Increased chromosome instability dramatically disrupts neural genome integrity and mediates cerebellar degeneration in the ataxia-telangiectasia brain

Ivan Y. Iourov1,2, Svetlana G. Vorsanova1,2, Thomas Liehr3, Alexei D. Kolotii2 and Yuri B. Yurov1,2*

1National Research Center of Mental Health, Russian Academy of Medical Sciences, Zagorodnoe sh. 2, Moscow 119152, Russia, 2Institute of Paediatrics and Paediatric Surgery, Rosmedtehnlogii, Moscow, Russia and 3Institute of Human Genetics and Anthropology, Jena, Germany

Received March 29, 2009; Revised and Accepted April 28, 2009

Ataxia telangiectasia (AT) is a chromosome instability (CIN) neurological syndrome arising from DNA damage response defects due to ATM gene mutations. The hallmark of AT is progressive cerebellar degeneration. However, the intrinsic cause of the neurodegeneration remains poorly understood. To highlight the relationship between CIN and neurodegeneration in AT, we monitored aneuploidy and interphase chromosome breaks (chromosomal biomarkers of genomic instability) in the normal and diseased brain. We observed a 2–3-fold increase of stochastic aneuploidy affecting different chromosomes in the cerebellum and the cerebrum of the AT brain. The global aneuploidization of the brain is, therefore, a new genetic phenomenon featuring AT. Degenerating cerebellum in AT was remarkably featured by a dramatic 5–20-fold increase of non-random DNA double-strand breaks and aneuploidy affecting chromosomes 14 and, to a lesser extend, chromosomes 7 and X. Novel recurrent chromosome hot spots associated with cerebellar degeneration were mapped within 14q12. In silico analysis has revealed that this genomic region contains two candidate genes (FOXG1B and NOVA1). The existence of non-random breaks disrupting specific chromosomal loci in neural cells with DNA repair deficiency supports the hypothesis that neuronal genome may undergo programmed somatic rearrangements. Investigating chromosome integrity in neural cells, we provide the first evidence that increased CIN can result into neurodegeneration, whereas it is generally assumed to be associated with cancer. Our data suggest that mosaic instability of somatic genome in cells of the central nervous system is more significant genetic factor predisposing to the brain pathology than previously recognized.

INTRODUCTION

Ataxia telangiectasia (AT; OMIM #208900) is an inherited neurodegenerative disease with onset in early childhood. Cerebellar ataxia, ocular telangiectasia, radiosensitivity, cancer predisposition, immunodeficiency, gonadal atrophy and premature aging characterize this presently incurable multi-system disorder. The cause of death in AT is ultimately linked to lung disease, cancer and/or neurological dysfunction. Mutations in the gene for AT (ATM—ataxia-telangiectasia, mutated) lead to the deregulation of DNA repair, genetic recombination, cell cycle checkpoints and apoptotic machinery. ATM is a protein kinase that is required for physiological processing of DNA double-strand breaks during somatic or meiotic recombination. The primary role of ATM is response to DNA damage and maintenance of genomic integrity. The hallmark of AT is progressive cerebellar degeneration. Paradoxically, other brain areas and tissues are less affected by progressive degeneration and atrophy (1,2).

DNA damage is an underlying cause of cellular dysfunction and death. Genomic instability due to DNA-damage and...
-repair defects can be considered as unified genetic mechanism leading to variety of neurological and neurodegenerative disorders, including AT (3). AT is a well-known chromosome instability (CIN) syndrome. The cytogenetic ‘marker’ of AT-associated CIN is the elevated level of non-random balanced chromosomal rearrangements in the immune system cells. Common breakpoints observed in lymphocytes are 14q11, 14q32, 7q35 and 7p14. These chromosomal regions contain genes encoding immunoglobulins and T-cell receptors, which have to be assembled from the multiple variable (V), joining (J) and diversity (D) gene segments present in the germ line loci. V(D)J recombination is the major source of the immense diversity of immune system cells (4). Thus, non-random breaks and rearrangements of chromosomes 14 and 7 reflect defective somatic DNA rearrangements by the V(D)J recombination mechanism during the development of immune system cells. However, the rearrangements present in lymphocytes are not usually observed in other cell types, i.e. fibroblasts (5). This suggests site- and chromosome-specific damage in different tissues of AT individuals to be responsible for pleiotropic effect of ATM deficiency (2,5).

To the best our knowledge, chromosomal instability in the cerebellum of the human AT brain (the target of progressive degeneration) has not been previously analyzed. It has been proposed that neuronal genome undergoes somatic recombination, which is responsible for the generation of neuronal diversity (6,7). Therefore, one might expect that ATM deficiency in developing and maturing neural system cells, affected by neurodegeneration, may be associated with increased genomic instability and aberrant somatic recombination similar to cells of immune system. The data concerning increased aneuploidy in cells of neuronal tissue has been obtained in genetic model organisms (Drosophila melanogaster and Mus musculus). Drosophila ATM homologue function is essential for normal development of the nervous system. Extensive, inappropriate apoptosis in atm-mutant neuronal tissues is associated with frequent mitotic defects and chromosomal abnormalities (8). In the Atm−/− mice, adult progenitor cells show an abnormally high rate of proliferation and genome instability (9). Atm-deficient mice display neurobehavioral deficits consistent with abnormal cerebellar function and possess a dramatic increase of the population of aneuploid neurons in the frontal cortex (10). Therefore, ATM is required to maintain genomic stability in proliferating neural progenitor cells. These data suggest that ATM gene deficiency may be associated with neural genome instability, including aneuploidy, and contribute to neurodegeneration not only in Atm−/− mice but also in the human AT brain. However, the substantial neuropathology including progressive cerebellar degeneration observed in humans lacks in Atm-null animals (11). Recently, increased aneuploidization of the human frontal cortex in AT has been demonstrated (12) using advanced single-cell molecular cytogenetic techniques (13,14). However, this brain area is not affected by progressive neuronal loss and atrophy in AT. Therefore, direct evaluation of CIN in the degenerating human cerebellum is required to delineate the ultimate reason for targeted neurodegeneration in AT. The present study was designed to determine whether CIN affects the cerebellum in the AT brain.

RESULTS
Aneuploidy in the normal human cerebellum
Using FISH with an extended panel of chromosome enumeration probes for 10 arbitrarily selected chromosomes: autosomes 1, 7, 8, 9, 11, 16, 17, 18 and sex chromosomes (X and Y), quantitative FISH (QFISH) and interphase chromosome-specific multicolor banding (ICS-MCB) probes for chromosomes 1, 7, 14, 21 and X, we assessed the rate of aneuploidy in the cerebellum and cerebral cortex of seven patients with AT and seven age- and sex-matched unaffected controls (Fig. 1). The results of aneuploidy scoring for the seven controls are shown in Figures 2A and C and 3A and B (FISH data) and 4A and C (MCB data). We found that aneuploidy frequency (losses + gains) vary in a wide range from 0 to 3% between chromosomes and individuals in the normal brain (Supplementary Material, Table S1). Aneuploidy was observed in both the neuronal population (NeuN-positive nuclei) and non-neuronal (NeuN-negative nuclei) with approximately similar frequency (data not shown). The mean frequency of stochastic aneuploidy per individual chromosome pair in the cerebrum and cerebellum was in the range of 0.3–0.9% for autosomes and 0.3–1.2% for sex chromosomes with the threshold levels between 0.6 and 3.7% (Fig. 3A and B). The ratio of chromosome losses and gains was approximately 2:1. No outliers of aneuploidy higher than the threshold level calculated for a definite chromosome pair was found among controls. The mean frequency of aneuploidy per ‘mean’ human chromosome pair (mean ± SD) was 0.6 ± 0.2% (95% CI 0.5–0.7%) for the cerebrum and 0.7 ± 0.2% (95% CI 0.6–0.8%) for the cerebellum of the normal brain (P = 0.77).

Aneuploidy in the AT cerebellum
The results of aneuploidy scoring after application of chromosome enumeration probe for the seven AT brain samples are shown in Figures 2B and D and 3A and B (FISH data) and MCB (Fig. 4B and D). We found that aneuploidy frequency (losses + gains) for chromosomes 1, 7, 8, 9, 11, 16, 17, 18, and sex chromosomes (X and Y) vary in a wide range from 0.2 to 9.5% between chromosomes and individuals in the cerebrum and cerebellum (Supplementary Material, Table S2). The mean frequency of stochastic aneuploidy per individual chromosome pair (without chromosome 14) was in the range of 1.0–3.1% for autosomes and 0.5–3.5% for sex chromosomes. The threshold levels were between 3.2 and 11.9% for different autosomes; 1.6 and 2.1% for chromosome Y and 3.9 and 8.2% for chromosome X. The occurrence of aneuploidy over the thresholds calculated for the normal brain was observed frequently in the AT brain. This indicates that an increased level of mosaic aneuploidy features the diseased brain. We found no remarkable difference in the percentage of aneuploidy between chromosomal 1, 7, 8, 9, 11, 16, 17, 18, X and Y in the cerebrum and cerebellum of the AT brain as well as multiple aneuploidy (aneuploidy simultaneously involves two or more non-homologous chromosomes). This indicates random involvement of different chromosomes in mitotic (post-meiotic) aneuploidization. The mean frequency of
aneuploidy was 2.2 ± 0.7% (95% CI 1.5–2.7%) for the cerebrum and 1.7 ± 0.8% (95% CI 1.2–2.3%) for the cerebellum. In spite of the high variability of aneuploidy frequencies between chromosomes and between individuals, the mean aneuploidy frequency was significantly lower in the diseased cerebellum versus the diseased cerebrum (P < 0.001). Notably, we have not found differences of the percentage of aneuploidy between the cerebrum and cerebellum in controls (P = 0.77). These results suggest the preferential loss of aneuploid neuronal cells in the degenerating cerebellum of AT individuals.

ICS-MCB with probes for chromosomes 1, 7, 21 and X has confirmed, in some extent, MFISH results. However, ICS-MCB analysis with an MCB probe for chromosome 14 revealed a unique and age-dependent pattern of increased aneuploidy specifically involving chromosome 14 in the cerebellum of AT patients (Fig. 4D). The percentage of aneusomy 14 gradually increased from 2.4 to 6.1% in patients from ages 8–24 years, reaching 19 and 42% in older patients (35 and 47 years, respectively).

ICS-MCB with probes for chromosomes 1, 7, 21 and X has confirmed, in some extent, MFISH results. However, ICS-MCB analysis with an MCB probe for chromosome 14 revealed a unique and age-dependent pattern of increased aneuploidy specifically involving chromosome 14 in the cerebellum of AT patients (Fig. 4D). The percentage of aneusomy 14 gradually increased from 2.4 to 6.1% in patients from ages 8–24 years, reaching 19 and 42% in older patients (35 and 47 years, respectively).

MCB analysis of interphase chromosome rearrangements in the normal and AT brain

Using ICS-MCB techniques, we were able to monitor the integrity of artificially ‘banded’ chromosomes 1, 7, 14, 21 and X in the interphase nuclei of neural cells (Fig. 5). Interphase chromosomes containing full set of hybridization signals according to the MCB labeling scheme were considered undamaged. The frequency of chromosome with partial absence of MCB hybridization signals was in the range of 0.0–1.0% in the cerebrum and the cerebellum of the normal controls as well as in the cerebrum of the AT patients (Fig. 6). We were not able to identify MCB labeled chromosomes that definitely represented structurally abnormal chromosomes in the cerebrum and the cerebellum of controls as well as in the cerebrum of AT patients. However, the cerebellum of AT patients possessed a dramatic and age-dependent increase of breaks within chromosome 14, and, to a lesser extent, in chromosomes 7 and X (Fig. 6). The cells of AT patients were used to determine the site of chromosome damage more precisely. We found that common breakpoint of chromosome 14 is located within 14q12 resulting to the loss of distal part of chromosome 14 in the majority of damaged cells (Fig. 5). Chromosome 7 has a common breakpoint within 7p14 with the loss of proximal part of the short arm from the band 7q14 to the telomere. Chromosome X has a breakpoint in Xp22.1 with loss of proximal part of the short arm including bands Xp22.2 and Xp22.3 (data not shown). The unrealized breaks in chromosomes 1 and 21 were not observed. The frequency of neural cells with chromosome 14 breaks was in the range of 1–4% in AT patients aged 8–24 years, with a dramatic increase up to 16 and 24% in patients aged 37 and 48 years, respectively (Fig. 6). Moreover, multiple aneuploidies involving undamaged and derivative chromosome 14 took place in neural cells of the diseased cerebellum. Chromosome 14 breaks were frequently associated with aneuploidy involving this chromosome, including monosomy, trisomy, tetrasomy and even pentasomy of derivative chromosome 14, der(14) (Fig. 5). This indicates that the occurrence,
transmission and accumulation of non-random interphase chromosome breaks may occur in proliferating neural precursor's cells. The primary mouse anti-NeuN was applied for immunophenotyping of neuronal cell populations in brain samples. The majority of neural cells (~80%) with chromosome breaks and aneuploidy were NeuN-negative (Fig. 7). The specific nuclear architecture of neural cells with chromosome breaks/aneuploidy (i.e. diffuse and lightly staining 4,6-diamidino-2-phenylindole (DAPI)-positive chromatin, relative large nuclear diameter, frequent somatic pairing of chromosomes) indicates for their relation to neuronal-like rather than glial-like cells (Figs 1A and 5B). We speculate that these NeuN-negative neural cells represent immature granular neurons—the major neuronal cell type in the cerebellum—as well as the precursors of Purkinje cells, which are normally NeuN-negative.

**In silico analysis of 14q12**

The locus of chromosome 14 recurrently disrupted in the AT cerebellum was additionally analyzed through genomic and epigenomic databases. Chromosomal region 14q12 contains 19 genes of known function and expression profiling. Two genes (*FOXG1B* and *NOVA1*) were found to be highly expressed in the human brain (especially, in the cerebellum) throughout ontogeny (Fig. 5C). This led us to suggest that *FOXG1B* and *NOVA1* are candidate genes for disruption produced by CIN in the AT cerebellum.
DISCUSSION
Molecular cytogenetic approaches to the evaluation of chromosomes in brain cells

FISH-based approaches are highly applicable for identification of CIN in tumor cytogenetics. However, a drawback of interphase FISH studying chromosomes in post-mitotic neural cells is associated with extreme cell-to-cell variability of genome organization in interphase. This can virtually be observed in all the interphase FISH studies, especially, when locus-specific, chromosome enumeration or microdissection whole chromosome painting probes are applied (See Supplementary Discussion). Because of FISH signal co-localization, scission or splitting, it is hard to assess the number of chromosomes in an interphase nucleus. Moreover, DNA replication observed in some areas of the brain tissues causes doubled FISH signals, which can be considered as a chromosome gain. To avoid considering FISH artifacts as false-positive chromosome imbalances, additional approaches were elaborated and used in this study: QFISH and ICS-MCB (12–14). It is noteworthy that ICS-MCB is the unique technique that allows analysis of interphase chromosome integrity and visualization of losses and gains of whole chromosomes as well as theirs specific bands (Supplementary Discussion).

Chromosome instability in the AT brain

AT is the result of mutations in the ATM gene. DNA double-strand breaks activate ATM, which participates in DNA repairs, chromatin remodeling, cell cycle checkpoint activation and initiation of apoptotic pathways. A paradoxical feature of AT is that the cerebellum is severely affected by the loss of Purkinje and granule neurons, whereas other brain regions are less affected (3,15,16). The molecular basis of neurodegeneration may be attributed to defects in the processing of DNA double-strands breaks during neuronal development, and therefore, may be intimately linked to the loss of genomic integrity in neural cells. However, the genetic signature of neuronal death in the affected AT cerebellum has not been as yet addressed. It has been hypothesized that AT is a disease exhibiting mosaic expression of genomic instability that selectively affects specific tissues of the body or even specific brain areas (16). Our comparative study of chromosomal instability manifested as aneuploidy and chromosome breaks in different areas of the post-mortem AT brain (the frontal cortex versus the cerebellum) has helped to solve the AT paradox.

The murine Atm<sup>−/−</sup> brain (specifically, the adult cerebral cortex) was shown to contain up to 40% of aneuploid cells (10). It was proposed that the maintenance of aneuploid cells can reflect altered neurogenesis and the failed apoptotic clearance of genetically defective (aneuploid) cells in the developing AT mouse brain. The failed clearance of presumably normal cells after irradiation agrees with these suggestions (17). These data support the hypothesis that ATM deficiency leads to the resistance of aneuploid neural cells to developmental clearance (9,10). Here we have observed a 2–3-fold increase of stochastic aneuploidy in the cerebellum and the cerebrum of AT individuals versus unaffected controls. Our estimations using extended set of DNA probes for different chromosomes (1, 7, 8, 9, 11, 14, 16, 17, 18, 21, X and Y) indicate that significant proportion of neural cells in the AT cerebellum may be affected by somatic aneuploidy. These data confirm our previous findings demonstrating genome-wide aneuploidy in the frontal cortex of the AT brain which affects up to 20–50% of cells (12). We propose that global neural aneuploidy in the AT brain is a secondary genetic mechanism associated with the brain pathology in AT. Interestingly, increased mosaic aneuploidy in AT might reflect the phenomenon of developmental chromosome instability, a normal characteristic feature of the developing mammalian brain (18,19). Meanwhile, the

Figure 3. Mean aneuploidy frequency (mean ± SD) per individual chromosome in the cerebrum (A) and the cerebellum (B) in the unaffected controls (black colons) and AT (white colons) revealed after aneuploidy scoring by FISH with chromosome enumeration DNA probes for chromosomes 1, 7, 8, 9, 11, 16, 17, 18, X and Y.
adult human brain contains less aneuploid neuronal and non-
neuronal cells (~10 versus 30–35% in the developing brain) (12,13). Therefore, AT is likely to be associated with abnor-
mal aneuploidy clearance during the early development of
the human brain. Aneuploidy as a highly pathogenic
genetic condition may significantly disturb proliferation,
differentiation and maturation of neural cells. Aneuploidy in
the diseased brain (AT cerebrum) affects simultaneously neur-
onal and non-neuronal (glial) cells (12). Similarly, we found
that aneuploidy affects both neuronal and non-neuronal cells
in the cerebellum. However, progressive degeneration in AT
occurs predominantly in the cerebellum with preferential
loss of granule and Purkinje neurons. Therefore, the phenom-
enon of mosaic neural aneuploidization cannot completely
explain the AT paradox. Other genetic factors are likely to
contribute to loss of neuronal cells in the AT cerebellum.

Non-random somatic rearrangements in the AT
cerebellum
Developing AT lymphocytes are characterized by non-random
chromosomal rearrangements, such as translocation and inver-
sions with breakpoints located at 14q11, 14q32, 7q35 and
7p14. These regions contain immunoglobulin (IgH) and T-
and B-cell receptor gene clusters, which must be assembled from the multiple variable (V), joining (J) and diversity (D) gene segments in the germ line loci. This process of somatic DNA rearrangements by V(D)J recombination is the major source of cellular diversity of immune system cells (4). The high incidence of chromosome 14 and chromosome 7...
The frequency of aneuploidy (A), chromosome breaks (B) and aneuploidy + chromosome breaks (C) involving chromosomes 1, 7, 14, 21 and X revealed by MCB in neural cells of the diseased cerebellum in seven AT patients with different age (seven bars arranged accordingly to the age of individuals analyzed, i.e. 8, 16, 19, 23, 24, 35 and 47 years, from the left to the right).

Figure 6. The frequency of aneuploidy (A), chromosome breaks (B) and aneuploidy + chromosome breaks (C) involving chromosomes 1, 7, 14, 21 and X revealed by MCB in neural cells of the diseased cerebellum in seven AT patients with different age (seven bars arranged accordingly to the age of individuals analyzed, i.e. 8, 16, 19, 23, 24, 35 and 47 years, from the left to the right).

rearrangements in lymphocytes of AT patients reflects the failed genomic arrangements during somatic DNA recombination in developing cells of the immune system. It has been proposed that the genome of human neural cells also undergoes somatic recombination (7). However, no evidence has been provided for the programmed genomic recombination in
neural cells of the human brain. The molecular cytogenetic study of interphase chromosome integrity in the AT brain may demonstrate the possible presence of specific chromosome breaks in neural cells.

This is the first study that analyzes structural rearrangements of interphase chromosomes in neuronal cells of the human brain. Application of ICS-MCB allowed us the identification of non-random breaks of chromosome 14 as well as...
chromosomes 7 and X in neural cells of the AT cerebellum. Additionally, an age-dependent increase in aneuploidy and chromosome breaks specifically involving these chromosomes was observed in the AT cerebellum.

In differentiating post-mitotic neurons, the repair of DNA double-strand breaks occurs via non-homologous end-joining (20). DNA strand breaks can arise during normal cellular metabolism and/or can be produced by exogenous agents from the environment. DNA-damage response defects in AT are a common feature underlying pathology of the disease. The presence of non-random chromosome breaks in neural cells may indicate that DNA double-strand break repair defects are likely to be confined to specific chromosomal loci. Interestingly, our findings demonstrate that AT cerebellar neural cells possess breakpoints within chromosomes 14 and 7 similar to the developing immune system cells. The breakpoint in 7p14 (near the common fragile site FRA7C and gamma-chain of the T-cell receptor) is likely to represent a common breakage site for both developing lymphocytes and cerebellar neural cells. However, it should be noted that neural interphase chromosomes demonstrate unrealized breaks in 14q12 and Xp22.1 (near the common fragile site FRAXC), while lymphocytes in AT patients usually demonstrate additional breaks at 14q11 (the T-cell receptor-alpha locus) and 14q32 [the B-cell receptor (IgH) locus]. We hypothesize that the chromosomal regions 14q12, and, probably, 7p14 and Xq22.1 should harbor genes that may play a critical role in AT-associated targeted cerebellar neurodegeneration.

**Genes of 14q12 that are probably disrupted in the degenerating AT cerebellum**

*In silico* analysis of 14q12 revealed the presence of two well-characterized genes highly expressed in the cerebellum (Fig. 5). One of these genes is *FOXG1B* (fork-head box G1B). *FOXG1B* encodes a brain-specific transcriptional repressor that is essential for early development of the telencephalon (21–23). The second gene is *NOVA1* (neuro-oncological ventral antigen 1), first identified as an antigen in a neurologic disorder known as paraneoplastic opticoclonus-mioclonus ataxia (24). *NOVA1*, targeted in patients with dysfunction of inhibitory motor control, is a neuron-specific protein that regulates alternative splicing of a discrete set of synaptic *NOVA1* transcripts. *NOVA1* is essential for neuronal viability in post-natal period (25–27).

**Somatic genome instability and neuronal death in the AT brain**

Chromosome imbalances due to aneuploidy and DNA double-strand breaks can have severe consequences on cellular physiology ranging from death to neoplastic transformation (6,28). Therefore, our finding of progressive aneuploidization and accumulation of unresolved chromosomal breaks in neural cells may serve as a missing links between somatic genome instability and neuronal death in the AT brain. Our results provide the first direct evidence that a significant proportion of neuronal and non-neuronal (probably, glial) cells in the developing AT brain are chromosomally abnormal. Therefore, secondary genetic lesions as gross chromosomal imbalances *per se* may contribute to abnormal neural cells behavior during proliferation, differentiation and maturation in the AT brain in addition to the primary genetic defect in DNA double-strand breaks repair.

A significant increase of the number of cerebellar cells with unresolved chromosome breaks is a remarkable genetic feature of cerebellar ataxia in aging AT individuals. Application of NeuN (nuclear neuron-specific) protein staining clearly indicates that stochastic aneuploidy affects both neuronal and non-neuronal (glial) cells in the cerebellum and the cerebrum (12). It is intriguing that ~80% of cerebellar cells in the AT cerebellum with unresolved chromosome 14 breaks and aneuploidy were NeuN-negative. We propose that these neuron-like cells correspond to Purkinje neurons and immature granule cells that normally do not express NeuN. The relatively large nuclear diameter and specific nuclear architecture of the cells with chromosome 14 breaks and imbalances (diffuse lightly DAPI-staining chromatin, presence of nucleoli, frequent chromosomal associations) indicate that these neuron-like nuclei originated from neuronal rather than glial precursor cells. We can only speculate that neural cells including granule and Purkinje neurons in the AT cerebellum may be more commonly affected by CIN as to other neural cells.

Interestingly, the increased longevity in AT patients was found to be in direct proportion to the percentage of neural cells with chromosome 14 imbalances in the cerebellum. We hypothesize that an age-dependent aneuploidization and of non-random chromosome breaks in the cerebellum could be explained by abnormal developmental neurogenesis. We cannot exclude the possibility that overproduction of immature neural cells with unresolved chromosome breaks and aneuploidy takes place exclusively during early brain development. These chromosomally aberrant cells might be resistant to clearance throughout ontogeny and, therefore, survive until the death of the AT patients.

**Is the adult neurogenesis altered in the human AT brain?**

Alternatively, an age-dependent accumulation of chromosome imbalances in the degenerating cerebellum could be explained by increased cell proliferation and abnormal neurogenesis in the AT cerebellum during aging. It is now accepted that adult neurogenesis occurs in the normal and diseased human brain (29–35). Therefore, we cannot completely exclude that adult neurogenesis occurs in the diseased AT cerebellum (Supplementary Discussion). We speculate here that the longer the lifetime of AT individuals, the bigger amount of immature neuronal-like cells is accumulated in the degenerating cerebellum. Thus, the persistence and age-dependent accumulation of neural cells with chromosome breaks during ontogeny (even immature and chromosomally abnormal) could protect the diseased cerebellum against dramatic cell loss and, therefore, partially prevents the progression of neurodegeneration.

**Genetic paradox of AT**

AT is a disorder that models pathogenic mechanisms of neurodegeneration, immune dysfunction and cancer predisposition in humans. The role of *ATM* in maintenance of genome integrity and proper somatic genome recombination in the
developing immune system is well documented. ATM deficiency and chromosome (genome) instability are two cooperating genetic mechanisms of immune system deficiency and increased tumorigenesis in AT (3,15). According to our data, progressive cerebellar neurodegeneration may be mediated by somatic genome instability in developing and maturing neural cells. The feature of genetic instability in the AT brain is gross genomic imbalance: aneuploidy and non-balanced chromosome rearrangements. Degenerating cerebellum, in contrast to the less affected areas of the frontal cortex, possessed non-balanced and unrepaired DNA double-strand breaks in chromosomes 14 and chromosomes 7 and X. These results indicate that chromosome damage may represent more important genetic phenomenon mediating cerebellar degeneration than aneuploidy. Nevertheless, the actual cause of neuronal death in the AT cerebellum is more likely the lack of proper gene regulation and loosing of cell balance mediated by somatic genome instability or CIN rather than the DNA or chromosome damage and aneuploidy themselves.

A paradoxical feature of AT is that neurodegeneration strongly affects the cerebellum, while other brain regions are less affected (3,15,16). Here, we show that diseased brain is chromosomal mosaic (or chromosomally instable) with the presence of sup-populations of neural cells with altered chromosomal complement. Moreover, the frontal cortex and the cerebellum in the AT brain, being chromosomally mosaics, featured by genomic (chromosomal) imbalances affecting different chromosomes in a tissue-specific manner. CIN or genome instability more severely affects the cerebellum and, therefore, has mosaic expression in the AT brain. Thus, chromosome-specific damage and imbalances confined to the cerebellum may help resolve the AT paradox, i.e. targeted cerebral atrophy.

CIN as a genetic cause of neurodegeneration

Aneuploidy and non-balanced and, probably, unrepaired chromosomal DNA breaks interfere with growth and development of an organism and a cell and is a common characteristic of chromosomal diseases and solid tumors. Molecular cytogenetic study of the AT brain demonstrates that the defects caused by chromosomal (genomic) instability may significantly contribute to cerebellar neurodegeneration. Although these data provide rationale for genome-based strategies in the brain studies, there are a large number of questions that are to be answered before the intrinsic role of neural genome (chromosome) instability in the pathogenesis of the neurological and psychiatric diseases will be established. The possible pathogenic role of neural aneuploidy in common brain disorders like Alzheimer’s disease, autism and schizophrenia have been recently highlighted (36–41). Here, using post-mortem brain tissues of patients with AT, we show that primary defect in a single-gene (ATM gene mutation) mediates secondary genetic defect, CIN or genomic instability (aneuploidy, chromosome breaks). This dysfunction leads to chromosomal (genomic) imbalances and might disrupt the integrity of neuronal genome or even specific genes highly expressed in the cerebellum. In replicating neuronal precursor cells, matured or terminally differentiated neuronal cells, such as Purkinje and granular neurons of the cerebellum, the loss of genome integrity leads to neuronal dysfunction and cells death via apoptosis and, consequently, to tissue degeneration and cerebellar atrophy. Neuronal cells are equipped with a myriad of genetically controlled DNA repairs, chromatin remodeling, cell cycle checkpoint and apoptotic pathways. Defects in one or more of these essential components can lead to propagation of genomic instability. This suggests that somatic genomic instability is a novel and, probably, common genetic mechanism of neuronal dysfunction and death in a variety of neurological and psychiatric disorders.

MATERIALS AND METHODS

Tissue collection and sample preparation

Fresh-frozen samples of post-mortem brain tissues, the cerebrum (cerebral cortex) and the cerebellum, from seven AT brains and seven age- and sex-matched controls were acquired from the NICHD Brain and Tissue Bank for Developmental Disorders, University of Maryland (Table 1). All AT cases were confirmed both clinically and histopathologically. The average age of AT patients and the controls was 24.6 ± 12.9 years in each group. The non-AT control individuals were free of known neurological diseases at the time of the death. The preparation of brain tissues from the cerebrum and cerebellum for molecular cytogenetic analysis was made according to a procedure described elsewhere (42). A suspension of nuclei was obtained through mechanical dissociation of the tissue brain samples (3 × 3 × 3 mm) in phosphate-buffered saline (pH 7.3) containing 0.1% (w/v) of Nonidet P-40 using a Teflon pestle and glass tube tissue homogenizer (No. 24010-035, Gibco Invitrogen, SARL; Cergy Pontois Cedex, France). Complete homogenization was achieved by microscopic control of the quality of suspension until the smallest visible tissue fragments or cell aggregates had been dissolved, but the nuclear envelope was intact in the majority of the interphase nuclei. The suspension was carefully filtered through nylon gauze (mesh diameter 100 µm) to remove debris and blood vessels. Then mixed neural and non-neural cell suspensions were processed in a manner similar to the common protocol for preparation of interphase nuclei and metaphase chromosome spreads for standard cytogenetic analysis, allowing for highly efficient in situ hybridization on the single cell level. Suspensions of nuclei were pre-fixed with acetic acid solution (45–60% w/v, 5 min) to remove the traces of cytoplasm contaminating some fraction of intact nuclei, and post-fixed with a methanol/acetic acid mixture (3:1, 30 min). The fixed nuclei were dropped onto wet slides dried at room temperature, dehydrated through ethanol series and processed for FISH.

DNA probes

To make a case–control study and to define the real rate of aneuploidy in the human brain, we have used a set of ten different DNA probes: (i) chromosome enumeration probes for chromosomes 1 (D1Z1), chromosome 7 (D7Z1), chromosome 8 (D8Z2), chromosome 9 (D9Z1), chromosome 11...
(D11Z1), chromosome 16 (D16Z3), chromosome 17 (D17Z1), chromosome 18 (D18Z1 and D18Z2), chromosome X (DXZ1) and chromosome Y (DYZ3) in three-color probe FISH assay (43–47); (ii) microdissection probes for multicolor banding (MCB) of chromosomes 1, 7, 14, 21 and X (48,49).

**FISH and MCB**

FISH with chromosome-enumeration and ICS-MCB probes was performed as described previously (13,14,45–49). High-resolution chromosome-specific multicolor banding patterns were generated with a set of human microdissection probes. Epifluorescence microscopy analysis was performed using an Axiosplan II microscope (Zeiss, Jena, Germany) equipped with a CCD camera (Sony), an HBO 100 mercury lamp, as well as filter sets for DAPI, diethylaminocoumarine, fluorescein isothiocyanate, Spectrum Orange, Texas Red and Cy5. Images were captured and analyzed using the ISIS digital imaging system (MetaSystems, Altusheim, Germany). Multicolor banding is a three-to-five-color FISH-based approach that produces a reproducible fluorochrome profile along interphase/metaphase chromosomal axes following determination of the number and structure of interphase chromosomes (12–14).

**Quantitative FISH**

Each nucleus exhibiting a number of signals that could be considered as aneuploidy or polyploidy were digitalized and evaluated by a QFISH technique described previously (50). The relative intensity of FISH signals was obtained by digital capturing of microscopic images by a CCD camera (Cohu, 4910 series, Cohu Inc., San Diego, CA), LG-3 grayscale scientific PCI frame grabber (Scion Corp., NIH, Frederick, MD). Signal intensity was measured using a Scion Image Beta 4.0.2 (Scion Corporation, National Institute of Health, Frederick, MD).

**Immunohistochemistry**

The primary mouse anti-NeuN (Neuronal Nuclei) biotin conjugated monoclonal antibody (Chemicon International, Inc., cat#MAB377B, lot LV1359481) was applied to phenotype neuronal and non-neuronal cell populations in cytological patons of the brain cells as previously described (12). Suspension of cells for immunohistochemistry were fixed in 0.4% paraformaldehyde, dropped on the slide and post-fixed by methanol (80%) for 30 min. The antibodies were diluted 1:400 and applied to cell suspensions after FISH with DNA probes, directly labeled by Spectrum Orange, Texas Red and Cy5. Detection of anti-NeuN was performed by application of FITC-avidin, 1:500 (Sigma-Aldrich Inc., cat# A2050).

**Data and image analysis**

The preparations were coded, scored and analyzed in a blinded study. The whole intermixed population of nuclei from both neural and non-neural cells was analyzed. In order to determine the threshold level for chromosome losses (monosomy), gains (trisomy and tetrasomy) and poly-ploidy, we scored no less than 3000 nuclei per chromosome/individual brain sample by Multiprobe FISH and 1000 nuclei by ICS-MCB. In total, approximately 490 000 nuclei from seven AT brains (the cerebrum and the cerebellum) and 490 000 nuclei from seven age- and sex-matched controls (the cerebrum and the cerebellum) were scored. The mean frequency, 95% CI, and the threshold or cut-off levels (the mean ± 3SD) for aneuploidy frequency were determined. The frequencies of aneuploidy over the cut-off (the threshold) levels were considered as outlier values representing the cases of low-level chromosome-specific mosaicism. Comparison of stochastic (or background) aneuploidy frequency between the two groups (AT and age-matched controls) was performed using non-parametric statistics (chi-square test).

**In silico analysis**

We addressed 14q21 by means of NCBI Build 36.3 database. Genes of this genomic locus were further analyzed using BioGPS (ver 1.0.0.4127) of Genomic Institute of the Novartis Research Foundation (http://biogps.gnf.org).

**ACKNOWLEDGEMENTS**

We gratefully acknowledge the NICHD Brain and Tissue Bank for Developmental Disorders, University of Maryland, USA for providing the brain tissues for the research. We thank research staff of laboratories at National Research Center of Mental Health and at Institute of Paediatrics and Paediatric Surgery, Moscow, Russia (Dr I.A. Demidova, Dr A.K. Beresheva, V.S. Kravetz, V.V. Monakov, O.S. Kurinnaya and M.K. Tagirova) for FISH scoring in blinded study. We also thank Cynthia Rothblum-Oviatt (AT Children’s Project, USA) for carefully reading the manuscript and helpful discussions.

**Conflict of Interest statement.** None declared.

**FUNDING**

This research was supported by AT Children’s Project (RUB1-1618-MO-06), RGNF (060600639a, Russian Federation), DFG 436 RUS 17/88/06 and DFG LI820/18-1. I.Y.I., S.G.V., A.D.K. and Y.B.Y. are supported by Philip Morris USA Inc.

**REFERENCES**

