Cerebellum
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ORIGINAL PAPER

A Majority of FXTAS Cases Present with Intranuclear Inclusions Within Purkinje Cells

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Abstract Fragile X-associated tremor/ataxia syndrome (FXTAS) is a progressive neurodegenerative disorder that affects carriers of a *FMR1* premutation. Symptoms include cerebellar ataxia, tremor, and cognitive deficits. The most characteristic pathology of FXTAS is the presence of eosinophilic ubiquitin-positive intranuclear inclusions in neurons and astrocytes throughout the nervous system and non-nervous tissues. Inclusions are present in neurons throughout the brain but are widely believed not to be present in the Purkinje cells (PCs) of the cerebellum. However, we analyzed 26 postmortem cases of FXTAS and demonstrated that 65 % of cases presented with inclusions within PCs of the cerebellum. We determined that the presence or absence of inclusions in PCs is correlated with age and that those cases with PC inclusions were overall 11 years older than those with no PC inclusions. Half of the cases with PCs with inclusions presented with twin nuclear inclusions. This novel finding demonstrating the presence of inclusions within PCs provides an insight into the understanding of the FXTAS motor symptoms and provides a novel target for the development of therapeutic strategies.

Keywords FXTAS · FRMP · Ubiquitin · Purkinje cells · Cerebellum

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Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a progressive neurodegenerative disorder that affects carriers of a *FMR1* premutation. Premutation alleles of 55–200 CGG repeats translate into a two to eight fold increase in *FMR1* mRNA, inducing a toxic RNA gain of function. Symptoms include cerebellar ataxia, tremor, cognitive deficits, peripheral neuropathy, autonomic dysfunction, and psychiatric involvement [1]. The most characteristic pathology of FXTAS is the presence of eosinophilic ubiquitin-positive intranuclear inclusions, which contain *FMR1* mRNA as well as numerous proteins [2]. These inclusions accumulate in neurons and astrocytes throughout the brain, in the autonomic nervous system, and in non-nervous tissues [3–6]. In the diagnostic criteria of FXTAS, the presence of intranuclear inclusions is a major postmortem criterion [7, 8]. Inclusions are present in neurons throughout the brain but are widely believed to be absent from the Purkinje cells (PCs) of the cerebellum [9]. However, magnetic resonance imaging (MRI) demonstrated moderate cerebellar atrophy and white matter changes in FXTAS patients, accompanied by histopathological findings of PC loss and Bergmann gliosis [4, 5, 10]. Inclusions have been described in both astrocytes and neurons of the cerebellar cortex and dentate nucleus of FXTAS subjects [4, 5].

Materials and Methods

Sample Collection Brain samples from 26 FXTAS subjects were obtained from the FXTAS brain repository at the School of Medicine, University of California Davis (Table 1). All tissue samples were obtained through consented autopsies with institutional review board approval. Control tissues were obtained from subjects who did not have any significant neurological history. All samples were fixed in 10 % buffered formalin.

Table 1 List of the 26 FXTAS subjects included in this study with their age, gender, and number of CGG repetitions

FXTAS Case	Age	Gender	CGGs
1	82	M	91
2	85	M	86
3	76	F	87
4	81	M	92
5	66	M	100
6	78	M	106
7	78	M	73
8	80	F	63
9	79	M	88
10	78	M	77
11	66	F	NK
12	72	M	79
13	76	M	87
14	68	M	112
15	79	F	94
16	85	M	85
17	58	M	97
18	69	M	98
19	34	M	73
20	84	F	59
21	54	M	88
22	70	M	113
23	52	F	75
24	69	M	96
25	66	M	105
26	88	M	NK

M male, *F* female, *NK* not known

Immunostaining Antigen retrieval was performed with 1× diva solution (heat-induced epitope retrieval [HIER] buffer; Biocare Medical, CA, US) at 80–110 °C (6 min), followed by pretreatment for 7 min with 3 % hydrogen peroxide (Fisher, CA, US), 10 % donkey serum, and 0.025 % Tween 20 (Fisher) for 1 h. Tissue samples were subsequently incubated in rabbit anti-ubiquitin primary antibody (1:200, Dako, Denmark) diluted in 10 % donkey serum, and 0.0025 % Tween 20 (4 °C, 15 h). After rinsing, samples were incubated in the donkey anti-rabbit secondary antibody diluted as above, for 1 h at room temperature (1:100, Jackson Immunoresearch, PA, US). Samples were developed using the DAB substrate kit (Vector Laboratories, CA, US), dehydrated with ethanol, cleared with xylene, and mounted with Permount® (Fisherbrand). We performed PBS rinsing between each step of the protocol.

Quantification of Inclusions To determine the number of PCs per length of PC layer, eight randomly selected microscopic views of the cerebellar cortex, including the PC layer, were measured for each case. The numbers of PCs containing or lacking nuclear inclusions were scored using a light microscope with a 100× objective, using BZ analyzer II software (Keyence BZ 9000 microscope) to determine the length of the PC layer analyzed in each section.

Statistical Analysis The number of FXTAS and control cases containing PC inclusions was compared using *t* tests. The influence of age and other variables on the percentage of PC inclusions was assessed using multiple-regression modeling, with regression models chosen using stepwise selection.

Results

Purkinje cells within the cerebellum were analyzed for 26 cases diagnosed with FXTAS. Subjects ranged from 52 to 87 years of age (mean 71.64 ± 2.2 years) and included 22 males and 6 females (Table 1). Three control subjects (males, mean age 73.5 years) were also analyzed. Initial immunostaining was performed with an antibody against ubiquitin using at least two tissue sections per case: one from the cerebral cortex and one from the cerebellar cortex. Each section was examined for the presence of intranuclear inclusions. No inclusions were detected in any of the three control cases. All cases presented with inclusions in the cerebral and cerebellar cortices. All 26 cases also presented with inclusions within astrocytes, but only 24 cases presented with inclusions within cortical neurons (Fig. 1a). Of the 26 FXTAS cases, 17 presented with inclusions within PC cells (65 %) and 9 did not (Figs. 1 and 2). The two cases without cortical neuronal inclusions did not present inclusions in cerebellar PCs. Inclusions in PCs were present within the nucleus and most often located near the nucleolus. Inclusions size varied from cell to cell and between cases. We calculated the ratio between the size of inclusions and the area occupied by the nucleus and found that inclusions occupied 5 ± 0.5 % of the nuclear area. Other cerebellar cell types with inclusions included granular cells, molecular cells, and astrocytes, presenting the molecular layer with a higher density of inclusions (Fig. 1b). The highest density of inclusions was present within the PC layer and the area surrounding the PC layer (Fig. 1b). Cells within the PC layer containing inclusions included basket cells, Golgi cells, and astrocytes. There also appeared to be a positive correlation between the number of neurons with inclusions in the cortex and the cerebellum, with those cases lacking inclusions in the PC layer having among the lowest counts in cerebral cortex.

The presence of inclusions in PCs was correlated with subject age, with those individuals with PC inclusions having a mean age 11 years older than those with no PC inclusions (with 75.81 ± 1.5 years; without 64.2 ± 2.9 years; $P = 0.008$). Furthermore, the number of PCs with inclusions (17 cases) was positively correlated with age (R square = 0.32; $P = 0.05$, Fig. 3a). For the 12/17 cases for which the number of CGG repeats is known (Table 1), there was a non-

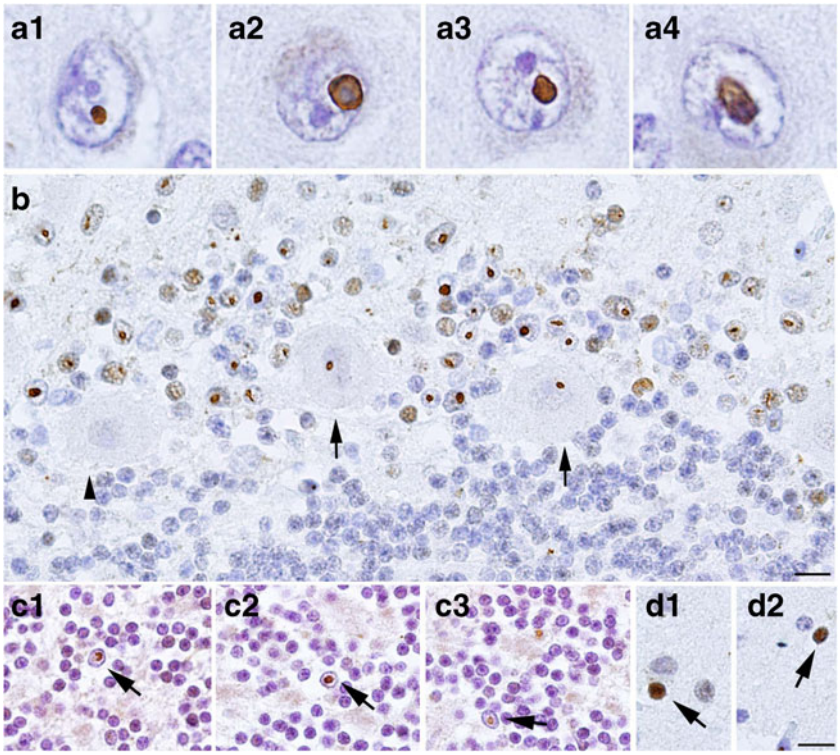

Fig. 1 **a** Ubiquitin inclusions in pyramidal cells on the prefrontal cortex in FXTAS; **b** PC layer containing Purkinje cells (PCs) and other neuronal types with ubiquitin-positive inclusions in FXTAS. PCs containing inclusions are indicated with an *arrow* and PCs with no inclusions with an *arrowhead*. **c** Granular layer of the cerebellum with a small number of cells containing inclusions (*arrows*). **d** Molecular layer of the cerebellum with a small number of cells containing inclusions (*arrowhead*)

significant trend toward lower number of PC inclusions with increasing number of repeats ($P=0.1$, Fig. 3b). There was also a non-significant correlation between the presence of PCs with inclusions and gender, although only six females were included in this study.

For the 17 cases with PC inclusions, there was an average of 9.1 ± 0.9 % of PC cells with inclusions (Fig. 2a). The number of PC cells containing inclusions was comparable to the number of inclusions previously reported in other cell types such as those in the cerebral cortex [5, 11]. There was also a reduction in the number of PC cells in FXTAS cerebellum samples when compared to the number in the control cerebellum, consistent with earlier reports [4]. The number of PCs in a given length of the PC layer for those cases without PC inclusions (4.64 ± 0.46 cells/mm) was slightly greater than the number of PCs for the cases with inclusions (3.89 ± 0.24 cells/mm), although this trend was not significant ($P=0.1$), nor was any significant correlation between the number of PC cells per millimeter and subject age ($P=0.2$). There was neither a correlation between the number of PCs and the number of PCs containing inclusions ($P = 0.3$; Fig. 3c). Surprisingly, in more than half of the cases (9/17), some nuclei possessed twin adjacent nuclear inclusions (Fig. 2b). Of these nine cases, PC cells with two inclusions comprised 16.7 ± 2.7 of all PCs with inclusions. These inclusions had a similar size, and they were always one adjacent to the other and near or in contact with the nucleolus.

Overall, we found that more than half of the FXTAS cases (65 %) present with nuclear inclusions, with some PCs possessing twin nuclear inclusions. Cases with PC inclusions came from older subjects compared to cases with no inclusions, and the number of PCs with inclusions increased with age. We need to take into account that, due to the reduced number of PCs that are present in each slide, we may be underestimating the number of cases with inclusions in PCs. We did not find a significant correlation between the number of PCs with inclusions and the number of CGG repeats, between the number of PCs with inclusions and the total number of PCs, or between the size of the inclusions and age of the subjects.

Discussion

The first description of the FXTAS cerebellum by Greco and colleagues reported a decrease in the number of PCs, the presence of PC axonal torpedoes, and Bergmann gliosis; however, no intranuclear inclusions were observed in PCs [5]. In the current study, 26 cases of FXTAS were examined, with more than half of these cases (65 %) presenting with ubiquitin-positive intranuclear inclusions within PCs. The loss or dysregulation of PCs is known to be central to the pathogenesis of tremor [12], acting through their influence on adjacent neuronal populations, giving rise to an aberrant cerebellar circuitry [12, 13]. The presence of inclusions in PCs is a further reflection of PC dysfunction prior to PC loss, with such altered function likely influencing cerebellar-cortical interactions and resulting in both the motor and cognitive impairments reported in FXTAS. The observation of inclusions in a significant fraction of PCs

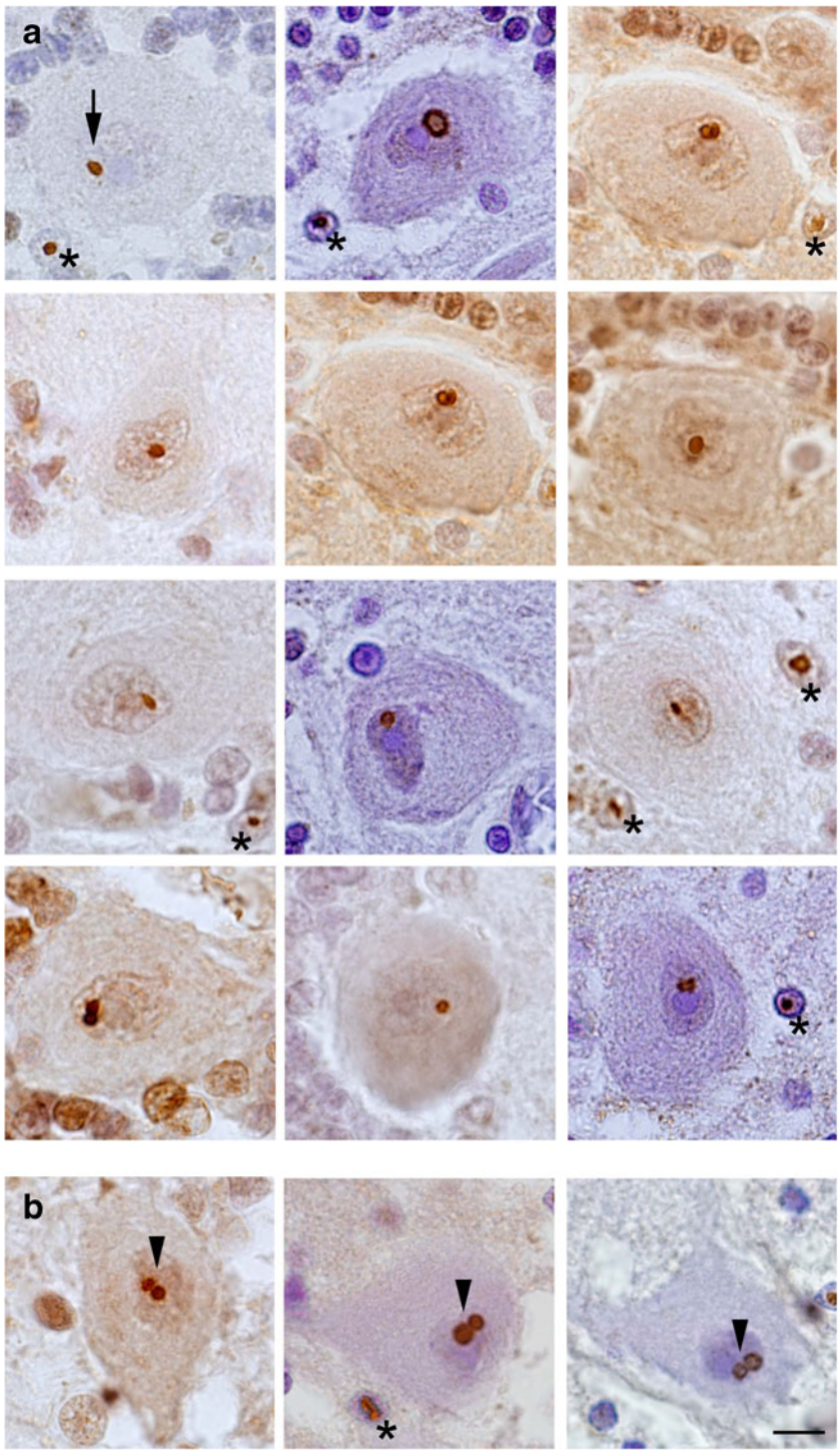

Fig. 2 PCs containing inclusions in the nucleus in tissue from FXTAS cases. **a** PCs in the cerebellum with a single nuclear inclusion. **b** PCs in the cerebellum with two nuclear inclusions. An inclusion is indicated with an *arrow* in **a**. Twin inclusions in **b** are indicated with *arrowheads*. Non-PC cells containing inclusions are indicated with an asterisk

differs from previous observations of "rare" inclusions in PCs and is likely due to the use of ubiquitin staining in the current instance, which renders inclusions more apparent than with the prior use of H&E. In addition, few laboratories have previously examined FXTAS cerebellar tissue, and they only analyzed a small number of cases [5, 6]. Our novel finding demonstrating the presence of inclusions within PCs, including the novel finding of twin inclusions, may provide a target for the development of novel therapeutic treatments mitigating the motor impairments resulting from FXTAS.

It has been speculated that the presence of neuronal inclusions in human FXTAS is correlated with age. The youngest case of human FXTAS (30s) ever analyzed presented with a number and size of inclusions that were within the normal range for older patients [11]. Perhaps the presence of

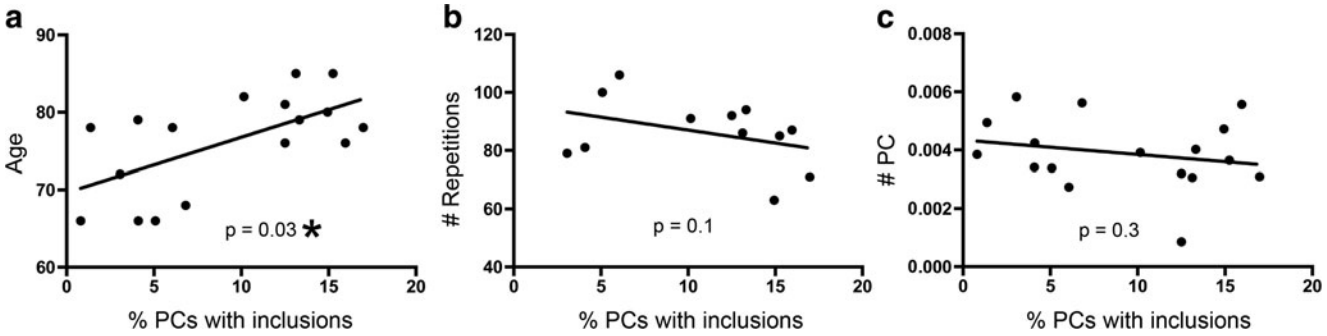

Fig. 3 **a** The percentage of PCs containing inclusions was positively correlated with age ($P = 0.03$). **b** The number of PCs with inclusions decreased as the number of repetitions increased; however, this correlation was not statistically significant ($P = 0.1$). **c** Non-significant correlation between the number of PCs and the number of PCs with inclusions ($P = 0.3$)

inclusions in this young patient was exacerbated by drug addiction, which has been shown to hasten the progression of FXTAS [14]. Therefore, this case may not be representative of the average case of FXTAS. Aside from this case, there are not any other reported case of human FXTAS obtained from subjects under the age of 55. In this report, we demonstrate that cases with inclusions in PCs were on average 11 years older than those with no inclusions, supporting the hypothesis that the presence of neuronal inclusions in human FXTAS subjects is correlated with age. It has been shown that "young" CGG knock-in mice (12–25 weeks of age) contain only a small number of neuronal intranuclear inclusions, while neurons containing intranuclear inclusions were found within most brain regions of "older" mice (52+ weeks). PC inclusions were also rarely observed in the original FXTAS mouse models [9, 15]. However, the most recent mouse models express higher levels of mutant CGG RNA and show higher numbers of inclusions in PC cells [16, 17], compared to the original KI models [9]. Overall, data obtained from human and mice subjects is in agreement with the hypothesis that the presence and number of inclusions is positively correlated with increasing age.

We previously observed that inclusions appear in higher numbers of astrocytes than neurons and postulated that intranuclear inclusions may form first in astrocytes and later in neurons [11]. We now propose than some types of neurons are more susceptible than others to the formation of nuclear inclusions. In this study, we demonstrated that cases with cortical neuron inclusions always contain inclusions in neurons (non-PC) of the cerebellum, but only 65 % contain inclusions in PCs. In addition, the fact that PC inclusions are only present in 65 % of FXTAS cases could explain why PC inclusions including twin PC inclusions have not been described before. We also showed that FXTAS cases with inclusions restricted to astrocytes in the cortex similarly had inclusions restricted to astrocytes in the cerebellum. These data corroborates our previous hypothesis stating that inclusions appear first in astrocytes and later in neurons, and supports our novel hypothesis stating that some neuronal types are more susceptible to the formation of inclusions than others. Accordingly, we show here that cortical pyramidal neurons and non-PC neurons in the cerebellum are more susceptible to the formation of inclusions than PC, and therefore inclusions may appear in these areas earlier in time.

Conclusions

Overall, we have demonstrated the presence of inclusions in PCs, a positive correlation between age and the number of PCs with inclusions, and a lower susceptibility of PCs to accumulate inclusions as compared to other neuronal types. Also, we demonstrated the occurrence of twin nuclear inclusions within the neuronal nuclei in FXTAS.

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Compliance with Ethical Standards All tissue samples were obtained through consented autopsies with institutional review board approval.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Hagerman PJ, Hagerman RJ. Fragile X-associated tremor/ataxia syndrome. Ann N Y Acad Sci. 2015;1338:58–70.
2. Iwahashi CK, Yasui DH, An HJ, Greco CM, Tassone F, Nannen K, et al. Protein composition of the intranuclear inclusions of FXTAS. Brain J Neurol. 2006;129(Pt 1):256–71.
3. Gokden M, Al-Hinti JT, Harik SI. Peripheral nervous system pathology in fragile X tremor/ataxia syndrome (FXTAS). Neuropathol Off J Jpn Soc Neuropathol. 2009;29(3):280–4.

4. Greco CM, Berman RF, Martin RM, Tassone F, Schwartz PH, Chang A, et al. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). Brain J Neurol. 2006;129(Pt 1):243–55.
5. Greco CM, Hagerman RJ, Tassone F, Chudley AE, Del Bigio MR, Jacquemont S, et al. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. Brain J Neurol. 2002;125(Pt 8):1760–71.
6. Louis ED, Vonsattel JP, Honig LS, Lawton A, Moskowitz C, Ford B, et al. Essential tremor associated with pathologic changes in the cerebellum. Arch Neurol. 2006;63(8):1189–93.
7. Hagerman PJ, Hagerman RJ. The fragile-X premutation: a maturing perspective. Am J Hum Genet. 2004;74(5):805–16.
8. Jacquemont S, Hagerman RJ, Leehey MA, Hall DA, Levine RA, Brunberg JA, et al. Penetrance of the fragile X-associated tremor/ataxia syndrome in a premutation carrier population. JAMA. 2004;291(4):460–9.
9. Willemsen R, Hoogeveen-Westerveld M, Reis S, Holstege J, Severijnen LA, Nieuwenhuizen IM, et al. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum Mol Genet. 2003;12(9):949–59.
10. Louis ED. Essential tremor. Lancet Neurol. 2005;4(2):100–10.
11. Martinez-Cerdeno V, Lechpammer M, Lott A, Schneider A, Hagerman R. Fragile X-associated tremor/ataxia syndrome in a man in his 30s. JAMA Neurol. 2015;72(9):1070–3.
12. Louis ED, Faust PL. Purkinje cell loss in essential tremor. Mov Dis Off J Mov Dis Soc. 2014;29(10):1329–30.
13. Babij R, Lee M, Cortes E, Vonsattel JP, Faust PL, Louis ED. Purkinje cell axonal anatomy: quantifying morphometric changes in essential tremor versus control brains. Brain J Neurol. 2013;136(Pt 10):3051–61.
14. Muzar Z, Lozano R, Schneider A, Adams PE, Faradz SM, Tassone F, et al. Methadone use in a male with the FMRI premutation and FXTAS. Am J Med Genet A. 2015;167(6):1354–9.
15. Hunsaker MR, Wenzel HJ, Willemsen R, Berman RF. Progressive spatial processing deficits in a mouse model of the fragile X premutation. Behav Neurosci. 2009;123(6):1315–24.
16. Hashem V, Galloway JN, Mori M, Willemsen R, Oostra BA, Paylor R, et al. Ectopic expression of CGG containing mRNA is neurotoxic in mammals. Hum Mol Genet. 2009;18(13):2443–51.
17. Hukema RK, Buijsen RA, Schonewille M, Raske C, Severijnen LA, Nieuwenhuizen-Bakker I, et al. Reversibility of neuropathology and motor deficits in an inducible mouse model for FXTAS. Hum Mol Genet. 2015;24(17):4948–57.