM1 cases, because the dominant cellular component of cerebellar cortex is granule cells. Further study will be needed to clarify this issue.

The expansion of the CTG repeats showed considerable homogeneity in all of the brain regions examined, except for the cerebellar cortex in the DM1 cases. On the other hand, the pathological changes were more heterogeneous, including ischemic changes of the white matter, tauopathy in the mesolimbic region, as well as Lewy bodies and NFT in the brainstem. The length of the CTG repeats was not parallel to the morphological changes, which suggested that the pathomechanisms of DM1 might be more complex and multifactorial rather than a toxic gain-of-function due to mutant RNA.

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