2011

Mosaicism for trisomy21: Utility of array-based technology for its detection and its influence on telomere length and the frequency of acquired chromosome abnormalities

Chariyawan Charalsawadi
Virginia Commonwealth University

MOSAICISM FOR TRISOMY 21: UTILITY OF ARRAY-BASED TECHNOLOGY FOR ITS DETECTION AND ITS INFLUENCE ON TELOMERE LENGTH AND THE FREQUENCY OF ACQUIRED CHROMOSOME ABNORMALITIES

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

CHARIYAWAN CHARALSAWADI
M.D. (Honors), Prince of Songkla University, THAILAND 2002
M.S. (Biomedical Sciences), Prince of Songkla University, THAILAND 2005

Director: Colleen Jackson-Cook, Ph.D.
Professor
Department of Pathology

Virginia Commonwealth University
Richmond, Virginia
2011

Acknowledgements

This project would not have been possible without all of the participating families of people with mosaic Down syndrome.

I would like to thank my best advisor, Dr. Colleen Jackson-Cook, for all of her guidance and help throughout the projects. She is a dedicated teacher, researcher and laboratory director. I would like to thank each member of my advisory committee, Dr. Catherine Dumur, Dr. Joy Ware, Dr. Joyce Lloyd, and Dr. Timothy York, for taking an interest in my project and for all help and suggestion regarding research methods and strategies. I would like to thank Dr. Carleton Garrett for constructive criticism and guidance regarding statistics for microarray.

While I was doing Ph.D., I had a chance to work part time in a clinical cytogenetic lab. I sincerely appreciate for all friendships and bonds that all people gave to me. Working in the clinical cytogenetic lab brought me a great experience for my future career in clinical cytogenetics. In addition, I would like to thank all the members of the cytogenetic research lab for their teamwork and knowledge sharing.

In particular, I sincerely thank to all my best friends, who supported me throughout the time I spent in the United States, who never left me when I needed help.

Most importantly, I am especially thankful to my family, for their unconditional love and support throughout my life. They taught me to appreciate the value of education. I dedicate this dissertation to my family, especially for my father.

Table of Contents

Page

List of Tables

Page

List of Figures

Page

Abstract

MOSAICISM FOR TRISOMY 21: UTILITY OF ARRAY-BASED TECHNOLOGY FOR ITS DETECTION AND ITS INFLUENCE ON TELOMERE LENGTH AND THE FREQUENCY OF ACQUIRED CHROMOSOME ABNORMALITIES

By Chariyawan Charalsawadi, M.D., M.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Major Director: Jackson-Cook, Ph.D., FACMG
Professor, Pathology

The primary aim of this study was to determine the effectiveness of array-based technology for detecting and quantifying the presence of mosaicism. This aim was achieved by studying individuals having mosaicism for Down syndrome. SNP arrays were performed on 13 samples from individuals with mosaicism for trisomy 21, 13 samples from individuals with normal chromosome 21complements (negative controls) and 5 samples from individuals with full or partial trisomy 21 (positive controls). In addition, BAC arrays were processed on 6 samples from individuals with mosaicism for trisomy 21, 3 negative controls and 1 positive control. These studies have shown that

array-based technology is effective for detecting mosaicism that is present in 20% or more cells with the results being consistent for both platforms. We also demonstrated the strength of array-based technology to identify previously unrecognized chromosomal mosaicism.

A second aim of this study was to gain insight regarding the effect that trisomy 21 has on telomere attrition and the frequency of chromosomal instability. This study provides the first reported measure of both chromosome-specific telomere lengths and the frequency of acquired chromosome abnormalities in trisomic cells and isogenic euploid cells obtained from the same individuals. A chromosome-specific telomere length assay was performed on lymphocytes obtained from 24 young individuals with mosaicism for Down syndrome. While differences in overall telomere signal intensities were observed between the euploid and trisomic cells within a person, strikingly similar profiles for chromosome-specific telomere intensities were observed between the cell types within a person. Analyses were also completed on lymphoblast samples obtained from 8 older individuals with mosaicism for Down syndrome, including 5 individuals without dementia and 3 individuals with dementia. In the older study subjects, a significant inverse correlation was observed between telomere length and the frequency of micronuclei, suggesting that telomeric shortening is leading to an increased frequency of chromosomal instability, possibly through dicentric chromosome formation. However, further studies of more individuals, especially additional analyses of older individuals, are needed. These future studies may help to identify genomic regions of interest and serve to inform investigators of potential candidate genes in the etiology of dementia.

Chapter 1

Introduction

Epidemiology and etiology of Down syndrome

Down syndrome (OMIM90685) is the best recognized and most common chromosomal disorder seen in live born individuals, affecting 1/700-1/800 live births (Sherman et al., 2007). Down syndrome is caused by a complete or partial triplication of chromosome 21. This condition was first described in 1866 by John Langdon Down, but the etiology of Down syndrome was not known until 1959, when Lejuene demonstrated the presence of 3 copies of chromosome 21 in cells from individuals with Down syndrome (Lejuene, 1959). It is known that the extra chromosome 21 originates from nondisjunction during gametogenesis. Nondisjunction can occur during meiosis I (MI), when the homologous chromosomes pairs fail to properly complete the reduction division or during meiosis II (MII) when the chromatids fail to correctly separate. The use of DNA polymorphic markers allowed geneticists to determine the parental and meiotic origin of the nondisjunctional error that resulted in the presence of the extra chromosome 21 in people having Down syndrome. In approximately 90% of individuals with Down syndrome, nondisjunction occurred in maternal meiosis, with the majority of these (~75%) arising during MI. Paternal nondisjunction and mitotic malsegregation are far

less prevalent, being seen in approximately 4 to 9% and 3 to 5% of cases, respectively (Yoon et al., 1996; Sherman et al., 2007).

Progress has been made to identify associated factors that increase the risk for chromosome 21 nondisjunction. By far, advanced maternal age is the most significant risk factor (Janerich and Bracken 1986). The birth rate of infants having Down syndrome is dramatically increased in women older than 35 years (1.8/1000 births) and older than 45 years (6.1/1000 births), when compared to the rates seen in 20- 24 years old women (0.4/1000 births) (Yoon et al., 1996). This observation, in part, can be explained by: (1) an accumulation of toxic effects/environmental insults/mitochondrial mutations during the period of oocyte arrest; (2) a decrease in ovarian reserve/limited oocyte pool; (3) hormonal imbalance; (4) impaired meiotic machinery; (5) altered meiotic recombination/instability during chromosome segregation; and/ or (6) accumulation of trisomy 21 oocytes due to preferential elimination of disomic oocytes (oocyte mosaicism selection model) (Sherman et al., 2007; Hultén et al., 2010).

It is estimated that 95 % of individuals with Down syndrome have full or partial trisomy 21. Of the remaining cases, approximately 2-4% are due to translocations between chromosome 21 and another chromosome [e.g., t(14;21), t(21;22)] (Pangalos et al., 1994; Devlin and Morrison, 2004; Shin et al., 2010). Approximately three fourths of these unbalanced translocations are de novo mutations, with one fourth being present as a result of malsegregation of a familial translocation (American Academy of Pediatrics,

Committee on Genetics, 2001). The remaining proportion of people having Down syndrome (1% to 4%) has mosaicism. Mosaicism is a condition in which an individual has two or more genetically distinct cell lines that develop from a single zygote (Thompson and Thompson, 2004). In the case of mosaicism for Down syndrome, an individual has at least 2 populations of cells: one that has trisomy 21 (47,XX,+21 or 47,XY,+21) and one with euploid cells (46,XX or 46,XY).

Overview of Down syndrome clinical features

The phenotype of people having Down syndrome, which is thought to result from the dosage imbalance of multiple genes, has been associated with more than 80 traits (Epstein 1986), including a small brachycephalic head, epicanthal folds, upward slanting palpebral fissures, Brushfield spots (speckling of iris), a small nose with a flat nasal bridge, small mouth, hypoplasic teeth, small ears, short neck, nuchal skin folds, single palmar creases, short metacarpals and phalanges, short fifth finger with clinodactyly, and wide spacing between the first and second toes. Besides cognitive impairment, the incidences of the most common clinical characteristics reported in people having Down syndrome are summarized in Table 1 (Committee on Genetics, American Academy of Pediatrics, 2001; Jones KL, 2005). Although individuals with Down syndrome are predisposed to develop childhood leukemia, epidemiologic studies revealed that individuals with Down syndrome have a lower incidence of developing solid tumors, especially breast cancer, when compare to the general population (Hasle et al., 2000).

Down syndrome has also been associated with primary gonadal deficiency, which contributes to their fertility reduction. Although, a number of females with Down syndrome have been reported to produce offspring (approximately 50% having a normal chromosomal complement and 50% having Down syndrome) (Sheridan et al., 1989), nearly all males with Down syndrome are infertile. Only a few case reports of males with non-mosaic Down syndrome have been reported to have offspring. Paternity studies completed for these cases confirmed that the men having Down syndrome were the biological fathers (Bobrow et al., 1992; Zuhlke et al., 1994; Pradhan et al., 2006).

Cognitive impairment in people having Down syndrome is variable, ranging from mild (IQ: 50-70) to moderate (IQ: 35-50), and occasionally severe (IQ: 20-35) (Committee on Genetics, American Academy of Pediatrics, 2001; Jones KL, 2005; Tarek 2005). The presence of visual and hearing impairments may further limit overall cognitive function, and language and psychosocial skill development. In addition, unrecognized thyroid dysfunction may compromise cognitive function. The development of seizures may also deteriorate cognitive function (Lott and Dierssen, 2010; Chen et al., 2011). An area of particular compromise for individuals with Down syndrome appears in their auditory short-term memory skills, which has been conjectured to cause expressive language skill impairment (Chapman and Hesketh, 2001).

Table 1: Clinical characteristics of individuals with Down syndrome

Characteristics	Percentage
Hypotonia	80%
Congenital heart defects, including endocardial cushion defect or atrioventricular canal defect, ventricular septal defect, patent ductus arteriosus, atrial septal defect, mitral valve prolapse with or without tricuspid valve prolapse and aortic regurgitation	40-58%
Hearing loss, including conductive, mixed, or sensorineural hearing loss	70-90%
Otitis media	50-70%
Eye diseases, including	
- Congenital cataracts	15%
- Acquired cataracts in adults	30-60%
- Severe refractive errors (mostly myopia)	50%
Obstructive sleep apnea	50-75%
Thyroid disease	15%
Seizures	<9%
Gastrointestinal tract anomalies, including	
- Duodenal atresia	12%
- Hirschsprung disease	<1%
Spine anomalies, including	
- Incomplete fusion of vertebral arches of lower spine	37%
- Atlantoaxial instability	12%
Hip dislocation	6%
Increased risk of leukemia and leukemoid reaction	<1%

Down syndrome: a syndrome of premature aging

Approximately 75% of concepti with trisomy 21 die prenatally. Approximately 85% of live born infants survive to age 1 year and 50% are expected to live longer than age 50 years (Tarek 2005). Congenital heart disease is the major factor that determines early survival. In addition, frequent infections that are presumably due to impaired immune responses and leukemia also contribute to high mortality (Chen et al., 2011). However, as a result of improvements in medical care, the survival of individuals with Down syndrome has markedly increased. Life expectancy estimates for people with Down syndrome have increased from 9 years old in 1929 to 60 years in 2000 (Bittles and Glasson, 2004). Age-related disorders in individuals with Down syndrome begin earlier than in the general population. Several precocious aging characteristics have been reported in 30 to 40 year-old individuals with Down syndrome, including acquired cataracts, alopecia, premature graying of hair, age-related hearing loss, skin atrophy, hypogonadism, early onset menopause, degenerative vascular disease senile dementia and an increased prevalence of Alzheimer disease (Potter, 1991; reviewed in Esbensen 2010).

How people with Down syndrome age prematurely is not known. The DNA damage theory had been proposed to explain precocious aging in Down syndrome. This theory, which postulates that aging is a consequence of accumulation of unrepaired DNA damage, is supported by the finding of increased sensitivity to the DNA-damaging agents and impaired cellular reaction to DNA damage in individuals with Down syndrome

(Morawiec et al., 2008). In addition, DNA damage has been found to be increased in individuals with Down syndrome in relation to control individuals (Maluf and Erdtmann, 2001). An alternative explanation that has been suggested to explain the precocious aging of people with Down syndrome focuses on free-radical metabolism. One of the key enzymes involved in free-radical metabolism is superoxide dismutase (*SOD-1*), which is encoded by the *SOD-1* gene on chromosome 21. This explanation is supported by the finding that cells from individuals with Down syndrome have a decreased ability to repair oxidative damage to mitochondrial DNA compared to age-matched controls (Druzhyna et al., 1998).

A relationship between the progression of aging and telomere length has been shown in chromosomally normal individuals from the general population (Wright and Shay, 1995; Fredrich et al., 2000; Stewart and Weinberg, 2006; Mayer, et al., 2006; Guan et al., 2007). A telomere is a specialized structure at the end of a chromosome that plays a role in ensuring chromosomal integrity. Several observations, both in vitro and in vivo, have shown that telomeres act as a mitotic clock; with the shortening of telomeres that occur with every cell division eventually causing cellular senescence and cell death (Herbert et al., 1999; Sherr and DePinho, 2000; Campisi, et al., 2001). Telomere shortening and a concomitant increase in genomic instability have also been described in older individuals having Down syndrome (Vaziri et al., 1993; Maluf and Erdtmann, 2001).

Given that individuals with Down syndrome exhibit similar neuropathological features to those observed in individuals acquiring Alzheimer disease, it has been speculated that the neurodegenerative courses in Alzheimer disease and Down syndrome are closely related. Alzheimer disease is the most common form of dementia in the elderly. It is characterized by progressive dementia associated with several neuropathologic findings, including cerebral cortical atrophy and the accumulation of intracellular neurofibrillary tangles harboring hyperphosphorylated tau and extracellular β-amyloid plaques (Kimura et al., 2007). In the general population, early-onset Alzheimer disease symptoms usually start before age 60 to 65 years and often before age 55 years, while the prevalence of clinical dementia/Alzheimer disease in individuals with Down syndrome present at an earlier age (the fourth and fifth decades of life) (Holland et al., 2000). However, neuropathologic characteristics consistent with Alzheimer disease have been observed (at autopsy) in the brains of individuals with Down syndrome as early as 30 years of age (Mann and Esiri 1989) and in the brains of all individuals with Down syndrome over the age of 40 years (Wisniewski et al., 1985). The diagnosis of Alzheimer disease in individuals with Down syndrome is complicated by their pre-existing developmental delay (Brugge et al., 1994). In addition, individuals with Down syndrome have limitations in motor, language, communication and intellectual abilities; therefore the detection of subtle changes in these functioning areas requires sensitive assessment scales. Furthermore, individuals with Down syndrome may also have other health problems associated with aging (e.g., hypothyroidism and depression) that may mimic or mask the presence of Alzheimer disease (Bush and Beail, 2004).

Alzheimer disease is a genetically heterogeneous condition. Most forms of familial Alzheimer disease (AD1) are caused by a mutation in the gene encoding the amyloid precursor protein (APP; OMIM 104760) on chromosome band 21q21.2 (http://www.ncbi.nlm.nih.gov/omim/104300). In these patients, the cerebral deposition of β-amyloid, the main component of amyloid plaques, has been associated with triplication of the APP gene (Hardy, 1992; Mann, 2004). These amyloid plaques are thought to lead to neuronal death and subsequently progressive signs and symptoms of Alzhemer disease. In addition to the APP gene, other genes on chromosome 21 that may be involved in the pathogenesis of Alzheimer disease include, but are not limited to, the superoxide dismutase (*SOD-1*) gene and the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (*DYRK1A*). It has been speculated that oxidative stress may be relevant to neurodegeneration in people with Down syndrome (Percy et al., 1990), as *SOD-1* is located on chromosome 21q22.1 and the activity of SOD-1 is elevated in their blood cells (De la Torre et al., 1996). SOD-1 is an enzyme that converts oxygen radicals to hydrogen peroxide and water. In people having Down syndrome SOD-1 activity is increased due to triplication of chromosome 21, with this increase being disproportionate to the activity of the downstream enzymes responsible for removal of hydrogen peroxide (e.g., glutathione peroxidase)(Brooksbank and Balazs, 1984; Dyer and Sinclair, 1998). This imbalance is thought to result in accumulations of hydrogen peroxide in the brain, causing neuronal damage which, in turn, results in the particularly rapid neurodegeneration with age that is similar to that seen in people having Alzheimer disease (De Haan et al., 1997). The *DYRK1A*, which is a candidate gene responsible for learning and memory deficit in

individuals with Down syndrome, has recently been demonstrated to be involved in the development of Alzheimer disease. The *DYRK1A* gene was found to be over expressed in the brains of individuals with Alzheimer disease with and without Down syndrome. Furthermore, an extra copy of the *DYRK1* gene has been observed to lead to an increased expression and activity of *DYRK1* kinase enzyme and has resulted in increased tau phosphorylation (Kimura et al., 2007; Liu et al, 2008; Wegiel et al., 2011).

Another biological factor that has been associated with the development of Alzheimer disease status in the general population is telomere attrition. The relationship between telomere shortening and Alzheimer disease has also been studied in individuals with Down syndrome. Jenkins et al. (2006) observed increased telomere shortening in adults with Down syndrome having dementia compared to age-matched individuals with Down syndrome who did not have dementia (Jenkins et al., 2006; Jenkins et al., 2008). Given that early clinical symptoms of Alzheimer disease can be very difficult to recognize, Jenkins et al. (2010) proposed that telomere shortening, especially for chromosome 21, may be used as a biomarker for early detection of Alzheimer disease in the Down syndrome population and could allow for benefits to be realized from early intervention before damage to the central nervous system occurred.

The biological basis for the role of the telomere in Alzheimer disease development has been proposed to arise from a decreased efficiency in DNA repair processes, leading to the accumulation of mutations which, in turn, result in an increased

level of DNA damage. Aviv and Aviv (1998) proposed that erosion of the telomere leads to chromosomal instability. At the chromosomal level, telomeric shortening may give rise to acentric chromosome fragments which would not be subsequently pulled toward the daughter nuclei at the time of nuclear division, being left in the cytoplasm as micronuclei (MN)(de Lange, 2005). In addition, MN may originate from whole chromosome lagging (reviewed in Fenech, 2007). A strong correlation between chromosomal aberrations and MN formation has been shown (Jones, et al., 1994). It has also been shown that MN frequency increases with age (Bolognesi, et al., 1999; Bonassi, et al.,2001); toxic substance exposure [e.g., lead (Kasuba, et al., 2010) and arsenic (Colognato, et al., 2007)]; and radiation exposure (Cho, et al., 2009; Banerjee et al., 2008); neurodegenerative diseases [e.g., Alzheimer disease (Migliore et al., 1997; Petrozzi et al., 2002), and Parkinson disease (Petrozzi et al., 2002; Migliore et al., 2002)]; obesity and metabolic syndromes (Andreassi et al., 2011); and cancer (Duffaud, et al., 1997; Bonassi et al.,2007; Milosević-Djordjević et al., 2010).

One of the methods for studying acquired chromosomal changes is through use of the cytokinesis-blocked micronucleus (CBMN) assay, which has been adopted by many laboratories. The CBMN assay, which was introduced by Fenech and Morley (1985), is a one of the most widely-used methods for measuring the frequency of MN. This cytome assay also allows for measuring other cytological structures that are indicative of chromosomal damage including nuclear buds (NBUD), which are thought to be a biomarker of eliminated amplified DNA and/or DNA repair complexes and

nucleoplasmic bridges (NPB), which serve as a biomarker of DNA misrepaired and/or telomere end-fusions. Interestingly, an increase in spontaneous MN frequency with age has been reported in buccal cells from individuals with Down syndrome (Thomas et al, 2008; Ferreira et al, 2009)., but there is a paucity of reports of MN frequencies in lymphocytes of people having trisomy 21.

Mosaicism for Down syndrome

The reported incidence of mosaicism for trisomy 21 may represent only a subset of individuals having mosaicism. One of the main reasons for this bias is that conventional cytogenetic technologies are limited in their ability to detect mosaicism, especially for cases having low levels of trisomic cell lines. In addition, the phenotypic appearance of individuals with low level mosaicism is often subtle, leading to a lack of recognition of the condition based on a physical examination. Therefore, the true prevalence of mosaicism for Down syndrome in the general population could be underestimated. It has been postulated that whenever a larger number of cells are studied, using fluorescent in situ hybridization (FISH) methodologies, trisomy 21 mosaicism may be surprisingly common in the general population (Hultén et al., 2010).

Trisomy 21 mosaicism can originate in two ways:

1) Somatic origin. After fertilization involving euploid gametes, a normal zygote with 46 chromosomes undergoes a mitotic nondisjunctional event (or anaphase lag) involving a chromosome 21 to result in a cell with 3 copies of chromosome 21. The cell

with three copies of chromosome 21 may continue to proliferate, giving rise to the trisomic cell line. However, the reciprocal daughter cell having only one copy of the chromosome is more often at a selective growth disadvantage and usually will not continue to reproduce (Gardner & Sutherland, 1996) (Figure 1a).

2) Meiotic origin. A meiotic error of chromosome 21 occurs during oogenesis or spermatogenesis which, following fertilization, results in an abnormal fertilized egg having 47 chromosomes (i.e., trisomic 21 zygote). A subsequent mitotic loss of the extra copy of chromosome 21 in one or more cells during embryogenesis, through a process called trisomy rescue, results in the presence of a normal cell line (Figure 1b).

(a) Somatic Origin

(b) Meiotic Origin

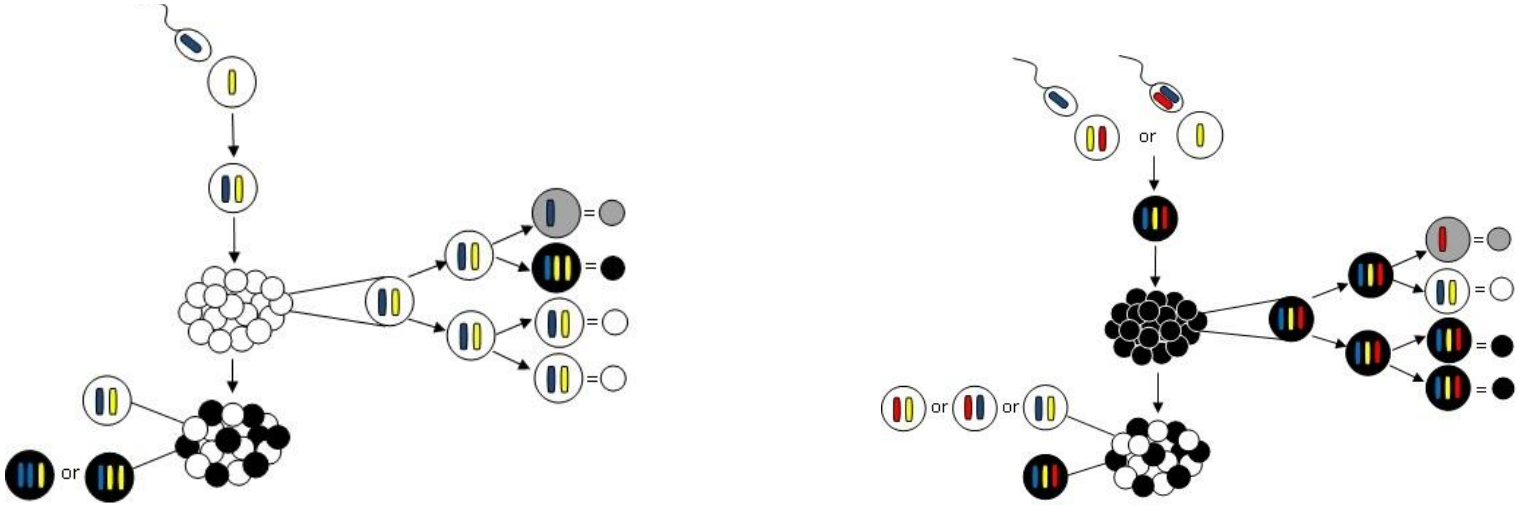


Figure 1: Origin of trisomy 21 mosaicism. (a) Somatic origin. After fertilization of euploid gametes, a normal zygote with 46 chromosomes undergoes a mitotic nondisjunctional event, resulting in a cell with 3 copies of chromosome 21 (black) and a cell with single copy of chromosome 21 (gray). The cell with one copy of chromosome 21 tends to have proliferative disadvantage, while the cell with 3 copies of chromosome 21 may continue to proliferate and gives rise to a mosaic zygote containing trisomy 21 cells and normal cell (white). (b) Meiotic origin. Following fertilization of a normal gamete with a gamete containing 2 copies of chromosome 21 due to a meiotic error, a trisomic zygote is formed. A subsequent mitotic loss of the extra copy of chromosome 21 in one or more cells occurs during embryogenesis, giving rise to the mosaicism.

The clinical manifestations of mosaicism for Down syndrome are highly variable, ranging from a phenotype comparable to that of individuals having "complete" trisomy 21 to a nearly normal phenotype. These phenotypic differences are thought to be due to variable numbers of trisomic cells in different people, as well as variation from tissue to tissue within a person (Papavassiliou, et al. 2009). The proportion of trisomic cells present may be influenced by the viability of trisomic cells in the specific cell lineages. Mosaicism originating from a meiotic error or a mitotic error that gives rise to a trisomic cell line that is present during early stages of embryogenesis, such as blastulation, may lead to generalized mosaicism in which most tissues are affected. An error that occurs at a later embryonic stage, such as during gastrulation, in which the 3 major cell lineages (i.e., ectoderm, mesoderm and endoderm) are being established, may affect a smaller proportion of the cells or result in mosaicism that is confined to a certain tissue(s). The type of cells that are affected may also determine the phenotypic outcome. If the genetic information on chromosome 21 is essential for the development of the affected tissue(s), it could either impair the overall function of that tissue(s) or lead to a selective disadvantage of the trisomic cells. Herein, certain mechanisms involved in cell selection help prevent the abnormal trisomic cells from reproducing, which in turn minimize or eliminate the effects of the genetic imbalances resulting from trisomy for chromosome 21.

As noted above, individuals having a higher frequency of trisomy 21 cells tend to have more clinical traits than those who have lower proportions of trisomic cells

(Papavassiliou, et al. 2009). Correlations have also been observed between phenotypic findings and level of trisomy 21 cells in different tissues. For example, IQ scores have been negatively correlated to the proportion of trisomic buccal cells, while the presence of congenital heart disease has been positively correlated to the proportion of trisomic lymphocytes (Papavassiliou, et al. 2009). This observation could be explained by the underlying embryonic origin of these tissues since both buccal cells and brain cells are ectodermal in origin and both lymphocytes and cardiac muscle cells are derived from the mesoderm. Children with mosaicism for Down syndrome have been shown to have a significantly lower prevalence of major congenital heart disease (36.4%) than children with non-mosaic Down syndrome (49.3%) (Shin et al., 2010). The types of congenital heart disease were also found to be different between individuals with mosaicism and non-mosaic or "complete" trisomy 21. The atrioventricular canal defect was found to be more common in the individuals having "complete" trisomy 21, whereas the less severe anomaly, atrial septal defect, was more prevalent in mosaic individuals (Papavassiliou et al., 2009).

Age-related changes leading to the acquisition of "mosaicism" have been documented in individuals having "complete" trisomy 21 (Jacob et al., 1961; Percy, et al., 1993; Jenkins, et al., 1997). The causes of chromosome 21 loss with advanced aging are not clear, but could be due to: (1) an increase in abnormal cell division (e.g., higher frequency of mitotic nondisjunction) with increasing age leading to loss of a chromosome 21; and (2) cell-line selection in the case of individuals having constitutional mosaicism

(e.g., diploid cells have proliferative advantage). Percy, et al. (1993), who observed a significantly increased proportion of diploid cells in older individuals having Down syndrome, hypothesized that the age-related loss of chromosome 21 could be related to the clinical expression of Alzheimer disease in these individuals, as well as individuals from the general population.

Diagnostic tools in cytogenetics and their abilities to detect mosaicism

While consistent constitutional mosaicism is a rare event, more recent studies have suggested that acquired chromosomal mosaicism may be a common event in the very early development of embryos (Vorsanova, 2005). Based on these new observations of "global" mosaicism, Iourov (2008) has speculated that residual somatic mosaicism may be a contributive factor affecting phenotypic expression variations in several age-related diseases, including, but not limited to cancer (Albertson and Pinkel, 2003; Albertson and Pinkel, 2005).

Chromosomal mosaicism can be detected by conventional Giemsa banding (GTG-banding) karyotype analysis, which is currently the standard diagnostic test used in clinical cytogenetic laboratories. This test allows for the whole genome identification of balanced and unbalanced numerical and structural chromosome aberrations. However, subtle cytogenetic aberrations may not be detected. At the level of routinely prepared metaphase chromosomes, which typically contain ~400-500 bands per haploid genome, deletions and duplications that are smaller than 5-10 Mb may not be reliably detected.

Deletions and duplications of 3-5 Mb can be detected by high resolution G-banding of prophase or early metaphase chromosomes, which contain ~800-1000 bands per haploid genome. However, this method, is not routinely used due to the fact that it is very labor intensive (Shaffer and Bejjani, 2004).

In the late 1980s and early 1990s, molecular genetics techniques, in particular, fluorescent in situ hybridization (FISH) (Pinkel et al., 1986), were implemented in clinical cytogenetic laboratories. FISH is currently one of the most widely used diagnostic molecular cytogenetic methods and has become an essential adjuvant assay. FISH is based on the hybridization of complementary fluorescent-labeled probe(s) to target DNA sequences. It can be performed on both metaphase chromosomes and interphase nuclei and can allow for the detection of submicroscopic rearrangements at the resolution of approximately 80-200 kb (Shaffer et al., 2001). A large number of probes are available for different diagnosis purposes (summarized in Table 2).

Table 2: Variations of commonly used FISH probes

Type of FISH probe	Diagnosis purpose	Reference
Chromosome enumeration probes (centromeric probes)	Detection of aneuploidy (e.g., trisomy 21, triomy 18 and trisomy 13)	Klinger et al., 1992
Locus-specific probes	Detection of particular microdeletions or duplications (e.g., RB1 locus)	Kallioniemi et al., 1992
Dual color fusion probes	Detection of gene rearrangements in cancer (e.g. BCR/ABL)	Dewald et al., 1993; Bentz et al., 1994
Dual color break apart probes	Detection of gene rearrangements in cancer (e.g. IGH/MYC)	Einerson, et al., 2006
Telomeric probes	Detection of cryptic deletions and translocations in the telomeric regions	NIH and Institute of Molecular Medicine Collaboration, 1996
Multicolor FISH (cenM-FISH) and/or spectral karyotyping (SKY)	Characterization of a multitude of alterations	Nietzel et al., 2001; Schrock et al., 1996; Schrock et al., 1997; Speicher et al., 1996; Chudoba, et al., 1999

The use of FISH methods for scoring targeted chromosomal regions in interphase nuclei, in particular, allows for assessments of chromosome copy numbers from large cell populations, making FISH a time efficient and sensitive method. Although FISH allows for higher resolution for detecting small genomic abnormalities than conventional G-banding, this approach is limited in that it lacks whole genome coverage and requires *a priori* knowledge (e.g., distinctive dysmorphic features for genetic syndromes or suspected diagnosis for hematologic malignancies) to identify the test(s) that will be of clinical value. While 24-color FISH methods (M-FISH or SKY) provide whole genome coverage, these methods have limitations akin to those noted for conventional GTG-banding, in that their ability to detect small deletions is limited. In addition, they do not allow for the recognition of intrachromosomal aberrations that do not result in a change in chromosome number or mortphology and are most effectively applied only for the interpretation of metaphase chromosome preparations (not interphase nuclei).

Comparative genomic hybridization (CGH), which was developed by Kallioniemi et al. (1992), is an alternative FISH-based methodology that uses DNA from the specimen being evaluated as the "probe" to determine chromosomal alterations. Briefly, in this method, two genomic DNA samples (test and reference) are differentially labeled with distinct fluorochromes and then competitively hybridized onto normal metaphase chromosomes. The ratio of the two fluorochromes present on the chromosomes are then quantified, using specialized computer software, to determine imbalances (gains and/or losses) in DNA sequences across the genome. However, since

conventional CGH is carried out on metaphase chromosomes, its resolution is limited to ~3-10 Mb (Pinkel et al., 1998). In addition, this method cannot detect balanced chromosome rearrangements, such as balanced reciprocal or Robertsonian translocations and inversions. Recently, genomic microarrays were developed for CGH applications. Array CGH shares the same principles as conventional CGH, except metaphase chromosomes have been replaced with DNA fragments (e.g., bacterial artificial chromosomes [BACs], oligonucleotides and/or PCR-generated sequences) as targets for the hybridization (Solinas-Toldo et al., 1997; Pinkel et al., 1998; Snijders et al., 2001; Pinkel and Albertson, 2001; Fiegler et al., 2003a; Fiegler et al., 2003b Veltman et al., 2003; Vissers et al., 2003; Albertson and Pinkel, 2003).

Currently there are 2 distinct types of microarray platforms: 1) single channel platform, in which test and reference samples are hybridized onto different matrices and 2) two-channel platform, in which both test and reference samples are co-hybridized on the same matrix (i.e., array CGH). Fundamentally, they operate by the same principle. For array CGH platforms, DNA from test and reference samples are labeled with different fluorophores and then competitively hybridized to a microarray including hundreds to millions DNA probes that are complementary to targeted genomic regions. The relative fluorescence intensities of the test DNA to the reference DNA is then calculated, with this value typically being transformed to a log2 ratio for assessment. A log2 ratio represents a fold change measurement of input signals for the test and reference samples and therefore reflects the copy number change.

The resolution of array CGH is limited by the size of the target sequences, as well as the distance between the BACs or oligos spotted onto the array. Recently, high density single nucleotide polymorphism (SNP) genotyping arrays, which were originally designed for whole genome association studies, have gained popularity for cytogenetic testing. This technology relies on hybridization of one sample to an array, with the results of that hybridization being compared *in silico* to a database of standard reference DNA to determine the presence of imbalances. SNP arrays have the advantage that they allow for the detection of long contiguous stretches of homozygosity (LCSH), in addition to recognizing copy number gains and losses. Thus, both imbalances and LCSH, the latter of which may be indicative of isodisomic uniparental disomy (UPD), identity by descent, or loss of heterozygosity (LOH), can be detected in a single experiment.

The clinical implementation of array-based technology has revolutionized cytogenetic diagnostic testing, being recently recommended as a first-tier assessment test for chromosomal imbalances (Miller et al., 2010). Array-based technologies have been developed for the analysis of clinically significant regions (targeted array) (Cheung et al., 2007) and the entire genome (whole genome array) (Snijders et al., 2001; Veltman et al., 2003; Vissers et al., 2003). The current limitations of array CGH include the inability to detect polyploidy and balanced chromosome rearrangements. Copy number alterations of unknown significance can also be problematic since significant knowledge regarding copy number variations (CNVs) throughout the genome and their exact roles are yet to be

determined. At this time, it is recommended that abnormalities or variations that are not recognized as variable regions in genome data bases, have reflex testing of parental samples to determine the clinical relevance, if any, of these findings (Manning and Hudgins 2007).

All of the cytogenetic tools described above can be used for mosaicism detection (Table 3). In general, when 20, 30 or 50 cells are evaluated without detection of mosaicism, the lowest level of mosaicism excluded with 95% confidence is 14, 10 and 6%, respectively (Hook, 1977). However, mosaicism that affects a small chromosomal region (i.e., less than 3Mb) or that is present in leukocytes other than T-cells may escape detection using conventional G-banding. Interphase FISH is a sensitive method for detecting low level mosaicism, allowing for the recognition of cell lines present in as low as 1% of the cell population (Dewald G, et al., 1998). However, this technology is limited because relatively few loci can be interrogated in a single experiment. Therefore, without prior knowledge of the chromosome or chromosomal region affected, mosaicism might be missed.

Table 3: Comparison of molecular cytogenetic techniques used for mosaicism detection

Cytogenetic techniques	Level of mosaicism detection
Chromosome G-banding	6-10% (Barch et al., 1997)
FISH	<1 % (Dewald et al., 1998)
Multiplex ligation-independent probe amplification (MLPA)	14% (Van Opstal et al., 2009)
Quantitative fluorescent polymerase chain reaction (QF-PCR)	10% (reviewed in Hulten et al., 2003)
Conventional CGH	16% (Lestou et al. 1999)
Array CGH	5-20% (Ballif et al., 2006; Cheung et al., 2007; Conlin et al., 2010)

Since microarray-based technology is increasingly being used in clinical diagnosis, an evaluation of its sensitivity for mosaicism detection is essential. The ability of array-based technology to detect the presence of multiple cell populations has also been shown through studies of *in vitro* contrived cellular admixture, constitutional mosaicism and acquired chromosome abnormalities in cancers (Table 4). Based on their *in vitro* contrived cellular admixture studies, Ballif et al. (2006) and Scott et al (2010) concluded that mosaicism levels of 20% to 40% could be consistently detected using array technology, but that values of 10% or less could not be unequivocally distinguished from non-mosaic cases. Using a SNP array, Conlin et al., 2010 reported detecting mosaicism for complements present is a low as 5% of cells. In this study, mosaicism was identified from a logR ratio and B allele frequency (BAF). The latter was also suggested to be useful for identifying mechanisms of mosaicism occurrence (i.e., origin of segregation error). However, when the proportion of abnormal cells was very low (e.g., at 5-10%), distinction between meiosis and mitosis origins were problematic.

Table 4: Summary of previous studies on mosaicism using array-based technologies

Manufacturer array	Array type	Specimen type	# mosaic cases studied	Chromosome(s) evaluated	% Mosaicism (gain/loss)		Reference
					FISH/GTG	Array (% minimum detected)	
GenoSensor Array 300	BAC/PAC (CGH)	frozen fetal lung tissue	1	i(18q)	17%(GTG),21%(FISH)	NA	Le Caignec et al., 2005
In house	BAC (CGH)	PB	1	monosomy 7	8%	5%	Manten et al., 2006
Signature Chip	BAC (CGH)	PB	18[a] 5[b]	vary[a] trisomy21[b]	10-50%(uncultured cells) 3-77% (cultured cells) 10-50% (artificial)	10% (subtle) 20% (clear)	Ballif et al., 2006
In house targeted	BAC (CGH)	PB	12	vary	1.5-31%(uncultured cells) 2-33%(cultured cells)	7.0%	Cheung et al., 2007
44k Agilent	Oligo (CGH)	PB	8	trisomy 13, trisomy 21	10.3-77.1%(QF-PCR)	10-12.3%	Hoang et al., 2007
In house targeted	BAC (CGH)	PB	5	trisomy 14	9.5-42% (uncultured cells) 2-15% (cultured cells)	12.4%	Shinawi et al., 2008
GeneChip Mapping 250K Nsp Array	SNP	? PB or BM	3	trisomy 21	25-50%	NA	Gondek et al, 2008
In house	BAC (CGH)	PB	48	vary	NA	10%	Neill et al., 2010
105K Agilent	Oligo (CGH)	PB	48[a] 4[b]	vary[a] trisomy 21[b]	10-30%[b]	21%[a] 10%-20% (subtle)[b] 30% (clear)[b]	Neill et al., 2010
Affymetrix 50K Xba Array	SNP	fibroblast	6	trisomy 8	0-100%	10% (subtle) 20% (clear)	Cross et al., 2007
Affymetrix 6.0 Array	SNP	PB	1	trisomy 21	8-13%	NA	Leon et al., 2010

Table 4: Summary of previous studies on mosaicism using array-based technologies (continued)

Manufacturer array	Array type	Specimen type	# mosaic cases studied	Chromosome(s) evaluated	% Mosaicism (gain/loss) detected		Reference
					FISH/GTG	Array	
44K Agilent	Oligo (CGH)	PB, POC, CV	7 [a] 28 [b]	vary [a] [trisomy 21, monosomy X, dup(3), del(15)] [b]	17-94% [a] 0-100% [b]	10% whole chromosome 20-30% segmental aneuploidy	Scott et al., 2010
Illumina HumanHap550 Bead Chip (V3)	SNP	PB, skin	21	vary	2-100%	5%	Conlin et al., 2010
Illumina Quad610 genotyping Bead Chip	SNP	UC	1	t(5;12) [del(5) and dup (12)]	87% (AF), 13-43% (postmortem tissues, various organs)	20% (UC)	Veenma et al., 2010
Illumina HumanHap 1M Bead Chip	SNP	PB	34	Vary	NA	10% (UPD), 18% (del), 23% (dup and/or trisomy)	Rodríguez-Santiago et al., 2010
244K Agilent	Oligo (CGH)	PB, BM	3	del(20), del(13), del(7) and dup(7)	11.5-14.5%	11.9%	Valli et al., 2011

[a] real specimen, [b] artificial specimen (a mixture of specimens or DNA from abnormal and normal cases), PB = peripheral blood, BM = bone marrow, UC = umbilical cord blood, POC = product of conceptus, CVS = chorionic villi, AF = amniotic fluid

Rationale for study

This review has shown the clinical relevance of constitutional (and acquired) mosaicism and highlighted the strengths and weaknesses of diagnostic methodologies used for mosaicism detection. While exciting results of case reports suggest that array technology can be useful for detecting constitutional mosaicism in patients presenting with clinical findings, to date, no systematic study of individuals having carefully documented proportions of mosaicism has been completed. Therefore, the first aim of this study was to determine the effectiveness of array-based technology for detecting levels of mosaicism. This aim was achieved by studying individuals having mosaicism for Down syndrome for whom the proportion of trisomic cells had been previously well documented using FISH methodology. The data obtained from this facet of this study allowed for testing the following hypotheses:

1) The relative fluorescence intensities obtained from microarray data, measured by the smoothed mean of log2 ratios of all probes across chromosome 21, are positively correlated with percentage of trisomic cells determined to be present in study samples using FISH methodology.

2) Array-based technology allows for the detection of a trisomic cell population that is present in 20% or more cells.

A second aim of this study was to gain insight on the effect that trisomy 21 has on telomere attrition. Given that people having Down syndrome show signs of premature aging and are at risk for developing Alzheimer disease, studies of their cells may provide knowledge regarding the relationship between telomere shortening, genomic instability, aging and Alzheimer disease. Studies of isogenic trisomic and normal cells from individuals having mosaicism for trisomy 21 provide a unique opportunity to evaluate effects of trisomy 21 on a trait without confounding influences attributable to differences due to age, genomic complement and environment exposure. This study provides the first reported measure of both chromosome-specific telomere lengths and the frequency of acquired chromosome abnormalities in trisomic cells and isogenic euploid cells obtained from the same individuals. The data obtained from this study allowed for testing of the following hypotheses:

1) There are differences in telomere lengths between trisomy 21 cells and their isogenic euploid cells.

2) These differences in telomere length affect a subset of chromosomes, rather than equally affecting all chromosomes.

3) There is an increased frequency of chromosomal instability in the trisomic cells compared to euploid cells.

Chapter 2

The use of array-based technology for mosaicism detection

Introduction

Mosaicism is a condition that denotes the presence of two or more cell lines that originated from a single zygote, but differ in their genetic make-up as a result of nondisjunction or mutation (Thompson and Thompson, 2004). Constitutional mosaicism has been observed in both somatic and germ-line tissues in humans. In addition to mosaicism, acquired somatic cell chromosomal changes can result in the presence of multiple cell lines. The identification of mosaicism/cellular admixture is clinically important, with its impact being especially relevant for evaluating cancer specimens, the latter of which are becoming one of the largest needs in diagnostic testing.

Despite its rare incidence, constitutional mosaicism is a formidable diagnostic challenge. Mosaicism has been reported for many different chromosomes and many different types of abnormalities including monosomy, trisomy, triploidy, deletions, duplications, translocations, rings and inversions (Schinzel, 2001). The clinical significance of mosaicism has been documented in humans from the prenatal to postnatal periods. During very early embryogenesis, chromosomal mosaicism has been shown to

be an unexpectedly common event, being seen in 50% to 90% of very early embryos studied through *in vitro* fertilization programs (Bielanska et al., 2002, Daphnis, et al., 2005; Vanneste et al., 2009; Santos, et al., 2010). Furthermore, about 50% of all spontaneous abortions have been conjectured to exhibit chromosomal mosaicism (Vorsanova et al., 2005), but this latter value has not been consistently seen by other investigators, with the variation in results possibly reflecting, at least in part, methodological differences. Mosaicism has also been associated with postnatal morbidity, including chromosomal syndromes, mental retardation and multiple congenital malformations, autism and schizophrenia. Multiple cell lines arising from acquired chromosomal changes have also been observed in a variety of health conditions, including but not limited to Alzheimer disease and neoplasia (Youssoufian and Pyeritz, 2000; Yurov et al., 2008; Schinzel, 2001).

The tissue-specific distribution and percentage of cells having constitutional mosaicism in an individual depends on the timing of the error, the cell lineage(s) involved and the survival potential of the cells (Kalousek et al., 2000). The consequences of mosaicism for an unbalanced cell line often are associated with greater clinical consequences when the error occurs earlier in embryogenesis, since these cases tend to have a higher percentage of abnormal cells and/or more tissues involved (especially if the error arose prior to cell lineage differentiation). The results of a study reported by Hsu et al., 1996 suggested that fetuses with a higher percentage of abnormal cells (>60 per cent) were at a higher risk for abnormal outcomes, compared to fetuses with a lower

percentage of abnormal cells (< 15 per cent). Often times, individuals with low-level mosaicism may be overlooked because they have subtle phenotypes. However, some patients with low-level mosaicism have been reported to have clinical outcomes (Yurov et al., 2007; Shinawi et al., 2008). In addition, individuals with low-level mosaicism could be at an increased risk for conceiving a child with a chromosomal imbalance since the aberrant cell line may be present in their gametes (Smith et al., 1962; Hsu et al., 1971; Mehes et al., 1973; Kaffe et al., 1974; Richards et al., 1974; Priest et al., 1977; Begleiter et al., 1977; Werner et al., 1982; Meschede et al., 1998; Wise et al., 2009; Kovaleva and Shaffer, 2003; Herrgård et al., 2007). Similarly, in conditions resulting from acquired somatic cell aneuploidy, such as the hematological malignancies, the presence of a small clone of cells having a chromosomal aneuploidy and/or rearrangement may be of great clinical relevance for the management of the patient's care, including decisions regarding their diagnosis, prognosis prediction and selection of targeted therapeutic options (Maciejewski et al., 2009).

Standard G-banding chromosomal tests may result in low-level mosaicism being missed or misinterpreted as a culture artifact, since this analysis is typically limited to the assessment of 20 metaphase spreads. G-banding analysis can also result in the failure to identify mosaicism due to selective *in vitro* growth pressure that may favor cells having a normal karyotype. When constitutional mosaicism is suspected, for example, due to a patient having variegated skin pigmentation, hypomelanosis of Ito and/or growth asymmetry (Donnai et al., 1988; Thomas et al., 1989; Woods et al., 1994), or when a

small number of cells with significant chromosome abnormalities are detected in the initial cytogenetic analysis, the examination of additional cells is usually indicated. In general, when 20, 30, or 50 cells are evaluated without detection of mosaicism, the lowest level of mosaicism excluded with at least 95% confidence is 14, 10 and 6%, respectively (Hook, 1977). Theoretically, fluorescent in situ hybridization (FISH) is considered to be an ideal laboratory technique for detecting mosaicism since cell lines that are present in 5% or fewer cells can be detected (Papavassiliou, et al., 2009; Dewald, et al., 1998). However, scoring FISH is labor intensive, requires precedent knowledge of specific chromosome abnormalities and also lacks whole genome coverage.

Recently, array-based technology has been developed and has shown several advantages when compared with other existing techniques used for the analysis of chromosomal abnormalities. It allows for genome-wide analysis at the highest resolution of less than 700 bp; however, in practice, other parameters may influence the resolution, such as experimental "noise" (which is often attributable to DNA fragmentation) and the sensitivity of copy number measurements (Bernardini et al., 2010.). In addition to improved resolution, another potential advantage of array-based diagnostic testing is that cells can be evaluated without potential growth selection that might arise from an *in vitro* culture system. In contrast, routine metaphase chromosome studies to detect constitutional chromosomal changes require three (blood) to seven or more (prenatal cases; products of conception) days of *in vitro* cell culture and aberrations that are smaller than 3-10 Mb cannot be reliably detected (Shaffer and Bejjani, 2004). Although array-

based technology has many strengths, it is limited in that it will not allow for the detection of balanced chromosomal findings or polyploidy, which can be detected using conventional G-banding methodologies.

Currently, two broad types of array-based technologies are used for clinical cytogenetic testing; comparative genomic hybridization arrays (CGH arrays) and single nucleotide polymorphism arrays (SNP arrays). In array CGH, patient DNA and control DNA are labeled with different fluorochromes and then competitively hybridized to arrays having DNA probes (e.g., bacterial artificial chromosomes array [BAC array] or synthesized DNA fragments [oligonucleotide array] that are immobilized on glass, chips, or beads). The fluorescent intensities of the case to control DNA values are then compared to determine copy number alterations across the entire genome. The other type of array, a SNP array, was originally designed for whole genome association studies, but has been adapted for cytogenetic testing. Given that SNPs are not distributed evenly across the genome, several of the original SNP-based microarray platforms were modified for cytogenetics testing by incorporating additional copy number probes, the latter of which allowed for increased genomic coverage of clinical relevant regions (Maciejewski et al., 2009) and better detection of copy number changes. When compared to array CGH platforms, SNP arrays have the additional advantage of allowing one to simultaneously analyze copy number changes, as well as copy number neutral loss of heterozygosity (LOH) and long contiguous stretches of homozygosity (LCSH), thereby allowing for the recognition of uniparental disomy (UPD). For assessments using SNP

arrays, patient DNA is labeled and hybridized to the microarray, with the results being compared with a database of standard reference DNA values.

While several geneticists have confirmed the ability of array-based technology to detect subtle or small abnormalities that were not perceived using conventional cytogenetic testing, the ability of array-based technologies to detect the presence of mosaicism remains controversial. A number of investigators have reported detecting a mosaic complement in blood specimens from individuals that was missed by traditional chromosomal analysis techniques (Table 3). Ballif, et al (2006) suggested that the array presented an advantage for mosaicism detection since all nucleated blood cell lineages could be evaluated (rather than just T-cells following mitogenic stimulation with phytohemaglutin, as is the case for conventional cytogenetic testing). The results of case reports, have led to a range of estimates regarding the lower detection limits of array-based technology. Systematic studies that were completed with the goal of evaluating the efficacy of array technology for mosaicism detection are few in number. The majority of these systematic studies have been performed on "artificial mosaicism" samples that were prepared by mixing blood or DNA samples from individuals having a known abnormal chromosome complement with normal reference DNA (Ballif et al., 2006; Cheung et al., 2007; Hoang et al., 2007). Investigators have often elected this approach due to the rarity of constitutional mosaicism, which makes it difficult to ascertain multiple patients having mosaicism for the same condition. While these laboratory created "mosaic" studies provide insight as to the technical strengths of the array assay, they cannot fully mimic

the experience that would be encountered when studying individuals having mosaicism, the latter of whom might have variable proportions of the different chromosomal complements in the total cell population (different cell types as noted above) that are present in clinical specimens. Therefore, we carried out a blinded study designed to test the efficacy of array-based technologies for detecting the trisomic cell lines present in individuals having mosaicism for trisomy 21. The data obtained from this study allowed for testing the following hypotheses: 1) The relative fluorescence intensities obtained from microarray data, as measured by the smoothed mean of log2 ratios of all probes across chromosome 21, are positively correlated with the percentage of trisomic cells determined to be present in study samples using FISH methodology (the latter of which is currently considered the "gold standard" for mosaicism assessment); and 2) Array-based technology allows for the detection of mosaicism of a trisomic cell population that is present in 15% or more cells.

Materials and Methods

Study Participants

The study participants having mosaicism for trisomy 21 were recruited through parental support groups [National Down Syndrome Congress (NDSC), the International Mosaic Down Syndrome Association (IMDSA) website, newsletters and conferences] and through clinical visitations. The only inclusion criterion was that the individual had a confirmed diagnosis of mosaicism for trisomy 21 (usually based on GTG-banding studies that were completed at or near the time of birth). After providing their informed assent (children)/consent (parents or competent adults having mosaicism) (Virginia Commonwealth University Institutional Review Board protocol #179), each study participant provided a peripheral blood specimen, with some individuals also electing to provide buccal smears for FISH studies. Confirmation and quantitation of the percentage of trisomic cells present was done using FISH on cultured and/or uncultured lymphocytes, as described below. Blood specimens were also evaluated from positive controls (individuals having "complete" trisomy 21) and negative controls (individuals or proficiency test specimens (College of American Pathology) having a previous diagnosis that showed either a normal complement or a chromosomal finding that did not involve chromosome 21). Specimens that were collected from control individuals followed the same informed assent /consent procedures as those used for the study participants having mosaicism (VCU IRB protocol #179). Prior to array specimen processing, the DNA samples were coded to ensure that the investigators were blinded to the karyotype status

of the specimens, thereby mimicking the scenario that would occur in the clinical evaluation of patients using array technology.

Sample Collection and DNA Extraction

Three to five milliliters of peripheral blood was collected from each participant, with DNA being extracted following standard procedures (Gentra Puregene, Qiagen, Valencia, CA; manufacturer protocol). Prior to microarray analysis, all DNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Gel electrophoresis was also used for monitoring potential DNA degradation. The quality control criteria used required that all DNA samples processed for microarray studies have an OD-260/280 ratio between 1.80 and 2.00, with a major band size range of approximately10-20 kb.

SNP Array Hybridization and Analysis

The Affymetrix Genome-Wide Human SNP Array 6.0 was used for the SNP array studies. This platform contains more than 906,600 SNP probes and more than 946,000 copy number (CN) probes. Each SNP probe contains 3-4 replicates per allele. The CN probes include markers that are distributed evenly across the genome. The median distance between probes for SNP and copy number assessments combined is less than 700 bp (Affymetrix Genome-Wide Human SNP Array 6.0 data sheet). Array experiments were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Briefly, a total of 500 ng of genomic DNA was digested with Nsp I and Sty I restriction

enzymes. All fragments resulting from restriction enzyme digestion were ligated to Nsp I and Sty I adaptors, which recognize the cohesive 4 base pair overhangs. The adaptor-ligated DNA fragments were subsequently amplified using a generic primer that recognizes the adaptor sequence. PCR amplification products for each restriction enzyme digest were combined and purified using magnetic beads (AMPure XP, Agencourt, Beckman Coulter, Brea, CA), with the purified PCR products being fragmented with DNaseI enzyme and end-labeled using a Terminal Deoxynucleotidyl Transferase (TdT) enzymatic reaction. The labeled DNA was hybridized to an Affymetrix Genome-Wide Human SNP Array 6.0 overnight. Following hybridization, the arrays were washed and stained with streptavidin phycoerythrin (SAPE) and a biotinylated antibody using a Fluidic Station 450. Following staining, the arrays were scanned using a GeneChip Scanner 3000 7G.

The sample files generated from the scanner were processed using the Genotyping Console Software Version 4.1.0 (Affymetrix, Santa Clara, CA) to assess data quality control (QC) and generate copy number files (CNCHP file) for further analysis in the Chromosome Analysis Suite Version 1.1 (ChAS) (Affymetrix, Santa Clara, CA). Mosaicism was determined by inspection of: 1) the CN value (falling between 2 and 3 for this cohort); 2) deviation of the log 2 ratio track from 0 and mean log2 ratio values between 0 (2/2 copies) and 0.58 (3/2 copies); 3) alteration of allele difference patterns (Figure 2); and 4) a smoothed log 2 ratio value that fell between 2 and 3 (Figure 2).

CGH Array Analysis

CGH array experiments were performed using a CytoChip array according to the manufacturer's protocol (BlueGnome, Cambridge, UK). Each CytoChip array was comprised of 4400 BAC clones with a median size of 565 kb and 1,357 subtelomeric clones having a median size of 250 kb. Each clone had 4 replicates via a dye-swap experimental design (for disease specific clones, each clone had 6 replicates). Random priming was used to label test and reference control DNA samples, according to the manufacturer protocol (BlueGnome). Briefly, a total of 800 ng of genomic DNA was used, with 400 ng of DNA being labeled with Cy3 and Cy5 dyes, respectively. The same quantity and labeling scheme was used for sex opposite reference DNA, the latter of which was purchased as a pooled human DNA sample (Promega G1471-male and G1521-female). After labeling, the test and reference DNA samples were run through an AutoSeqTM G50 column and then checked for DNA yield and dye incorporation using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). After combining the test and reference DNA samples (i.e., Cy3-labeled test DNA and Cy5-labeled reference DNA), the DNAs were ethanol precipitated and suppression hybridized using human COT-1 DNA and herring sperm DNA in 10% dextran sulphate. Following suppression hybridization, the DNAs were hybridized to the CytoChip array and incubated at 37°C in a humidified hybridization chamber for 21-24 hours. Following hybridization, non-specifically bound and unbound DNA was removed by washing in a 2X SSC/0.05% Tween-20 twice at room temperature for 10 minutes each, followed by serial washing in 2X SSC/0.05% Tween-20 at 60°C for 5 minutes, 1XSSC at 60°C for 5

minutes, 0.1X SSC at 60°C for 5 minutes, and 0.1X SSC at room temperature for 10 minutes. The array was immediately centrifuged to dry and scanned using a PerkinElmer ScanArray Gx PLUS (PerkinElmer Life and Analytical Sciences, Shelton, CT) at 5 µm resolution. The intensity data files were analyzed using the BlueFuse for Microarrays software (BlueGnome, Cambridge, UK). Mosaicism was recognized by a deviation of the log2 ratio values. The negative control specimens are expected to have a mean log2 ratio for chromosome 21 equal to zero, while the expected copy number gain for positive controls is 0.58 (a 3/2 ratio).

FISH Methodology

FISH was performed using probes specific for chromosome 21 (test probe) and chromosome 13 (control probe) to determine the proportion of cells having trisomy for chromosome 21. These studies were completed on cultured (72 hours) and uncultured (blood smears) leukocyte nuclei as described previously (Papavassiliou, et al., 2009). Briefly, for the cultured cell preparations, the slides were serial dehydrated 2 minutes each in cold ethanol series (70%, 85%, and 100%). After air-drying, a 10µl aliquot of the probe mixture (chromosome 21q22.13-21q22.2 - D21S259\D21S341\D21S1432; chromosome 13q14 – RB1)(Abbott, IL) was added to the slides, with the target chromatin and probes being co-denatured at 73°C for 2 minutes. Following hybridization (at 37ºC for 4-16 hours), the non-specifically bound and excess probes were removed by washing (0.4X SSC/0.3% NP-40 solution at 72°C for 2 minutes, followed by 2X

SSC/0.1% NP-40 wash solution for 1 minute). The nuclear chromatin was then stained using a DAPI/antifade solution (Abbott, IL).

For the uncultured preparations, an aliquot of blood was smeared on the slides (20 µl of blood per slide) and air-dried. The slides were serially fixed in a modified Carnoy's fixative (3 parts of methanol and 1 part of acetic acid) at –20°C for 30 minutes. The slides were then placed in 90% formamide in 2XSSC solution at 37°C for 5 minutes. The slides were serial dehydrated for 2 minutes each in an ethanol series (70%, 85%, and 100%). After air-drying, a 10µl aliquot of the probe mixture (chromosome 21q22.13-21q22.2 and chromosome 13q14) (Abbott, IL) was added to the slides. The target chromatin and probes were co-denatured at 75°C for 10 minutes. Following overnight hybridization, excess and unbound probes were removed by serial washing in 0.4XSSC at 72 °C for 2.5 minutes followed by 0.1% NP-40 in PBS at room temperature for 2 minutes. The nuclei were counterstained with DAPI/antifade solution (Abbott, IL).

Probe signals were visualized using a Zeiss Axiskop equipped with single (Spectrum Orange, Spectrum Green) and triple band pass filters. In order to detect mosaicism levels as low as 5% with greater than 99% power, a total of 1000 cultured blood lymphocyte nuclei, and 500 uncultured blood nuclei were scored for each study participant (Dewald et al., 1998).

Results

SNP arrays were processed for 31 DNA samples, including 13 samples from individuals with mosaicism for trisomy 21, 13 samples from individuals having a previous diagnosis that showed either a normal complement or a chromosomal finding that did not involve chromosome 21 (negative controls) and 5 samples with a previous diagnosis of full or partial (due to a structural change that was in every cell) trisomy 21 by G-banding analysis (positive controls). BAC arrays were processed on 10 DNA samples, including 6 samples from individuals with mosaicism for trisomy 21, 3 samples from individuals with chromosome abnormalities that did not involve chromosome 21 (negative controls) and 1 individual with a previous diagnosis of full trisomy 21 (positive control). In a Table 5 a summary of the microarray results obtained from both array platforms is presented. The results of each case are compared to previous cytogenetic analyses and indicate the percent mosaicism observed by the FISH compared to microarray methodologies.

As expected, the CN for chromosome 21 detected in each of the negative control cases was equivalent to 2 (no aberrations noted). In addition, abnormalities involving other chromosomes that were present in these cases were correctly identified with no additional clinically relevant aberrations being detected. The background level of cells having gains involving chromosome 21 based on the SNP microarray analysis in the negative control group ranged from 0% to 4%. Using only the CN state values calculated

by the ChAS software (which has criteria requiring 30% or higher mosaicism for detection), only 5 of the 13 mosaic cases were detected, with their estimated percentage of trisomic cells ranging from 28% to 100%. By expanding the ChAS software analysis to include an assessment of the smoothed signal, a total of 8 of the 13 individuals with mosaicism were readily identified as having mosaicism, with a case having 15% trisomic cells being detected. A mosaic case having 10% trisomic cells showed subtle changes from the non-mosaic cases, but yielded a value that was equivocal and thus not clearly defined as mosaicism (Figure 2). In two individuals having approximately 19% trisomic cells as determined by FISH, the SNP array patterns were within normal limits (false negative). Interestingly, two individuals with mosaicism involving approximately 80% trisomic cells, as determined by FISH, had an array value that was consistent with non-mosaic ("full") trisomy 21.

Table 5: Summary of microarray results in 30 study subjects

Karyotype	%FISH		Affymetrix Genome-Wide Human SNP 6.0								BlueGnome CytoChip		
			Gain for ch.21 detected				%Array	Smooth signal of ch.21		Mean log2 ratio of ch.21	Gain for ch.21 detected	log2 ratio of ch.21	
	CPB	UPB	Log2 ratio	Allele difference	Smooth signal	CN calling		Mean	SD			Mean	SD
Negative Controls (2 copies ch.21 in all cells)													
46,XY,del(3)(q29)			N	N	N	N	3	2.025	0.124	0.008	N	0.003	0.058
46,XX,dup(3)(q26.1),del(8)(p23.2)			N	N	N	N	0	1.980	0.127	-0.010	N	0.015	0.048
46,XY,dup(16)(q22.2q23.2)			N	N	N	N	4	2.036	0.233	0.009	N	0.009	0.057
46,XY,dup(10)(p13p15.3)			N	N	N	N	0	1.995	0.386	-0.003			
46,XY,del(18)(p11.2)			N	N	N	N	2	2.016	0.089	0.006			
46,XY,del(8)(p23.1p23.3),dup(8)(p21.1p22)			N	N	N	N	0	1.907	0.123	-0.040			
46,XX			N	N	N	N	0	1.895	0.155	-0.045			
46,XY			N	N	N	N	0	1.941	0.153	-0.026			
46,XX,del(17)(p11.2p12)			N	N	N	N	3	2.030	0.139	0.010	N	-0.024	0.075
46,XX			N	N	N	N	3	2.028	0.153	0.009	N	-0.030	0.059
46,XY,del(2)(q14.1q14.3),dup(22)(q11.21q11.21)			N	N	N	N	2	2.023	0.121	0.008			
46,XY,dup(5)(q33.3q35.3),del(13)(q34)			N	N	N	N	1	2.014	0.123	0.004			
46,XY,dup(17)(p11.2p12),del(17)(q11.2q12)			N	N	N	N	3	2.033	0.112	0.012	N	0.012	0.041
Mosaic Tri 21													
mos 47,XX,+21/46,XX	93.6	80.4	Y	Y	Y	Y	100	3.088	0.178	0.347	Y	0.381	0.064
mos 47,XY,+21/46,XY	50.5	63.2	Y	Y	Y	Y	50	2.498	0.147	0.177	Y	0.216	0.083
mos 47,XY,+21/46,XY	48	26	Y	Y	Y	N	21	2.212	0.155	0.079	N	-0.049	0.102
mos 47,XX,+21/46,XX	90.5	78.5	Y	Y	Y	Y	100	3.160	0.183	0.365			
mos 47,XX,+21/46,XX	24.9	28	N	N	S	N	7	2.068	0.150	0.025			
mos 47,XX,+21/46,XX	21	19.4	N	N	N	N	0	1.970	0.140	-0.014			
mos 47,XX,+21/46,XX	18.7	19	N	N	N	N	0	1.960	0.151	-0.018			
mos 47,XY,+21/46,XY	29.5		Y	Y	Y	N	20	2.199	0.146	0.074			

Table 5: Summary of microarray results in 31 study subjects (continued)

Karyotype	%FISH		Affymetrix Genome-Wide Human SNP 6.0								BlueGnome CytoChip		
			Gain for ch.21 detected				% Array	Smooth signal of ch.21		Mean log2 ratio of ch.21	Gain for ch.21 detected	log2 ratio of ch.21	
	CPB	UPB	Log2 ratio	Allele difference	Smooth signal	CN calling		Mean	SD			Mean	SD
Mosaic Tri 21													
mos 47,XY,+21/46,XY	62	53	Y	Y	Y	Y	66	2.659	0.157	0.227			
mos 47,XX,+21/46,XX	23.2	20	S	N	Y	N	15	2.147	0.141	0.055			
mos 47,XY,+21/46,XY	6.5	17	N	N	S	N	8	2.079	0.131	0.030			
mos 47,XX,+21/46,XX	43	41	Y	Y	Y	Y	28	2.279	0.223	0.101			
mos 47,XY,+21/46,XY	10.4	8.5	N	N	S	N	10	2.099	0.150	0.037			
Positive control (3 copies of ch.21 in all cells)													
47,XX,+21	96.8	89.4	Y	Y	Y	Y	100	3.181	0.272	0.369	Y	0.357	0.047
47,XY,der(21)(q10;q10)	81.5		Y	Y	Y	Y	51	2.511	0.282	0.177			
47,XY,+21	97.6		Y	Y	Y	Y	100	3.162	0.236	0.365			
47,XX,+21	97.2		Y	Y	Y	Y	100	3.072	0.236	0.342			
46,XX,der(21)(qter→q21::p11.1→qter)			Y	Y	Y	Y	100	3.051	0.188	0.242			

ch.21 = chromosome 21, Tri 21 = trisomy 21, CPB = cultured peripheral blood, UPB = uncultured peripheral blood, CN = copy number, SD = standard deviation, Y = yes, N = no, S = suspicious

In practice, the manufacturer recommended protocol for detecting mosaicism in a specimen is to combine the data collected from all analyses, including the log2 ratios, smoothed signals and especially the allelic patterns. By expanding the assessments to include each of thesfore data points, the current version of the ChAS software consistently allowed for the detection of a minor cell population that was present in approximately 20% of cells (20% to 80% trisomic complement)(Figure 2).

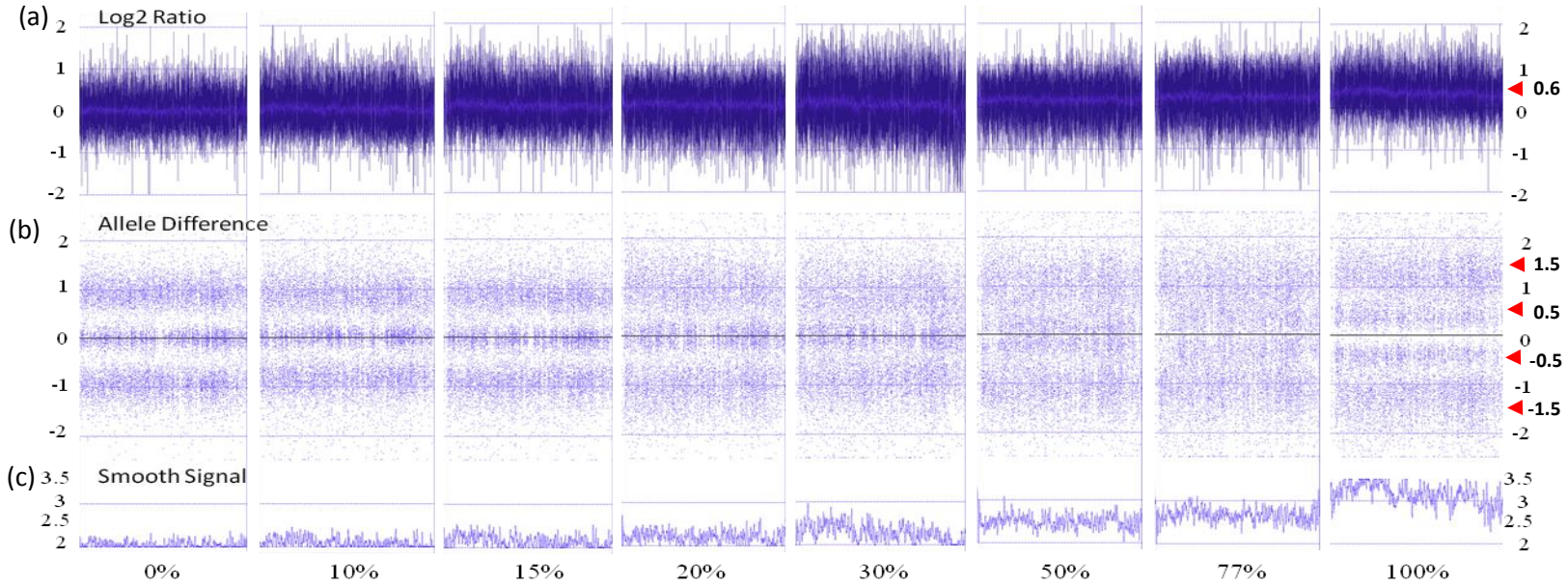


Figure 2: Composite array results of representative cases having mosaicism for trisomy21. Each box in this figure represents a different individual, with a total of 8 patients being shown. The percentage values below the data indicate the level of trisomic cells present in each person, with 0% representing an individual having a normal (2 copies) complement for chromosome 21 and 100% representing an individual with "complete" trisomy 21. The results of the ChAS findings from different analyses are shown, including the log2 ratio (a); the allelic patterns (b) and the smoothed signal (c). The mean log 2 ratios (a) are shown as a light blue line in the middle of the marker data values and range from 0 (0% trisomy) to 0.6 (100% trisomy). (b). The allele difference track shows allele patterns observed in euploid (where A allele = 0.5 and B allele = -0.5 and AA = 1; AB = 0; and BB = -1) compared to trisomic (AAA = 1.5, AAB = 0.5, ABB = -0.5 and BBB = -1.5) cells. (c) The smooth signal of log2 ratio track shows an increase of the signal from 2 to 3, corresponding with 2 to 3 copies of chromosome 21.

For the positive control cases, the SNP array evaluation of 4 of the 5 specimens was consistent with the GTG banding karyotypic results. However, one case, who was referred for assessment due to a phenotype suggestive of mosaicism (presence of hypomelanosis of Ito), had an array result that was discrepant to the findings of the conventional cytogenetic analysis. The chromosome analysis showed the presence of secondary trisomy for chromosome 21 [der(21)(q10;q10); either an isochromosome or a Robertsonian translocation] in each of the 30 metaphase spreads examined (100%). Interestingly, the array result was consistent with the presence of trisomy mosaicism for both chromosome 14 (62% of cells) and chromosome 21 (51% of cells). To confirm/refute these array findings, additional analyses were completed using FISH with two probe sets. Probe set one included a probe that was specific for the long arm of chromosome 14 (specific for 14q.32; spans the IGH region), with a control probe also being evaluated (specific for band 11q13; spans the CCND1 breakpoint region). Probe set two included a probe that is specific for band 21q22 (D21S259\D21S341\D21S1432), along with a control probe from chromosome 13 (spans the 13q14; RB1locus) (Figure 3). An assessment of 500 interphase nuclei per probe set confirmed the presence of mosaicism for both chromosomes, with three signals being present for the chromosome 14 probe in 22% of intephase nuclei and three signals for the chromosome 21 probe in 81.5% of interphase nuclei.

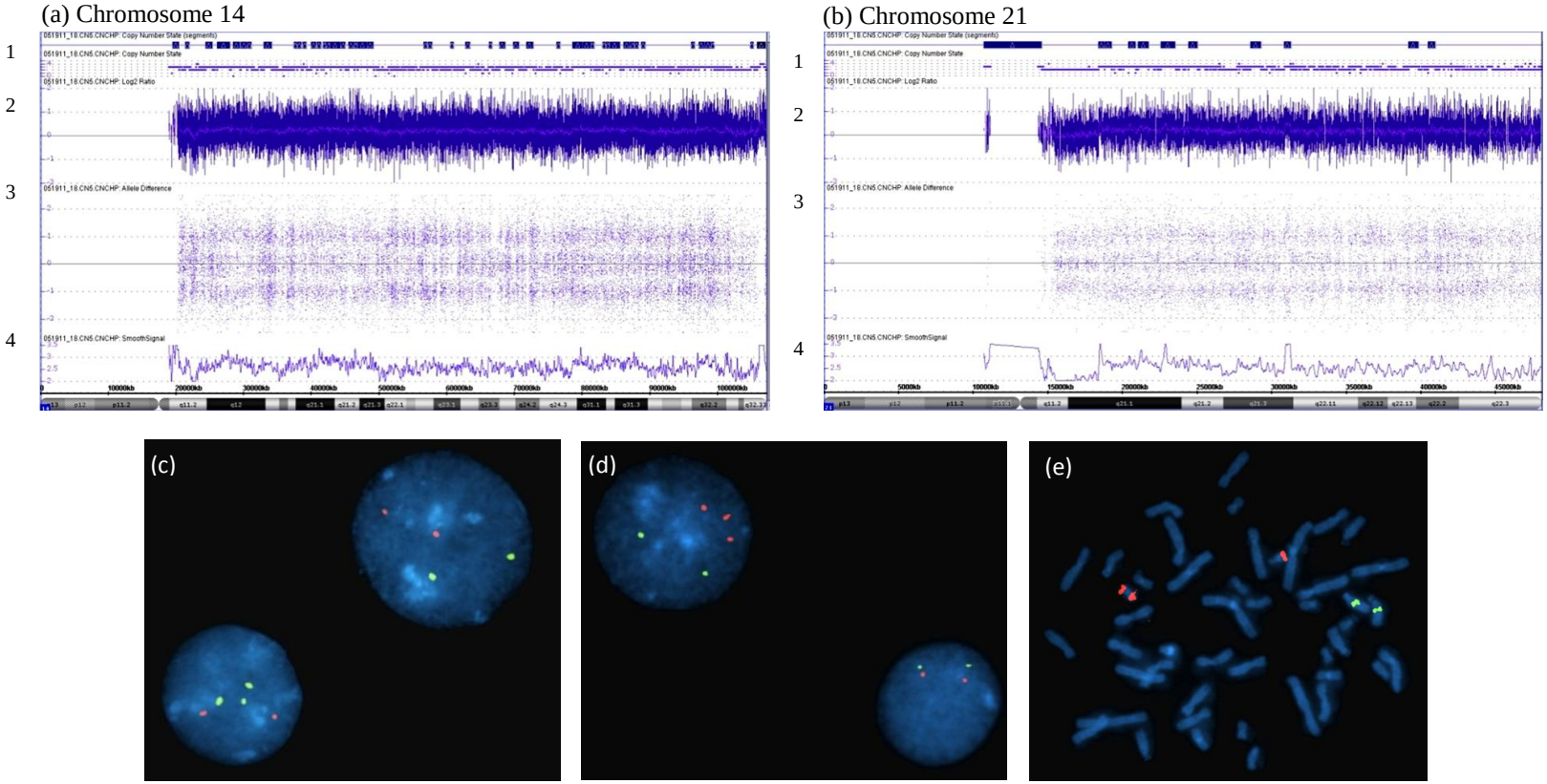
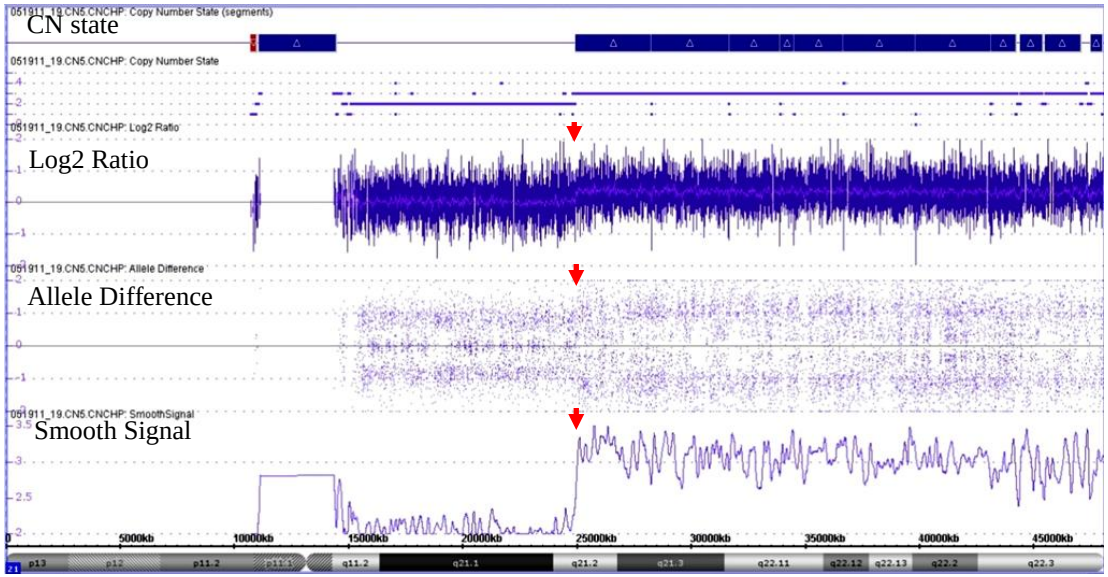


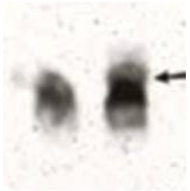
Figure 3: Detection of mosaicism for trisomy 14 and a trisomic dose of 21q. Mosaicism was determined from CN state (1), log2 ratio (2), allele difference (3) and smooth signal (4) tracks for chromosome 14 (a) and chromosome 21 (b). Interphase analysis on cultured lymphocytes (c), using a locus specific probe mixture for chromosome 14 (green) and for chromosome 11 (red) showed cells having 3 (lower left) or 2 (upper right) signals for the chromosome 14 probe. Interphase analysis on cultured lymphocytes (d), using a locus specific probe mixture for chromosome 21 (red) and for chromosome 13 (green) showed cells with 3 (lower right) and 2 (upper left) signals for chromosome 21. (e) Metaphase spread showing a trisomic dose of the long arm of chromosome 21 due to a der(21)(q10;q10).

A SNP array was also processed using a DNA sample from an individual with partial trisomy for chromosome 21, which resulted in her having a trisomic dose of 21q21 to 21qter (Figure 4). The array result was consistent with the findings of the GTG-banding and allowed for refinement of the breakpoint in the rearrangement to band 21q21.2 (Figure 4).

A potential advantage for using a SNP array was illustrated by a case having mosacism with 20% of cells that were trisomic based on the array analysis (Figure 5). In this specimen an allelic pattern consistent with the presence of 3 distinct chromosomes 21 was seen for the proximal long arm markers, with the distal long arm markers showing a shift in the allele pattern that could indicate the location of a meiotic recombinational event (Figure 5).

The collective information gained from the assessments of the mosaic probands and the positive and negative control subjects, was used to determine if the proportion of trisomic cells estimated from the SNP array, as measured by the smoothed mean of log2 ratios of all probes across chromosome 21, correlated with the percentage of trisomic cells determined by the "gold standard" FISH methodology (Figure 6). These values were positively correlated, with no significant difference being detected in the percentage of trisomic cells quantified using the SNP array compared to the FISH analysis that was completed on uncultured lymphocytes (p-value = 0.80, paired t-test), or on the cultured lymphocytes (p-value = 0.30, paired t-test).





46,XX,der(21)(qter→q21::p11.1→qter)

Figure 4: SNP array result of an individual with partial trisomy for chromosome 21 due to a structural abnormality. Note the increased log2 ratio, and smooth signal, and altered allele difference pattern at 21q21.2 (position 24,959,394) (arrow) with 3 doses continuing for the rest of the long arm. The SNP array result is in agreement with the findings of the GTG-banding analysis which showed a partial trisomy, with a breakpoint at 21q21 (as evidenced by the reduced thickness of the 21q21 dark band in the upper portion of the derivative chromosome 21 when compared to the bottom portion of the derivative chromosome).

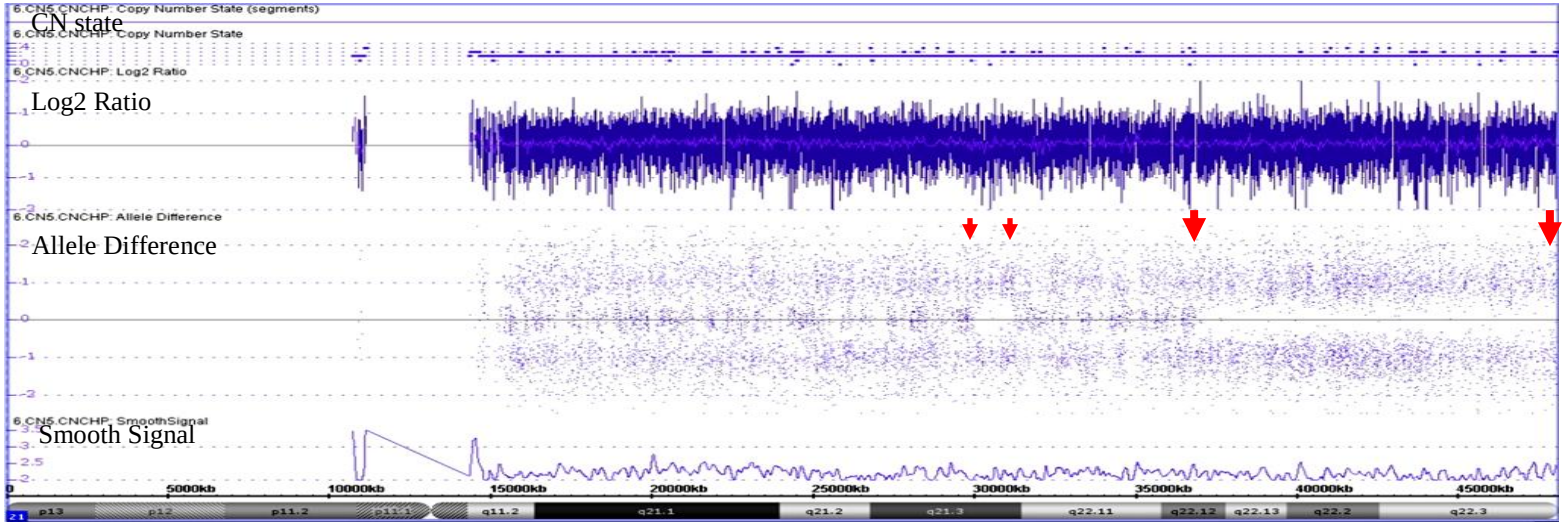


Figure 5: SNP array result of chromosome 21 showing an altered allelic pattern. Probable meiotic recombination events involving loci at 21q21.3 (position 29,989,702-31,288,209) (small arrows), and 21q22.12 (position 37,050,935-47,983,657) (large arrows) occurred during meiosis I, leading to alterations in the allelic patterns in this individual with 20% of trisomy 21cells.

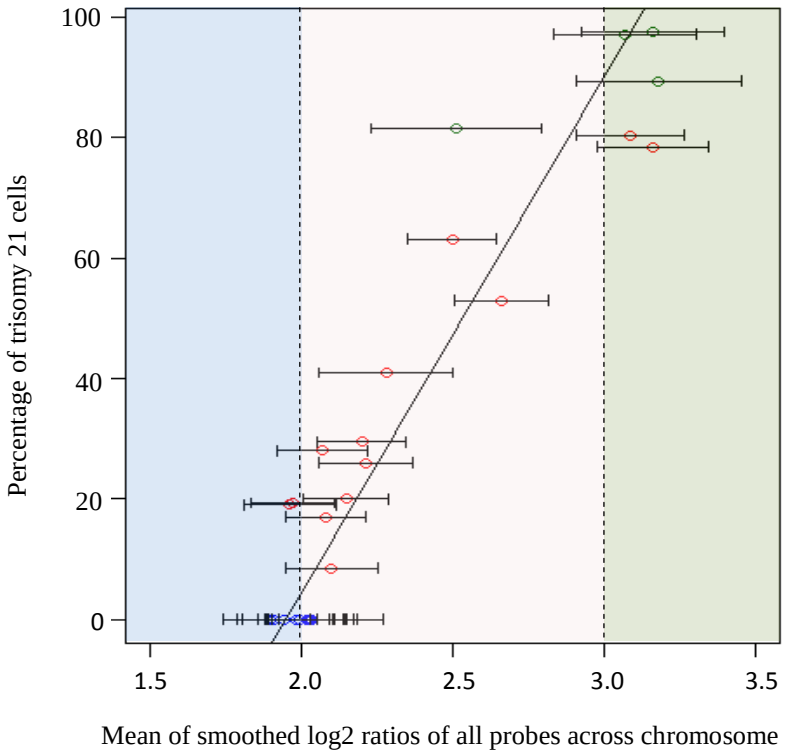


Figure 6: Mean of smoothed log2 ratios of all probes across chromosome 21 and percentage of trisomy 21 cells as determined by FISH. The data are shown with the mean and SD of the smoothed log2 ratios for the negative controls (blue), individuals with mosaicism for trisomy 21 (red) and positive trisomic controls (green). The blue box shows zone of normal copy number (CN of 2), the pink box shows the mosaicism zone and the green box shows the zone of complete trisomy 21 (CN of 3). Note the positive correlation between mean of the smoothed log2 ratios and percentage of trisomic cells (Pearson correlation, r = 0.88, p-value <0.00001).

The BAC array CGH platform was used to process a total of 10 DNA samples, including 6 negative control samples, 3 samples from individuals with mosaicism for trisomy 21 and 1 sample from an individual with full trisomy 21 (Table 5 and Figure 7). The BAC array provided findings that were consistent with those of conventional G-banding results for each of the negative control cases, with no gain of chromosome 21 being detected in any of these samples. A gain of chromosome 21 was detected in the full trisomy 21sample and in 2 of the 3 mosaic cases. The mosaic case that was not readily identified had 26% trisomic cells in the FISH assay and did show a subtle deviation of the log2 ratio, but this variance was too equivocal to allow for clear categorization as a mosaic case (Figure 7b). This same patient was correctly categorized as a mosaic using the SNP array.

A comparison of the potential correlation between trisomic values detected using the BAC array compared to FISH analyses and the SNP array was completed (Figure 8). The estimates of trisomic cells present in individuals (based on the mean log2 ratio of all probes across chromosome 21) from both array platforms were positively correlated with the values obtained in the FISH assay. Furthermore, for 2 of the 3 mosaic cases evaluated with both platforms, there was good agreement for the estimated proportion of trisomic cells present.

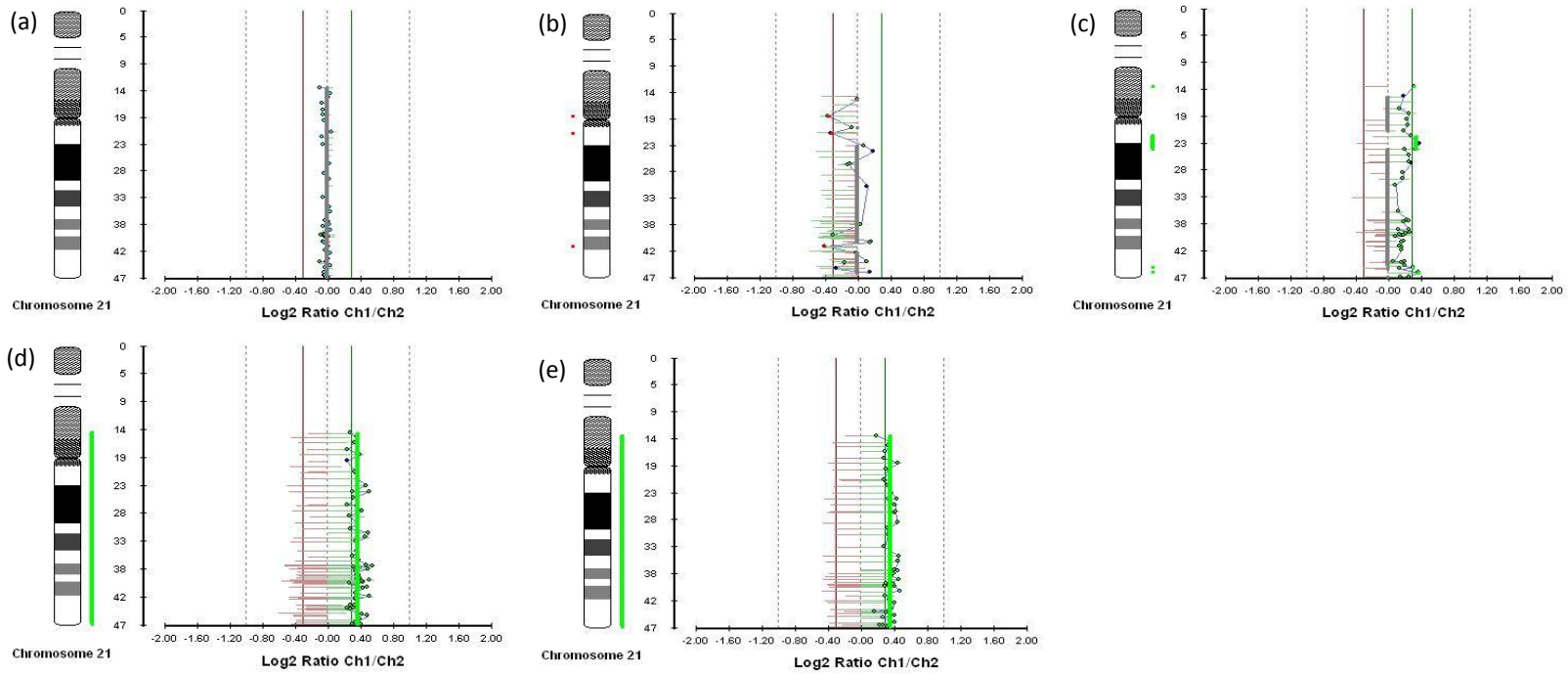


Figure 7: Array CGH results for chromosome 21 using a BAC array platform. This figure shows the results from 5 subjects including: (a) an individual with a normal chromosome 21 complement (log 2 ratio of 0 as expected with 2 copies); (b) an individual with 26% trisomic cells determined by FISH on uncultured lymphocytes, which shows subtle deviations from the log2 ratio (suggestive of mosaicism but not definitive); (c) an individual with 50% trisomic cells as determined using FISH on uncultured lymphocytes, which shows gains (log 2 ratio of 0.3 or more) for several of the BACs evaluated from chromosome 21 (green line); (d) an individual with 80% trisomic cells on uncultured lymphocytes, showing a value consistent with "full" trisomy 21; and (e) an individual with "full" trisomy 21 that has a log 2 ratio consistent with trisomy 21 (but less than the theoretical value of 0.58 as expected for 3/2 copies).

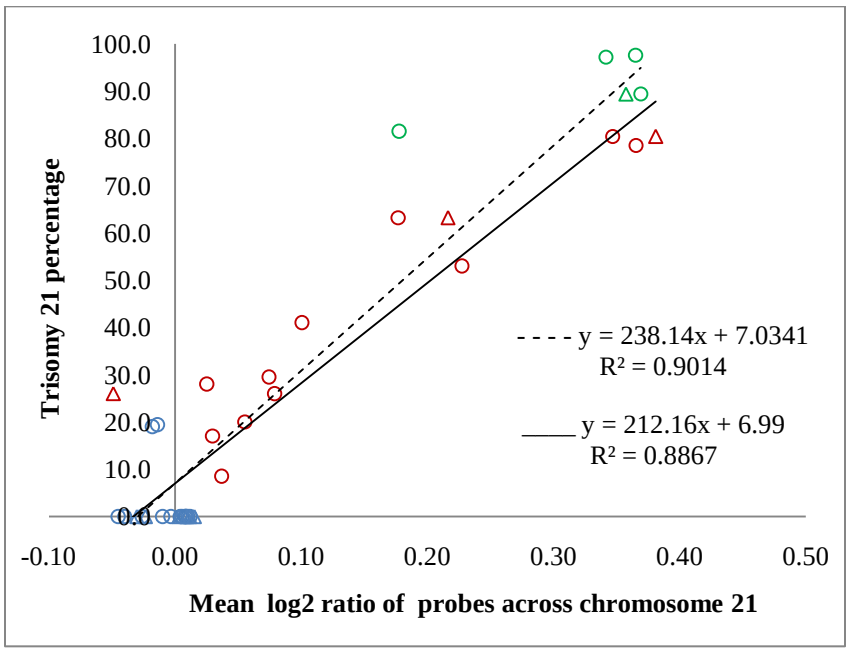


Figure 8: Comparison of the proportion of trisomic cells detected using SNP array, BAC array and FISH methodologies. The mean log2 ratios for chromosome 21 from SNP (○) or BAC (Δ) arrays are presented on the X axis, with the percentage of trisomic cells as determined using FISH, being presented on the Y axis. The data points shown for each individual are the mean values for the negative controls (blue), individuals with mosaicism for trisomy 21 (red) and positive controls (green). Note the nearly parallel trend lines of the SNP (dashed line) and BAC (solid line) platforms.

Discussion

These studies have shown that array-based technology has both strengths and weaknesses in its ability to detect the presence of chromosomal mosaicism. Using the default software analysis setting for CN state, we were unable to detect trisomic cell populations that were present in less than 30%, due to the software categorization of log2 ratio changes from 0-30% as copy number neutral findings. Thus, it is important to include analyses of mosaic cases in laboratory validation studies to ensure that the software criteria are defined in a manner that is congruent with the level of detection for mosaicism that is desired by the lab. In addition to log2 ratio values, SNP arrays provide information about the allelic patterns present in each case. While these patterns were very useful for confirming suspected cases of mosaicism, in our experience the use of allele pattern assessments did not allow for the recognition of additional cases that were undetected using the log2 ratios, or the smoothed signal values.

For the SNP microarray platform (Affymetrix 6.0) and analysis software (ChAS) used, the smooth signal assessment tool provided the most efficient means for detecting mosaicism. Based on the collective information gained from all assessment tools for the 13 cases evaluated, no significant difference was detected in the quantitative percentage of trisomic cells estimated using microarray compared to FISH technologies. However, categorically, the microarray studies resulted in 2 false negative diagnoses (with both cases having less than 20% trisomic cells) and 2 cases that would have been misclassified

as “full” trisomy that were truly mosaic (80% or more trisomic cells). Furthermore, for 3 cases (2 of which had approximately 10% trisomic cell populations), detection of the trisomic cell line was subtle/equivocal and limited to an assessment of the smooth signal value and would have been missed using the other analysis assessments. Nonetheless, these specimens were distinguished as candidates for additional reflex testing with FISH to confirm/refute the possible presence of low level mosaicism. However, without experience in scoring mosaic cases, these subtle results may well have been misclassified as normal, which would have reduced the sensitivity to 0.62. Therefore, as noted above, it is important that geneticists who are interpreting the results of microarray findings gain experience in the assessment of cases having mosaicism to improve the likelihood that they will recognize cases.

There are several explanations for the incongruity between microarray and FISH and/or G-banding studies. Firstly, the DNA used for the microarray studies is collected from all types of leukocytes, while the G-banding studies are performed on T lymphocytes, (following ohytohemaglutinin [PHA] stimulation). Secondly, it is possible that *in vitro* selective growth differentials contribute to the discrepancy. Thirdly, the cells evaluated in the microarray studies represent a composite of the total cell population (average) value, whereas the FISH analyses allow for the recognition of single cell aberrations, making FISH the more sensitive technology for detection of mosaicism if a known target can be anticipated.

Using the current software analysis tool sets supplied by the array vendors, this study showed that levels of mosaicism that were greater than 15% to 20% were consistently detected (Figure 2). This result is in close agreement with the findings of previous investigators who denoted the ability to detect cell mixtures synthesized to have 10% to 20% "mosaicism" using array CGH platforms (Ballif et al., 2006; Cheung et al., 2007; Hoang et al., 2007). In one investigation, a SNP array was reported to be have the ability to detect mosaic cell lines that were present in as few as 5% of the total cell population (Conlin, et al., 2010). These investigators suggested that a key advantage of using a SNP array for mosaicism detection is that one has allelic patterns, as well as copy number changes, to aid in the interpretation of cell lines having mosaicism (Conlin, et al., 2010).

In this study, we demonstrated that both the BAC and SNP platforms could detect mosaicism, with their results being consistent for two of the three cases evaluated using both platforms. This inability to identify the one case having mosaicism using a BAC array could be explained by the smaller number of probes analyzed for chromosome 21 (52 BAC clones versus 24,170 SNP and CN probes). However, this study was not designed to compare the performance between SNP and BAC arrays, since the number of study subjects (31 cases versus 10 cases) varied between platforms, being limited to only 3 cases having mosaicism for the BAC array (due to cost limitations).

While limitations of arrays for identifying mosaicism were observed in this study, it also showed the strength of array-based technology to identify previously unrecognized chromosomal mosaicism. Specifically, the array studies allowed for the detection of mosaicism for trisomy 14 that was not identified in a conventional GTG-banding study. However, an assessment of the percentage of trisomic cells present in this specimen varied between the FISH and SNP array assays, with the percentage in arrays being higher for trisomy 14 (62% compared to 22%) and lower for trisomy 21 (51% arrays; 81.5% FISH; 100% GTG-banding). Possible explanations for the observed variations in frequencies of cell lines include: (1) selective growth pressure against the trisomy 14 cells in the *in vitro* culture system used for the FISH and GTG-banding studies; (2) a higher proportion of trisomy 14 complements in the total leukocyte cell population when compared to the T-cell population, the latter of which is preferentially present in the *in vitro* cultures due to stimulation using PHA; or (3) a potential influence of having used an archival heparinized blood specimen for the DNA extraction.

One major advantage for using a SNP array is that, when combined with parental studies, one can infer the origin of the segregation error (i.e., meiosis I versus meiosis II and mitosis) that resulted in the presence of the extra chromosome in the trisomic individuals. However, these patterns were difficult to distinguish when the percentage of trisomic cells was low. When the proportion of trisomic cells was 50% or more, one could confidently differentiate between meiosis I and meiosis II/mitosis errors. Also, an analysis of alleleic patterns can allow for the recognition of recombination events, which

can be helpful for confirming the presence of mosaicism in specimens having less than 50% trisomic cells, as seen in Figure 5.

In summary, our study demonstrates that array-based technology is effective for detecting mosaicism that is present in 20% or more cells. However, FISH remains the "gold standard" for mosaicism detection and should be considered for confirmation when low level mosaicism is suspected and/or to confirm/refute equivocal array-based results.

Chapter 3

Chromosome-specific telomere length profiles in euploid and trisomic cells obtained from individuals having mosaicism for Down syndrome

Introduction

The growing number of elderly individuals in our population has caused an increased concern about the management of future healthcare needs and costs. It has been estimated that the number of people aged 65 years or older will increase from approximately 35 million in 2000 to 71 million in 2030, with the number of people who are age 80 years or older being expected to increase from 9.3 million in 2000 to 19.5 million in 2030 (www.cdc.gov/mmwr/).This increase in our need for services related to aging individuals underscores the necessity to have a better understanding of the mechanisms of aging and age-related health conditions, with the ultimate goal of improving our ability to diagnosis, treat and possibly prevent age-related diseases such as cancers, and neurodegenerative diseases (i.e., Alzheimer disease). To better understand the mechanisms underlying aging and age-related diseases, different study models have been utilized. For example, investigators have studied biological measures collected from centenarians or other older individuals compared to those observed in young individuals. However, interpretation of the results of these studies can be confounded by differences

in genetic backgrounds, environmental exposures and co-existing health conditions. One approach that can be used to test the contribution of genes on the aging phenotype is to study individuals who have mutations that cause them to have premature aging, such as Hutchinson–Gilford Progeria syndrome and Werner syndrome (Crabbe, et al., 2007; Ariyoshi, et al., 2007; Cao, et al. 2011). Individuals with Down syndrome have also been observed to age prematurely. In particular, people with Down syndrome have an increased risk for developing an early onset of Alzheimer disease (Potter, 1991; Roth et al., 1996).

The results of the many studies focused on understanding aging have shown that both genetic and environmental factors play an important role in the etiology of normal aging and the acquisition of age-related conditions in humans. One genetic factor that has been implicated in the aging process and development of age-related diseases is the shortening of telomeres. A telomere is a specialized structure at the end of a chromosome that consists of tandem repeats $(TTAGGG/CCCTAA)_n$ and telomere associated proteins, including TRF1, TRF2, TIN2, TPP1, Rap1 and POT1 (Aubert and Lansdorp, 2008). Telomeres play an important role in maintaining structural integrity of chromosomes by keeping the chromosome ends intact and preventing the single stranded tip that results from incomplete replication from being recognized as DNA damage (Blackburn, 2005).

The protective function of the telomere was first recognized by Muller (1938) and McClintock (1941). McClintock noted that without a telomere, a chromosome's ends

would fuse and causes genomic instability. In 1961, Hayflick and Moorhead demonstrated that cultured cells have a limited number of divisions they complete before entering a senescence phase. This phenomenon is known as the "Hayflick limit". In 1973, Olovnikov was the first to recognize the "end replication problem". He proposed that following each round of replication, cells lose small segments of DNA due to DNA polymerase being unable to fully replicate the chromosome ends. This phenomenon is ultimately thought to lead to cell death when telomeres reach critically short lengths. At the cellular level, the mechanism whereby telomere shortening triggers replicative senescence and cell death is unclear. It has been speculated that telomere shortening may trigger a TP53 DNA damage response (Davis et al., 2003).

Telomere attrition has been conjectured to play a causal role in aging (Mayer et al., 2006) and has also been associated with a number of health conditions, including, but not limited to, neoplasms (de Lange, 1994; Shay, et al., 1993;Autexier and Greider, 1996; Blackburn, 2005; Gerashchenko, 2010; Ma, et al., 2011, Donate and Blasco, 2011); atherosclerosis (Benetos et al., 2004); heart failure (Wong et al., 2008); obesity (Valdes, et al., 2005); rheumatoid arthritis (Schonland et al., 2003); stress (Epel et al., 2004); chronic schizophrenia (Yu et al. 2008); dyskeratosis congenita (Vulliamy et al., 2004); Alzheimer disease (Panossian et al. 2003); premature aging syndromes (such as Hutchinson–Gilford Progeria syndrome and Werner syndrome [Crabbe et al., 2007; Ariyoshi et al., 2007; Cao, et al., 2011]); chromosome instability syndromes (such as ataxia telangiectasia, Bloom syndrome and Fanconi anemia [reviewed in Callén and

Surrallés, 2004]); and Down syndrome (Vaziri et al., 2003; Jenkins et al., 2006; Jenkins et al., 2008). Telomere length has also been speculated to influence mortality (Cawthon, et al., 2003).

In most somatic cells, the telomere shortens with each cell division due to the end replication problem. This shortening is thought to occur in cells that lack telomerase. Telomerase, which was discovered by Blackburn and colleagues (Greider and Blackburn,1985; Shampay and Blackburn 1988; Greider and Blackburn, 1989), is a specialized enzyme that maintains telomere length by adding TTAGGG repeat sequence to the 3' end of DNA strands in the telomere regions, thus maintaining telomere length (reviewed in Chan and Blackburn, 2004; Aubert and Lansdorp 2008). Telomerase activity is present in germ cells, but not in most somatic cells, leading to somatic cells having a limited lifespan. However, in some cell types, telomere length can be maintained by an alternative pathway (ALT) involving homologous recombination between telomeric or subtelomeric sequences (reviewed in Mefford and Trask, 2002).

Besides the end replication problem, investigators have shown that oxidative damage of the telomeric sequence could be a major cause of telomere shortening. This finding was supported by antioxidant treatment with a free radical scavenger, which was able to reduce telomere shortening in cultured fibroblasts (Von Zglinicki, 2000). One of the key enzymes involved in free-radical metabolism is superoxide dismutase (SOD-1), which is encoded by the *SOD*-1 gene on chromosome 21. In individuals with Down

syndrome, SOD-1 activity is increased due to triplication of chromosome 21, with this increase being disproportionate to the activity of the downstream enzymes responsible for removal of hydrogen peroxide (e.g., glutathione peroxidase). It has been speculated that this imbalance may contribute to premature telomere damage in trisomy 21 cells by an accumulation of hydrogen peroxide.

To date, only a few investigators have studied the relationship between telomere length and Down syndrome. Using a terminal restriction fragment (TRF) telomere assay, which provides an overall average telomere length, Vaziri et al. (1993) observed a significantly higher rate of telomere loss (133 ± 15 bp/year) in people having Down syndrome compared with age-matched controls (41 ± 7.7 bp/year). In addition, individuals with Down syndrome who have dementia/Alzheimer disease or mild cognitive impairment have been reported to have shorter telomeres than individuals with Down syndrome without these conditions (Jenkins et al, 2006; Jenkins et al., 2008). One limitation in interpreting the results from these previous studies is that the observed differences in telomere length (which is a heteromorphic trait) reflect variations between unrelated individuals who, in addition to having Down syndrome, also have differences in their genetic make-up and environmental exposure histories.

Twin studies, comparing identical to non-identical twins or identical twins who are discordant for a phenotype/exposure, are one of the most powerful model systems for teasing apart the contribution of genetic versus environmental influences on a trait.

Another interesting experimental approach for recognizing genetic differences attributable to genetic imbalance is to study individuals having mosaicism, because an individual with mosaicism has 2 or more genetically distinct cell lines that develop within a single zygote and differ only by chromosomal nondisjunction. Despite it is rarity, mosaicism for trisomy 21 is a fascinating condition to study for gaining insight about aging and Alzheimer disease since individuals with mosaicism have two types of cells (i.e., euploid and trisomy 21) that are identical for environmental exposure and nearly identical for their genetic background (i.e., only different by the number of chromosomes 21).

Therefore, this study was performed to evaluate the impact of having a trisomic dose of chromosome 21 on telomere length. By comparing trisomic to euploid cells from individuals having mosaicism for Down syndrome, one could minimize inter-individual differences arising from other genetic/environmental influences. In addition, by utilizing FISH methodology with a telomere-specific probe on metaphase chromosomes, combined with comparative genomic hybridization (CGH) technology, one can compare "chromosome-specific" telomere lengths from euploid cells to trisomic cells obtained from individuals having mosaicism for trisomy 21. Lastly, while there have been previous reports of telomere lengths in older individuals having Down syndrome, there have been no reports of the telomere lengths in children. The data derived from this study allowed for a direct testing of the following hypotheses: (1) Differences in telomere length can be detected between cell types based on their genetic make-up; (2) Telomeres

are shorter in cells having a trisomic dose of chromosome 21 compared to cells having a euploid (2 copies) dose; (3) Telomere attrition can be observed as a biological change that occurs during early childhood in a cell having a trisomy 21 complement; (4) Telomere attrition associated with trisomy 21 affects all chromosome equally, rather than having a targeted effect on a subset of chromosomes.

Materials and Methods

Study Participants

Participants were recruited through the National Down Syndrome Congress (NDSC) meeting (Washington D.C., 1995), announcements made in national and local Down syndrome support groups, newsletters, the International Mosaic Down Syndrome Association (IMDSA) (via their website and IMDSA conferences) and through visits for genetic counseling. The only inclusion criterion for study participation was that the individual had a confirmed diagnosis of mosaicism for trisomy 21. All families have given informed consent to participate in this study, which has been approved by the Virginia Commonwealth University Institutional Review Board (protocol #179).

Cell Cultures

Duplicate stimulated lymphocyte cultures were established and harvested according to standard protocols [RPMI 1640 media, supplemented with 15% fetal bovine serum (FBS) and phytohemaglutinin (PHA)]. A total of 72 hours after culture initiation, the lymphocyte cultures were harvested as described previously, with colcemid being added 15 minutes prior to harvest to enrich the specimens for cells that were in the mitosis portion of the cell cycle (Leach and Jackson-Cook, 2001).

The lymphocyte chromosome preparations that were used in this study were obtained from archival cell pellets that were harvested from 2004 to 2009. These archival

pellets were kept in a modified Carnoy's fixative (3 parts of methanol and 1 part of acetic acid) at -20^{o}C. To reduce cytoplasm, which might compromise probe hybridization, the cell pellets were washed in Carnoy's fixative twice before the cell solutions were dropped onto the slides using a Thermatron (temperature of 22±1°C and humidity of 48±2%). The slides were reviewed with a phase contrast/bright field microscope to ensure that the quality of the preparation was adequate for the FISH study (number of metaphases/interphase nuclei and quality of preparation [lack of cytoplasm]). After review, the slides were placed on a hot plate at 60°C for an hour, followed by aging at room temperature for 1-2 weeks. Alternatively, rather than aging at room temperature, for a portion of the cells the aging was induced by soaking the slides in 2xSSC for 10 minutes prior to the FISH experiment. If cytoplasm was present, the slides were soaked in Carnoy's fixative for 1 additional hour before proceeding with probe hybridization.

Chromosome-Specific Telomere Length Assay

Metaphase chromosomes were hybridized with a telomere-specific FITC-labeled synthetic peptide nucleic acid (PNA) probe following the manufacturer's protocol (DakoCytomation, Denmark). In addition, an FITC-labeled probe that is specific for the pericentromeric region of chromosome 2 was simultaneously hybridized to the metaphase spreads as a control (and to serve for standardization of intensity values from cell to cell) (Mayer et al., 2006). Briefly, slides were fixed in cold Carnoy's fixative for 1 hour. After air-drying, the slides were rinsed with 1xTBS (Tris-Buffered Saline, pH 7.5) for 2 minutes, fixed in 3.7% formaldehyde in 1xTBS for 2 minutes and then rinsed (twice in

1xTBS for 5 minutes). The slides were then immersed in a pre-treatment solution containing proteinase K, for 10 minutes, rinsed (twice in 1xTBS for 5 minutes) and dehydrated (using a cold ethanol series [70%, 85% and 100%]). After air-drying, a cocktail probe mixture (11µl of FITC-labeled telomere specific probe and 1 µl of FITC-labeled centromere-2 probe per subject [a half slide area]) was added to each slide and the probe and metaphase spreads co-denatured in a thermocycler at 80^{o}C for 3 minutes. After hybridization in a dry hybridization chamber at room temperature for 2 hours, the excess and unbound probe was removed by rinsing (once in a manufacturer provided rinse solution at room temperature for 1 minute, followed by 5 minutes in a manufacturer provided wash solution at 65^{o}C). Following serial dehydration in a cold ethanol series (70%, 85% and 100%), the slides were air-dried and counterstained with a 5:1 DAPI/propidium iodide solution.

The telomere lengths of each chromosome were assessed using a semi-quantitative FISH method (CGH software from Applied Imaging Cytovision System) as described by Leach et al. (2004). Briefly, three images were captured with a CCD camera for each metaphase: (1) a reverse DAPI image, which allows for chromosome identification and subsequent karyotyping; (2) a test/ FITC image, showing telomeric and centromeric probe signals; and (3) a reference/ propidium iodide image that allows for visualization of the chromosome body. Fluorescent intensities obtained from the "test" and "reference" images were used for calculating the ratio profiles of relative telomere intensity for each chromosome arm. Overlapping telomeres or telomeres that were in

close proximity were excluded from the analysis. For each person the intensity values were averaged over 20 homologs from 10 euploid cells and 20 homologs from trisomic cells (30 homologs for chromosome 21). A representative metaphase stained with telomeric probe and centromeric probe for chromosome 2 with DAPI/PI counterstain is shown in Figure 9.

Cen-2 intensities of all individuals were standardized to a value of 4, and the respective telomere lengths for each person adjusted proportionally. All statistical analyses were performed using the R statistical software program.

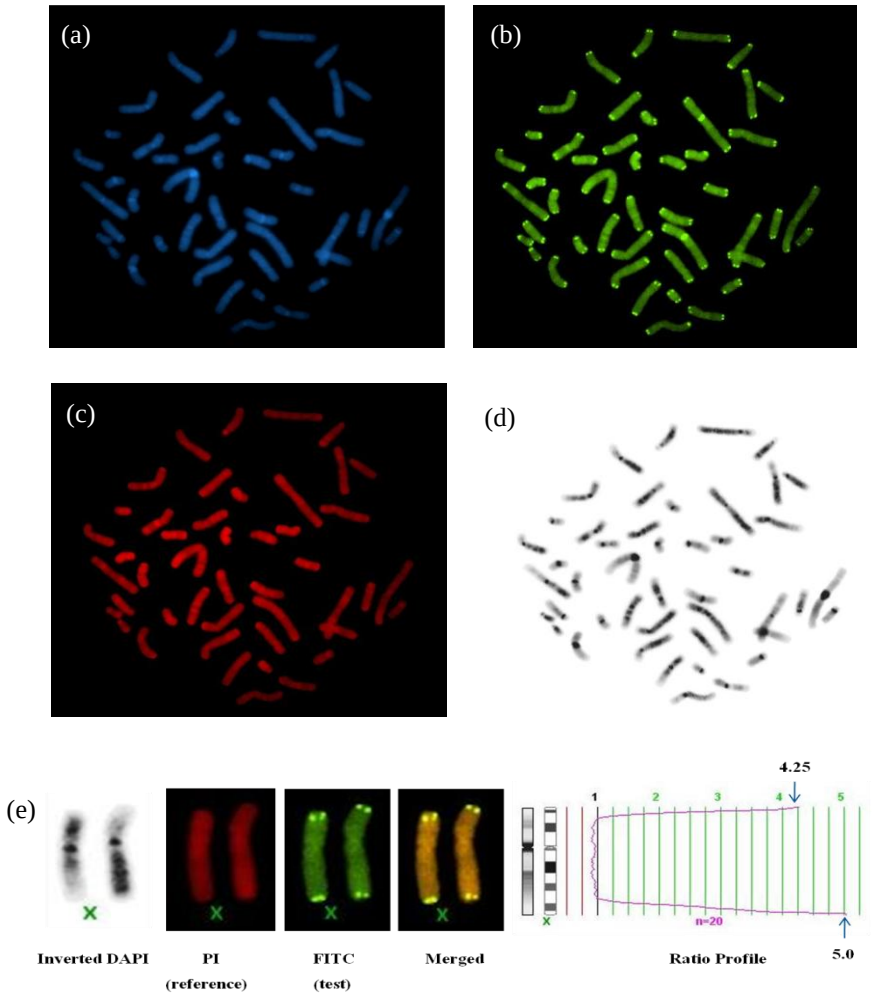


Figure 9: Representative images showing FISH-based quantification of chromosome-specific telomere intensity. Image (a) is a metaphase spread stained with DAPI, which allows for chromosome identification as shown in a reverse DAPI image (d). Image (b) shows the FITC-labeled PNA telomeric signals and the PNA centromeric signal for chromosome 2 (test image). Frame (c) shows this same metaphase as it appears with a PI-stain (reference image). For each chromosome, CGH software transformed the intensities of telomere signals into ratio profiles averaged over the 20 (or 30) homologs. These ratios were based on the florescence intensities of the test and reference images. As seen in (e), telomeres of chromosome X, as identified by the inverted DAPI banding pattern, showed a short arm telomere relative fluorescence unit (RFU) value of 4.25, while the long arm telomere value was 5.00. Note that signal intensities between the replicate sister chromatids of each homolog were very similar.

Results

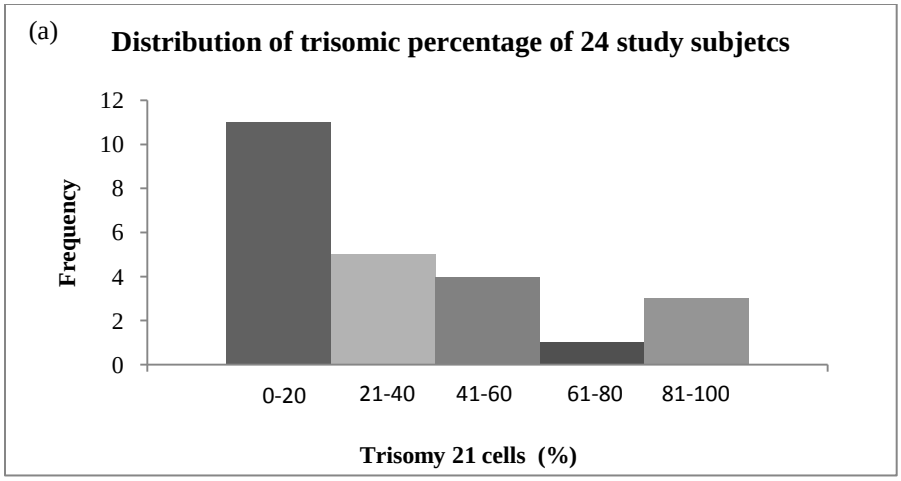
Chromosome-specific telomere length assays were performed on lymphocyte samples obtained from 24 individuals with mosaicism for trisomy 21, including 12 males and 12 females. These study participants ranged in age from 3 weeks old to 28 years old, with a median age of 3.5 years old. The age, gender and percentage of trisomy 21 cells for each proband are given in Table 6. The distribution of the percentage of trisomic cells and the age of all study subjects are shown in Figure 10.

Overall telomere length in euploid and trisomic cells

Estimates of the overall telomere intensity in each individual were obtained by averaging the intensity values of all chromosomes, except chromosome Y (which was excluded from the analysis since the females would not have a correlate chromosome). This analysis showed no significant correlation between overall telomere intensity values and the percentage of trisomic cells, which was log transformed values present in the probands (Pearson correlation, $r = -0.15$, p-value = 0.485). In addition, no correlation between overall telomere intensity values and age was observed in the study cohort, which was comprised of predominantly young individuals (Pearson correlation, $r = -0.117$, p-value = 0.587) as shown in Figure 11a and 11b.

Table 6: Age, gender and percentage of trisomy 21 cells of 24 study subjects

Case	Age	Gender	Percentage of trisomy 21 cells
1	0.4	F	24.9
2	4.0	F	19.0
3	0.1	M	50.5
4	12.0	F	26.5
5	3.0	M	23.8
6	0.3	M	48.0
7	2.0	F	9.4
8	28.0	F	90.5
9	5.0	M	12.0
10	25.0	M	8.4
11	14.0	F	10.3
12	7.0	F	91.6
13	3.0	M	29.5
14	11.0	F	17.6
15	0.9	M	62.0
16	4.0	M	10.8
17	0.3	F	56.8
18	0.5	M	10.9
19	11.0	M	6.55
20	21.0	F	92.7
21	2.5	F	43.0
22	3.0	F	23.2
23	18.0	M	11.0
24	2.9	M	11.7



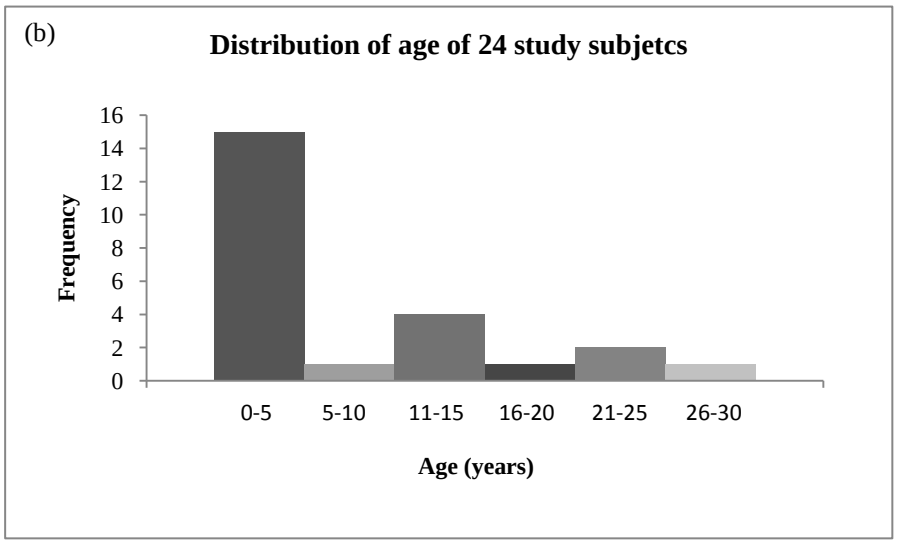


Figure 10: Distribution of the percentage of cells having trisomy 21 and age for the 24 study subjects. Note the skewing of distribution of trisomic cells toward low levels (less than 20%) (a), and the skewing of the study participants' age toward very young individuals (less than 5 year old) (b).

To test the primary hypothesis of this study, the overall telomere intensity values between euploid and trisomic cells "within a person" were compared. Telomere intensities for the short arm of the 23 different chromosomes (1-22 and X) in females and 24 different chromosomes (1-22, X and Y) in males were compared between the two cell types using a paired t-test. Analyses were performed in the same manner for long arm of the chromosome. A Bonferroni correction for multiple comparisons was applied for these tests [the p-value was set at < 0.001 (i.e., 0.05/46 and 0.05/48)]. The mean, standard deviation, mean of the differences and p-values for these individual-specific comparisons are summarized in Table 7. A significant difference between telomere intensity values present in euploid compared to trisomic cells was observed for 7 individuals for the short arms of chromosomes and in 13 individuals for the long arms of chromosomes. A total of 7 individuals had significantly different values for both their short arms and long arms. For each of these cases, there was consistency in the directionality of the observed differences (i.e., shorter in the trisomic cells for both long arm/short arm or longer in the trisomic cells for both long arm/short arms). No clear ascertainment pattern was observed for the probands who had shorter telomeres in their trisomic cells versus those who had longer telomeres in their trisomic cells (not apparently related to age or the proportion of trisomic cells).

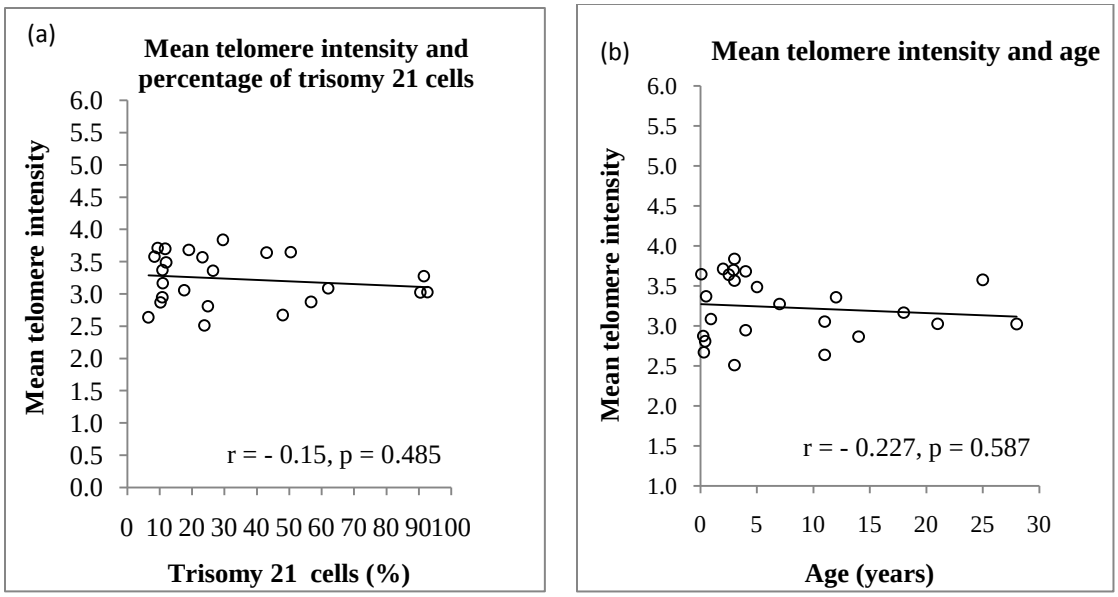


Figure 11: Overall mean telomere intensity compared to (a) the probands' percentage of cells with trisomy 21 and (b) the proband's age. Each data point represents an individual (○). No significant correlation was observed for the trisomic percentage (Pearson correlation, r = -0.15, p-value = 0.485) or age (Pearson correlation, r = -0.117, p-value = 0.587).

Table 7: Telomere intensity values in euploid compared to trisomic cells obtained from lymphocytes of individuals having mosaicism for trisomy 21.

Age (years)	Short arm						Long arm					
	Euploid cells		Trisomy 21 cells		Mean of the differences (e-t)	p-value	Euploid cells		Trisomy 21 cells		Mean of the difference (e-t)	p-value
	Mean	SD	Mean	SD			Mean	SD	Mean	SD		
0.1	2.883	0.544	3.188	0.809	-0.305	0.027	2.789	0.416	2.933	0.548	-0.144	0.095
0.3	2.780	0.519	2.223	0.361	0.556	**<0.0005**	2.568	0.502	2.148	0.405	0.420	**<0.0005**
0.3	4.916	1.149	4.349	1.068	0.568	0.006	4.526	1.097	4.013	0.939	0.513	0.003
0.4	3.630	0.846	3.851	0.937	-0.221	0.137	3.707	0.883	3.397	0.774	0.311	0.957
0.5	4.883	1.287	4.589	1.147	0.294	0.092	4.443	0.860	4.551	0.923	-0.108	0.490
0.9	4.206	1.015	3.883	1.004	0.322	0.027	4.008	0.816	3.610	0.838	0.399	0.004
2.0	2.848	0.792	3.376	0.829	-0.529	**<0.0005**	2.469	0.495	3.152	0.745	-0.683	**<0.0005**
2.5	2.774	0.544	2.100	0.647	0.674	**<0.0005**	2.489	0.419	1.739	0.237	0.750	**<0.0005**
2.9	4.297	1.019	4.485	0.979	-0.189	0.078	3.993	0.740	3.954	1	0.009	0.956
3.0	2.000	0.352	2.155	0.277	-0.154	0.017	2.021	0.272	2.249	0.392	-0.228	**0.0004**
3.0	6.587	1.374	5.856	1.397	0.731	0.058	6.166	1.379	5.608	1.256	0.558	0.076
3.0	3.559	0.504	2.666	0.482	0.892	**<0.0005**	3.227	0.556	2.606	0.568	0.622	**<0.0005**
4.0	2.917	0.443	2.580	0.412	0.337	0.001	2.857	0.369	2.472	0.361	0.385	**<0.0005**
4.0	3.157	0.733	2.764	0.472	0.394	0.006	3.229	0.705	2.565	0.435	0.664	**<0.0005**
5.0	4.256	1.009	4.485	0.979	-0.228	0.034	3.956	0.733	3.984	1	-0.0282	0.870
7.0	2.363	0.586	2.917	0.523	-0.554	**<0.0005**	2.291	0.389	2.678	0.387	-0.389	**<0.0005**
11.0	3.426	0.811	2.737	0.681	0.690	**<0.0005**	3.296	0.688	2.581	0.550	0.716	**<0.0005**
11.0	2.533	0.527	2.879	0.741	-0.345	0.001	2.410	0.592	2.844	0.832	-0.434	0.036
12.0	2.759	0.432	3.575	0.597	-0.816	**<0.0005**	2.646	0.490	3.415	0.497	-0.769	**<0.0005**
14.0	2.295	0.589	2.197	0.337	0.098	0.249	2.019	0.352	2.090	0.390	-0.070	0.332
18.0	2.948	0.750	3.360	1.819	-0.412	0.052	2.575	0.492	2.942	0.625	-0.368	**0.0004**
21.0	2.819	0.621	3.166	0.702	-0.347	0.029	2.779	0.740	2.983	0.493	-0.204	0.210
25.0	2.887	0.909	2.795	0.568	0.091	0.606	2.577	0.524	2.846	0.614	-0.269	**0.0003**
28.0	2.266	0.412	2.730	0.565	-0.464	0.001	2.102	0.324	2.552	0.488	-0.450	**<0.0005**

Chromosome-specific differences in telomere length

The telomere intensity values of each of the autosomal and sex chromosomal arms of all individuals (n = 24 for chromosomes 1-22 and X; n = 12 for chromosome Y) were visualized using boxplots of the raw data for euploid and trisomic cells (Figure 12). In this figure, telomere intensities were not equal among all chromosome arms. The shortest telomeres were found on 9q for both trisomic and euploid cells, with 1p, 2q, 4p, 16q, 17p, 17q, 19p and 22q also tending to have relatively short telomeres. The longest telomeres were found on 3p for both trisomic and euploid cells, with the Y chromosome also tending to have longer telomeres in the males evaluated.

A striking similarity of the telomere length profiles of the euploid and trisomic cells was also observed (Figure 13a). The difference in telomeric values in the euploid compared to trisomic cells resulted in positive values (shorter in trisomic cells) for 26 chromosomal arms and negative values (longer in trisomic cells) for 21 chromosomal arms (Figure 13b). However, the majority of these values were not significant using a paired t-test, except for the difference for 2q, and 6q (Table 8). However, if one applies a Bonferroni correction for multiple tests (48 tests); none of the observed difference values reach statistical significance.

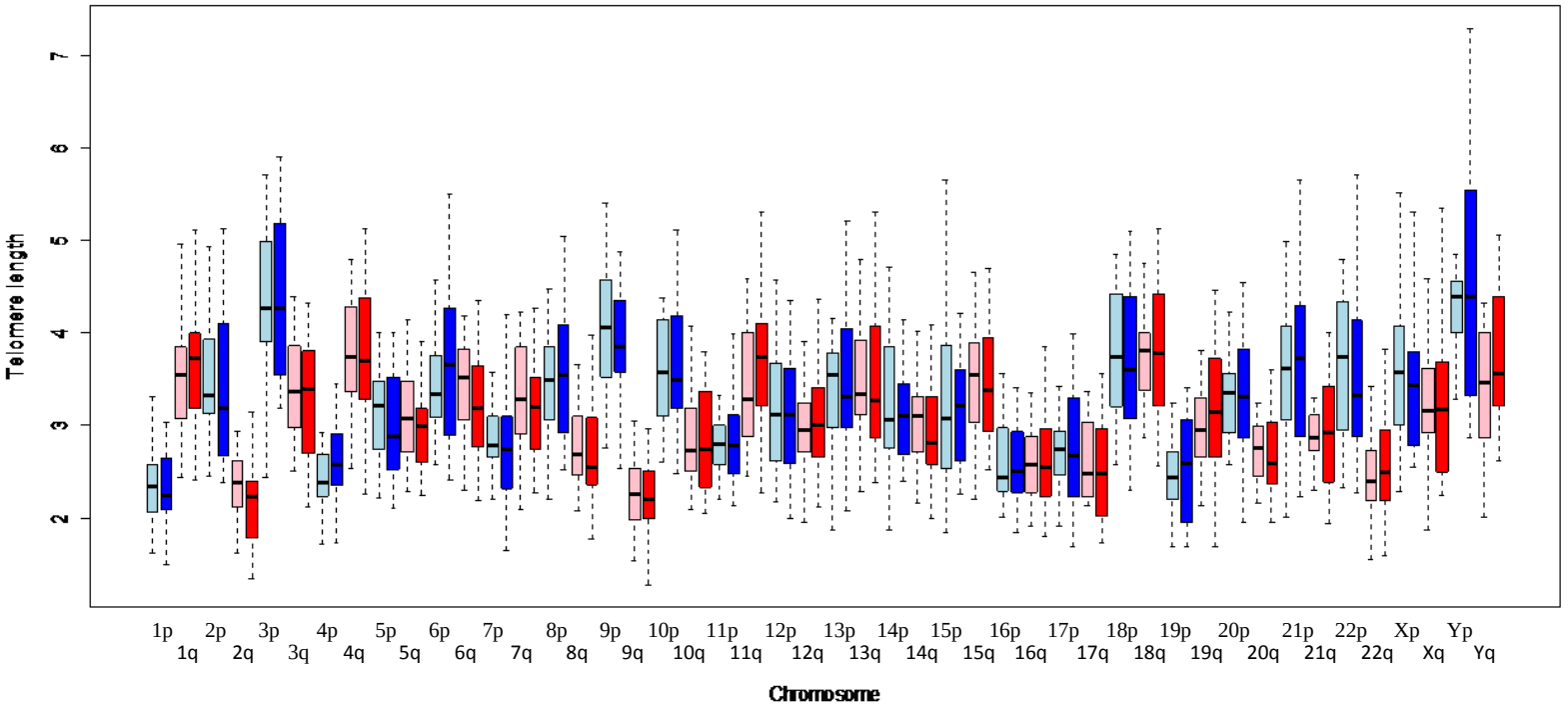


Figure 12: Boxplot distribution of chromosome-specific telomere lengths in euploid and trisomic cells of all study subjects (n=24). The data for the individual chromosomes in euploid cells (light blue = short arm, pink= long arm) and trisomic cells (dark blue =short arm, red = long arm) is shown by the minimum (lower bar), 25th percentile (lower box boundary), median (line), 75th percentile (upper box boundary) and maximum (upper bar).

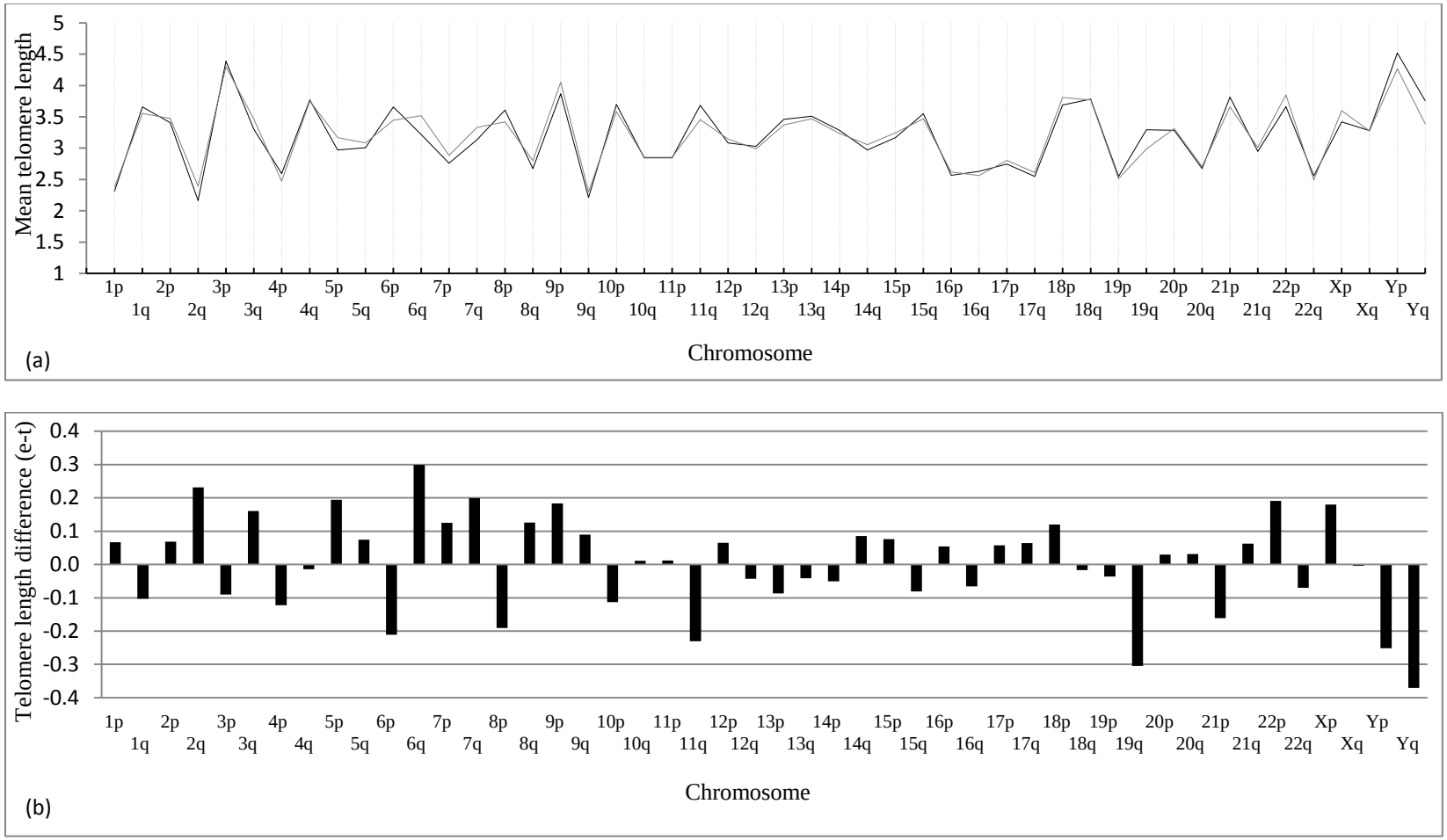


Figure 13: Mean telomere lengths in euploid and trisomic cells of all study subjects (n=24). (a) The data from individual chromosome arms in euploid cells (gray) and trisomic cells (black) reveal a nearly parallel course for all chromosomes. (b) The difference (euploid-trisomic) in telomere intensity values are shown for each chromosome.

Table 8: Chromosome-specific telomere lengths in euploid and trisomy 21 cells obtained from lymphocytes of individuals with mosaicism for Down syndrome (n=24)

Chromosome	Short arm						Long arm					
	Eup cells		Tri21cells		Mean difference (e-t)	p-value	Eup cells		Tri 21 cells		Mean difference (e-t)	p-value
	Mean	SD	Mean	SD			Mean	SD	Mean	SD		
1	2.381	0.471	2.314	0.431	0.067	0.532	3.554	0.7250	3.657	0.687	-0.103	0.562
2	3.474	0.682	3.405	0.838	0.068	0.683	2.392	0.376	2.160	0.463	0.231	**0.044**
3	4.300	0.899	4.391	0.920	-0.090	0.635	3.463	0.685	3.303	0.623	0.160	0.327
4	2.471	0.380	2.593	0.423	-0.122	0.317	3.753	0.649	3.767	0.736	-0.013	0.935
5	3.166	0.589	2.972	0.579	0.194	0.197	3.081	0.485	3.006	0.506	0.074	0.538
6	3.448	0.553	3.658	0.802	-0.211	0.181	3.518	0.707	3.220	0.641	0.299	**0.049**
7	2.885	0.359	2.760	0.569	0.125	0.238	3.331	0.615	3.132	0.512	0.199	0.232
8	3.418	0.554	3.608	0.733	-0.190	0.198	2.798	0.499	2.672	0.556	0.126	0.282
9	4.051	0.697	3.868	0.669	0.183	0.285	2.303	0.444	2.214	0.413	0.090	0.310
10	3.585	0.572	3.698	0.792	-0.113	0.441	2.856	0.512	2.845	0.590	0.011	0.931
11	2.855	0.472	2.843	0.489	0.012	0.912	3.454	0.669	3.685	0.814	-0.231	0.172
12	3.146	0.636	3.081	0.633	0.065	0.702	2.988	0.509	3.031	0.528	-0.043	0.752
13	3.371	0.615	3.458	0.821	-0.087	0.574	3.467	0.619	3.509	0.859	-0.041	0.818
14	3.233	0.741	3.284	0.782	-0.050	0.769	3.057	0.500	2.972	0.589	0.085	0.568
15	3.242	0.964	3.166	0.603	0.075	0.743	3.469	0.659	3.550	0.773	-0.081	0.699
16	2.621	0.471	2.568	0.457	0.053	0.628	2.562	0.462	2.628	0.519	-0.066	0.578
17	2.806	0.651	2.748	0.611	0.057	0.738	2.612	0.396	2.547	0.565	0.064	0.611
18	3.810	0.684	3.691	0.799	0.120	0.503	3.768	0.579	3.785	0.745	-0.017	0.923
19	2.513	0.441	2.550	0.555	-0.036	0.784	2.988	0.483	3.293	0.991	-0.304	0.188
20	3.313	0.480	3.283	0.667	0.030	0.861	2.704	0.311	2.672	0.470	0.032	0.770
21	3.655	0.829	3.816	1.240	-0.161	0.548	3.012	0.553	2.950	0.649	0.063	0.718
22	3.851	1.306	3.660	1.120	0.191	0.465	2.490	0.430	2.560	0.517	-0.070	0.475
X	3.600	0.851	3.420	0.681	0.180	0.352	3.277	0.705	3.281	0.844	-0.005	0.982
Y	4.269	0.473	4.520	1.345	-0.251	0.470	3.387	0.710	3.758	0.737	-0.371	0.124

Discussion

Telomere shortening has been previously observed in older individuals with Down syndrome, compared to normal age-matched controls, using terminal restriction fragment (TRF) methodology (Vaziri et al., 1993). The TRF assay is one of the first techniques used to assess telomere length. Based on the fact that that telomeres lack cleavage sites for restriction enzymes, the TRF assay is implemented by cutting genomic DNA using a common 4 base-cutter restriction enzyme, followed by gel electrophoresis and hybridization to a probe with telomere specific sequence, to obtain an average (pooled over all chromosomes) telomere length based on the size parameters of the resulting DNA smear. However, if a subset of chromosomes has short telomeres, or possibly have elongated telomeres, this information could be missed using the TRF assay. Therefore, in this study, we elected to investigate the length of telomere repeats using a chromosome-specific assay. Also, by studying individual chromosome arms in people who have mosaicism for trisomy 21, we were able to measure telomere lengths in normal and trisomic cells obtained from the same individual, thereby controlling for the potentially confounding effects of heritable variation in telomere lengths and different environmental exposures between people.

This is the first study to use quantitative FISH methodology to measure chromosome-specific telomere length profiles of trisomic and euploid cells. However, this chromosome-specific assay has been previously shown to be a reliable measure of telomere length in studies completed on a variety of cell types from different individuals (Lansdorp et al., 1996; Martens et al., 1998; Graakjaer et al., 2003).

Specifically, we have shown that the overall average of telomere length estimates obtained from our adaptation of the quantitative FISH assay correlate with the estimates obtained from the "gold standard" TRF assay (Leach et al., 2004).

Of twenty-four subjects evaluated in this current study, we observed a weak negative correlation between telomere intensity values and the percentage of trisomic cells present in the probands. However, our observation failed to show a significant correlation between percentage of trisomy 21 cell and telomere length. This observation could be attributable to the small sample size. In addition, given that the individuals with mosaicism who were evaluated in this project had a skewing of their distribution of trisomic cells toward low levels (less than 20%), as shown in Figure 10a, it is difficult to conclude that dosage of trisomy 21 cells has no impact on telomere length. The lack of a clear effect of the proportion of trisomic cells on telomere length could reflect the low percentage of trisomic cells in these individuals, who also showed fewer other phenotypic traits seen in people with Down syndrome, suggesting they had a lower "threshold" of imbalance.

We also observed a weak, non-significant negative correlation between telomere intensity and age. However, the skewing of the study participants' age toward very young individuals (less than 5 year old) is a possible explanation for this observation. Therefore, since most of study subjects were very young, we cannot conclude that in general, age has no effect on telomere length in individuals with mosaicism for Down syndrome. However, this study is the first to show that the

presence of a trisomic complement in young individuals has a minimal impact on telomere length.

While no generalized effect of trisomy 21 was observed, differences in overall telomere intensity between euploid and trisomic cells "within a person" were detected for a subset of people. Approximately 50% of all individuals studied had telomere intensity values in euploid cells that were brighter than those in the trisomic cells for both arms of their chromosomes. However, significant differences were found in only 16.7% for short arms and 25% for the long arm. Unexpectedly, the young adults in this study (18 to 28 years old) tended to have longer telomeres in their trisomic cells longer compared to their euploid cells.

These observations suggest that the telomere length dynamics in young individuals having low levels of trisomic cells are not clearly different from those of young people having normal chromosomal complements, the latter of whom have been shown to have the greatest decrease in their telomere length during the first years of life, with little additional attrition occurring until they reach middle age (Zeichner et al., 1999).

We found that telomere lengths were not equally distributed among all chromosome arms. We also observed that while each person has his/her own specific telomere length profile; there was a common profile of telomere length shared between different individuals. This observation is also consistent with results of

previous reports in people with normal chromosomal complements (Graakjaer et al, 2003, Mayer et al., 2006).

The finding that both trisomic and euploid cells had strikingly similar chromosome-specific telomere length profiles (Table 8 and Figure 13a) implies that: (1) dosage of trisomy 21 cells has a minimal impact on telomere lengths in this young population; and (2) the impact, if any, tends to be generalized for all chromosomes.

In summary, the results of this study failed to detect a clear influence of a trisomy 21 complement in telomere length in young children and young adults. However, this conclusion does not seem to apply to older people having trisomy 21. Telomeres in euploid and trisomic cells may be subjected to different regulatory control processes and such processes may be acquired later in life. In addition, sensitivities to mutations that are accumulated over time, which could result in different in telomere attrition rates, may be different between euploid and trisomic cells. Furthermore, the cell cycle in people having trisomy 21 has been shown to be shorter than that of normal people (Leonard and Merz, 1983). Given this observation, it is feasible that the telomeric attrition observed in older individuals having Down syndrome simply reflects the fact that their cells have completed more rounds of replication. The shortening of the cell cycle may also explain, at least in part, the premature aging phenotype associated with Down syndrome since trisomic individuals complete more cell cycles in a smaller amount of time. Clearly, additional studies should be carried out in extended populations with individuals from older ages. In addition, it would be very interesting to perform a longitudinal study in this

young population, to measure their rate of telomere shortening in trisomic and euploid cells compared to that of age-matched controls having a normal complement. Collectively, further studies of telomere length variation in people having mosaicism could provide insight about the association between telomere attrition and the premature aging phenotype in Down syndrome.

Chapter 4

Frequency of chromosomal instability and chromosome-specific telomere length profiles in individuals with mosaicism for Down syndrome

Introduction

Acquired chromosomal changes have been associated with the development of several diseases including, but not limited to, solid tumors [such as malignant glioma (Lindström et al., 1991), prostate cancer (Zitzelsberger et al., 1994), renal cell carcinoma (Kuroda et al., 2010), malignant melanoma (Balaban et al., 1986)]; hematological disorders [such as leukemia and lymphoma (Kaneko et al.,1982, Clare et al., 1982, Hagemeijer et al., 1981; Wisniewski and Hirschhorn,1983)]; and neurological disorders [such as Alzheimer disease (Migliore et al., 1997; Petrozzi et al., 2002; Zekanowski and Wojda, 2009) and Parkinson disease (Petrozzi et al., 2002; Migliore et al., 2002; reviewed in Migliore et al., 2011)]. Several investigators have shown the frequencies of acquired chromosomal abnormalities involving the sex chromosomes to be increased in cultured lymphocytes from healthy older individuals (Jacobs et al, 1961; Jacob et al., 1963; Fitzgerald and McEwan, 1977; Martin et al, 1980; Guttenbach et al, 1995; Richard et al, 1994; Catalan et al, 2000), but there is a paucity of information available regarding the frequency of acquired **autosomal** abnormalities and their clinical consequences. Furthermore, while sex chromosome

loss has been clearly shown to be correlated with age (Bolognesi et al., 1999; Bonassi et al., 2001), the influence of age on acquired **autosomal** aneuploidy is not well established. Evidence for a relationship between aging and an increased frequency of acquired chromosomal instability comes from studies of individuals having premature aging syndromes, such as Werner syndrome. Cells from individuals having Werner syndrome have shown a higher incidence of chromosomal abnormalities than cells from normal controls (Crabbe et al., 2007; Ariyoshi et al., 2007). These studies also showed a relationship between telomere dysfunction and acquired chromosome abnormalities (Crabbe et al., 2007; Ariyoshi et al., 2007), which is a finding that supports the previous hypotheses of Barbara McClintock (McClintock, 1941). Aviv and Aviv (1998) proposed that erosion of the telomere leads to genomic instability. At the chromosomal level, the resultant abnormalities may give rise to chromosome fragments lacking a centromere (acentric fragments), which would subsequently not be pulled toward the daughter nuclei at the time of nuclear division and would either randomly segregate to the nuclei of daughter cells or be excluded into a small cytological structure called a micronucleus/micronuclei (MN).

It is well documented that people with Down syndrome age prematurely, as they show signs of degenerative changes in their physical appearance, including premature graying and loss of hair, age-related visual and hearing loss, skin atrophy and neuropathologic features identical to those observed in people having Alzheimer disease (Potter, 1991; Esbensen, 2010). However, little is known about the frequency of acquired chromosome abnormalities in people having Down syndrome.

In addition to the standard G-band karyotyping, the cytokinesis-blocked micronucleus (CBMN) assay is one of the preferred methods for assessing acquired DNA damage at the chromosome level (Fenech et al., 2003). It allows for the measurement of acquired whole chromosomal loss and/or chromosomal structural abnormalities (i.e., acentric fragments; dicentrics, etc.), with the aberrant chromatin being excluded into MN. Different mechanisms may be involved in the formation of MN, including (but not limited to): 1) misrepaired or unrepaired DNA double-strand breaks resulting in acentric chromosome fragments; 2) hypomethylation of centromeric and pericentromeric repeat sequences leading to malsegregation of chromosomes and subsequent loss; and 3) mutations leading to defects in kinetochores or microtubules, defects in mitotic spindle assembly, mitosis check point genes; and/or abnormal centrosome amplification. A strong correlation between chromosomal aberrations, as assessed using the gold standard of metaphase chromosome studies and MN formation has been shown (Ramalho et al., 1988). The CBMN assay also allows for measurement of other biological markers which appear as distinctive cytological structures, including nuclear buds (NBUD), which are thought to be biomarkers of elimination of amplified DNA and/or DNA repair complexes, and nucleoplasmic bridges (NPB), which are thought to be biomarkers of DNA misrepaired and/or telomere end fusions (Fenech et al., 2011).

NPB are thought to originate primarily from dicentric chromosomes that are pulled to opposite poles of the cell during anaphase. In turn, dicentric chromosomes are thought to arise from misrepair of chromosome breaks or telomere end fusions. In addition, defects in protein complexes involved in sister chromatid separation during

anaphase may result in NPB formation. NBUD are thought to occur as a means for elimination of amplified DNA, but may also be present as a remnant of NPB breakage (Fenech et al., 2011).

MN frequencies have not been extensively studied in individuals with Down syndrome (Table 9). Investigators who evaluated buccal cells showed a significant increase in MN frequencies in individuals with Down syndrome compared to normal healthy controls (Thomas et al., 2008; Ferreira et al., 2009). However, the studies completed in lymphocytes were discrepant, with one group of investigators finding a decrease in MN frequencies in people having Down syndrome (Scarfi et al., 1990), while the other group saw no significant difference in MN frequencies for people with Down syndrome compared to age-matched controls (Maluf and Erdtmann, 2001). Similar discrepancies have been observed when comparing younger people having Down syndrome to older trisomic individuals, with older subjects tending to have a higher frequency of spontaneous MN than younger individuals for buccal cells (Ferreira et al., 2009), but not lymphocytes. However, MN frequencies in older individuals with Down syndrome have been noted to be higher than those observed in younger individuals with Down syndrome when their lymphocytes were treated with mitomycin-C (MMC), which is a DNA cross-linking agent (Scarfi et al., 1990). In addition, individuals with Down syndrome appeared to be more sensitive to MMC, as their MMC-induced MN frequency was higher than healthy age-matched controls.

Table 9: **Summary of previous studies assessing MN frequencies in individuals with Down syndrome**

Cell type	Individuals with Down syndrome						Normal healthy controls						Reference
	Older group			Younger group			Older group			Younger group			
	Age[†](years)	n	MN (%)	Age[†](years)	n	MN (%)	Age[†](years)	n	MN (%)	Age[†](years)	n	MN (%)	
Lymphocytes[*]	37-55, 44.7±3.8	4	0.86±0.1	9-16, 13.0±1.7	3	0.64±0.06	41-54, 45.8±2	6	1.39±0.11	21-33, 27.6±1.5	8	1.02±0.1	Scarfi et al., 1990
Lymphocytes	-	-	-	0.7±1.8	30	10.17±3.64	-	-	-	3.5±4.9	30	9.3±3.1	Maluf et al, 2001
Buccal cells	-	-	-	5-20, 10.4±5.6	21	0.25[**]	64-75, 67.1±2.6	31	0.14[**]	18-26, 22.5±2.2	30	0.03[**]	Thomas et al.,2008
Buccal cells	≥21,30.8±.4	10	1.00[**]	<10, 5.5±2.6	10	0.45[**]	≥21, 31.6±8.8	10	0.25[**]	<10, 5.7±3.2	10	0.2[**]	Ferreira et al., 2009
				<20, 14.1±3.5	10	0.55[**]				<20, 13.9±3	10	0.15[**]	

[†]range, mean ± S.D., [*]Overall MN frequency in spontaneous and mitomycin-C (MMC)-induced MN, [**] approximated percentage from published histograms

Clearly, there is a lack of consensus regarding the influence, if any, that a trisomy 21 complement may have on the propensity for a cell to acquire chromosomal instability. Furthermore, it is not known if the premature aging present in people with trisomy 21 is associated with an increased frequency of acquired chromosomal abnormalities. One potential mitigating factor in the interpretation of the previous data is that the observed difference in response between people having Down syndrome could reflect differences in their genetic make-up (genes involved in check points, DNA repair, etc), as well as differences in environmental exposures. Therefore, in this study, we used the CBMN assay in combination with interphase FISH technology to determine the frequency of MN in isogenic trisomic compared to euploid cells obtained from older individuals who were described as having "mosaicism" for trisomy 21. In addition, to determine if the chromosomal content of the MN and NBUDs had a non-random pattern, we used Spectral Karyotyping (SKY) technology. To the best of our knowledge, this is the first study to determine the chromosomal content of MN (using SKY) from the cells of individuals having Down syndrome.

Materials and Methods

Study Participants

Lymphoblast cell lines from 8 individuals with mosaic Down syndrome were ascertained through collaboration with Dr. Edmund Jenkins, who has amassed a collection of lymphoblast cell lines from individuals who were seen through the New York State Developmental Disability Service System (Table 10). Upon receipt in our lab, the cells were thawed and established in culture for at least one week to ensure that the cells demonstrated growth.

Cell Cultures

Upon the sample arrival, frozen lymphoblast cell lines were quickly warmed to 37^{o}C, washed twice in sterile 1XPBS and established in culture using a lymphoblast media (RPMI 1640) containing 10% FBS and antibiotic (100 U of penicillin and 100 µg of streptomycin). Cell cultures were maintained at 37^{o}C, in 5% CO_2 until they demonstrated adequate growth. At that time the cell solutions were sub-cultured to encourage log growth of the cells, with the harvest occurring 24 hours following sub-culture initiation. Approximately 30 minutes before harvesting, colcemid was added to the cultures to enrich the cultures for cells at metaphase. Chromosomes were harvested using standard procedures (Moorhead, et al., 1960).

CBMN and FISH Assays

After successful establishment in culture, cytochalasin B (Cyt-B) was added to a final concentration of 3µg/ml, 44 hours after sub-culturing. Cyt-B, which inhibits cytokinesis by blocking microfilaments, captures cells at the telophase portion of the cell cycle, thereby preventing the cytoplasm from dividing and causing the resultant cell to appear binucleated. Twenty-eight hours after the addition of Cyt-B, lymphoblast cultures were harvested as described previously (Leach and Jackson-Cook, 2001). Cell pellets were dropped onto slides. The slides were then placed on a hot plate at 60°C for 1 hour, followed by aging at room temperature for 4 days. FISH was performed using probes specific for chromosome 21 (test probe) and chromosome 13 (control probe). The test probe used was one that is localized to 21q22.13-21q22.2 (D21S259\D21S341\D21S1432) (Abbott, IL). The control probe was specific for band 13q14 (RB1 locus) (Abbott, IL) and served as an internal control for hybridization efficiency. These probes were hybridized onto cytokinesis-blocked cells to determine their trisomic versus euploid status. Briefly, prior to hybridization the slides were dehydrated in a cold ethanol (series of 70%, 85% and 100% ethanol). Following dehydration, the slides were air-dried. A total of 10µl of the 13/21 probe mixture was added to appropriate hybridization areas on the slides. The target chromatin and probes were then co-denatured at 73°C for 2 minutes and hybridized in a pre-warmed, humidified chamber at 37ºC for 4-16 hours. Upon completion of hybridization, the excess and non-specifically bound probes were removed by washing (0.4X SSC/0.3%NP-40 solution at 72°C for 2 minutes, followed by 2X SSC/0.1% NP-40 wash solution for 1

minute). The chromatin in the binucleates was visualized by staining with a DAPI/antifade solution (Abbort, IL). Probe signals were visualized using a Zeiss Axiskop equipped with single (spectrum Orange, Spectrum Green) and triple band pass filters. For each individual, a total of 1000 cells were randomly scored to determine the percentage of trisomic cells present. In addition, a total of 500 binucleated cells were randomly scored for frequencies of MN, NPB and NBUD in trisomy 21 compared to euploid cells. The criteria for recognition of binucleated cells, MN, NPB and NBUD followed the guidelines by Fenech, et al. (2003). Representative images were documented using a Cytovision Imaging system from Applied Imaging.

SKY analysis of micronuclei

Slides were aged at room temperature for 4 days prior to the SKY experiment. SKY was performed according to the manufacturer's protocol (Applied Spectral Imaging, CA) as adapted by Leach and Jackson-Cook (2001). Briefly, slides were denatured in a 70% formamide/2XSSC solution (pH 7.0) at 73˚C for 2 minutes. Following denaturation, the slides were briefly rinsed in cold water and then dehydrated in an ethanol series (70%, 85% and 100% for 2 minutes each at room temperature). The SKY probe (Applied Spectral Imaging, CA) was denatured at 75˚C for 10 minutes and suppression hybridized at 37˚C for 60 minutes to bind repetitive sequences. After suppression hybridization, the denatured probe was added to the denatured slides, hybridizion occurring in a humidified chamber and at 37˚C for approximately 44 hours. At the completion of hybridization, the excess and non-specifically bound probe was removed by washing (using a

0.4xSSC/0.3%NP-40 solution at 73˚C for 2 minutes, followed by a one minute wash at room temperature in a 2xSSC/0.1%NP-40). Indirectly labeled probes (biotin and digoxigenin) were detected using buffers with avidin-Cy5, mouse anti-digoxin and goat anti-mouse conjugated to Cy5.5 (provided by manufacturer). All incubations were for 40 minutes at 37˚C. The binucleates were counterstained with DAPI/antifade (Applied Spectral Imaging, CA) to allow for their visualization. A total of 100 MN and their contiguous interphase nuclei were identified per study subject using a Zeiss Axioskop equipped with a DAPI filter and a custom triple-band pass filter (Chroma, VT). Each MN (with adjacent interphase cells) was captured with a SpectraCube system (Applied Spectral Imaging, CA). The images were processed using the vendor supplied software (Applied Spectral Imaging, CA), which classifies the information obtained by using an algorithm that assigns a spectra-specific pseudocolor to all pixels in the image.

Chromosome-Specific Telomere Length Assay

Slides were aged (either at room temperature for 1-2 weeks or by soaking in 2xSSC for 10 minutes) prior to the FISH experiment. Metaphase chromosomes were hybridized with a telomere-specific FITC-labeled PNA probe following the manufacturer's protocol (DakoCytomation, Denmark). In addition, an FITC-labeled probe that is specific for the pericentromeric region of chromosome 2 was simultaneously hybridized to the metaphase spreads as a control (and to serve for standardization of intensity values from cell to cell) (Mayer et al., 2006). Briefly, slides were fixed in cold Carnoy's fixative for 1 hour. After air-drying, the slides were rinsed with 1XTBS (Tris-

Buffered Saline, pH 7.5) for 2 minutes, fixed in 3.7% formaldehyde in 1XTBS for 2 minutes, and then rinsed (twice in 1XTBS for 5 minutes). The slides were then treated in pre-treatment solution, containing proteinase K, for 10 minutes, rinsed (twice in 1xTBS for 5 minutes) and dehydrated (using a cold ethanol series [70%, 85% and 100%]). After air-drying, the a cocktail probe mixture (11µl of FITC-labeled telomere specific probe and 1 µl of FITC-labeled centromere-2 probe per subject [a half slide area]) was added to each slide and the probe and metaphase spreads co-denatured in a thermocycler at 80°C for 3 minutes. After hybridization in a dry hybridization chamber at room temperature for 2 hours, the excess and unbound probes were removed by rinsing (once in manufacturer provided rinse solution at room temperature for 1 minute, followed by 5 minutes in a manufacturer provided wash solution at 65°C). Following serial dehydration in a cold ethanol series (70%, 85% and 100%), the slides were air-dried, and then counterstained with a 5:1 DAPI/propidium iodide solution.

The telomere lengths of each chromosome were assessed using a semi-quantitative FISH method (CGH software from Applied Imaging Cytovision System) as described by Leach et al., (2004). Briefly, three images were captured with a CCD camera for each metaphase: (1) a reverse DAPI image, which allows for chromosome identification and subsequent karyotyping; (2) a test/ FITC image, showing telomeric and centromeric probe signals; and (3) a reference/ propidium iodide image. Fluorescent intensities obtained from the "test" and "reference" images were used for calculating the ratio profiles of relative telomere intensity for each chromosome arm. Overlapping

telomeres or telomeres that were in close proximity were excluded from the analysis. For each person the intensity values were averaged over 20 homologs from 10 euploid cells, and 20 homologs (30 homologs for chromosome 21) from trisomic cells.

Results

Analyses were completed on lymphoblast samples obtained from 8 individuals who were previously categorized (by Jenkins, et al) as having two cell lines (acquired mosaicism for trisomy 21 for 5 cases and constitutional mosaicism for 3 cases), as summarized in Table 10. Prior to our analyses, the percentages of trisomy 21 cells from all lymphoblast cultures were re-evaluated. A total of 5 individuals had trisomic cells predominate, while 2 individuals had euploid cells predominate in the current cultures. Therefore, chromosomal instability frequencies and telomere length values could be analyzed in either trisomic or euploid cells from these individuals, respectively, but not both as was initially planned. For one individual both trisomic (10.7%) and euploid (89.3%) cells were present.

Cellular distribution of chromosome 21 and 13 probe signals

The proportion of binucleated cells having MN, NPB, NBUD (Figure 14), as well as the total frequency of cytome assay aberrations (MN + NPB + NBUD) was determined for each case, along with information regarding their dementia status (Table 10). The results of the FISH scoring were used to categorize the binucleates (with or without cytome aberrations) into cells having or lacking signals for chromosomes 21 and/or 13. These categorizations included cells having numerical abnormalities (i.e., hyperdiploidy and hypodiploidy) and unequal segregation (i.e., nondisjunction) of the chromosomes 21 and 13 into the daughter nuclei and/or cytological structures as summarized in Table 11.

Hyperdiploidy of chromosome 21and/or 13 was found to be the most frequent cellular alteration. Chromosome 21 and 13 were not frequently excluded in MN. Of the 430 binucleates scored that had MN, only 11 (2.56%) contained signals having chromatin from the region targeted by the probe for chromosome 21, with only 10 MN (2.33%) having a signal for the chromosome 13 probe. From these binucleates, "corrective" cells, which showed the exclusion of chromosome 21 or 13 into MN (possible trisomy rescue) resulting in a balanced complement for the binucleates, were not frequently seen. The ratio of the corrective cells to non-corrective cells, the latter of which contained an imbalance of chromosome 21 or 13 (either hyperdiploidy or hypodiploidy) was 1 to 2.67 and 1 to 9 for chromosomes 21 and 13, respectively. Interestingly, 98 of the 430 cells that contained MN (23%) had numerical aberrations for chromosomes 13 and/or 21 that were not included in the MN. In comparison, only 115 of the 3,536 binucleates without MN (3.3%) had acquired numerical aberrations involving chromosomes 13 and/or 21. Therefore, even though the cytome aberrations did not reflect all chromosomal anomalies that were present in the cells, the observation of a MN (or other cytome structures) was a good indicator that the parent cell had chromosomal instability.

Table 10: Frequencies of MN, NBUD and NPB observed from CBMN and FISH assays and overall telomere length obtained from 8 individuals with mosaicism for Down syndrome, according to their age, gender and dementia status.

Case	Age at sample collection (years)	Gender	% Tri21		Dementia Status	% MN		% NBUD and NPB		% Total Cytome Abnormalities*		Mean telomere intensity value	
			Ascertainment	Current cultures		Tri 21	Eup	Tri 21	Eup	Tri 21	Eup	Tri 21	Eup
25	47.7	M	96.0	97.1	No	11.4 (57/500)		1.0 (5/500)		12.4 (62/500)		1.97	
26	57.8	M	92.0	93.4	No	8.4 (42/500)		0.4 (2/500)		8.8 (44/500)		2.30	
27	55.1	F	94.0	98.0	No	7.4 (37/500)		2.2 (11/500)		9.6 (48/500)		1.79	
28	60.4	M	92.0	97.8	No → dementia (at age 63)	13.4 (67/500)		0.6 (3/500)		14.0 (70/500)		1.77	
29	78.1	F	90.0	94.6	Yes	14.0 (70/500)		0.6 (3/500)		14.6 (73/500)		1.59	
30	48.6	M	78.0	1.0	No		7.6 (38/500)		0.6 (3/500)		8.2 (41/500)		2.63
31	57	F	16.0	1.2	MCI → dementia (at age 59)		13.2 (66/500)		1.0 (5/500)		14.2 (71/500)		1.37
32	43.1	F	32	10.7	No	34.0 (17/50)	8.0 (36/450)		0.67 (3/450)	34.0 (17/50)	8.7 (39/450)	2.96	3.32

MCI = mild cognitive impairment, Tri21= trisomy 21cells, Eup = euploid cells, RFU = relative fluorescent unit, *Total cytome abnormalities determined by the sum of MN, NBUD and NPB

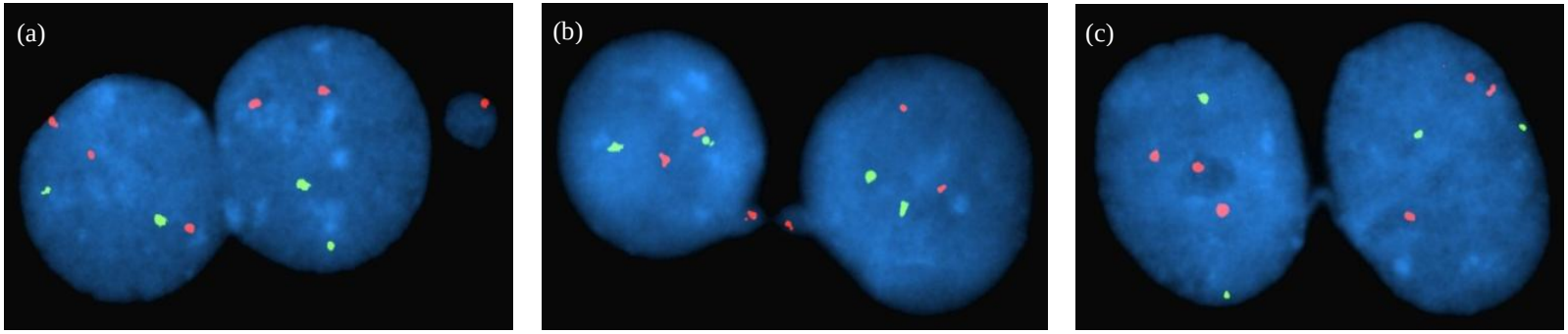
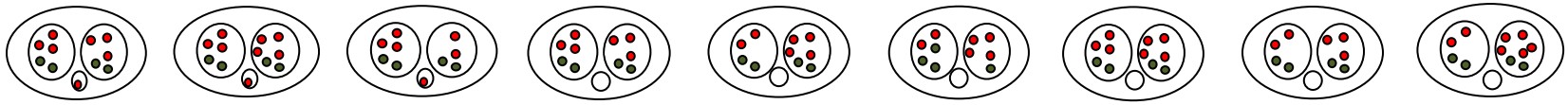


Figure 14: Representative images of cytological structures observed in binucleated cells following CBMN and FISH assays using probes specific for chromosome 21 (red) and chromosome 13 (green). (a) A binucleated cell with a trisomy 21 complement that had a MN containing a signal for chromosome 21, suggesting a "trisomy rescue" corrective event occurred in the right binucleate to give rise to a euploid cell; (b) a binucleated cell with a trisomy 21 complement that had nuclear buds containing chromatin from chromosome 21, suggesting that a corrective event (trisomy corrected to euploid complement) occurred for both daughter cells; and (c) a trisomy 21 binucleated cell with a NPB that does not contain chromatin for the targeted regions evaluated with these probes.

Table 11: Segregation of chromosomes 21 and 13 into MN and acquired aneuploidy for chromosomes 21 and 13 in binucleates lacking cytome abnormalities

Case	BN with MN											BN without MN						
	MN with 21		MN with 13		MN without 21 or 13							NL	NDJ21	NDJ13	NDJ21,13	Hypo	Hyper	Multiple
	C	NC	C	NC	Typical	NDJ21	NDJ13	NDJ21,13	Hypo	Hyper	Multiple							
25	0	2	0	0	43	0	0	1	5	5	1	421	4	2	0	4	6	1
26	0	0	0	0	20	5	1	3	2	8	3	440	1	1	0	6	8	0
27	0	1	0	2	30	0	0	0	0	3	1	426	1	1	1	2	20	1
28	1	1	1	2	40	1	1	1	7	12	0	419	1	2	0	2	6	0
29	0	0	0	0	57	1	0	0	1	11	0	404	2	1	0	8	11	1
30	0	0	0	0	33	1	0	0	1	2	1	446	1	0	0	3	8	1
31	1	3	0	1	52	2	1	2	0	3	1	423	0	0	0	0	6	0
32	1	1	0	4	36	0	1	1	2	6	1	441	0	2	0	0	1	0
Total	3	8	1	9	311	10	4	8	18	50	8	3421	10	9	1	25	66	4

C = Corrective, NC = Non-corrective, NDJ = Nondisjunction, Hypo = Hypodiploidy, Hyper = Hyperdiploidy, Multiple = Hypodiploidy and hyperdiploidy



MN with 21 corrective | MN with 21 hyperdiploidy | MN with 21 hypodiploidy | MN without 21 typical | MN without 21 with NDJ 21 | MN without 21 with NDJ 21, 13 | MN without 21 hyperdiploidy | MN without 21 hypodiploidy | MN without 21 multiple

Telomere length and correlations of telomere length with the frequency of MN, NBUD and NPB

Figure 15 shows representative images of metaphase chromosomes from an individual without dementia and an individual with dementia and illustrates the overall trend that was observed for telomeres having less intensity (shorter) in individuals with dementia as compared to individuals without dementia.

For these analyses, the individuals studied were categorized into 2 groups: non-demented (n = 5) and demented (n = 3), regardless of their cell types. Age ranges were 43.1-57.8 years old (median = 48.6) and 57-78.1 (median=60.4) for non-demented and demented groups, respectively. The frequency of cytome abnormalities (MN, NBUD, and NPB), as well as the overall telomere lengths are shown in Figure 16a and 16b, respectively. To compare the frequency of cytome abnormalities and the overall telomere length between the non-demented and demented group, the Mann-Whitney U test was applied. This statistic involves ranking all the observations, from the smallest to the largest values regardless the dementia status. A U statistic was then calculated using the following formula:

$U = R - \frac{n(n+1)}{2}$, where n = sample size and R = sum of the rank

This test was performed using the R statistical software program. We found that individuals who had dementia had a significantly higher frequency of cytome abnormalities (10.0±1.7% *vs* 14.3±0.3%, p-value = 0.036, Mann-Whitney U test). A regression analysis showed that the observed increased frequency of cytome

abnormalities significantly co-varied with age (p-value = 0.003), but age effects alone were not attributable for the observed cytome frequency differences (p-value = 0.59). Telomere intensity scores between cells from individuals with and without dementia were also compared. The telomere intensity values of the cells from people with dementia were significantly lower than those observed in cells from people without dementia (1.58±0.20 *vs* 2.37±0.54; p-value =0.036, Mann-Whitney U test). A regression analysis showed a significance co-variance of dementia status and age on telomere intensity values (p-value = 0.04), but neither attribute independently accounted for a significant portion of the observed variation (age, p-value = 0.5; dementia, p-value = 0.4). We also observed a significant negative correlation between telomere length and frequency of MN, NBUD and NPB (Spearman correlation, r = - 0.785, p-value = 0.028) over all study participants (Figure 17).

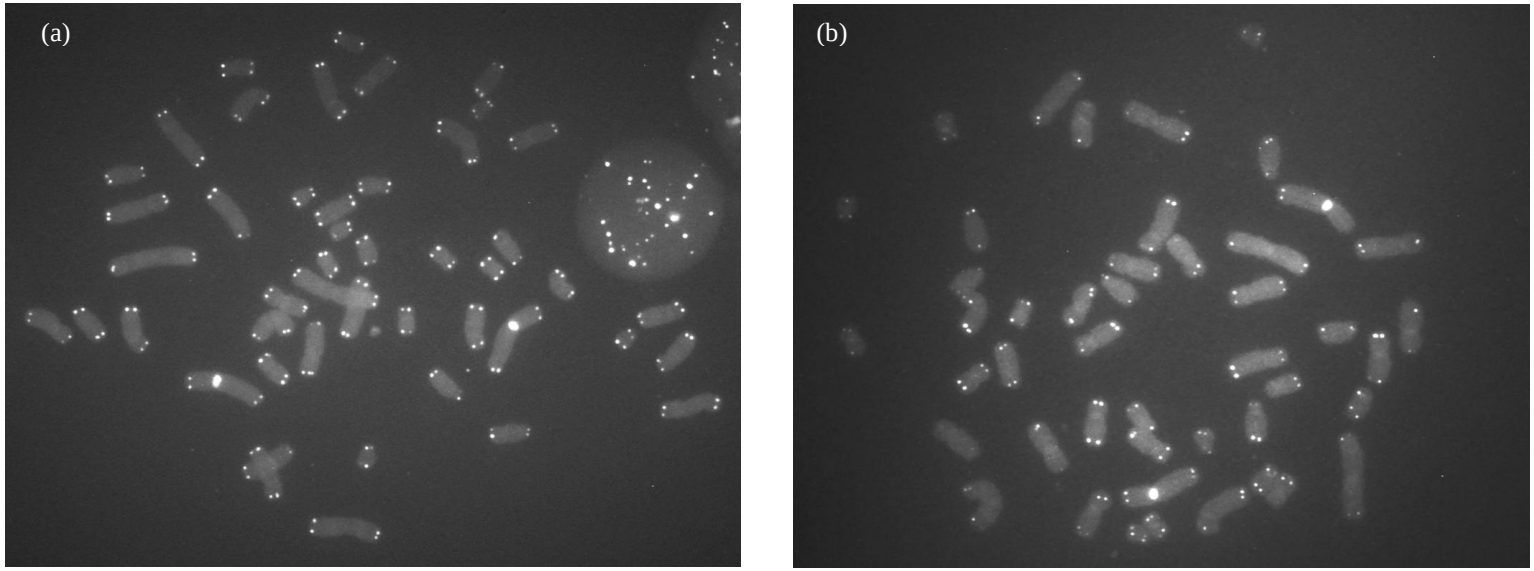


Figure 15: Representative images of metaphase spreads following the chromosome-specific telomere length assay. In image (a) are metaphase chromosomes from an individual without dementia; with image (b) showing metaphase chromosomes from an individual with dementia. Note the overall decreased fluorescent intensity of the metaphase chromosomes from the individual with dementia [most telomeres are not readily visible and less intense than the centromeric control probe] as compared to the individual without dementia [all telomeres are easily visualized with bright signals that are comparable in intensity to the signal of the centromeric control probe].

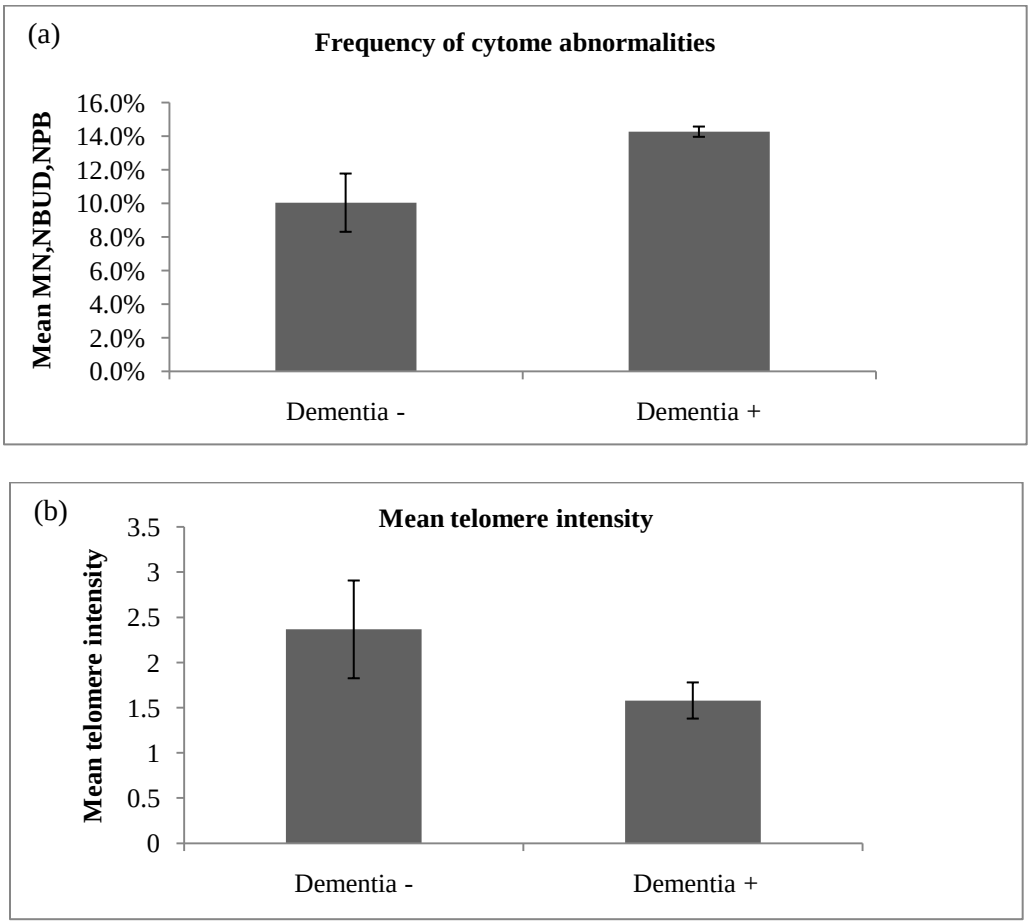


Figure 16: Frequencies of cytome abnormalities (total MN, NBUD and NPB) and overall telomere length (signal intensity). Individuals without dementia (n = 5) and individuals with dementia (n = 3) are shown. The data is presented as the mean and standard deviation for each group. (a) The frequency of cytome aberrations in individuals with dementia was significant higher than those observed in individuals without dementia (p-value = 0.036, Mann-Whitney U test). (b) Telomere lengths (signal intensities) in individuals with dementia were significant higher than those observed in individuals without dementia (p-value = 0.036, Mann-Whitney U test).

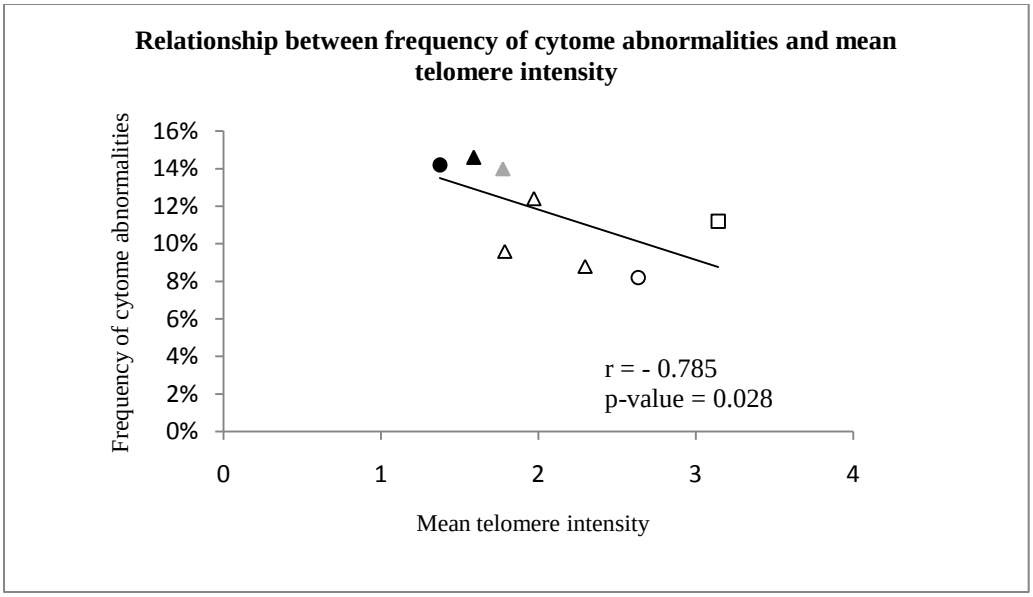


Figure 17: Relationship between frequency of cytome abnormalities and mean telomere length (signal intensity). A significant negative correlation between telomere signal intensity (length) and the frequency of cytome abnormalities for individuals without dementia (white) and individuals with dementia (black) was detected (Spearman correlation, r = - 0.785, p-value = 0.028). An individual who was later diagnosed as having dementia is also shown (gray). The data are shown as frequencies/intensities in trisomic (triangle) and euploid cells (circle). An individual with mosaicism is represented by □ with their values being the average of the two cell populations.

Chromosomal contents in MN

The chromatin present in the MN from the trisomic and euploid binucleates was determined using SKY (Figures 18 and 19). Of the total of 777 MN scored, the majority (85.71%) contained chromatin from a single chromosome. However, the most frequent category of MN that was observed contained chromatin from more than one chromosome, with these MN accounting for 12.4±3.6% of the total number of MN evaluated from individuals without dementia, and 17.7±0.4% in individuals with dementia. The frequencies of autosomal and sex chromosome exclusion into micronuclei were not significantly different between the dementia groups. However, a non-random pattern of chromosomes present in MN was observed for both groups, with chromatin from chromosome 16 being present most frequently and chromatin from chromosome 17 being present least frequently. A Mann-Whitney U test with Bonferroni correction for multiple comparisons was applied for these tests [the p-value was set at < 0.002 (i.e., 0.05/24)]. The chromosome-specific statistical comparisons between the MN from individuals with and without dementia are summarized in Table 12.

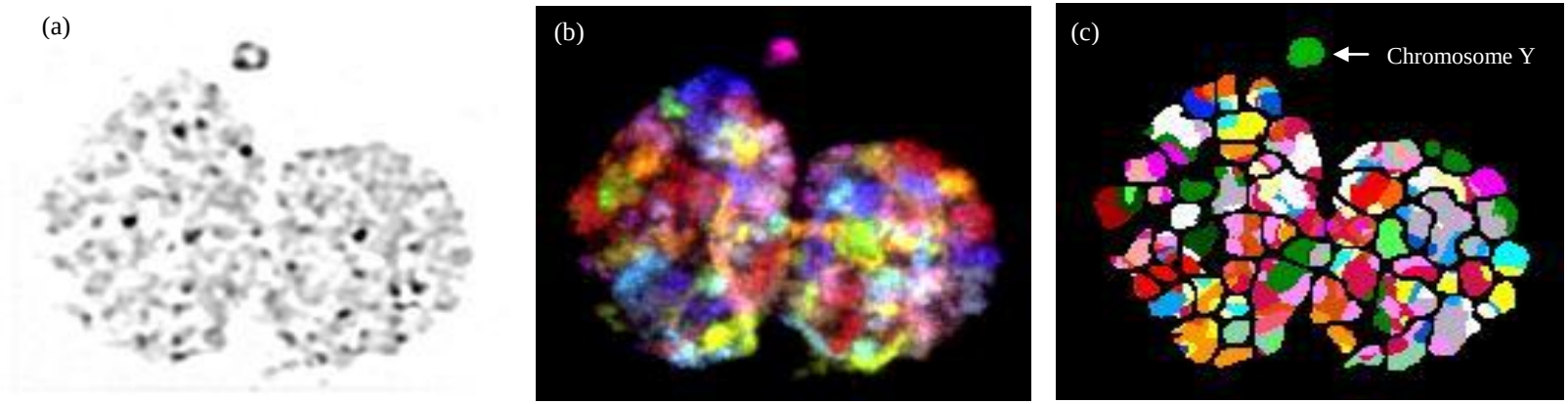


Figure 18: Representative images of a binucleated cell and micronucleus following the CBMN and SKY assays. (a) A reverse DAPI image; (b) spectral image; and (c) classified images. In this cell the chromatin content of the micronucleus originated from a Y chromosome.

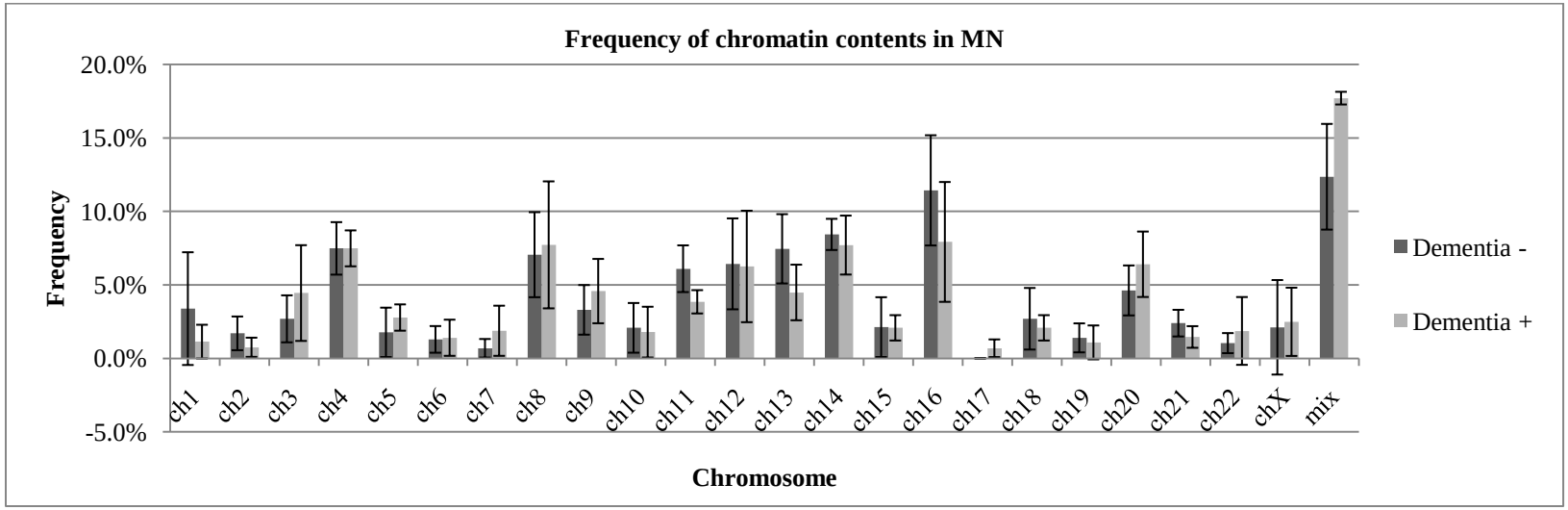


Figure 19: Frequency of chromatin contents in MN observed from individuals based on their dementia status. The proportion of MN containing each of the chromosomes is shown for individuals without dementia (dark gray) and individuals with dementia (light gray), pooled over all cell types. The data are shown as the mean (histogram) and standard deviation (error bar). The frequencies of MN that contain chromatin from more than one chromosome are shown in the "mix" category.

Chromosome-specific telomere length profiles

Chromosome-specific (averaged over the short arm and long arm) telomere lengths (probe signal intensities) were also determined for the study participants with and without dementia (Figure 20). Individuals with dementia tended to have shorter telomeres than the people without dementia for all chromosomes studied. A Mann-Whitney U test with Bonferroni correction for multiple comparisons was applied for these tests [the p-value was set at < 0.002 (i.e., 0.05/24)]. None of the observed difference values reach statistical significance. Chromosomes 16 and 17 were found to have the shortest telomeres in both the non-demented and dementia group. In this study, chromosome 18 was found to have the longest telomeres in both groups. Statistical comparisons between the individuals from the non-demented and demented groups are summarized in Table 12. Individuals without dementia appeared to have nearly the same pattern of chromosome-specific telomere length (Figure 20b).

We were able to measure chromosome-specific telomere lengths of euploid compared to isogenic trisomic cells in the one individual who had two cell populations present in their lymphoblast culture (Figure 21). The telomere intensities tended to be shorter for nearly all chromosomes in the trisomic cells. However, none of the telomeric values were significantly different between the cell types (Figure 21).

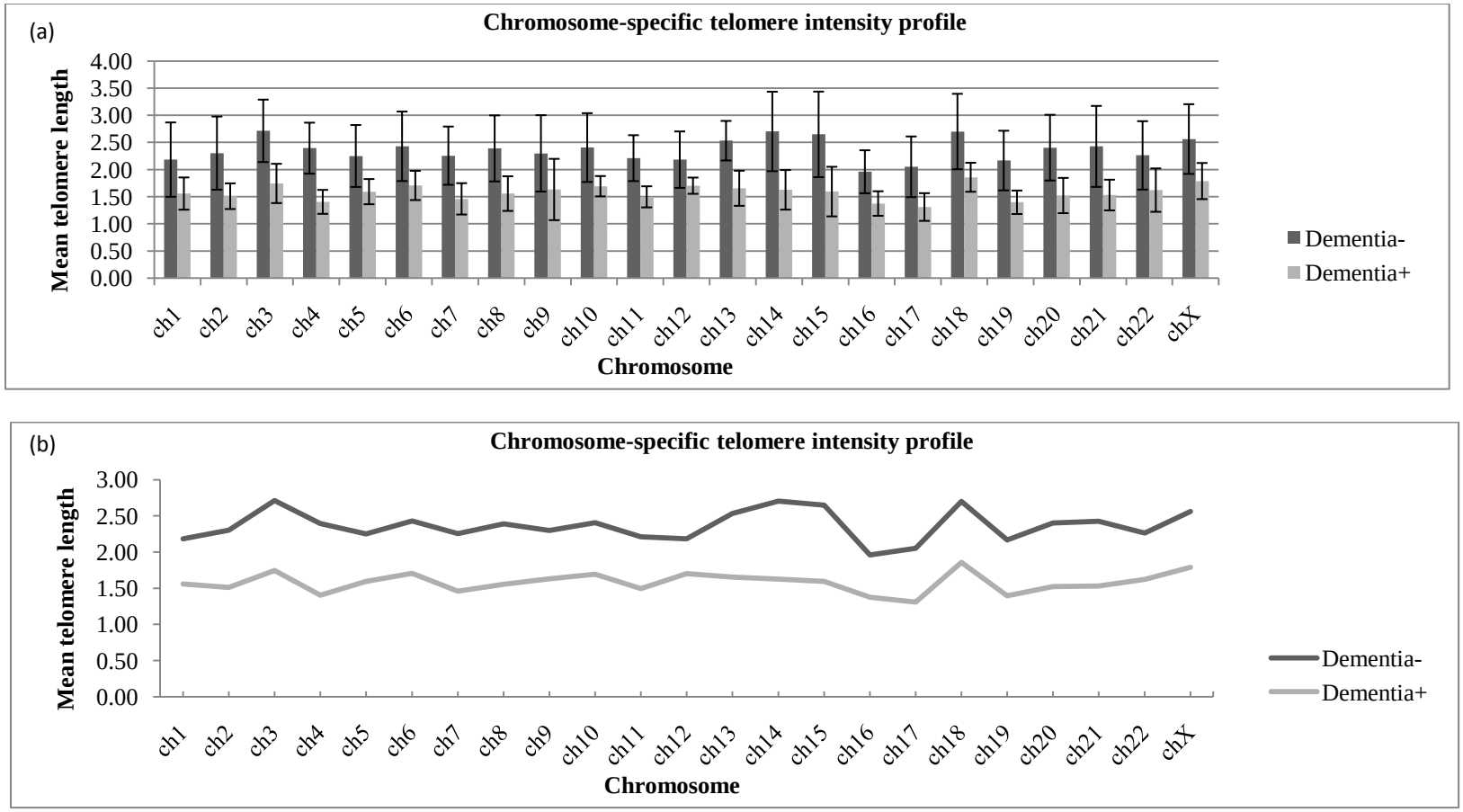
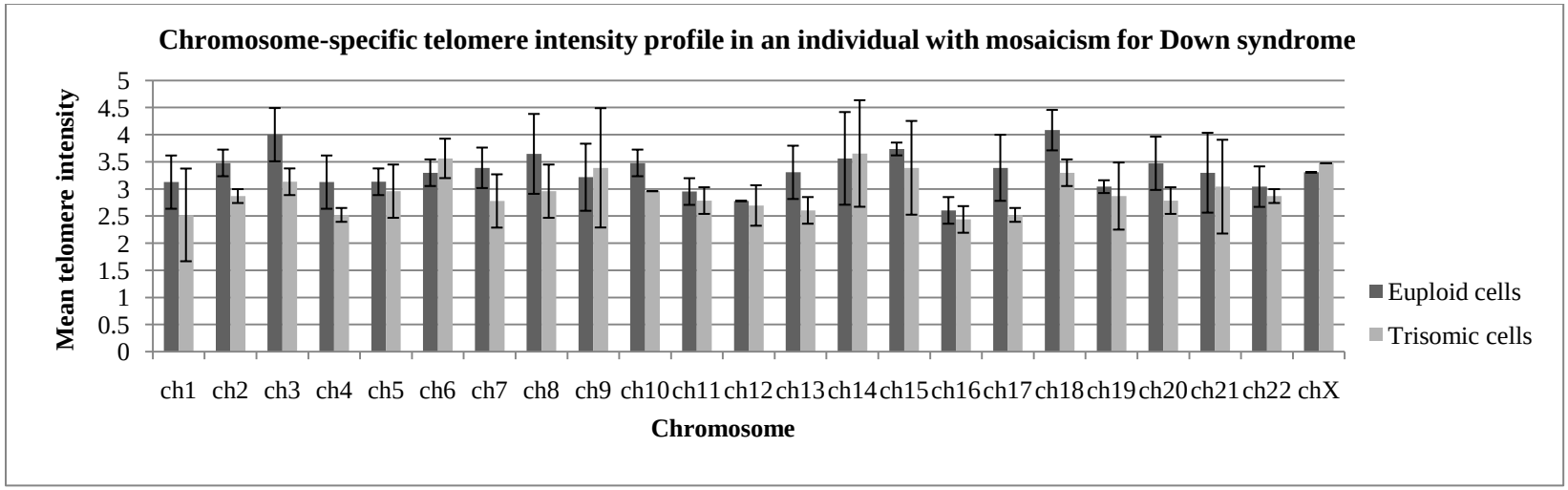


Figure 20: Chromosome-specific telomere intensity values in individuals without dementia (dark gray) and with dementia (light gray). The data are represented by the median (histogram) and interquatile range (error bar). Note that all telomeres of individuals with dementia are shorter compared to those without dementia. (b) Trend lines of the mean of the telomere intensity values are shown for each chromosome in the individuals without dementia (dark gray) and with dementia. Note the nearly parallel course of lines between individuals without dementia (dark gray) and individuals with dementia (light gray).

Table 12: Summary of statistical analysis for MN content and telomere length

Chromosome	MN Content					Telomere Intensities				
	Non-demented		Demented		p-value*	Non-demented		Demented		p-value*
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
1	3.4%	3.8%	1.1%	1.1%	0.55	2.18	0.69	1.56	0.30	0.14
2	1.7%	1.1%	0.8%	0.7%	0.27	2.30	0.67	1.51	0.24	0.14
3	2.7%	1.6%	4.4%	3.3%	0.39	2.71	0.58	1.74	0.36	0.04
4	7.5%	1.8%	7.5%	1.2%	1	2.39	0.47	1.40	0.22	0.04
5	1.8%	1.7%	2.8%	0.9%	0.52	2.25	0.57	1.59	0.23	0.14
6	1.3%	0.9%	1.4%	1.2%	0.88	2.43	0.64	1.71	0.27	0.07
7	0.7%	0.6%	1.9%	1.7%	0.38	2.26	0.54	1.46	0.29	0.04
8	7.1%	2.9%	7.7%	4.3%	1	2.39	0.61	1.56	0.32	0.07
9	3.3%	1.7%	4.6%	2.2%	0.39	2.30	0.70	1.63	0.56	0.14
10	2.1%	1.7%	1.8%	1.7%	1	2.40	0.63	1.69	0.19	0.25
11	6.1%	1.6%	3.8%	0.8%	0.07	2.21	0.42	1.50	0.19	0.04
12	6.4%	3.1%	6.3%	3.8%	1	2.18	0.52	1.70	0.15	0.4
13	7.5%	2.4%	4.5%	1.9%	0.14	2.53	0.36	1.65	0.32	0.04
14	8.4%	1.1%	7.7%	2.0%	0.79	2.70	0.73	1.63	0.37	0.07
15	2.1%	2.0%	2.1%	0.9%	1	2.65	0.79	1.59	0.46	0.07
16	11.4%	3.7%	7.9%	4.1%	0.25	1.96	0.40	1.37	0.23	0.07
17	0.0%	0.0%	0.7%	0.6%	0.11	2.05	0.56	1.31	0.26	0.07
18	2.7%	2.1%	2.1%	0.9%	1	2.70	0.70	1.86	0.27	0.14
19	1.4%	1.0%	1.1%	1.2%	0.63	2.17	0.55	1.40	0.22	0.07
20	4.6%	1.7%	6.4%	2.2%	0.39	2.40	0.61	1.52	0.32	0.07
21	2.4%	0.9%	1.5%	0.7%	0.25	2.43	0.75	1.53	0.28	0.07
22	1.0%	0.7%	1.9%	2.3%	0.88	2.26	0.63	1.62	0.40	0.14
X	2.1%	3.2%	2.5%	2.3%	0.79	2.56	0.64	1.79	0.33	0.04
mix	12.4%	3.6%	17.7%	0.4%	0.14					

* p-value prior to Bonferroni



ch	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
mean difference	0.60	0.61	0.87	0.60	0.17	-0.26	0.61	0.69	-0.17	0.52	0.17	0.08	0.70	-0.09	0.34	0	0.87	0.78	0.17	0.69	0.26	0.18	0
p-value	0.26	0.09	0.34	0.26	0.80	0.65	0.08	0.16	0.70	0.20	0.72	0.80	0.15	0.5	0.70	NA	0.24	0.32	0.71	0.16	0.22	0.5	NA

Figure 21: Chromosome-specific telomere intensity profiles in the individual with mosaicism for trisomy 21. **C**hromosome-specific telomere length profiles in euploid and trisomic cells obtained from an individual with mosaicism (but not dementia) are shown. (a) The data represent mean (histogram) and standard deviation (error bar) values. (b) The comparisons of the mean differences, using a paired t-test, showed no significant differences.

Discussion

In this study, we originally intended to evaluate the frequency of chromosomal instability and chromosomal contents of MN between isogenic trisomic and euploid cells obtained from individuals with mosaicism for Down syndrome. Of eight individuals who were ascertained for having "mosaicism", we were only able to obtain both trisomic and euploid cells for one of these individuals. Of the 8 cases, 5 lymphoblast cultures had nearly all trisomic cells. This change in the proportion of cells could be due to selection against the euploid cells that happened either at the time that lymphocytes were transformed to lymphoblasts (clonal like selection) at the time of freezing/thawing, or during the lymphoblast culture maintenance (differential growth). It is likely that these individuals had full trisomy 21 with an "acquired" loss of chromosome 21 that occurred later in their lives. It has been reported previously that acquired loss of chromosome 21 happens in elderly people with Down syndrome (Percy et al., 1993; Jenkins et al., 1997). For those 2 individuals who had euploid cells that predominated, they are likely to have had "constitutional" mosaicism for Down syndrome, with loss of the trisomic line due to cell culture selection as described above.

Considering the parallel between the increase in MN frequency and Alzheimer type of dementia (Migliore et al., 2011), one could hypothesize that the increase in MN frequency could be related to the early development of Alzheimer symptoms in people having Down syndrome. In this study, we observed significantly higher frequencies of

chromosomal instability in individuals who had dementia, compared to those without dementia, which supports the findings of Migliore and colleagues (2011). We also observed that individuals who had dementia had significantly shorter telomere than those without dementia, which is in agreement with the previous report by Jenkins and colleagues (2008) who looked at mean telomere lengths, but not chromosome-specific telomere lengths. Given that free radicals have been purported to play a role in the shortening of telomeres (Von Zglinicki, 2000) and that the superoxide dismutase-1 (*SOD-1*) gene is located on chromosome 21, which encodes an enzyme that is responsible for destroying free radicals, the observation of telomere shortening associated with a trisomic complement is a bit counter-intuitive. Increased expression of *SOD-1* has been documented in individuals with Down syndrome. However, H_2O_2, a product of the catalytic reaction by SOD-1, will break down to a hydroxyl radical (OH^-). OH^- is highly toxic, which can result in profound cellular damage (reviewed in Capone, 2001). Therefore, individuals with Down syndrome are believed to be vulnerable to oxidative damage to DNA, including telomeres. The imbalance between oxygen free radical production and scavenging leads to cellular dysfunction, which in turn, has been postulated to result in elevated genomic instability (Mancuso et al., 2006). In addition, it has been reported that individuals with Down syndrome have an accelerated decline in DNA repair capacity with age (Raji and Rao, 1998). This could also contribute to chromosomal and/or genomic instability in elderly individuals with Down syndrome.

In addition to observing generalized trend toward shortened telomeres in the older group having dementia, we observed that chromosome 16 had the shortest telomeres for both the demented, as well as the non-demented individuals. While the telomere intensity values were not significantly different between the two groups, it is of interest to note whether there might be genes localized near the heterochromatic telomere whose activity could be altered by telomeric attrition. Two such genes were identified to be of interest. One of these genes, called partner and localizer for BRCA2 (*PALB2*), located on chromosome band 16p12.2 and encodes a protein that stabilizes and anchors the BRCA2 protein to structures within the nucleus. Therefore, PALB2 is essential for allowing the DNA double-strand break repair functions of BRCA2, which, in turn, prevents cells from accumulating genetic damage that can trigger genomic/chromosomal instability (http://omim.org/entry/6103555). Given that MN could be formed as a result of misrepaired and unrepaired DNA double strand breaks, shortening of the telomere on chromosome 16 could be a factor contributing to the higher frequency of MN observed in individuals with Down syndrome when compared to age-matched controls having a normal complement, the latter of which is a finding reported by Ferreira, et al. (2009). The CREB binding protein (*CREBBP* or *CBP*) gene is another gene that is located on chromosome 16 (band 16p13.3). The *CREBBP* gene has been shown to have an essential role in long term memory formation in mice (Bourtchouladze et al., 2003) (http://omim.org/entry/600140). Given that shortening of the heterochromatic telomeric region could alter the chromatin conformation of the distal long arm, thereby potentially

altering the function of *CREBBP* gene, this is an interesting gene to consider for future studies of the progression of dementia in individuals with Down syndrome.

In this study, we found a significant correlation between telomere shortening and increased frequency of MN, NBUD and NPB. It is thought that excessive telomere shortening can eventually result in telomere to telomere end fusions and the formation of dicentric chromosomes via inappropriate assembly of the telomeric protein structures. Using the CBMN and FISH assays, we found that hyperdiploidy, hypodiploidy and NDJ were more common alterations than "corrective" changes leading to MN formation for chromosome 21 and 13. From MN content analysis using SKY, we found that MN containing more than one chromosome (mix) was seen more frequently than MN containing a single chromosome. Taken together, one could speculate that telomeric shortening is leading to an increased frequency of dicentric chromosome formation in older individuals with Down syndrome and that the dicentric chromosomes may contribute to the observed increased frequency of chromosomal instability as shown in Figure 22.

While we found that the overall telomere lengths (inferred from the reduced intensity of signal for the telomeres) of individuals with dementia were significant shorter than individuals without dementia, we observed that the telomeres tended to be shorter across all chromosomes and the patterns of chromosome-specific telomere length were very similar for both groups. However, further studies of more individuals, including

individuals with non-mosaic and mosaic Down syndrome, are needed, which ultimately may help to identify genomic regions of interest and serve to inform investigators of potential candidate genes for future investigations in the etiology of dementia.

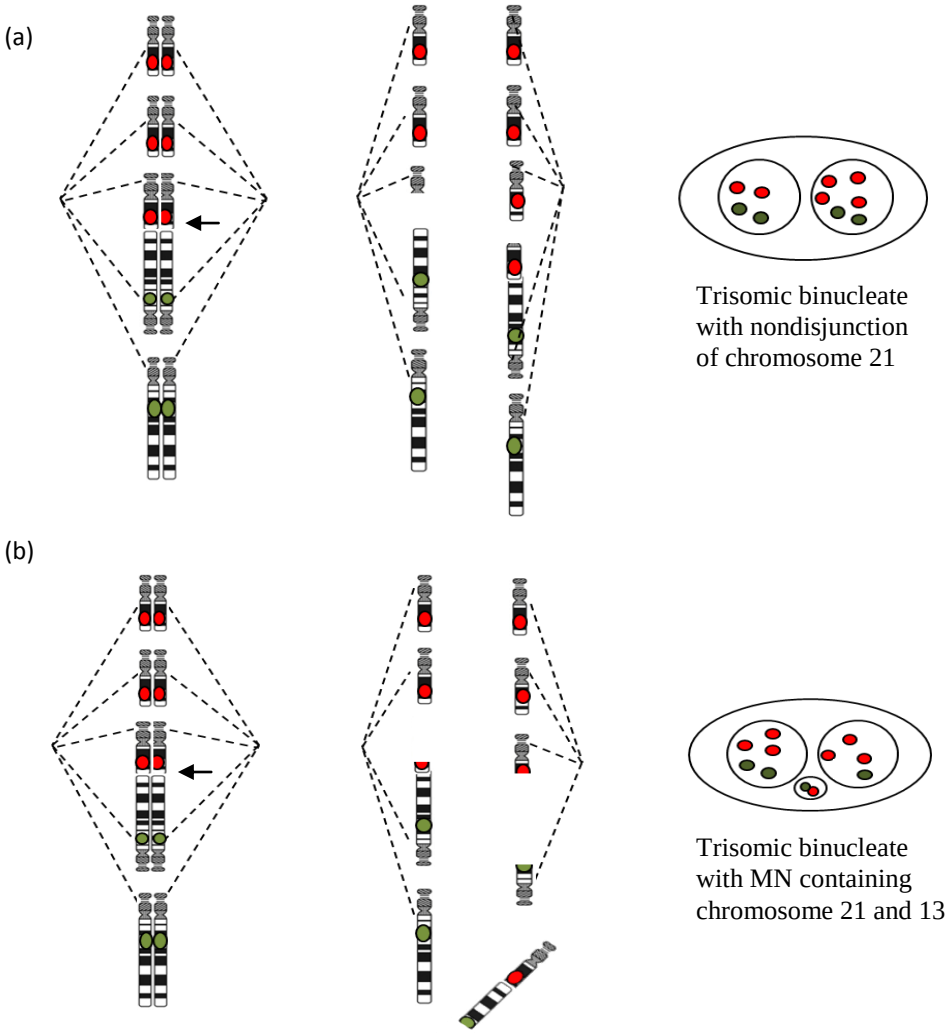


Figure 22: Diagram showing link between telomere shortening and chromosomal instability. Chromosome 21 is shown in red and chromosome 13 is shown in green. The left side of the diagram shows the replicated chromosomes' alignment during metaphase. Dicentric chromosomes, which form from end-to-end fusion as a result of telomeric shortening, are highlighted with an arrow. The middle of the diagram shows the chromosomes following the separation of the sister chromatids during anaphase. As the chromosomes are pulled toward the spindle poles, the tension exerted by spindle fibers can cause breakage of the dicentric chromosome, which could result in (a) nondisjunction; or (b) MN formation following anaphase lagging. The right side of the diagram shows patterns that would result from these abnormal segregation events in cells evaluated after the CBMN and FISH assays. Note the content of the MN in (b) is comprised of a combination of chromatin from chromosomes 21 and 13 (mix).

Chapter 5

Summary, conclusions, and future directions

A. Array-based technology for mosaicism detection.

- It is important that geneticists who are interpreting the results of microarray studies gain experience in the assessment of cases having mosaicism. Using the default software analysis setting for copy number calling alone, is not sufficient to recognize individuals with mosaicism involving a minor cell population that is present in less than 30% of cells since the software routinely categorizes log2 ratio changes from 0-30% as copy number neutral findings. By expanding our analysis to include an assessment of the smooth signal of the log2 ratio, cases having a minor cell population in as few as 15-20% of cells could be detected. However, FISH remains the "gold standard" for mosaicism detection. In addition, FISH should be considered for confirmation when low level mosaicism is suspected and/or to confirm/refute equivocal array-based results.

- Array-based technology has limitations in identifying mosaicism, and is unable to detect unbalanced structural chromosome rearrangements, and ploidy changes; however, it shows strengths in its ability to identify previously unrecognized chromosomal abnormalities. In addition, due to its high resolution compared to the standard G-banding analysis, array-based technology allows for refinement of breakpoinst of unbalanced

structural chromosome abnormalities. Using the SNP array, the origin of a segregation error (meiosis or mitosis, when coupled with an assessment of parental patterns), and long contiguous stretches of homozygosity (LCSH), the latter of which may be indicative of isodisomic uniparental disomy (UPD) or loss of heterozygosity (LOH), can be detected.

- The relative fluorescence intensities obtained from microarray data were positively correlated with the percentage of trisomic cells determined by the "gold standard" FISH methodology. Thus, for specimens in which mosaicism is detected, array-based technology appears to yield reliable estimates of the proportion of cell populations present

- A further study including more individuals with various levels of mosaicism for trisomy 21 syndrome, mosaicism involving other constitutional chromosome abnormalities, and multiple clonal cell lines from cancer specimens should be evaluated to assess reliability and limitations of this assay by laboratories before it is used in a diagnostic setting (i.e., the validation should include mosaic cases) .

B. Chromosome-specific telomere length profiles in euploid and trisomic cells from younger individuals with mosaicism for trisomy 21.

- A weak, non-significant negative correlation between telomere intensity values and the percentage of trisomic cells present in the mosaic probands was observed in this study. The lack of a clear effect of the proportion of trisomy cells could be attributable to the small sample size, or a skewing of the distribution of trisomic cells in the study participants toward low levels (less than 20%). Alternatively, the influence of the trisomic imbalance could be limited to the cellular boundaries, with the euploid cells showing no influence from the trisomic cells.

- A weak, non-significant negative correlation between telomere intensity and age was also observed. The lack of an age effect on telomere intensity values in this young study cohort of individuals having mosaicism (majority of participants were less than 5 years old) is consistent with expectations from studies of young individuals having euploid (normal) chromosomal complements.

- Both trisomic and euploid cells had strikingly similar chromosome-specific telomere length profiles, which imply that the trisomy 21 imbalance has a minimal impact on telomere lengths in this young population, with any influences that were present tending to be generalized for all chromosomes. Although no clear generalized effect of trisomy 21 was observed in this young study cohort, case by case differences in overall telomere intensity between euploid and trisomic cell "within a person" were detected for a subset of individuals.

- Telomere lengths were not equally distributed among all chromosome arms, with a common pattern of telomere length being observed between genetically unrelated individuals. In addition, the chromosome-specific telomere length profiles in this cohort tended to parallel the profiles that have been reported in normal populations.

- Further studies should be carried out in extended populations with individuals from older ages. In addition, a longitudinal study in this young cohort may provide information regarding a potential differential rate of telomere shortening in trisomic compared to euploid cells.

C. Frequency of chromosomal instability and chromosome-specific telomere length profiles in older individuals with Down syndrome with and without dementia.

- Significantly higher frequencies of cytome abnormalities and significantly shorter telomere lengths were observed in individuals with Down syndrome who had dementia, compared to those without dementia. In addition, a significant correlation between telomere shortening and an increased frequency of cytome abnormailties was observed. Other cellular alterations, such as hyperdiploidy, hypodiploidy, and nondisjuction (imbalances) were more common than "corrective" changes leading to micronuclei (MN) formation. Using SKY, MN containing more than one chromosome were noted to arise more often than MN containing a single chromosome. Taken together, these findings suggest that telomere shortening may lead to an increased frequency of dicentric

chromosome formation and that the dicentric interchromosomal abnormalities may be an intermediate between the observed increased frequency of hyperdiploidy, hypodiploidy, NDJ, and MN containing more than one chromosome.

- Further studies, especially the CBMN in combination of FISH and SKY, should be carried out in an extended population of older individuals having mosaicism for trisomy 21 with and without dementia to further clarify the impact of the trisomic imbalance on the acquisition dementia. In addition, investigations in young and older individuals with mosaicism for Down syndrome may provide insight about the effects of constitutional aneuploidy on the frequency of age-related, acquired chromosomal instability.

List of references

Albertson DG, Pinkel D. Genomic microarrays in human genetic disease and cancer. Hum Mol Genet. 2003; 12: R145-52.

American Academy of Pediatrics. Committee on Genetics. American Academy of Pediatrics: Health supervision for children with Down syndrome. Pediatrics. 2001; 107: 442-9.

Andreassi MG, Barale R, Iozzo P, Picano E. The association of micronucleus frequency with obesity, diabetes and cardiovascular disease Mutagenesis.2011 26:77-83.

Ariyoshi K, Suzuki K, Goto M, Watanabe M, Kodama S. Increased chromosome instability and accumulation of DNA double-strand breaks in Werner syndrome cells. J Radiat Res (Tokyo). 2007; 48: 219-31.

Aubert G, Lansdorp PM. Telomeres and aging. Physiol Rev. 2008; 88: 557-79.

Autexier C, Greider CW. Telomerase and cancer: revisiting the telomere hypothesis. Trends Biochem Sci. 1996; 21: 387-91.

Aviv A, Aviv H. Telomeres, hidden mosaicism, loss of heterozygosity, and complex genetic traits. Hum Genet. 1998; 103: 2-4.

Balaban GB, Herlyn M, Clark WH Jr, Nowell PC. Karyotypic evolution in human malignant melanoma. Cancer Genet Cytogenet. 1986; 19: 113-22.

Ballif BC, Rorem EA, Sundin K, Lincicum M, Gaskin S, Coppinger J, Kashork CD, Shaffer LG, Bejjani BA: Detection of low-level mosaicism by array CGH in routine diagnostic specimens. Am J Med Genet A. 2006; 140: 2757-67.

Banerjee B, Sharma S, Hegde S, Hande MP. Analysis of telomere damage by fluorescence in situ hybridisation on micronuclei in lymphocytes of breast carcinoma patients after radiotherapy. Breast Cancer Res Treat. 2008; 107: 25-31.

Barch MJ, Knutsen T, Spurbeck JL, et al. The AGT Cytogenetics Laboratory Manual. 3rd ed. Philadelphia: Lippincott-Raven Publishers, 1997.

Begleiter ML, Harris D J, Magenis RE. Maternal trisomy 21mosaicism in a family with three children with trisomy 21. Am J Hum Genet. 1977; 29: 28.

Benetos A, Gardner JP, Zureik M, Labat C, Xiaobin L, Adamopoulos C, Temmar M, Bean KE, Thomas F, Aviv A. Short telomeres are associated with increased carotid atherosclerosis in hypertensive subjects. Hypertension. 2004; 43: 182-5.

Bentz M, Cabot G, Moos M, Speicher MR, Ganser A, Lichter P, Döhner H. Detection of chimeric BCR-ABL genes on bone marrow samples and blood smears in chronic myeloid and acute lymphoblastic leukemia by in situ hybridization. Blood. 1994; 83: 1922-8.

Bernardini L, Alesi V, Loddo S, Novelli A, Bottillo I, Battaglia A, Digilio MC, Zampino G, Ertel A, Fortina P, Surrey S, Dallapiccola B. High-resolution SNP arrays in mental retardation diagnostics: how much do we gain? Eur J Hum Genet. 2010; 18: 178-85.

Bielanska M, Tan SL and Ao A. Chromosomal mosaicism throughout human preimplantation development in vitro: incidence, type, and relevance to embryo outcome. Hum Reprod. 2002; 17: 413-9.

Bittles AH, Glasson EJ. Clinical, social, and ethical implications of changing life expectancy in Down syndrome. Dev Med Child Neurol. 2004; 46: 282-6.

Blackburn EH.Telomerase and Cancer: Kirk A. Landon--AACR prize for basic cancer research lecture. Mol Cancer Res. 2005; 3: 477-82.

Blackburn EH.Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. FEBS Lett. 2005; 579: 859-62.

Bobrow M, Barby T, Hajianpour A, Maxwell D, Yau SC. Fertility in a male with trisomy 21. J Med Genet. 1992; 29: 141.

Bolognesi C, Lando C, Forni A, Landini E, Scarpato R, Migliore L, Bonassi S. Chromosomal damage and ageing: effect on micronuclei frequency in peripheral blood lymphocytes. Age Ageing. 1999; 28: 393-7.

Bonassi S, Fenech M, Lando C, Lin YP, Ceppi M, Chang WP, Holland N, Kirsch-Volders M, Zeiger E, Ban S, Barale R, Bigatti MP, Bolognesi C, Jia C, Di Giorgio M, Ferguson LR, Fucic A, Lima OG, Hrelia P, Krishnaja AP, Lee TK, Migliore L, Mikhalevich L, Mirkova E, Mosesso P, Müller WU, Odagiri Y, Scarffi MR, Szabova E, Vorobtsova I, Vral A, Zijno A. HUman MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. Environ Mol Mutagen. 2001; 37: 31-45.

Bonassi S, Znaor A, Ceppi M, Lando C, Chang WP, Holland N, Kirsch-Volders M, Zeiger E, Ban S, Barale R, Bigatti MP, Bolognesi C, Cebulska-Wasilewska A, Fabianova E, Fucic A, Hagmar L, Joksic G, Martelli A, Migliore L, Mirkova E, Scarfi MR, Zijno A, Norppa H, Fenech M. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis. 2007; 28: 625-31.

Bourtchouladze R, Lidge R, Catapano R, Stanley J, Gossweiler S, Romashko D, Scott R, Tully T. A mouse model of Rubinstein-Taybi syndrome: defective long-term memory is ameliorated by inhibitors of phosphodiesterase 4. Proc Natl Acad Sci U S A. 2003; 100: 10518-22.

Brooksbank BW, Balazs R. Superoxide dismutase, glutathione peroxidase and lipoperoxidation in Down's syndrome fetal brain. Brain Res. 1984; 318: 37-44.

Brugge KL, Nichols SL, Salmon DP, Hill LR, Delis DC, Aaron L, Trauner DA. Cognitive impairment in adults with Down's syndrome: similarities to early cognitive changes in Alzheimer's disease. Neurology. 1994; 44: 232.

Bush A, Beail N. Risk factors for dementia in people with down syndrome: issues in assessment and diagnosis. Am J Ment Retard. 2004; 109: 83-97.

Callén E, Surrallés J. Telomere dysfunction in genome instability syndromes. Mutat Res. 2004; 567: 85-104.

Campisi J, Kim SH, Lim CS, Rubio M. Cellular senescence, cancer and aging: the telomere connection. Exp Gerontol. 2001; 36: 1619-37.

Cao K, Blair CD, Faddah DA, Kieckhaefer JE, Olive M, Erdos MR, Nabel EG, Collins FS. Progerin and telomere dysfunction collaborate to trigger cellular senescence in normal human fibroblasts. J Clin Invest. 2011; 121: 2833-44.

Capone GT. Down syndrome: advances in molecular biology and the neurosciences. J Dev Behav Pediatr. 2001; 22: 40-59.

Catalán J, Autio K, Kuosma E, Norppa H. Age-dependent inclusion of sex chromosomes in lymphocyte micronuclei of man. Am J Hum Genet. 1998; 63: 1464-72.

Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA. Association between telomere length in blood and mortality in people aged 60 years or older. Lancet. 2003; 361: 393-5.

Chan SR, Blackburn EH. Telomeres and telomerase. Philos Trans R Soc Lond B Biol Sci. 2004; 359: 109-21.

Chapman RS, Hesketh LJ. k, cognition, and short-term memory in individuals with Down syndrome. Down Syndrome Research and Practice. 2001; 7: 1-7.

Chen H, Bowman J, Windle ML, Flannery D, Petry PD, Buehler B. Genetics of Down Syndrome. http://emedicine.medscape.com/article/943216. Updated Feb4, 2011.

Cheung SW, Shaw CA, Scott DA, Patel A, Sahoo T, Bacino CA, Pursley A, Li J, Erickson R, Gropman AL, Miller DT, Seashore MR, Summers AM, Stankiewicz P, Chinault AC, Lupski JR, Beaudet AL, Sutton VR: Microarray-based CGH detects chromosomal mosaicism not revealed by conventional cytogenetics. Am J Med Genet A 2007; 143A: 1679-86.

Cho YH, Kim YJ, An YS, Woo HD, Choi SY, Kang CM, Chung HW. Micronucleus-centromere assay and DNA repair gene polymorphism in lymphocytes of industrial radiographers. Mutat Res. 2009; 680: 17-24.

Chudoba I, Plesch A, Lorch T, Lemke J, Claussen U, Senger G High resolution multicolour-banding: a new technique for refined FISH analysis of human chromosomes. Cytogenet Cell Genet .1999; 84: 156–60.

Clare N, Elson D, Manhoff L. Cytogenetic studies of peripheral myeloblasts and bone marrow fibroblasts in acute myelofibrosis. Am J Clin Pathol. 1982; 77: 762-6.

Colognato R, Coppedè F, Ponti J, Sabbioni E, Migliore L. Genotoxicity induced by arsenic compounds in peripheral human lymphocytes analysed by cytokinesis-block micronucleus assay. Mutagenesis. 2007; 22: 255-261.

Conlin LK, Thiel BD, Bonnemann CG, Medne L, Ernst LM, Zackai EH, Deardorff MA, Krantz ID, Hakonarson H, Spinner NB. Mechanisms of mosaicism, chimerism and uniparental disomy identified by single nucleotide polymorphism array analysis. Hum Mol Genet. 2010; 19: 1263-75.

Crabbe L, Jauch A, Naeger CM, Holtgreve-Grez H, Karlseder J. Telomere dysfunction as a cause of genomic instability in Werner syndrome. Proc Natl Acad Sci U S A. 2007; 104: 2205-10.

Cross J, Peters G, Wu Z, Brohede J, Hannan GN. Resolution of trisomic mosaicism in prenatal diagnosis: estimated performance of a 50K SNP microarray. Prenat Diagn. 2007; 27: 1197-204.

Daphnis DD, Delhanty JD, Jerkovic S, Geyer J, Craft I, Harper JC. Detailed FISH analysis of day 5 human embryos reveals the mechanisms leading to mosaic aneuploidy. Hum Reprod. 2005; 20: 129-37.

Davis T, Singhrao SK, Wyllie FS, Haughton MF, Smith PJ, Wiltshire M, Wynford-Thomas D, Jones CJ, Faragher RG, Kipling D. Telomere-based proliferative lifespan barriers in Werner-synsdrome fibroblasts involve both p53-dependent and p53-independent mechanisms. J Cell Sci. 2003; 116: 1349-57.

de Haan JB, Wolvetang EJ, Cristiano F, Iannello R, Bladier C, Kelner MJ, Kola I. Reactive oxygen species and their contribution to pathology in Down syndrome. Adv Pharmacol. 1997; 38: 379-402.

De La Torre R, Casado A, López-Fernández E, Carrascosa D, Ramírez V, Sáez J. Overexpression of copper-zinc superoxide dismutase in trisomy 21.Experientia. 1996; 52: 871-3.

de Lange T. Activation of telomerase in a human tumor. Proc Natl Acad Sci U S A. 1994; 91: 2882-5.

de Lange T. Telomere-related Genome Instability in Cancer. Cold Spring Harb Symp Quant Biol 2005; 70: 197-204.

Devlin L, Morrison PJ. Mosaic Down's syndrome prevalence in a complete population study. Arch Dis Child. 2004; 89: 1177-8.

Dewald G, Stallard R, Al Saadi A, Arnold S, Bader PI, Blough R, Chen K, Elejalde BR, Harris CJ, Higgins RR, Hoeltge GA, Hsu WT, Kubic V, McCorquodale DJ, Micale MA, Moore JW, Phillips RM, Scheib-Wixted S, Schwartz S, Siembieda S, Strole K, VanTuinen P, Vance GH, Wiktor A, Zinsmeister A, et al. A multicenter investigation with interphase fluorescence in situ hybridization using X- and Y-chromosome probes. Am J Med Genet. 1998; 76: 318-26.

Dewald GW, Schad CR, Christensen ER, Tiede AL, Zinsmeister AR, Spurbeck JL, Thibodeau SN, Jalal SM. The application of fluorescent in situ hybridization to detect Mbcr/abl fusion in variant Ph chromosomes in CML and ALL. Cancer Genet Cytogenet. 1993; 71: 7-14.

Donate LE, Blasco MA. Telomeres in cancer and ageing. Philos Trans R Soc Lond B Biol Sci. 2011; 366: 76-84.

Donnai D, Read AP, McKeown C, Andrews T. Hypomelanosis of Ito: a manifestation of mosaicism or chimerism. J Med Genet. 1988; 25: 809–18.

Down JL. Observations on an ethnic classification of idiots. London Hospital Reports.1866; 3: 259-262.

Druzhyna N, Nair RG, LeDoux SP, Wilson GL. Defective repair of oxidative damage in mitochondrial DNA in Down's syndrome. Mutat Res. 1998; 409: 81-9.

Duffaud F, Orsière T, Villani P, Pelissier AL, Volot F, Favre R, Botta A. Comparison between micronucleated lymphocyte rates observed in healthy subjects and cancer patients. Mutagenesis. 1997; 12: 227-31.

Dyer CA, Sinclair AJ. The premature ageing syndromes: insights into the ageing process. Age and Ageing. 1998; 27: 73-80.

Einerson RR, Law ME, Blair HE, Kurtin PJ, McClure RF, Ketterling RP, Flynn HC, Dogan A, Remstein ED. Novel FISH probes designed to detect IGK-MYC and IGL-MYC rearrangements in B-cell lineage malignancy identify a new breakpoint cluster region designated BVR2. Leukemia. 2006; 20: 1790-9.

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. Accelerated telomere shortening in response to life stress.Proc Natl Acad Sci U S A. 2004; 101: 17312-5.

Epstein CJ. Consequences of chromosome imbalance: Principles, mechanisms and models. New York: Cambridge University Press. 1986.

Esbensen AJ. Health conditions associated with aging and end of life of adults with Down syndrome. Int Rev Res Ment Retard. 2010; 39: 107-26.

Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E; HUman MicronNucleus project. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat Res. 2003; 534: 65-75.

Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, Norppa H, Eastmond DA, Tucker JD, Thomas P. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis. 2011; 26: 125-32.

Fenech M, Morley AA. Measurement of micronuclei in lymphocytes. Mutat Res. 1985; 147: 29-36.

Fenech M. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2007;2:1084-104.
Ferreira FL, Prá D, Martino-Roth MG, Garcias GL. Buccal micronucleus frequency is associated with age in Down syndrome. Genet Mol Res. 2009; 8: 1231-7.

Fiegler H, Carr P, Douglas EJ, Burford DC, Hunt S, Scott CE, Smith J, Vetrie D, Gorman P, Tomlinson IP, Carter NP. DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones. Genes Chromosomes Cancer. 2003; 36: 361-74.

Fitzgerald PH, McEwan CM. Total aneuploidy and age-related sex chromosome aneuploidy in cultured lymphocytes of normal men and women. Hum Genet. 1977; 39: 329-37.

Friedrich U, Griese E, Schwab M, Fritz P, Thon K, Klotz U. Telomere length in different tissues of elderly patients. Mech Ageing Dev. 2000; 119: 89-99.

Gardner RJM and Sutherland GR. Chromosome Abnormalities and Genetic Counseling. Oxford monographics on Medical Genetics No.29. 2nd ed. Oxford University Press, New York. 1996.

Gerashchenko BI. At a crossroads of cancer risk and aging: the role of telomeres. Exp Oncol. 2010; 32: 224-7.

Gondek LP, Tiu R, O'Keefe CL, Sekeres MA, Theil KS, Maciejewski JP. Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, and MDS-derived AML. Blood. 2008; 111: 1534-42.

Graakjaer J, Bischoff C, Korsholm L, Holstebroe S, Vach W, Bohr VA, Christensen K, Kølvraa S. The pattern of chromosome-specific variations in telomere length in humans is determined by inherited, telomere-near factors and is maintained throughout life. Mech Ageing Dev. 2003; 124: 629-40.

Greider CW, Blackburn EH. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature. 1989; 337: 331-7.

Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell. 1985; 43: 405-13.

Guan JZ, Maeda T, Sugano M, Oyama J, Higuchi Y, Makino N. Change in the telomere length distribution with age in the Japanese population. Mol Cell Biochem. 2007; 304: 353-60.

Guttenbach M, Koschorz B, Bernthaler U, Grimm T, Schmid M. Sex chromosome loss and aging: in situ hybridization studies on human interphase nuclei. Am J Hum Genet. 1995; 57: 1143-50.

Hagemeijer A, Hählen K, Abels J. Cytogenetic follow-up of patients with nonlymphocytic leukemia. II. Acute nonlymphocytic leukemia. Cancer Genet Cytogenet. 198; 3: 109-24.

Hardy J. Framing beta-amyloid. Nat Genet. 1992; 1: 233-4.

Hasle H, Clemmensen IH, Mikkelsen M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. Lancet. 2000; 355:165-9.

Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961; 25: 585-621.

Herbert B, Pitts AE, Baker SI, Hamilton SE, Wright WE, Shay JW, Corey DR. Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. Proc Natl Acad Sci U S A. 1999; 96: 14276-81.

Herrgård E, Mononen T, Mervaala E, Kuusela L, Aikiä M, Stenbäck U, Pääkkönen L, Airaksinen RL, Kälviäinen R. More severe epilepsy and cognitive impairment in the offspring of a mother with mosaicism for the ring 20 chromosome. Epilepsy Res. 2007; 73: 122-8.

Hoang S, Ahn J, Mann K, Bint S, Mansour S, Homfray T, Mohammed S, Ogilvie CM. Detection of mosaicism for genome imbalance in a cohort of 3,042 clinical cases using an oligonucleotide array CGH platform. Eur J Med Genet. 2011; 54: 121-9.

Holland AJ, Hon J, Huppert FA, Stevens F. Incidence and course of dementia in people with Down's syndrome: findings from a population-based study. J Intellect Disabil Res. 2000; 44: 138-46.

Hook EB. Exclusion of chromosomal mosaicism: Tables of 90%, 95%, and 99% confidence limits and comments on use. Am J Hum Genet. 1977; 29: 94-7.

Hsu LY, Yu MT, Richkind KE, Van Dyke DL, Crandall BF, Saxe DF, Khodr GS, Mennuti M, Stetten G, Miller WA, Priest JH. Incidence and significance of chromosome mosaicism involving an autosomal structural abnormality diagnosed prenatally through amniocentesis: a collaborative study. Prenat Diagn. 1996 ; 16: 1-28.

Hsu LYF, Gertner M, Leiter E, Hirschhorn K. Paternal trisomy 21mosaicism as a cause of Down syndrome. Am J Genet 1971; 23: 592-601.

http://www.ncbi.nlm.nih.gov/omim/104300

http://omim.org/entry/600140

http://omim.org/entry/6103555

Hultén MA, Dhanjal S, Pertl B. Rapid and simple prenatal diagnosis of common chromosome disorders: advantages and disadvantages of the molecular methods FISH and QF-PCR. Reproduction. 2003; 126: 279-97.

Hultén MA, Jonasson J, Nordgren A, Iwarsson E. Germinal and Somatic Trisomy 21 Mosaicism: How Common is it, What are the Implications for Individual Carriers and How Does it Come About? Curr Genomics. 2010; 11: 409-19.

Hultén MA, Patel S, Jonasson J, Iwarsson E.On the origin of the maternal age effect in trisomy 21 Down syndrome: the Oocyte Mosaicism Selection model. Reproduction. 2010; 139: 1-9.

Iourov IY, Vorsanova SG, Yurov YB. Chromosomal mosaicism goes global. Mol Cytogenet. 2008; 1: 26.

Jacobs PA, Brunton M, Court Brown WM, Doll R, Goldstein H. Change of human chromosome count distribution with age: evidence for a sex differences. Nature. 1963; 197: 1080-1.

Jacobs PA, Court Brown WM, Doll R. Distribution of human chromosome count distributions with age. Nature. 1961; 191: 1178-80.

Janerich DT, Bracken MB. Epidemiology of trisomy 21: a review and theoretical analysis. J Chronic Dis. 1986; 39: 1079-93.

Jenkins EC, Schupf N, Genovese M, Ye LL, Kapell D, Canto B, Harris M, Devenny D, Lee JH, and Brown WT. Increased low-level chromosome 21 mosaicism in older individuals with Down syndrome. Am J Med Genet 1997; 68: 147-51.

Jenkins EC, Velinov MT, Ye L, Gu H, Li S, Jenkins EC Jr, Brooks SS, Pang D, Devenny DA, Zigman WB, Schupf N, Silverman WP. Telomere shortening in T lymphocytes of older individuals with Down syndrome and dementia. Neurobiol Aging. 2006; 27: 941-5.

Jenkins EC, Ye L, Gu H, Ni SA, Duncan CJ, Velinov M, Pang D, Krinsky-McHale SJ, Zigman WB, Schupf N, Silverman WP. Increased "absence" of telomeres may indicate Alzheimer's disease/dementia status in older individuals with Down syndrome. Neurosci Lett. 2008; 440: 340-3.

Jenkins EC, Ye L, Gu H, Ni SA, Velinov M, Pang D, Krinsky-McHale SJ, Zigman WB, Schupf N, Silverman WP. Shorter telomeres may indicate dementia status in older individuals with Down syndrome. Neurobiol Aging. 2010; 31: 765-71.

Jones KL. Smith's Recognizable Patterns of Human malformation, 6th ed. Philadelphia: Elsevier Inc.2005.

Jones LA, Clegg S, Bush C, McMillan TJ, Peacock JH. Relationship between chromosome aberrations, micronuclei and cell kill in two human tumour cell lines of widely differing radiosensitivity. Int J Radiat Biol. 1994; 66: 639-42.

Kaffe S, Hsu LYF, Hirschhorn K. Trisomy 21 mosaicism in a woman with two children with trisomy 21 Down's syndrome. J Med Genet 1974; 11: 378-81.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science. 1992; 258: 818-21.

Kallioniemi A, Kallioniemi OP, Waldman FM, Chen LC, Yu LC, Fung YK, Smith HS, Pinkel D, Gray JW. Detection of retinoblastoma gene copy number in metaphase chromosomes and interphase nuclei by fluorescence in situ hybridization. Cytogenet Cell Genet. 1992; 60: 190-3.

Kalousek DK, Vekemans M. Baillieres. Confined placental mosaicism and genomic imprinting. Best Pract Res Clin Obstet Gynaecol. 2000; 14: 723-30.

Kaneko Y, Larson RA, Variakojis D, Haren JM, Rowley JD. Nonrandom chromosome abnormalities in angioimmunoblastic lymphadenopathy. Blood; 60: 877-87.

Kasuba V, Rozgaj R, Milić M, Zeljezić D, Kopjar N, Pizent A, Kljaković-Gaspić Z. Evaluation of lead exposure in battery-manufacturing workers with focus on different biomarkers. J Appl Toxicol. 2010; 30: 321-8.

Kimura R, Kamino K,Yamamoto M, Nuripa A, Kida T, Kazui H, Hashimoto R, Tanaka T, Kudo T, Yamagata H, Tabara Y, Miki T, Akatsu H, Kosaka K, Funakoshi E, Nishitomi K, Sakaguchi G, Kato A, Hattori H, Uema T, Takeda M. The DYRK1A gene, encoded in chromosome 21 Down syndrome critical region, bridges between b-amyloid production and tau phosphorylation in Alzheimer disease. Hum Mol Genet. 2007; 16: 15-23.

Klinger K, Landes G, Shook D, Harvey R, Lopez L, Locke P, Lerner T, Osathanondh R, Leverone B, Houseal T, et al. Rapid detection of chromosome aneuploidies in uncultured amniocytes by using fluorescence in situ hybridization (FISH). Am J Hum Genet. 1992; 51: 55-65.

Kovaleva NV, Shaffer LG. Under-ascertainment of mosaic carriers of balanced homologous acrocentric translocations and isochromosomes. Am J Med Genet A. 2003; 121A: 180-7.

Kuroda N, Shiotsu T, Hes O, Michal M, Shuin T, Lee GH. Acquired cystic disease-associated renal cell carcinoma with gain of chromosomes 3, 7, and 16, gain of chromosome X, and loss of chromosome Y. Med Mol Morphol. 2010; 43: 231-4.

Lansdorp PM, Verwoerd NP, van de Rijke FM, Dragowska V, Little MT, Dirks RW, Raap AK, Tanke HJ. Heterogeneity in telomere length of human chromosomes. Hum Mol Genet. 1996; 5: 685-91.

Le Caignec C, Boceno M, Saugier-Veber P, Jacquemont S, Joubert M, David A, Frebourg T, Rival JM. Detection of genomic imbalances by array based comparative genomic hybridisation in fetuses with multiple malformations. J Med Genet. 2005; 42: 121-8.

Leach NT, Jackson-Cook C.The application of spectral karyotyping (SKY) and fluorescent in situ hybridization (FISH) technology to determine the chromosomal content(s) of micronuclei. Mutat Res. 2001; 495: 11-9.

Leach NT, Rehder C, Jensen K, Holt S, Jackson-Cook C. Human chromosomes with shorter telomeres and large heterochromatin regions have a higher frequency of acquired somatic cell aneuploidy. Mech Ageing Dev. 2004; 125: 563-73.

Lejeune,J, Gautier M, Turpin R. Ctude des chromosomes somatiques de neuf enfants mongoliens. C.R. Acad. Sci. 1959; 248: 1721-2.

Leon E, Zou YS, Milunsky JM. Mosaic Down syndrome in a patient with low-level mosaicism detected by microarray. Am J Med Genet A. 2010; 152A: 3154-6.

Leonard JC, Merz T. The influence of cell cycle kinetics on the radiosensitivity of Down's syndrome lymphocytes. Mutat Res. 1983; 109: 111-21.

Lestou VS, Lomax BL, Barrett IJ, Kalousek DK. Screening of human placentas for chromosomal mosaicism using comparative genomic hybridization. Teratology. 1999; 59: 325-30.

Lindström E, Salford LG, Heim S, Mandahl N, Strömblad S, Brun A, Mitelman F.Trisomy 7 and sex chromosome loss need not be representative of tumor parenchyma cells in malignant glioma. Genes Chromosomes Cancer. 1991; 3: 474-9.

Liu F, Liang Z, Wegiel J, Hwang YW, Iqbal K, Grundke-Iqbal I, Ramakrishna N, Gong CX. Overexpression of Dyrk1A contributes to neurofibrillary degeneration in Down syndrome. FASEB J. 2008; 22: 3224-33.

Lott IT, Dierssen M. Cognitive deficits and associated neurological complications in individuals with Down's syndrome. Lancet Neurol. 2010; 9: 623-33.

Ma H, Zhou Z, Wei S, Liu Z, Pooley KA, Dunning AM, Svenson U, Roos G, Hosgood HD 3rd, Shen M, Wei Q. Shortened telomere length is associated with increased risk of cancer: a meta-analysis. PLoS One. 2011; 6: e20466.

Maciejewski JP, Tiu RV, O'Keefe C. Application of array-based whole genome scanning technologies as a cytogenetic tool in haematological malignancies. Br J Haematol. 2009; 146: 479-88.

Maluf SW, Erdtmann B. Genomic instability in Down syndrome and Fanconi anemia assessed by micronucleus analysis and single-cell gel electrophoresis. Cancer Genet Cytogenet. 2001; 124: 71-5.

Maluf SW, Erdtmann B. Genomic instability in Down syndrome and Fanconi anemia assessed by micronucleus analysis and single-cell gel electrophoresis. Cancer Genet Cytogenet. 2001; 124: 71-5.

Mancuso M, Coppede` F, Migliore L, Siciliano, G. and Murri, L.Mitochondrial dysfunction, oxidative stress and neurodegeneration. J. Alzheimers Dis 2006; 10: 59–73.

Mann D. Down's syndrome and Alzheimer's disease. In: Esiri M, Lee VM-Y, Trojanowski JQ, eds. The Neuropathology of Dementia. Cambridge, England: Cambridge University Press; 2004.

Mann DMA, Esiri MM. The pattern of acquisition of plaques and tangles in Down's syndrome patients under 50 years of age. J Neuro Sci. 1989, 89: 169-79.

Manning M, Hudgins L. Use of array-based technology in practice of medical genetics. Genetics in Medicine. 2007; 9: 650-3.

Martens UM, Zijlmans JM, Poon SS, Dragowska W, Yui J, Chavez EA, Ward RK, Lansdorp PM. Short telomeres on human chromosome 17p. Nat Genet. 1998; 18: 76-80.

Martin JM, Kellett JM, Kahn J. Aneuploidy in cultured human lymphocytes: I. Age and sex differences. Age Ageing. 1980; 9: 147-53.

Mayer S, Brüderlein S, Perner S, Waibel I, Holdenried A, Ciloglu N, Hasel C, Mattfeldt T, Nielsen KV, Möller P. Sex-specific telomere length profiles and age-dependent erosion dynamics of individual chromosome arms in humans. Cytogenet Genome Res. 2006; 112: 194-201.

McClintock, B. The stability of broken ends of chromosomes in Zea mays. Genetics. 1941; 26: 234-282.

Mefford HC, Trask BJ. The complex structure and dynamic evolution of human subtelomeres. Nat Rev Genet. 2002; 3: 91-102. Erratum in: Nat Rev Genet. 2002; 3: 229.

Mehes K. Paternal trisomy 21 mosaicism and Down's anomaly. Humangenetik. 1973; 17: 297-300.

Menten B, Maas N, Thienpont B, Buysse K, Vandesompele J, Melotte C, de Ravel T, Van Vooren S, Balikova I, Backx L, Janssens S, De Paepe A, De Moor B, Moreau Y,

Marynen P, Fryns JP, Mortier G, Devriendt K, Speleman F, Vermeesch JR: Emerging patterns of cryptic chromosomal imbalance in patients with idiopathic mental retardation and multiple congenital anomalies: a new series of 140 patients and review of published reports. J Med Genet. 2006; 43: 625-33.

Meschede D, Lemcke B, Exeler JR, De Geyter C, Behre HM, Nieschlag E, Horst J. Chromosome abnormalities in 447 couples undergoing intracytoplasmic sperm injection--prevalence, types, sex distribution and reproductive relevance. Hum Reprod. 1998; 13: 576-82.

Migliore L, Coppedè F, Fenech M, Thomas P. Association of micronucleus frequency with neurodegenerative diseases. Mutagenesis. 2011; 26: 85-92.

Migliore L, Petrozzi L, Lucetti C, Gambaccini G, Bernardini S, Scarpato R, Trippi F, Barale R, Frenzilli G, Rodilla V, Bonuccelli U. Oxidative damage and cytogenetic analysis in leukocytes of Parkinson's disease patients. Neurology. 2002; 58: 1809-15.

Migliore L, Testa A, Scarpato R, Pavese N, Petrozzi L, Bonuccelli U. Spontaneous and induced aneuploidy in peripheral blood lymphocytes of patients with Alzheimer's disease. Hum Genet. 1997; 101: 299-305.

Miller DT, Adam MP, Aradhya S, Biesecker LG, Brothman AR, Carter NP, Church DM, Crolla JA, Eichler EE, Epstein CJ, Faucett WA, Feuk L, Friedman JM, Hamosh A, Jackson L, Kaminsky EB, Kok K, Krantz ID, Kuhn RM, Lee C, Ostell JM, Rosenberg C, Scherer SW, Spinner NB, Stavropoulos DJ, Tepperberg JH, Thorland EC, Vermeesch JR, Waggoner DJ, Watson MS, Martin CL, Ledbetter DH. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. Am J Hum Genet. 2010; 86: 749-64.

Milosević-Djordjević O, Grujiciĉ D, Vaskoviĉ Z, Marinkoviĉ D. High micronucleus frequency in peripheral blood lymphocytes of untreated cancer patients irrespective of gender, smoking and cancer sites. Tohoku J Exp Med. 2010; 220: 115-20.

Moorhead PS, Nowell PC, Mellman WJ, Battips DM, Hungerford DA. Chromosome preparations of leukocytes cultured from human peripheral blood. Exp Cell Res. 1960; 20: 613-6.

Morawiec Z, Janik K, Kowalski M, Stetkiewicz T, Szaflik J, Morawiec-Bajda A, Sobczuk A, Blasiak J. DNA damage and repair in children with Down's syndrome. Mutat Res. 2008; 637: 118-23.

Muller, H. J. The remaking of chromosomes. Collecting Net. 1938; 13: 182-198.

National Institutes of Health and Institute of Molecular Medicine collaboration. A complete set of human telomeric probes and their clinical application. National Institutes of Health and Institute of Molecular Medicine collaboration. Nat Genet. 1996; 14: 86-9.

Neill NJ, Torchia BS, Bejjani BA, Shaffer LG, Ballif BC. Comparative analysis of copy number detection by whole-genome BAC and oligonucleotide array CGH. Mol Cytogenet. 2010; 3: 11.

Nietzel A, Rocchi M, Starke H, Heller A, Fiedler W, Wlodarska I, Loncarevic IF, Beensen V, Claussen U, Liehr T. A new multicolor-FISH approach for the characterization of marker chromosomes: centromere-specific multicolor-FISH (cenM-FISH). Hum Genet. 2001; 108: 199-204.

Nussbaum RL, McInnes RR, Willard HF, Thompson MW. Thompson and Thompson genetics in medicine. 6th ed. Philadelphia: W.B. Saunders Company. 2004.

Olovnikov, A. M. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol 1973; 41: 181-190.

Pangalos C, Avramopoulos D, Blouin JL, Raoul O, deBlois MC, Prieur M, Schinzel AA, Gika M, Abazis D, Antonarakis SE.Understanding the mechanism(s) of mosaic trisomy 21 by using DNA polymorphism analysis. Am J Hum Genet. 1994; 54: 473-81.

Panossian LA, Porter VR, Valenzuela HF, Zhu X, Reback E, Masterman D, Cummings JL, Effros RB. Telomere shortening in T cells correlates with Alzheimer's disease status. Neurobiol Aging. 2003; 24: 77-84.

Papavassiliou P, York TP, Gursoy N, Hill G, Nicely LV, Sundaram U, McClain A, Aggen SH, Eaves L, Riley B, Jackson-Cook C. The phenotype of persons having mosaicism for trisomy 21/Down syndrome reflects the percentage of trisomic cells present in different tissues. Am J Med Genet. 2009; 149A: 573-83.

Percy ME, Dalton AJ, Markovic VD, McLachlan DR, Hummel JT, Rusk AC, Andrews DF. Red cell superoxide dismutase, glutathione peroxidase and catalase in Down syndrome patients with and without manifestations of Alzheimer disease. Am J Med Genet. 1990; 35: 459-67.

Percy ME, Markovic VD, Dalton AJ, McLachlan DRC, Berg JM, Rusk ACM, Somerville MJ, Chodakowski B, and Andrews DF. Age associated chromosome 21 loss in Down syndrome: Possible relevance to mosaicism and Alzheimer disease. Am J Med Genet. 1993; 45: 584-8.

Petrozzi L, Lucetti C, Scarpato R, Gambaccini G, Trippi F, Bernardini S, Del Dotto P, Migliore L, Bonuccelli U. Cytogenetic alterations in lymphocytes of Alzheimer's disease and Parkinson's disease patients. Neurol Sci. 2002; 23 Suppl 2: S97-8.

Pinkel D, Albertson DG. Assembly of microarrays for genome-wide measurement of DNA copy number. Nat Genet. 2001; 29: 263-4.

Pinkel D, Albertson DG: Array comparative genomic hybridization and its applications in cancer. Nat Genet 2005; 37: S11-S17.

Pinkel D, Segraves R, Sudar D, Clack S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, Albertson DG. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 1998; 20: 207-11.

Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci U S A. 1986; 83: 2934-8.

Potter H. Review and hypothesis: Alzheimer disease and Down syndrome--chromosome 21 nondisjunction may underlie both disorders. Am J Hum Genet. 1991; 48: 1192-200.

Powis Z, Kang SH, Cooper ML, Patel A, Peiffer DA, Hawkins A, Heidenreich R, Gunderson KL, Cheung SW, Erickson RP: Mosaic tetrasomy 12p with triplication of 12p detected by array-based comparative genomic hybridization of peripheral blood DNA. Am J Med Genet. 2007; 143A: 2910-5.

Pradhan M, Dalal A, Khan F, Agrawal S. Fertility in men with Down syndrome: a case report. Fertil Steril. 2006; 86: 1765.e1-e3.

Priest JH, Brantley KE, Blackston RD. Parental mosaicism as a cause of Down snydrome. J Pediatr. 1977; 90: 786-8.

Raji NS, Rao KS. Trisomy 21 and accerelated aging: DNA repair parameters in peripheral lymphocytes of Down’s syndrome patients. Mech. Ageing Dev1998; 100: 85-101.

Ramalho A, Sunjevaric I, Natarajan AT. Use of the frequencies of micronuclei as quantitative indicators of X-ray-induced chromosomal aberrations in human peripheral blood lymphocytes: comparison of two methods. Mutat Res. 1988; 207: 141-6.

Richard F, Muleris M, Dutrillaux B. The frequency of micronuclei with X chromosome increases with age in human females. Mutat Res. 1994; 316: 1-7.

Richards BW. Investigation of 142 mosaic mongols and mosaic parents of mongols; cytogenetic analysis and maternal age at birth. J Ment Defic Res. 1974; 18: 199-208.

Rodríguez-Santiago B, Malats N, Rothman N, Armengol L, Garcia-Closas M, Kogevinas M, Villa O, Hutchinson A, Earl J, Marenne G, Jacobs K, Rico D, Tardón A, Carrato A, Thomas G, Valencia A, Silverman D, Real FX, Chanock SJ, Pérez-Jurado LA. Mosaic uniparental disomies and aneuploidies as large structural variants of the human genome. Am J Hum Genet. 2010; 87: 129-38.

Roth GM, Sun B, Greensite FS, Lott IT, Dietrich RB. Premature aging in persons with Down syndrome: MR findings. AJNR Am J Neuroradiol. 1996; 17: 1283-9.

Santos MA, Teklenburg G, Macklon NS, et al. The fate of the mosaic embryo: chromosomal constitution and development of Day 4, 5 and 8 human embryos. Hum Reprod. 2010; 25: 1916-26.

Scarfí MR, Cossarizza A, Monti D, Bersani F, Zannotti M, Lioi MB, Franceschi C. Age-related increase of mitomycin C-induced micronuclei in lymphocytes from Down's syndrome subjects. Mutat Res. 1990; 237: 247-52.

Schinzel A: Catalogue of Unbalanced Chromosome Aberrations in Man. Berlin, New York: De Gruyter, Walter, Inc.; 2001.

Schönland SO, Lopez C, Widmann T, Zimmer J, Bryl E, Goronzy JJ, Weyand CM. Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages.Proc Natl Acad Sci U S A. 2003; 100: 13471-6.

Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T. Multicolor spectral karyotyping of human chromosomes. Science. 1996; 273: 494-7.

Schröck E, Veldman T, Padilla-Nash H, Ning Y, Spurbeck J, Jalal S, Shaffer LG, Papenhausen P, Kozma C, Phelan MC, Kjeldsen E, Schonberg SA, O'Brien P, Biesecker L, du Manoir S, Ried T. Spectral karyotyping refines cytogenetic diagnostics of constitutional chromosomal abnormalities. Hum Genet. 1997; 101: 255-62.

Scott SA, Cohen N, Brandt T, Toruner G, Desnick RJ, Edelmann L: Detection of low-level mosaicism and placental mosaicism by oligonucleotide array comparative genomic hybridization. Genet Med. 2010; 12: 85-92.

Shaffer LG, Bejjani BA. A cytogeneticist's perspective on genomic microarrays. Hum Reprod Update. 2004; 10: 221-6.

Shaffer LG, Ledbetter DH, Lupski JR: Molecular cytogenetics of contiguous gene syndromes: mechanisms and consequences of gene dosage imbalances. In The Metabolic and Molecular Basis of Inherited Disease. Edited by Scriver C, Beaudet A, Sly W, Valle D, Childs B, Kinzler K, et al. New York: McGraw Hill; 2001.

Shampay J, Blackburn EH. Generation of telomere-length heterogeneity in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A. 1988; 85: 534-8.

Shay J, Wright W, Werbin H Loss of telomeric DNA during aging may predispose cells to cancer (review). Int J Oncol. 1993; 3: 559-63.

Sheridan R, Llerena J Jr, Matkins S, Debenham P, Cawood A, Bobrow M. Fertility in a male with trisomy 21. J Med Genet. 1989; 26: 294-8.

Sherman SL, Allen EG, Bean LH, Freeman SB. Epidemiology of Down syndrome. Ment Retard Dev Disabil Res Rev. 2007; 13: 221-7.

Sherr CJ, DePinho RA. Cellular senescence: mitotic clock or culture shock? Cell. 2000; 102: 407-10.

Shin M, Siffel C, Correa A. Survival of children with mosaic Down syndrome. Am J Med Genet Part A. 2010; 152A: 800–1.

Shinawi M, Shao L, Jeng LJ, Shaw CA, Patel A, Bacino C, Sutton VR, Belmont J, Cheung SW. Low-level mosaicism of trisomy 14: phenotypic and molecular characterization. Am J Med Genet A. 2008; 146: 1395-405.

Smith DW, Therma EM, Pfitau KA, Inhorn SL. Mosaicism in mother of two mongoloids. Am J Dis Child. 1962; 104: 534.

Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N, Conroy J, Hamilton G, Hindle AK, Huey B, Kimura K, Law S, Myambo K, Palmer J, Ylstra B, Yue JP, Gray JW, Jain AN, Fiegler H, Gribble SM, Burford DC, Carr P, Prigmore E, Porter KM, Clegg S, Crolla JA, Dennis NR, Jacobs P, Carter NP. Array painting: a method for the rapid analysis of aberrant chromosomes using DNA microarrays. J Med Genet. 2003; 40: 664-70.

Solinas-Toldo S, Lampel S, Stilgenbauer S, Nickolenko J, Benner A, Döhner H, Cremer T, Lichter P Matrix-based comparative genomic hybridization: biochips to screen for genomic imbalances. Genes Chromosomes Cancer. 1997; 20: 399-407.

Speicher MR, Gwyn Ballard S, Ward DC. Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet. 1996; 12:368-75.

Stewart SA, Weinberg RA. Telomeres: cancer to human aging. Annu Rev Cell Dev Biol. 2006; 22: 531-57.

Tarek M. The baby with Down syndrome. ASJOG 2005; 2: 362-365.

Thomas IT, Frias JL, Cantu ES, Lafer CZ, Flannery DB, Graham Jr JG. Association of pigmentary anomalies with chromosomal and genetic mosaicism and chimerism. Am J Hum Genet. 1989; 45: 193–205.

Thomas P, Harvey S, Gruner T, Fenech M. The buccal cytome and micronucleus frequency is substantially altered in Down's syndrome and normal ageing compared to young healthy controls. Mutat Res. 2008; 638: 37-47.

Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. Lancet. 2005; 366: 662-4.

Valli R, Maserati E, Marletta C, Pressato B, Lo Curto F, Pasquali F. Evaluating chromosomal mosaicism by array comparative genomic hybridization in hematological malignancies: the proposal of a formula. Cancer Genet. 2011; 204: 216-8.

Van Opstal D, Boter M, de Jong D, van den Berg C, Brüggenwirth HT, Wildschut HI, de Klein A, Galjaard RJ. Rapid aneuploidy detection with multiplex ligation-dependent probe amplification: a prospective study of 4000 amniotic fluid samples. Eur J Hum Genet. 2009; 17: 112-21.

Vanneste E, Voet T, Le Caignec C, Ampe M, Konings P, Melotte C, Debrock S, Amyere M, Vikkula M, Schuit F, Fryns JP, Verbeke G, D'Hooghe T, Moreau Y, Vermeesch JR. Chromosome instability is common in human cleavage-stage embryos. Nat Med. 2009; 15: 577-83.

Vaziri H, Schächter F, Uchida I, Wei L, Zhu X, Effros R, Cohen D, Harley CB. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. Am J Hum Genet. 1993; 52: 661-7.

Veenma D, Beurskens N, Douben H, Eussen B, Noomen P, Govaerts L, Grijseels E, Lequin M, de Krijger R, Tibboel D, de Klein A, Van Opstal D. Comparable low-level mosaicism in affected and non affected tissue of a complex CDH patient. PLoS One. 2010; 5: e15348.

Veltman IM, Veltman JA, Arkesteijn G, Janssen IM, Vissers LE, de Jong PJ, van Kessel AG, Schoenmakers EF. Chromosomal breakpoint mapping by arrayCGH using flow-sorted chromosomes. Biotechniques. 2003; 35: 1066-70.

Vissers LE, de Vries BB, Osoegawa K, Janssen IM, Feuth T, Choy CO, Straatman H, van der Vliet W, Huys EH, van Rijk A, Smeets D, van Ravenswaaij-Arts CM, Knoers NV, van der Burgt I, de Jong PJ, Brunner HG, van Kessel AG, Schoenmakers EF, Veltman JA. Array-based comparative genomic hybridization for the genomewide detection of submicroscopic chromosomal abnormalities. Am J Hum Genet. 2003; 73: 1261-70.

von Zglinicki T. Role of oxidative stress in telomere length regulation and replicative senescence. Ann N Y Acad Sci. 2000; 908: 99-110.

Vorsanova SG, Kolotii AD, Iourov IY, Monakhov VV, Kirillova EA, Soloviev IV, Yurov YB. Evidence for high frequency of chromosomal mosaicism in spontaneous abortions revealed by interphase FISH analysis. J Histochem Cytochem. 2005; 53: 375-80.

Vulliamy T, Marrone A, Szydlo R, Walne A, Mason PJ, Dokal I. Disease anticipation is associated with progressive telomere shortening in families with dyskeratosis congenita due to mutations in TERC. Nat Genet. 2004; 36: 447-9.

Wegiel J, Kaczmarski W, Barua M, Kuchna I, Nowicki K, Wang KC, Wegiel J, Yang SM, Frackowiak J, Mazur-Kolecka B, Silverman WP, Reisberg B, Monteiro I, de Leon M, Wisniewski T, Dalton A, Lai F, Hwang YW, Adayev T, Liu F, Iqbal K, Iqbal IG, Gong CX. Link between DYRK1A overexpression and several-fold enhancement of neurofibrillary degeneration with 3-repeat tau protein in Down syndrome. J Neuropathol Exp Neurol. 2011; 70: 36-50.

Werner W, Herrmann FH, John B. Cytogenetic Studies of a Family with Trisomy 21 Mosaicism in Two Successive Generations as the Cause of Down's Syndrome. Hum Genet 1982; 60: 202-4.

Wise JL, Crout RJ, McNeil DW, Weyant RJ, Marazita ML, Wenger SL. Cryptic subtelomeric rearrangements and X chromosome mosaicism: a study of 565 apparently normal individuals with fluorescent in situ hybridization. PLoS One. 2009; 4: e5855.

Wisniewski KE, Dalton AJ, McLachlan C, Wen GY, Wisniewski HM. Alzheimer's disease in Down's syndrome: clinicopathologic studies.Neurology. 1985; 35: 957-61.

Wisniewski LP, Hirschhorn K. Acquired partial deletions of the long arm of chromosome 5 in hematologic disorders. Am J Hematol. 1983; 15: 295-310.

Wong LS, de Boer RA, Samani NJ, van Veldhuisen DJ, van der Harst P. Telomere biology in heart failure. Eur J Heart Fail. 2008; 10: 1049-56.

Woods CG, Bankier A, Curry J, Sheffield LJ, Slaney SF, Smith K, Voullaire L, and Wellesley DJ. Asymmetry and skin pigmentary anomalies in chromosome mosaicism. Med Genet. 1994; 31: 694–701.

Wright WE, Shay JW. Time, telomeres and tumours: is cellular senescence more than an anticancer mechanism? Trends Cell Biol. 1995; 5: 293-7.

Yoon PW, Freeman SB, Sherman SL, Taft LF, Gu Y, Pettay D, Flanders WD, Khoury MJ, Hassold TJ. Advanced maternal age and the risk of Down syndrome characterized by the meiotic stage of chromosomal error: a population-based study. Am J Hum Genet. 1996; 58: 628-33.

Youssoufian H, Pyeritz RE. Mechanisms and consequences of somatic mosaicism in humans. Nat Rev Genet. 2002; 3: 748-58.

Yu WY, Chang HW, Lin CH, Cho CL. Short telomeres in patients with chronic schizophrenia who show a poor response to treatment.J Psychiatry Neurosci. 2008; 33: 244-7. Erratum in: J Psychiatry Neurosci. 2008; 33: 343.

Yurov YB, Iourov IY, Vorsanova SG, Demidova IA, Kravetz VS, Beresheva AK, Kolotii AD, Monakchov VV, Uranova NA, Vostrikov VM, Soloviev IV, Liehr T. The schizophrenia brain exhibits low-level aneuploidy involving chromosome 1. Schizophr Res. 2008; 98: 139-4.

Yurov YB, Vorsanova SG, Iourov IY, Demidova IA, BereshevaAK, Kravetz VS, Monakhov VV, Kolotii AD, Voinova-Ulas VY, Gorb achevskaya NL. Unexplained autism is frequently associated with low-level mosaic aneuploidy. J Med Genet 2007; 44: 521-25.

Zeichner SL, Palumbo P, Feng Y, Xiao X, Gee D, Sleasman J, Goodenow M, Biggar R, Dimitrov D. Rapid telomere shortening in children. Blood. 1999; 93: 2824-30.

Zekanowski C, Wojda U. Aneuploidy, chromosomal missegregation, and cell cycle reentry in Alzheimer's disease. Acta Neurobiol Exp (Wars). 2009; 69: 232-53.

Zitzelsberger H, Szücs S, Weier HU, Lehmann L, Braselmann H, Enders S, Schilling A, Breul J, Höfler H, Bauchinger M. Numerical abnormalities of chromosome 7 in human prostate cancer detected by fluorescence in situ hybridization (FISH) on paraffin-embedded tissue sections with centromere-specific DNA probes. J Pathol. 1994; 172:325-35.

Zühlke C, Thies U, Braulke I, Reis A, Schirren C. Down syndrome and male fertility: PCR-derived fingerprinting, serological and andrological investigations. Clin Genet. 1994; 46: 324-6.

Vita

Chariyawan Charlasawadi was born on December 12, 1977 in Thailand.

She earned a Doctor of Medicine (M.D.) degree from Prince of Songkla University, Thailand in 2002. She worked as a medical practitioner and earned a Master of Sciences (Biomedical Sciences) from Prince of Songkla University, Thailand in 2005. She is currently a medical lecturer in the Department of Pathology, Faculty of Medicine, Prince of Songkla University, Thailand.