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Premature centromere division of the X chromosome in neurons in Alzheimer's disease

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Abstract

Premature centromere division (PCD) represents a loss of control over the sequential separation and segregation of chromosome centromeres. Although first described in aging women, PCD on the X chromosome (PCD,X) is markedly elevated in peripheral blood lymphocytes of individuals suffering from Alzheimer disease (AD). The present study evaluated PCD,X, using a fluorescent *in situ* hybridization method, in interphase nuclei of frontal cerebral cortex neurons from sporadic AD patients and age-matched controls. The average frequency of PCD,X in AD patients (8.60 ± 1.20%) was almost three times higher ($p < 0.01$) than in the control group (2.96 ± 1.20). However, consistent with previous studies, no mitotic cells were found in neurons in either AD or control brain, suggesting an intrinsic inability of post-mitotic neurons to divide. In view of the fact that it has been well-documented that neurons in AD can re-enter into the cell division cycle, the findings presented here of increased PCD advance the hypothesis that deregulation of the cell cycle may contribute to neuronal degeneration and subsequent cognitive deficits in AD.

Keywords: Alzheimer's disease, cell cycle, fluorescent *in situ* hybridization, frontal cerebral cortex, premature centromere division.

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Alzheimer disease (AD) is the most common cause of senile dementia (Smith 1998) and represents a complex and progressive neurodegenerative disorder of the human brain. The great majority of patients are classified as sporadic (90–95%) with predominating degenerative brain disorder in old age (Schellenberg 1995; Cruts *et al.* 1996; Blacker and Tanzi 1998; Hoyer 2006). The cause of sporadic AD is still unknown, however, age, or more specifically age-related alterations, are likely key. In relation to the latter regard, among the first genetic lesions found to be specifically associated with aging were aberrations in chromosome number and/or structure, primarily the sex chromosomes (Wojda *et al.* 2006).

One chromosomal alteration, premature centromere division (PCD), a phenomenon representing the loss of control over the sequential separation and segregation of chromosome centromeres, is characterized by distinctive and easily recognizable separation of chromatids occurring earlier than usual (Fitzgerald *et al.* 1986; Mehes and Buhler 1995; Spremo-Potparević *et al.* 2000; Spremo-Potparevic *et al.* 2004). Conditions which express PCD include Robert's syndrome, Down's syndrome, neoplasias, and exposure to toxic chemicals (Major *et al.* 1999) and PCD can affect many chromosomes. PCD, as a potential cause of improper chromosome segregation, is one of the genetic mechanisms related to increased aneuploidy, and many studies have shown a significant increase in chromosome loss in peripheral blood lymphocytes and a high percentage of PCD, in both men and women of advanced age (Ward *et al.* 1979; Migliore *et al.* 1999; Wojda *et al.* 2006; Zivkovic *et al.* 2006). The most frequently investigated chromosomes in AD patients are the sex chromosomes because of their frequent aneuploidy rates

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Abbreviations used: AD, Alzheimer's disease; DAPI, diamindino phenylindole; FISH, fluorescent *in situ* hybridization; H&E, hematoxylin and eosin; PCD, premature centromere division; PCD,X, premature centromere division on the X chromosome.

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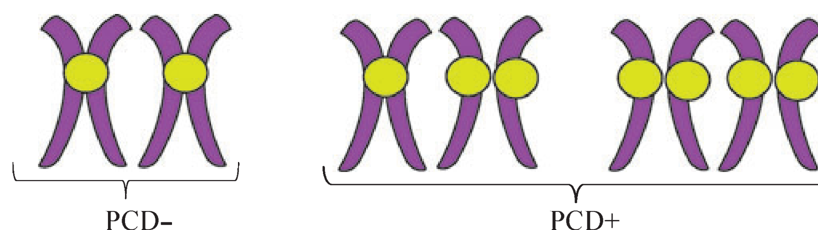
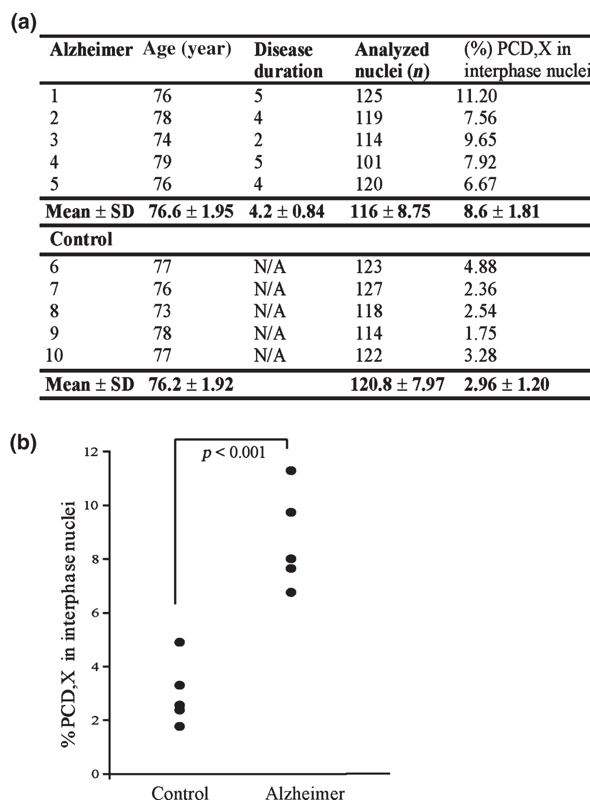


Fig. 1 Schematic representation of FISH visualization of PCD using a specific probe to the centromeric region of the X chromosomes, DXZα (●). Normal cells (PCD–) show two distinct signals, whereas premature centromere division (PCD+) results in three or four signals.

which are correlated with age (Fitzgerald and McEwan 1977; Bajnoczky and Mehes 1988; Mosch *et al.* 2007).

Ectopic neuronal cell cycle re-entry has been well-documented in the brain of AD patients as well as in mouse models of AD (McShea *et al.* 1997, 2007; Herrup and Arendt 2002; Yang *et al.* 2006; Arendt and Bruckner 2007). In addition, shortened telomeres (Franco *et al.* 2006), neuronal binucleation events (Zhu *et al.* 2008), and increased aneuploidy (Geller and Potter 1999) provide further evidence for chromosomal instability in AD. While we have previously shown that the X chromosome is susceptible to the phenomenon of PCD in peripheral blood lymphocytes in AD patients (Spremo-Potparevic *et al.* 2004), whether this is also evident in the brain is unknown. As highly differentiated cells, neurons are not thought to undergo cell division. Therefore, in female cerebral cortex, it was expected to obtain two dot-like signals in each nucleus, i.e., one dot per each centromere of X chromosome. However, if PCD of X chromosome is present, bipartite signals will appear in one or both X centromeres, resulting in 3 or 4 dot signals per nucleus (Fig. 1).



(a)

Alzheimer	Age (year)	Disease duration	Analyzed nuclei (*n*)	(%) PCD,X in interphase nuclei
1	76	5	125	11.20
2	78	4	119	7.56
3	74	2	114	9.65
4	79	5	101	7.92
5	76	4	120	6.67
Mean ± SD	**76.6 ± 1.95**	**4.2 ± 0.84**	**116 ± 8.75**	**8.6 ± 1.81**
Control				
6	77	N/A	123	4.88
7	76	N/A	127	2.36
8	73	N/A	118	2.54
9	78	N/A	114	1.75
10	77	N/A	122	3.28
Mean ± SD	**76.2 ± 1.92**		**120.8 ± 7.97**	**2.96 ± 1.20**

Fig. 2 (a) Clinical characteristics and quantification of PCD,X in five AD female patients and five female controls. In all patients, as well as in controls, at least 100 interphase nuclei from frontal cerebral cortex neurons were analyzed. (b) The percentage of nuclei displaying PCD,X is significantly higher in the AD cases analyzed when compared with age-matched controls ($p < 0.01$; Mann–Whitney).

Materials and methods

Subjects

Frontal cortical brain tissue was collected at autopsy from five sporadic female AD patients (ages 74–79 years), and five age-matched female controls (ages 73–78 years) following approved protocols and with written family consent. Figure 2 includes a further description of the cases used. In all cases, a pathohistological cross section of tissue from the frontal cerebral cortex was used for diagnosis according to established criteria.

Slide preparation

Brain tissue was routinely formalin fixed, paraffin embedded, and sectioned at 4 μm. Slides were dewaxed in xylol for 30 min, dehydrated in absolute ethanol for 5 min and air-dried at 22°C. Slides were than treated with 0.2 N HCl for 20 min, deionized H_2O for 3 min and 2x SSC (1 × SSC: 0.15 M NaCl and 0.15 M sodium citrate pH 7.0) 1 and 10 min, respectively. Protease treatment was performed with pepsin (4 mg/mL) for 10 min at 37°C, followed by washing in 2 × SSC for 10 min. The slides were fixed in 10% formalin for 10 min, washed in 2 × SSC for 10 min and air-dried. Adjacent serial sections from each case were stained with hematoxylin and eosin (H&E). Cellular size and morphology, as well as nuclear size, were used to confirm the location of the pyramidal neurons in the adjacent sections for application of fluorescent *in situ* hybridization (FISH) for analysis of PCD on the X chromosome (PCD,X) in the neuronal nuclei.

Application of FISH for analysis of PCD on the X chromosome in interphase nuclei

The probe specific for the X centromeric α repetitive sequences, locus DXZ (DXZα) was used (Cooke and Hindley 1979). The probe was labeled with biotin-16 dUTP in nick-translation reaction using a bio-nick labeling system (Gibco-BRL, Paisley, UK). For each slide, 200 ng of the probe was mixed in 16 μL of hybridization buffer,

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consisting of 50% formamide, 10% dextran-sulfate, 1% sodium dodecyl sulfate, 1× Denhardt's, 2 × SSC and 0.04 M Sodium phosphate pH 7.0, denatured for 10 min at 68°C, and applied to slides.

Hybridization and detection

After overnight hybridization at 37°C, detection was performed essentially as previously described (Verbić *et al.* 2000). Briefly, the biotinylated probe was detected with Fluorescein Avidin DCS (Vector Laboratories, Peterborough, UK) and Biotinylated anti-avidin D (Vector Laboratories). For amplification of signals, three layers of Fluorescein Avidin DCS were applied. The slides were mounted in 0.4 μg/mL diamindino pheniylindole (DAPI) and 0.4 μg/mL propidium iodide, counterstained in Vectashield Antifade Buffer (Vector Laboratories). The slides, blinded as to diagnosis, were viewed under an Olympus BX 50 (Olympus Optical Co., GmBH, Hamburg, Germany) epifluorescent microscope with an appropriate filter combination for detecting fluorescein (Spectrum Green) and DAPI and analyzed using Cytovision 3.1 (Applied Imaging Corporation, Santa Clara, CA, USA).

Centromere analysis

FISH analysis of PCD on the X chromosome was analyzed in interphase nuclei from neurons of the frontal cerebral cortex using the FISH centromere assay (Fig. 1). In addition to nuclear size, the location of all neurons within each field was determined by direct comparison with the adjacent H&E stained sections. Only nuclei contained within neurons were evaluated by FISH on adjacent sections. In both groups, AD patients and controls, at least 100 interphase nuclei were analyzed per patient.

Statistical analysis

Statistical analysis was performed by Mann–Whitney test using the Statgraph 4.2 software.

Results

After analyzing neuronal nuclei from all AD and control samples, the average frequency of PCD,X in AD group was found to be 8.60 ± 1.81%, whereas, in a group of five age-matched female controls, the average frequency of PCD,X was found to be 2.96 ± 1.20% (Fig. 2). In both AD and control cases, in all analyzed nuclei, PCD,X was present on only one of two X chromosomes, resulting in three dots. No cell showed four dots. The bipartite signal of X chromosome where PCD was verified was scored as PCD+ (Fig. 3a and c), while the X chromosome where PCD was not present was scored as PCD- (Fig. 3a and b). No mitotic neuronal cells were found in either AD or control brain (Fig. 3a).

The presented results show almost three times higher incidence ($p < 0.01$) of PCD,X in the neurons of the frontal lobe cortex in AD patients than in age-matched controls.

Discussion

A long standing dogma in neuroscience is that neurons in the adult CNS are in the terminal stage of differentiation. However, over the last decade, accumulating evidence indicates that neurons may be capable of re-entering the cell division cycle under pathological conditions (Arendt *et al.* 1995, 1996, 1998; Vincent *et al.* 1996, 1997; McShea *et al.* 1997, 2007; Nagy and Esiri 1997; Nagy *et al.* 1998, 2000; Raina *et al.* 2004) and in rare instances display binucleation (Zhu *et al.* 2008). This capability likely depends on extra-cellular signals, i.e., on the balance between mitogenic stimuli and differentiating factors (Hengst and Reed 1996; Lavoie *et al.* 1996; McShea *et al.* 1999; Nagy *et al.* 2000; Zhu *et al.* 2004), and various mitogenic signals cause cell cycle re-entry of neurons in the CNS of AD patients, including loss of synaptic connections (Nagy *et al.* 2000) and cerebral hypoxia (Smith *et al.* 1999). Furthermore, there is evidence that amyloid-β protein is mitogenic in cultured neurons (Schubert *et al.* 1989; McDonald *et al.* 1998; Pyo *et al.* 1998). Interestingly, AD affects twice as many women as men, indicating that hormonal factors may also play an important role in the loss of the differentiated phenotype in neurons (Bernal and Nunez 1995; Singer *et al.* 1998; Denver *et al.* 1999; Perez-Juste and Aranda 1999; Pike 1999; Webber *et al.* 2006, 2007). Additionally, genetic influences are also involved since mutations in the presenilin 1 gene, resulting in abnormal presenilin function, have been found to lead to chromosome missegregation (Boeras *et al.* 2008). The present study, by employing a novel method that enables a direct visual proof of centromere division, further suggests that neurons of cerebral cortex begin to re-enter into the cell division cycle.

Using the FISH method, the presence of PCD,X was verified in frontal cerebral cortex cells of all analyzed individuals. For this study, female subjects were analyzed, because in earlier studies, PCD, X was found in both male and in female lymphocytes, yet was only significantly different between AD and control in female population (Spremo-Potparevic *et al.* 2004). In sporadic AD patients, the frequency of PCD,X was significantly higher than in control group. To generate PCD+ signals, the cell must first transit from G_0 to G_1 phase of the cell cycle, complete DNA replication (S phase) and go further to G_2 phase. Only a chromosome that has completed replication can generate two signals from one centromere, i.e., each chromatid from chromosome with PCD behaves like a separate chromosome (Fig. 1).

Although our results corroborate DNA replication in the neurons of the frontal cerebral cortex (Mosch *et al.* 2007), in no cases were mitotic cells evident. Therefore, it is conceivable that neurons do not pass the G_2-M transition but rather, after G_2 phase, neurons may undergo cell death (Zhu *et al.* 1999). One mechanism that may drive cell death is the expression of the cell cycle-dependent kinase cdc2 which, when activated, promotes phosphorylation of BCL2-antagonist of cell death (Zhu *et al.* 1999; Konishi *et al.* 2002). Of note, cdc2 is expressed at higher levels in AD and is

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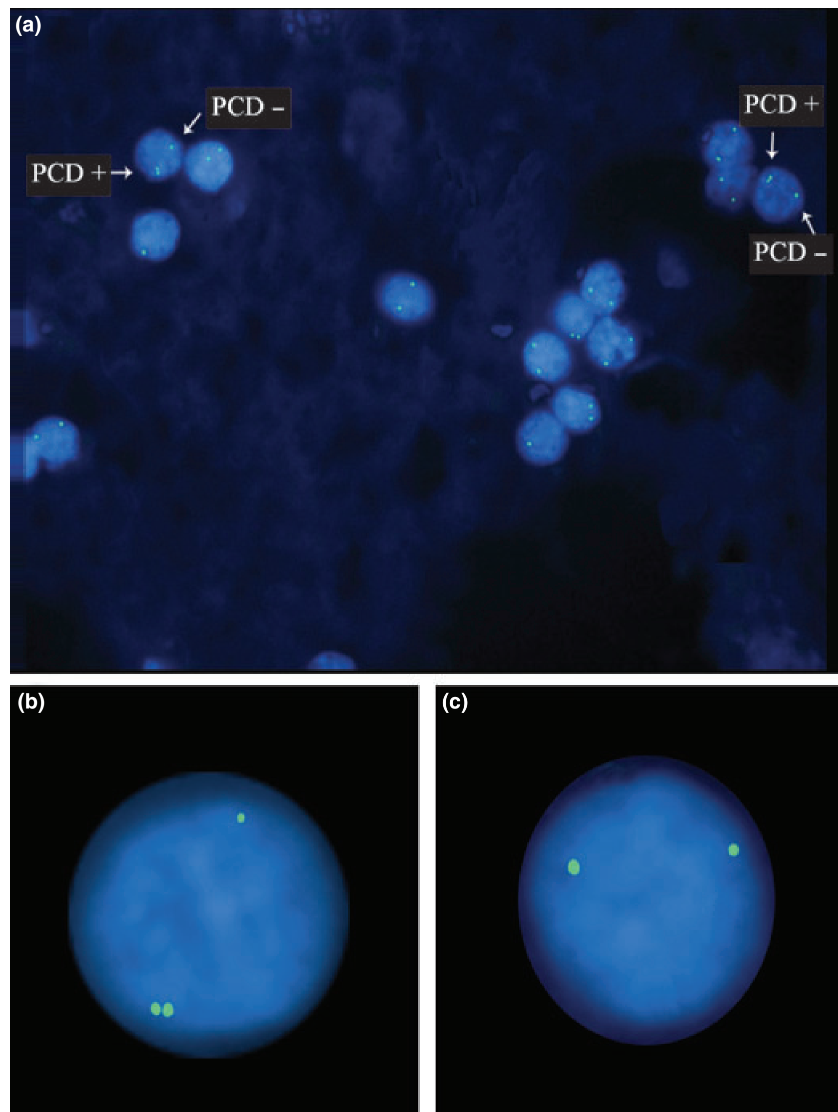

Fig. 3 Configurations of fluorescent hybridization signals identifying the X chromosome specific α satellite loci (DXZα) in interphase nuclei of neuronal cells of female AD patients. (a) Interphase nuclei of neurons from the frontal cerebral cortex of a female AD patient. Original magnification 1000 ×. Arrows show premature centromere division of one X chromosome (PCD+), and normal centromere of the other X chromosome in the same nucleus (PCD−). Other nuclei have two dot signals, one for each X chromosome, which represent normal centromeres of both X chromosomes (PCD−). At higher magnification (b), AD female patient nucleus with one dot like signal for one X chromosome (PCD−), and one bipartite signal for the other X chromosome (PCD+); (c) AD female patient nucleus with two dot like signals, each for one X chromosome (PCD−).

localized within glia and neurofibrillary tangles (Vincent *et al.* 1997).

One of the first results based on FISH method for the analysis of centromere regions of chromosomes 18 and 21 in hippocampal interphase nuclei, pointed to an ultimate cell death as a consequence of genetic misbalance caused by a 'supposed' tetraploid state of their genome (Yang *et al.* 2001). However, considering the data generated here, these findings may represent interphase PCD, also observable as two dot-like signals per each analyzed chromosome, rather than tetraploidy. Using another novel slide-based cytometry method, others suggested that some neurons in AD can pass through a functional interphase with a complete DNA replication (Mosch *et al.* 2007). Here we also demonstrated that only one of two X chromosomes generates PCD+ signals and the question arises which of the two X chromosomes undergoes PCD. Soon after the discovery of PCD,X in peripheral blood lymphocytes of elderly women, autoradiographic studies reveled that PCD predominantly occurs on a partially inactive X chromosome (Fitzgerald and McEwan 1977; Galloway and Buckton 1978; Abruzzo *et al.* 1985). Therefore, one significant consequence of inactivation of excess centromeres is a differential pattern of replication versus separation when compared to the active centromere. Inactivation destabilizes the time pattern of centromere replication between two X chromosomes, leading to genome instability, i.e., aneuploidy (Litmanovitch *et al.* 1998). Inactivated centromeres exhibit early replication and, interestingly, PCD (Litmanovitch *et al.* 1998). In fact, a dysregulated centromere segregation has been hypothesized as one pathway leading to the neurodegeneration in diseases such as AD (Bajic *et al.* 2008).

The interaction between cell cycle re-entry and other disease parameters such as oxidative stress (Barlow *et al.*

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1999; Nunomura *et al.* 1999) is likely critical in the development of the disease phenotype (Zhu *et al.* 2004). In this regard, it is of note that mutations associated with the familial forms of AD are not only associated with alterations in oxidative stress (Nunomura *et al.* 2004) but also cell cycle alterations (Prat *et al.* 2002).

In conclusion, the results of this work provide compelling evidence for re-entry into the cell cycle of cerebral cortical neurons leading to PCD in the interphase of the cell cycle immediately after replication. This pattern of genome instability can be viewed as a disorder of the hierarchical control of the sequence of centromere separation and segregation. Based on the findings of PCD in the neurons as well as peripheral blood lymphocytes (Spremo-Potparevic *et al.* 2004), PCD,X may be a possible cytogenetic biomarker in patients with AD.

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