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Age-related genetic and epigenetic chromosomal changes: A twin study

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

by

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Abstract

AGE-RELATED GENETIC AND EPIGENETIC CHROMOSOMAL CHANGES: A TWIN STUDY

Kimberly Haydu Jones, B.S.

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

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The primary aims of this study were to examine acquired genetic and epigenetic changes that occur in individuals with increasing age and to determine how these changes are influenced by genetic/environmental factors. Acquired genetic changes were assessed by determining the frequency and chromosomal contents of spontaneously occurring micronuclei in identical and fraternal twins. A total of 115 individuals (48 twin pairs and 19 singletons) were evaluated, ranging in age from 7 to 85 years. As expected, micronuclei frequencies, which are indicative of genomic damage, significantly increased with age ($p<0.0001$, $r=0.446$). The majority of micronuclei (32%) contained sex chromosomes and the frequency of sex chromosome-bearing micronuclei significantly increased with age ($p<0.0001$). The frequency of autosome-containing micronuclei was not significantly influenced by age or gender. However, some autosomes were seen more (chromosomes 4, 8, and 9) or less (chromosomes 17 and 22) frequently than expected by

chance ($p<0.05$). An evaluation of the numerical contents of the sex chromosome-containing micronuclei and their corresponding binucleates showed that the majority of the binucleates had an abnormal chromosomal complement (either hypodiploid or hyperdiploid), with the subset of binucleates having a normal chromosomal complement decreasing with age for both the Y chromosome in males and the X chromosome in females. Model fitting, implemented in Mx, showed the variation in the frequency of micronuclei to be best explained by either additive genetic and unique environmental components, or common and unique environmental factors. Specific environmental exposures and health conditions that were shown to influence micronuclei frequencies, included: multivitamins, leafy green vegetables, fruit, vitamin E supplements, arthritis, heart disease, allergies, and alcohol.

To assess acquired epigenetic changes, global methylation profiles of two identical twin pairs were compared and found to differ, indicating that individuals do develop alterations in their methylation profiles with age. Furthermore, the twin pair having a significant difference in their micronuclei frequencies and environmental exposures had more differences in their methylation pattern compared to the twin pair whose micronuclei frequencies and environmental factors did not differ. Overall, genetic and epigenetic changes were shown to occur with age and to be influenced by genetic and lifestyle factors.

Chapter 1.

Background and Significance

It has long been known that constitutional chromosomal abnormalities can have a dramatic effect on an individual's health. Although acquired chromosomal anomalies have been associated with many disease states, such as cancer, little is known about their etiology. In addition to cancer, new evidence is rapidly accumulating to suggest that there may also be a link between acquired chromosomal changes and other age-related disease states, such as Alzheimer's disease (Zekanowski and Wojda 2009), rheumatoid arthritis (Tascioglu et al., 2005), and osteoarthritis (Castellanos et al. 2004, Dahlen et al., 2001, Kinne et al., 2001, Mertens et al., 1996, Broberg et al., 1998). Before the significance of this relationship can be understood, more information needs to be gathered regarding which specific chromosomal changes occur with age and how these changes vary in the normal population.

The idea that an individual may acquire chromosomal abnormalities with age was first proposed in 1961 by Jacobs et al., who first discovered an increase in somatic cell chromosome loss with age. Since then, research studies have led to the conclusion that sex chromosomes are most often involved in acquired aneuploidy and that an age effect exists, with sex chromosomes being lost with increasing age (Fitzgerald and McEwan, 1977; Hando et al., 1994; Mukherjee et al., 1995). Interestingly, this age-related loss of sex chromosomes has been noted to be most pronounced in females (Fitzgerald and McEwan, 1977; Wojda and Witt, 2003).

Much less information is available regarding the frequency of autosomal abnormalities that are acquired with age, with the existing data leading to conflicting conclusions (Mukherjee et al., 1997; Hando et al., 1994). The results of the few studies that have directly evaluated specific autosomal involvment in acquired aneuploidy frequencies (using fluorescence in situ hybridization (FISH) techniques on interphase cells) have supported the premise of a non-random pattern of autosomal loss. The non-random trend of autosomal involvement seems to favor the loss of chromosomes with large blocks of heterochromatin (Stacey et al., 1995; Mukherjee et al., 1996; Fauth and Zankl 1999; Leach et al., 2004), which may provide valuable insight into the mechanism of chromosome loss.

An alternative method for studying acquired chromosomal changes is through use of the cytokinesis-blocked micronucleus (CBMN) assay. Micronuclei are small round bodies, containing whole chromosomes or chromosome fragments left behind during anaphase and thus not included into the daughter nuclei (Figure 1). The frequency of micronuclei serves as an indication of DNA damage having been shown to provide estimates of chromosomal abnormalities that are comparable to those quantified using traditional metaphase analysis for detecting genomic instability (Miller et al., 1998; Bonassi et al., 2001). Similar to the results of FISH studies on interphase cells, much of the information collected regarding micronuclei, to date, has shown that the majority of micronuclei contain sex chromosomes (Guttenbach et al., 1994; Hando et al., 1994; Richard et al., 1994; Zijno et al., 1996; Catalan et al., 1995 and 1998; Norppa and Falck, 2003). Furthermore, these frequencies have been shown to increase with age (Bolognesi

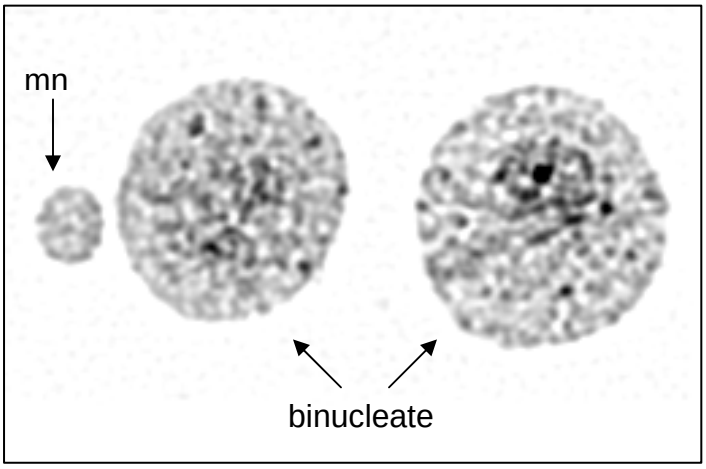

Figure 1. The appearance of a Giemsa stained micronucleus (mn) and corresponding binucleate from a lymphocyte culture.

et al., 1999; Bonassi et al., 2001), with the most dramatic increase in micronuclei being apparent between the ages of 50 and 69, followed by a decrease in micronuclei in individuals who live beyond age 69 (Wojda and Witt 2003; Bolognesi et al., 1997). Micronuclei frequencies have been shown to be higher in females, but this observation has not been consistently seen in all studies (Bonassi et al., 2001).

While numerous studies have been completed for adults, there is a paucity of information available about micronuclei frequency rates in children. Meta- and pooled analyses have been performed to estimate a baseline level of micronuclei frequencies in children with no observable gender effect (Neri et al., 2005). The small amount of current information regarding acquired autosomal abnormality frequencies in children also suggests a non-random pattern of chromosome involvement in micronuclei (Fauth et al., 1998; Norppa and Falck, 2003). However, researchers have reported mixed results as to which autosomes are excluded into micronuclei most frequently and whether or not there is an increase in autosome-containing micronuclei with age. Among the autosomes that have been suggested to show an over-representation in micronuclei in children are chromosomes 1, 9, and 16, which are chromosomes that have large blocks of heterochromatin (Sawyer et al. 1995; Stacey et al. 1995; Norppa and Falck, 2003).

In addition to studies of acquired numerical abnormalities, investigators have also evaluated the frequency of acquired structural changes that are present in micronuclei. Although the majority of micronuclei in normal, aged individuals contain what appear to be intact, structurally normal chromosomes (Wojda and Witt, 2003), this observation has not been consistently observed in the micronuclei seen in people having various health conditions. In Parkinson's disease and systemic lupus erythmatosus, there is an increase

in micronuclei containing acentric fragments, yet Alzheimer's disease patients have been shown to have an increase in micronuclei containing whole chromosomes (Migliore et al., 1997; 1999a; 1999b). Although there have been some studies regarding the frequencies of micronuclei, information regarding the chromosome-specific contents and structure is far from complete.

Several investigators have reported an increase in micronuclei frequencies in patients having cancer when compared to healthy individuals (Tucker and Preston, 1996; Duffaud et al., 1997; Venkatachalam et al., 1999; Znoar et al., 2003; Olaharski et al., 2006; Bonassi et al., 2007). Micronuclei frequencies have also been found to be increased in patients with Alzheimer's and Parkinson's disease (Migliore et al., 1997; Migliore et al., 2002). The cause of this increase in peripheral blood micronuclei frequencies remains controversial, and seems to vary with the specific disease. For example, the increase seen in Parkinson's patients has been speculated to arise from the "endogenously produced or exogenous toxins capable of damaging, via oxidative mechanisms, nigral dopaminergic neurons as their principal target" (Migliore et al., 2002). Alternatively, the increased frequency of micronuclei in Alzheimer's patients has been linked to a defect in microtubule assembly (Migliore et al., 1997). In cancer, one theory is that the correlation of lymphocyte micronuclei frequencies reflects genomic damage that also contributes to cancer initiating events in other tissues (Bonassi et al., 2007).

Some researchers have reported that environmental factors influence the frequency of acquired chromosomal abnormalities. The factors that have been shown to influence micronuclei frequencies include diet, hormone supplementation, and tobacco

usage, with the association of tobacco usage and micronuclei frequencies varying from no effect to increased frequencies (reviewed in Battershill et al., 2008; Fenech 1993; Schneider et al., 2001; Bonassi et al., 2001).

Differences in micronuclei frequencies observed between individuals have also been reported, with genetic influences being postulated to impact the frequency of acquired chromosomal abnormalities. Of particular interest are the results of a study reported by Bonassi and Fenech (2004), who found that chronic *in vitro* alcohol treatment induced an increase in micronuclei frequencies in cell lines having a BRCA1 gene mutation. Researchers have also consistently found an increased frequency of micronuclei with alcohol use in individuals having genetic variants in the alcohol metabolizing enzyme, alcohol dehydrogenase (Ishikawa et al., 2006; Kim et al., 2005). The exact role genetics and/or environment play in acquired chromosomal abnormalities is still largely unknown.

Twin studies are a useful way to determine the role that environment and genetics play in determining observed phenotypic variations. Phenotypic differences in monozygotic twins are usually treated as evidence of an environmental influence. To date, very few studies have examined the frequency of acquired chromosomal abnormalities in twins. One such study, by Jarvik and Kato (1970), found a genetic contribution to aneuploidy in 61 adult twins studied. However, this investigation was performed using metaphase cells, prior to the advent of chromosome banding technology. Thus, the completion of a twin study analyzing the role of genetics and/or environment on acquired chromosomal abnormalities, using today's technology, is long overdue.

While acquired chromosomal changes in DNA (aneuploidy) have been noted for almost 50 years, it was only recently recognized that chromatin has the potential to undergo epigenetic changes with advancing age. Epigenetic modifications, such as DNA methylation, play an important role in the control of gene expression and chromosome structure (Frigola et al. 2002; Peaston and Whitelaw, 2006). Simplistically, methylation typically prevents gene expression, while loss of methylation allows transcription. Some researchers report that methylation increases with age, while others report an overall decrease in methylation with age (Golbus et al., 1990; Cooney, 1993; Vertino et al., 1994; Toyota et al., 1999; Geigl et al., 2004; Clayton et al., 2005; Wojdacz and Hansen, 2006). The cause of these changes in methylation patterns with age is unknown. Some believe it is a function of diet and environment, while others believe it to be a random process (Cooney, 1993; Fenech 2002; Jaenisch et al., 2003; Bjornsson et al., 2004; Peaston and Whitelaw, 2006). It has been hypothesized that as humans age, they accumulate epigenetic and/or chromosomal changes over time (Wong et al, 2005; Petronis, 2006). Once multiple changes are acquired, they may result in the development of an age-related complex disease. In fact, many cancer cells have been shown to develop epigenetic changes (reviewed by Iacobuzio-Donahue, 2009). Some scientists have argued that epigenetic changes are more clearly associated with tumor progression than specific mutations (Feinberg and Tycko, 2004). However, the role these changes play in the genesis of disease has not been fully resolved. The possible randomness and unpredictable nature of these changes in DNA methylation has been suggested as an explanation for the observations in monozygotic twins, who have no evident differences in environmental exposure, being discordant for certain diseases (schizophrenia at 50%

discordance for MZ twins, 70% for rheumatoid arthritis, and 80% for breast cancer) (King et al., 1992; Petronis, 2001; Singh et al., 2002). Studies showing no difference in the frequency of phenotypic discordance between MZ twins raised apart when compared to MZ twins raised together suggest that this discordance reflects a mechanism other than environment (Wong et al. 2005). Large amounts of epigenetic changes can accumulate over thousands of mitotic divisions in cells from two genetically identical individuals (Petronis 2001). In their paradigm shifting study, Fraga et al. (2005) found that patterns of epigenetic modifications in monozygotic twin pairs diverge as they become older, which has been described as epigenetic drift (Martin, 2005). These alterations in an individual's epigenetic profile appear to be much more prevalent than DNA mutations (Bennett-Baker, 2003). In contrast to the study by Fraga et al. (2005), which examined global epigenetic changes, studies by Petronis et al. (2003) and Agrelo et al. (2006) examined local epigenetic changes of single, disease-related genes. In these cases, it was discovered that epigenetic changes in these gene regions appear to be linked to the disease state. Increasing numbers of autosomal genes were affected by methylation changes that occur during aging (Wojdacz and Hansen, 2006). Methylation specific arrays have also been used to evaluate changes in methylation with age. Using this technology, specific genes that seem to vary in methylation status with age and gender have been identified and include activin A receptor type I, interleukin 6, caspase recruitment domain-containing protein 15, platelet-derived growth factor receptor alpha, nuclear factor kappa-B subunit 1, and ETS-domain protein (Boks et al., 2009).

Since epigenetic mechanisms play a key role in establishing and maintaining chromosomal structure, specifically heterochromatin and euchromatin, it is possible that

epigenetic changes could be an underlying mechanism causing the structural and numerical chromosomal abnormalities associated with aging. Methylation differences that occur with age have the potential to contribute to somatic mutations, altering normal chromosome structure (Singh et al., 2002; Jones and Takai, 2001). Chromosomal translocations have been found to occur in hypomethylated regions (Richardson 2002). Fauth et al. (1998) found that treatment with the drug 5-azacytidine, which induces hypomethylation, increased the frequencies of micronuclei containing the chromosomes 1, 9, and 16, which have large blocks of heterochromatin. They concluded that undermethylation of heterochromatin may be associated with loss of specific chromosomes.

Thus, while much has been learned regarding the occurrence of acquired chromosomal abnormalities that arise in humans, many questions remain. Are individual's genetically predetermined to acquire these chromosomal changes or are these chromosomal changes more strongly affected by an individual's environment? Are these changes acquired randomly or do commonalities exist between individuals? The primary aims of this study were to better characterize the chromosome-specific frequencies of acquired chromosomal abnormalities as assessed through the CBMN assay, and to determine the extent to which the variation in the frequency of micronuclei was determined by genetics and/or environment. A secondary aim was to determine if global methylation patterns varied between twin pairs and with age. These aims were achieved by testing the following hypotheses:

1. Micronuclei frequencies increase with age.
2. Micronuclei frequencies are influenced by both genetic and environmental factors.

3. There is a nonrandom pattern of chromosomal exclusion into micronuclei, with this pattern being age-related.
4. Methylation patterns change with increasing age.
5. The acquisition of changes in the pattern of methylation within an individual are positively correlated with their frequency of micronuclei.

Chapter 2.

Genetic and environmental factors contributing to spontaneous micronuclei frequencies in children and adults: A twin study.

Introduction

Acquired chromosomal abnormalities were first reported to occur in human somatic cells in 1961 (Jacobs et al.). Since that initial discovery, the majority of investigations of acquired chromosomal changes have centered on their association with particular types of neoplasia. Sandberg (1990) reported that approximately 85% of the affected tissues from patients with cancer have acquired chromosomal changes. In addition to cancer, new evidence is rapidly accumulating to suggest that there may also be a link between acquired chromosomal changes and other age-related disease states, such as Alzheimer's disease (Zekanowski and Wojda 2009), rheumatoid arthritis (Tascioglu et al., 2005), and osteoarthritis (Castellanos et al. 2004; Dahlen et al., 2001; Kinne et al., 2001; Mertens et al., 1996; Broberg et al., 1998). However, little is known about the frequency or types of acquired chromosomal changes that spontaneously arise in healthy humans, especially in children.

One approach for understanding the etiology of acquired chromosomal changes has been to delineate their frequency of occurrence and to see if consistent patterns emerge that might suggest a causative factor(s). The "gold standard" for scoring acquired chromosomal abnormalities has been the evaluation of metaphase chromosomes. While

this technique allows for full characterization of all cytogenetic findings present, it is limited in that it is: (1) labor intensive, thereby reducing the number of observations that can be collected; and (2) at risk for producing "artifactual" anomalies as a result of the necessary cell culture, harvesting, and slide making procedures. The cytokinesis-block micronucleus (CBMN) assay has been used as an alternative approach to estimate the frequency of acquired chromosomal changes. This CBMN assay provides information regarding the previous interphase/mitotic division of a somatic cell prior to the presence of selective pressure on the resultant daughter cells. Given that this assay allows for an assessment of a large number of cells (1000 or more) and is less labor intensive than conventional cytogenetic studies to perform, it has potential for use as a high throughput assay.

A micronucleus is defined as a small structure that is juxtaposed to the main daughter nuclei following mitosis. Micronuclei are thought to contain chromatin (from one or more chromosomes) that was not incorporated into the daughter nuclei ("lagging" or "lost") following cell division (Fenech and Morley, 1985). Micronuclei frequencies have been shown to increase with both age and DNA damage, providing data that closely parallels that of metaphase chromosomal analyses (Miller et al., 1998; Bonassi et al., 2001). Thus, the assessment of micronuclei frequencies has become a very attractive biosurveillance tool for quantifying genomic damage associated with environmental insults and occupational exposures, as well dietary and lifestyle habits (Battershill et al., 2008). As anticipated from the above noted observations regarding chromosomal changes and age-related conditions, researchers have also reported micronuclei frequencies to be increased in individuals with different health conditions,

such as cancer, Alzheimer's disease, and Parkinson's disease (Tucker and Preston, 1996; Duffaud et al., 1997; Venkatachalam et al., 1999; Znoar et al., 2003; Olaharski et al., 2006; Migliore et al. 1997; Migliore et al. 2002). Given the potential use of this assay for recognizing individuals who may have an increased risk for developing health problems, it is important to understand the factors that influence alterations in micronuclei frequencies. In particular, it is not known if most of the variation observed for micronuclei frequencies reflects environmental exposures, a predisposition based on one's genetic make-up, or an interaction between heritable genetic and environmental factors.

One of the most robust methods for identifying sources of variation in humans is to study specific traits in twins. Thus, the primary aim of this study was to measure the frequency of spontaneously occurring micronuclei in twins of differing ages and to determine the extent to which the variation in the frequency of micronuclei was determined by genetics and/or environment.

Materials and Methods

Sample Ascertainment

A total of 48 twin pairs and 19 singletons, whose co-twin did not complete the sample collection process, were studied. The twins were ascertained through the Mid-Atlantic Twin Registry (MATR). Of the 115 participants, 52 were males and 63 were females. The only study inclusion criterion was that the twins be of the same gender (to eliminate confounding effects in data interpretation that might arise from potential gender differences). After providing their informed consent (VCU IRB protocol #179), the participants submitted blood samples and a completed health history questionnaire. Blood samples were collected by the participants' health care providers. Following their collection, the samples were shipped to our laboratory via an overnight delivery carrier.

DNA Isolation and Zygosity Determination

Genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Qiagen). Zygosity was determined using 13 highly polymorphic short tandem repeat sequences (AmpFlSTR Profiler Plus and Cofiler kits, Applied Biosystems, Foster City, CA) according to standard procedures. Twins were classified as monozygotic if the marker data for the co-twins matched at all 13 loci. Co-twins having marker differences were identified as a dizygotic pair.

Cell Culture

Lymphocytes were isolated using Histopaque-1077 (Sigma) and lymphocyte cultures were established according to the protocol of Fenech (1993). Briefly, cytochalasin B (Sigma, 14930-92-2) was added to the cells 44 hours after culture initiation at a concentration of 3.0 μg/ml. At 72 hours, binucleate interphase cells were harvested using standard techniques, which included a 10-minute incubation in hypotonic solution (0.075 M KCl), and serial fixation (three times using a 3:1 methanol: acetic acid solution). Slides were made following standard procedures (Leach and Jackson-Cook, 2001).

Micronuclei Analysis

The prepared slides were stained for 4 minutes in a 4% Harleco Giemsa solution. Micronuclei frequencies were determined by replicate scoring (2 times per slide) of the number of micronuclei observed per 1000 cytochalasin-B blocked binucleates, following the criteria established by Fenech et al. (2003). The frequency of micronuclei was calculated by averaging the values obtained from the 2 replicate scores (2000 total binucleates counted).

Statistical Analysis

Non-parametric Spearman's correlation coefficients were calculated for the association comparisons using either the JMP 8 (Version 8.0.1) or R (Version 2.9.1) statistical analysis packages. The impact of additive genetic (A), common environmental (C), and/or unique environmental (E) factors on micronuclei frequencies was evaluated

using an ACE model fitting approach (Neale and Cardon, 1992) (Figure 2). This analysis, which was implemented in Mx, allowed for an evaluation of the fit of full (ACE) and reduced (AE, AC, or CE) models with log-likelihood ratio tests. A univariate regression analysis, with and without correction for age, was used to assess the effects of health history, lifestyle, and medication usage on micronuclei frequencies. When indicated by the univariate analysis, general linear modeling was used to assess the age-corrected variation in micronuclei frequencies seen for other potential predictive factors (such as alcohol use, calcium or multivitamin consumption). In order to limit type I errors, an alpha level of $p<0.05$ was used for all statistical tests.

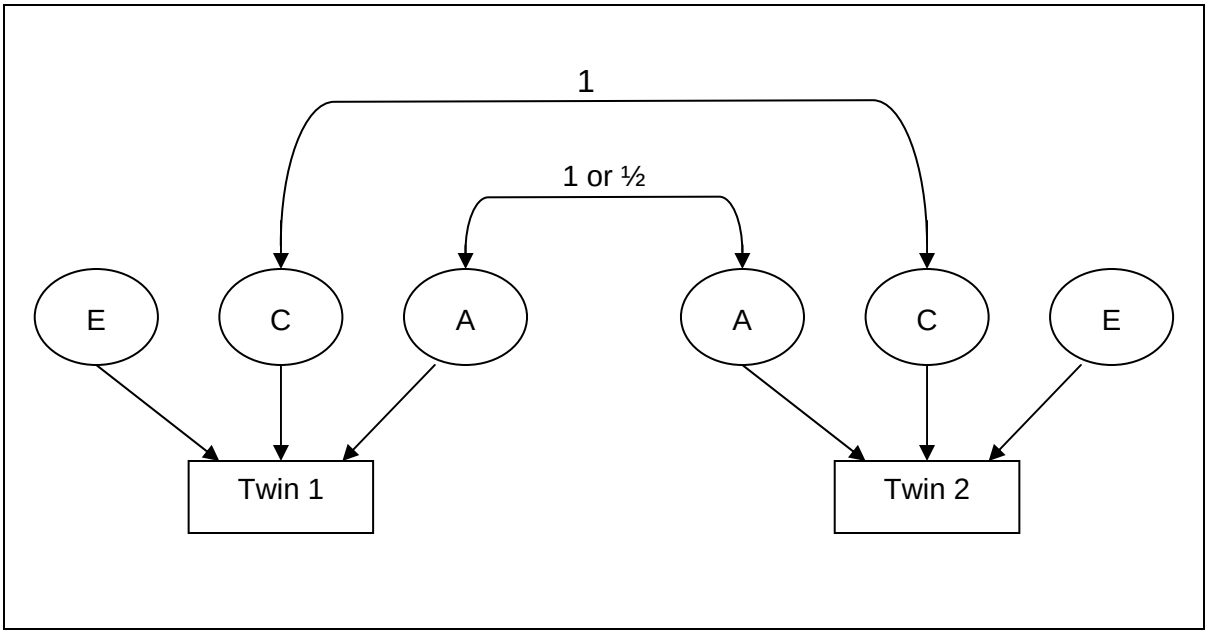

Figure 2. Path diagram illustrating the potential sources of variance on acquired chromosomal abnormalities for MZ and DZ twin pairs, additive genetics (A), common environmental (C), and unique environmental (E). The MZ additive genetic correlation is 1. The DZ additive genetic correlation is ½. The common environmental correlation is equal to 1 for both DZ and MZ twins.

Results

Sample Distribution

The ages of the study participants ranged from 7 to 85 years, and included 52 male and 63 female participants (Table 1). The average age of the male and female participants was 47.5 and 41.3, respectively. Of the 48 complete twin pairs collected, 33 were determined to be monozygotic, with 15 being dizygotic. The majority of samples were submitted from individuals living in the Mid-Atlantic region (Virginia and North Carolina were most common). However, samples were collected from individuals residing in locales throughout the United States (including California, Colorado, Florida, Indiana, Michigan, Missouri, North Dakota, Pennsylvania, South Carolina, Texas, Tennessee, and Washington).

Micronuclei frequencies

No significant difference in micronuclei frequencies was observed between males (1.39% ± 0.11% [mean ± standard error]) and females (1.22% ± 0.10%) ($p=0.87$), regardless of their age ($p=0.254$) (Figure 3). As a result, the data from males and females were pooled for all other statistical comparisons. A significant positive correlation was detected between micronuclei frequency and age ($p<0.0001$, $r=0.446$), as shown in Figures 3 and 4. Interestingly, the highest frequency of micronuclei was observed for the twins who were 60 to 69 years of age, with the lowest frequencies being noted for the twins who were 19 years of age or younger. The variation in micronuclei frequencies observed for the children (ages 7 to 18) ranged from 0.1% to 2%, with variation in

Table 1. Sample distribution and micronuclei frequencies by age group

Age Group	Males	Females	Total	MN Frequency per 1000 binucleates (mean ± s.e.)	MZ pairs	DZ pairs	Singletons
7-9	4	2	6	5.6±1.1	3	0	0
10-19	6	18	24	7.6± 0.9	7	5	0
20-29	1	5	6	14.2±3.7	2	0	2
30-39	7	2	9	12.8±1.4	3	0	3
40-49	6	13	19	12.9±1.3	6	1	5
50-59	6	6	12	11.3±1.3	2	2	4
60-69	11	9	20	22.5±2.5	5	3	4
70-79	9	6	15	13.3±1.7	3	4	1
80+	2	2	4	14.3±1.1	2	0	0
Total	**52**	**63**	**115**	**13.0 ± 0.8**	**33**	**15**	**19**

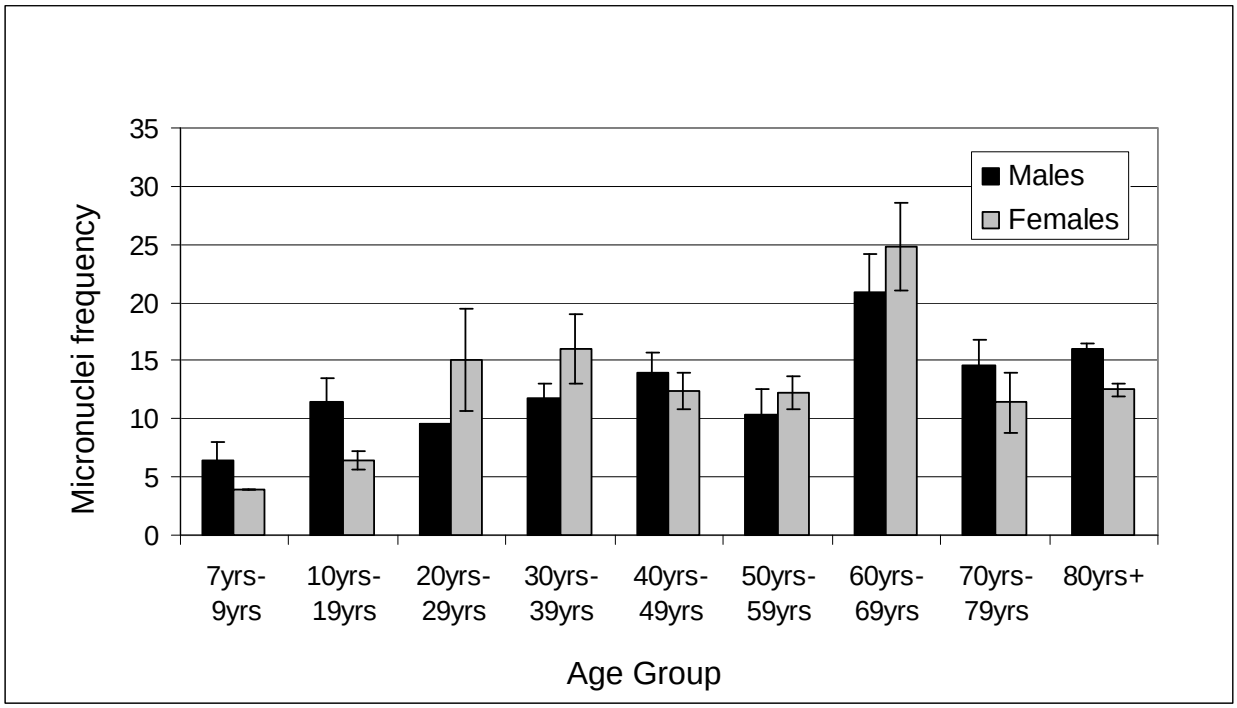

Figure 3. Mean micronuclei frequency by age decade and gender. The micronuclei frequency is presented as the number of micronuclei scored per 1000 binucleates $\pm$ standard error. The micronuclei values for males (black histograms) and females (grey histograms) are shown.

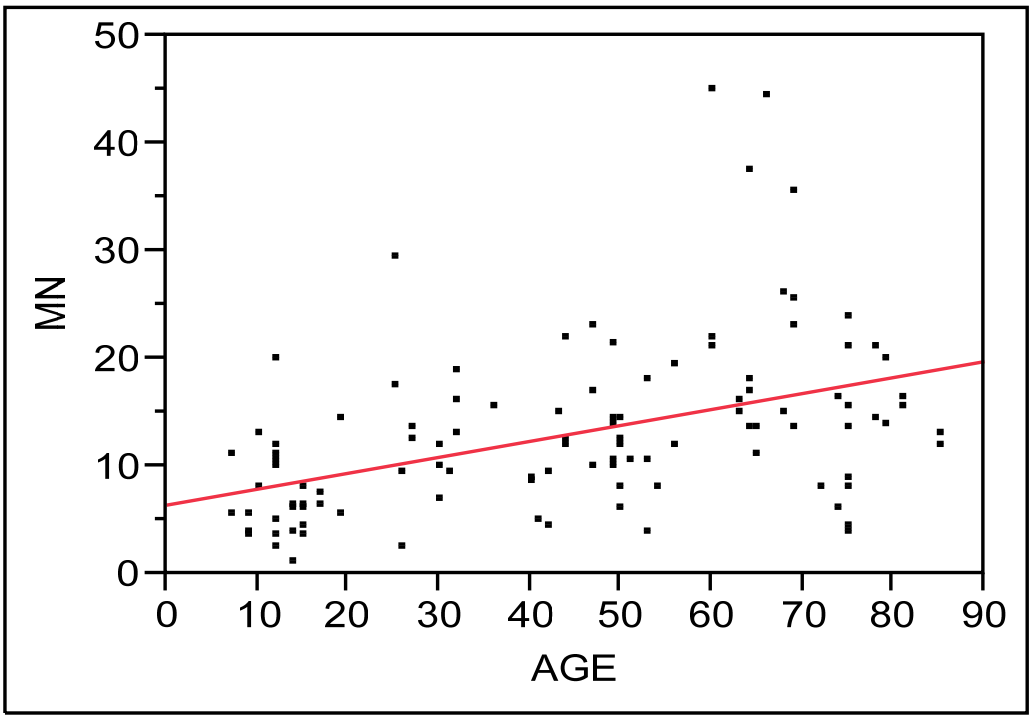

Figure 4. **Correlation of micronuclei frequency with age.** Micronuclei frequencies (Y axis) are presented as the mean number of micronuclei per 1000 binucleates. The spontaneous frequency of micronuclei, plotted in relation to the 115 study participants' age (X axis), showed a significant positive correlation (r=0.446).

younger adults (19 to 49) and middle-aged to older twins (50 or older) ranging from 0.3% to 3% and, 0.4% to 4.5%, respectively.

Micronuclei frequencies and genetics

The correlation coefficient for micronuclei frequencies between monozygotic twins (who share all of their genes) was 0.5 ($p=0.003$) compared to 0.42 ($p=0.118$) for the dizygotic twins (who share half of their genes). Given that the correlation between identical twins was only 0.5, one can infer that there are unique environmental factors influencing the frequency of micronuclei. However, the ACE model fitting analysis showed that unique environmental effects alone (E) provided a significantly poor fit to the data ($p<0.0004$). While none of the models evaluated showed a strong fit to the data, the best fitting models were those that included influences from both additive genetic and unique environment (AE), or both common and unique environmental effects (CE) (Table 2).

Micronuclei frequencies and health history questionnaire information

Given that unique environmental effects, in conjunction with either additive genetic and/or common genetic effects, appear to contribute to the observed variation in micronuclei frequencies, we assessed the effect of various unique environmental and health components on micronuclei frequencies (Tables 3 and 4). An initial univariate

Table 2. Mx ACE model fitting.

Model	-2LnL	k	AIC	chi^2	df	p-value
ACE	285.099	4	81.099	2.454	3	0.4837
CE	285.099	3	79.099	0	1	1.000
AE	286.794	3	80.794	1.695	1	0.1929
E	300.657	2	92.697	15.558	2	0.0004

k= parameters
AIC= Akaike's Information Criterion
Chi^2= Chi-square value
Df= Degrees of freedom

analysis showed that age had the most significant influence on micronuclei frequencies ($p<0.00000043$). Therefore, an age-corrected univariate assay was performed to evaluate the effect of other potential influences. The age-corrected analysis showed a significantly increased frequency of micronuclei to be associated with a history of allergies (Table 3) and with the consumption of vitamin E supplements (Table 4), with a borderline significance value being observed for body mass index (BMI) (which led to a tendency for increased micronuclei frequencies). In contrast, a significantly decreased frequency of micronuclei was associated with eating fruit (at least 5 days per week); green vegetables (at least 5 days per week); or folate-supplemented bread (at least 5 days per week) (Table 4).

Surprisingly, a significantly decreased frequency of micronuclei was also noted for people reporting a history of heart disease and arthritis (Table 3). Given that most of the individuals who reported having heart disease and arthritis also reported taking medication to treat these conditions, the effect that these medications might have on micronuclei frequencies was evaluated. Medication groups used by less than 4 individuals were not analyzed due to the small sample size. Individuals who reported taking angiotensin converting enzyme (ACE) inhibitors for treatment of their heart disease had a significantly lower frequency of micronuclei compared to individuals who did not report taking these medications (Table 5). In contrast, diuretics usage was associated with a significantly higher frequency of micronuclei (Table 5).

Table 3. Age-corrected univariate regression analysis of association of health history factors with lymphocyte micronuclei frequencies.

Health History Predictor	N	MN Frequency/1000 binucleates (mean ± s.e.)	MN Age adjusted	Beta Estimate	Std. Error	p-value
Arthritis						
Yes	15	12.3 ± 1.3	-3.9 ± 1.2	-0.4348	0.1242	0.0005*
No	84	12.3 ± 0.8	0.2 ± 0.7			
Migraines						
Yes	17	12.6 ± 1.6	-0.4 ± 0.7	0.0537	0.1167	0.645
No	82	12.2 ± 0.8	-0.3 ± 1.2			
High Blood Pressure						
Diagnosed	27	17.9 ± 1.8	1.7 ± 1.8	0.1103	0.1414	0.435
Not Diagnosed	78	11.2 ± 0.8	-0.7 ± 0.7			
Cancer						
History of cancer	16	16.3 ± 2.3	-0.3 ± 2.4	-0.0814	0.1192	0.495
Never had cancer	89	12.2 ± 0.8	-0.1 ± 0.7			
Heart Disease						
Diagnosed	11	16.8 ± 1.2	-0.7 ±1.4	-0.1535	0.074	0.0381*
Not Diagnosed	94	12.4 ± 0.9	0 ± 0.8			
Allergies						
Yes	44	14.1 ± 1.3	0.8 ± 1.2	0.1306	0.042	0.0019*
No	55	10.8 ± 0.7	-1.4 ± 0.6			
BMI						
Obese	19	14.8 ± 1.9	0.8 ± 1.2	0.1045	0.0585	0.0742
Overweight	21	16.5 ± 1.8	1.3 ± 1.8			
Normal weight	43	10.6 ± 0.8	-1.8 ± 0.7			
Underweight	14	8.25 ± 1.3	-0.2 ± 1.3			

*Statistically significant ($p \leq 0.05$)

Table 4. Age-corrected univariate regression analysis of association of lifestyle factors with lymphocyte micronuclei frequencies.

Lifestyle Predictor	N	MN Frequency/1000 binucleates (mean ± s.e.)	MN Age adjusted	Beta Estimate	Std. Error	p-value
Tobacco						
Past and current users	23	16.0 ± 2.1	1.5 ± 1.9	0.0004	0.1002	0.996
Non-users	82	12.0 ± 0.8	-0.5 ± 0.7			
Alcohol						
Alcoholic	2	15 ± 0.5	0.1 ± 3.6	-0.0032	0.0443	0.942[a]
Everyday	6	14 ± 3.7	0.4 ± 4.6			
Few times per week	8	11.7 ± 1.5	-1.8 ± 2.0			
One per week or less	23	14.9 ± 1.6	1.2 ± 1.4			
Never	59	10.9 ± 0.9	-1.1 ± 0.8			
Fruit						
5 days per week or more	64	11.3 ± 0.7	-1.5 ± 0.6	-0.211	0.0877	0.0162*
less than 5 days per week	35	14.1 ± 1.6	1.5 ± 1.4			
Folate enriched bread						
5 days per week or more	49	10.6 ± 0.8	-1.9 ± 0.7	-0.1377	0.0743	0.064
less than 5 days per week	50	13.9 ± 1.2	1.0 ± 1.1			
Leafy Green Vegetables						
5 days per week or more	37	10.6 ± 0.8	-3.0 ± 0.7	-0.2979	0.088	0.0007*
less than 5 days per week	62	13.3 ± 1.0	1.1 ± 0.9			
Vitamin C supplement						
5 days per week or more	12	16.1 ± 2.5	0.2 ± 2.4	0.1283	0.1135	0.258
less than 5 days per week	87	11.8 ± 0.7	-0.5 ± 0.7			
Calcium Supplement						
5 days per week or more	21	14.1 ± 1.3	-1.0 ± 1.1	0.0389	0.0985	0.693[a]
less than 5 days per week	78	11.8 ± 0.8	-0.3 ± 0.8			
Vitamin E supplement						
5 days per week or more	10	18.4 ± 4.1	2.6 ± 4.1	0.264	0.1179	0.0251*
less than 5 days per week	89	11.6 ± 0.6	-0.8 ± 0.6			
Vitamin D supplement						
5 days per week or more	14	15.5 ± 2.2	0.3 ± 2.0	0.082	0.1143	0.473
less than 5 days per week	85	11.8 ± 0.8	-0.5 ± 0.7			
Fish Oil supplement						
5 days per week or more	15	14.8 ± 2.4	-0.1 ± 2.3	0.0928	0.1067	0.384
less than 5 days per week	84	11.8 ± 0.7	-0.5 ± 0.6			
Multivitamin						
5 days per week or more	35	15.3 ± 1.1	-0.1 ± 1.1	0.3149	0.1114	0.0047*[a]
less than 5 days per week	64	10.6 ± 0.9	-0.6 ± 0.8			

*Statistically significant ($p \leq 0.05$)

[a] association varies with age, see covariate model Table 6

To determine if the observed differences in micronuclei frequencies of people having allergies or arthritis might also be confounded by medications used for their treatment, lists of medication usage for these conditions were also tabulated. However, the sample sizes of individuals taking medication for these conditions (only 4 of the 44 individuals having allergies and only 4 of the 15 individuals having arthritis) was too small to allow for an unbiased statistical assessment.

The micronuclei frequencies associated with three of the variables tested (alcohol use, multivitamin supplements, and calcium supplements) varied with age. As a result, a covariate model was used to test for potential associations for these variables. These covariate assays showed both alcohol intake (moderate) and multivitamin use to be associated with decreased micronuclei frequencies (Table 6). However, no significant change in micronuclei frequencies was detected for calcium supplement usage (borderline value).

Table 5. Age-corrected univariate regression analysis of association of medications with lymphocyte micronuclei frequencies.

Medication	N	MN Frequency/1000 binucleates (mean ± s.e)	MN Age Adjusted	Beta estimate	Std. Error	p-value
ACE inhibitor						
Yes	7	18.1 ± 1.0	-0.59 ±1.4	-0.4467	0.1802	0.0132*
No	92	11.6 ± 0.8	-0.41 ± 0.7			
β Blocker						
Yes	11	16.3 ± 1.2	1.72 ± 1.3	0.2746	0.1630	0.0920
No	88	12.1 ± 0.7	-0.69 ± 0.7			
Calcium Channel Blocker						
Yes	4	15.3 ± 3.1	-0.05 ± 1.3	0.1672	0.1772	0.3453
No	95	12.1 ± 0.7	-0.43 ± 0.7			
Angiotensin II Receptor Blocker						
Yes	5	21.3 ± 3.0	-0.58 ± 3.5	-0.1142	0.1794	0.5243
No	94	11.3 ± 0.7	-0.41 ± 0.7			
Diuretic						
Yes	10	17 ± 1.5	-6.07 ± 2.8	0.4638	0.1261	0.0023*
No	89	12.0 ± 0.8	-1.14 ± 0.6			

* statistically significant ($p \leq 0.05$)

Table 6. Covariate regression analysis of association of health history/lifestyle factors with lymphocyte micronuclei frequencies and age.

Health History or Lifestyle Predictor	Beta Estimate	Std. Error	z-value	p-value
Calcium Supplement	0.013	0.007	1.818	0.069
Alcohol	-0.008	0.002	-3.390	0.001*
Multivitamin	-0.012	0.006	-1.954	0.050*

* Statistically significant

Discussion

The frequencies of spontaneous micronuclei observed in the adults (mean age of 55) of this twin population (which ranged from 0.25% to 4.5%) were comparable to the frequencies observed in singletons having a similar mean age (58) that were studied by other investigators (range of 0.2% to 4.2%) (Bonassi et al., 2001), suggesting that the twins who were evaluated in this investigation are representative of the general population. As noted by others, a strong positive correlation was present between micronuclei frequencies and age (Bolognesi et al., 1999; Bonassi et al., 2001). Interestingly, our observation of a peak in micronuclei frequencies in the members of our 60-69 year-old age group (1.1% to 4.5%) is also a finding that has been seen by other investigators, who have shown that the frequency of micronuclei appears to peak between ages 50-69, remaining either unchanged or slightly lower in the individuals who live to be 80 or older (Wojda and Witt 2003; Bolognesi et al., 1997). In contrast to our data, which showed no effect of sex on the micronuclei frequencies observed over all the individuals studied, several researchers have reported a difference in micronuclei frequencies between adult men and women, with women usually having higher micronuclei frequencies than men (Bonassi et al., 2001). The reason for this contrasting observation is not clear, but may reflect the fact that our data set included individuals from a broader range of ages than those typically studied. For example, 9 of the 22 study groups included in the Human Micronucleus Project (HUMN) found a higher frequency of micronuclei in males, and two of those studies also included children (Bonassi et al.,

2001). Additionally, a study by Bonassi et al. (1995) saw a dramatic increase in female micronuclei frequencies, but this increase was limited to women in age groups over 44 years. When one examines the mean micronuclei frequencies of the 20 to 40 year-olds and the 50 to 70 year-olds from our study, which are the age groups most frequently included by others, the micronuclei frequencies of the females tend to be higher than those of males.

Little information is available about micronuclei frequency rates in children. However, in their meta- and pooled analyses of baseline levels of micronuclei frequencies, Neri, et al. (2005) found the average micronuclei frequency in children age 0-18 years to be 0.52% $\pm$ 0.51%. The average micronuclei frequency in the children participating in our study was 0.70% $\pm$ 0.07%. However, our study included children ages 7 to 18 years, while approximately 30% of the children in their study were younger than 7 years. Given that an age effect on micronuclei has been shown, even in the first two decades of life, this variation in frequencies seems likely to reflect the variation in study subject ages between investigations (Neri et al., 2003). Neri, et al. (2005) also found no gender effect on micronuclei frequencies in children, consistent with our observations.

Since micronuclei frequencies are increasingly used as a biomarker, it is important to know what factors influence their spontaneous frequency. By studying twins, we were able to provide the first estimate of the proportion of variation in micronuclei frequencies that was attributable to unique environmental, common environmental, and/or additive genetic effects. Interestingly, the ACE model fitting analyses, which were completed on age-adjusted data to account for the strong

correlation present with advancing age, showed the best fit for reduced models, in which the variation was best explained by both common environmental and unique environmental factors (CE); or by additive genetic and unique environmental factors (AE). Both of these models (AE or CE) provided a significantly better fit than models attributing the observed variation to only additive genetic effects or only unique or common environmental exposures. Similarly, the MZ (0.5) and DZ (0.42) twin correlation coefficients provided values that clearly showed the presence of an environmental influence, but did not readily allow one to discriminate between the additional effects arising from additive genetic versus common environmental influences. Thus, the frequency with which an individual forms micronuclei appears to be a complex trait that is determined by a mixture of unique environmental influences, as well as either additive genetic or common environment exposures. Our continued studies of a larger number of twins should allow us to better recognize the contribution of environmental and/or genetic influences to the formation of micronuclei in humans.

Although unique environmental effects do not appear to solely influence micronuclei frequencies, they were consistently shown to contribute to the observed variation in frequencies. Therefore, data from the health history questionnaires were analyzed to determine if specific environmental/health factors could be identified that were consistently associated with an increased or decreased frequency of micronuclei. Of the 7 health conditions that were assessed (Table 3), only three conditions (heart disease, arthritis, and allergies) were shown to be significantly associated with micronuclei frequencies after age adjustment. Specifically, individuals with allergies had substantially larger micronuclei frequencies compared to individuals without allergies,

while individuals who reported arthritis or heart disease had significantly lower micronuclei frequencies compared to individuals without these conditions.

The assessment of heart disease in this study cohort was compromised because it was self-reported and the study participants differed in their inclusion criteria. Some individuals reported having heart disease because they had high cholesterol levels or high blood pressure, or both, while others only reported heart disease if they had experienced a heart attack. Because of this complication, the data were less subjectively analyzed by evaluating these individuals based on the medications that were prescribed for treatment by their physician. The medications that were used by 4 or more individuals included ACE inhibitors, beta blockers, calcium channel blockers, angiotensin II receptor blockers, diuretics, statins, and aspirin. Of the more commonly used medications, ACE inhibitors were found to be associated with significantly decreased micronuclei frequencies ($p=0.013$), while diuretics were associated with significantly increased micronuclei frequencies ($p=0.0002$) (Table 5). These findings are in agreement with previous results that showed the use of diuretics *in vitro* (specifically hydrochlorothiazide) was associated with increased micronuclei frequencies (Andrianopoulos et al. 2006), while ACE inhibitors (specifically Captopril) significantly decreased micronuclei frequencies (Hosseinimehr et al., 2007). However, in this latter study, micronuclei frequencies were assessed in bone marrow cells from mice following irradiation, with no other studies in humans being reported.

Although the sample size of this study ($n=115$ individuals) was smaller than that used by some other investigators whose primary aim was to identify potential dietary and

lifestyle influences on micronuclei frequencies (reviewed in Battershill et al., 2008), significant differences were observed for both dietary patterns and alcohol usage.

The regular inclusion (at least 5 days per week) of fruit and leafy green vegetables in the study participants' diet was associated with a significant decrease in their micronuclei frequencies. In addition, a trend toward a reduced frequency of micronuclei was observed for people reporting a consistent intake of folate-enriched bread. Collectively, these data suggest that folate may be important for regulating micronuclei frequencies, providing a "protective" effect that is associated with a decrease in frequency. This finding confirms the observations of other researchers, who have also reported folate usage to be associated with decreased micronuclei frequencies (Fenech 2001; Fenech 2002; Fenech et al., 2005; Fenech and Rinaldi, 1994; Abramsson-Zetterberg et al., 2006; Fenech 1998; Titenko-Holland et al., 1998). Given that folate is required for DNA synthesis and repair, with it acting as a methyl donor for thymidylate synthesis, it is understandable that increased folate ingestion is associated with decreases in micronuclei frequencies (Fenech 2001).

The influence of ingesting supplemental vitamins was also evaluated. Of the vitamins assessed (C, E, D, calcium, fish oil/omega 3 fatty acids, and multivitamins) only vitamin E and multivitamin ingestion were found to significantly influence micronuclei frequencies. The use of vitamin E supplements was associated with an increased frequency of micronuclei. Other investigators who have studied this effect have reported varied results, with increases, decreases and no effect being seen for Vitamin E and C ingestions and micronuclei frequencies (Battershill 2008). Interestingly, Fenech, et al. (2005), observed a decreased frequency of micronuclei with vitamin E intake, but did

note an increased frequency of micronuclei with biotin, riboflavin and pantothenic acid ingestion. It is also interesting to note that vitamin E is found in many fruits and leafy green vegetables (along with folate, iron, calcium, and vitamins A, K, C), yet our sample showed a decreased frequency of micronuclei associated with their consumption. Thus, the protective influence conferred from the ingestion of these fruits/vegetables may arise primarily from the other components (such as folate), or the effect of vitamins may be varied depending on whether they are ingested as a supplement (perhaps over usage) versus ingested as a component of natural foods.

In addition to looking at individual vitamins, multivitamin use was evaluated and found to be associated with a decreased frequency of micronuclei (with the effects co-varying with age). The individual components of the daily multivitamins were not reported, so we are unable to directly associate the positive effects to a key component(s).

The effect of tobacco and alcohol usage on micronuclei frequencies of the adults who participated in this study was also evaluated. Like several investigators (specifically the HUMN project which included over 5000 individuals), we found tobacco usage to have no significant influence on micronuclei frequencies (Bonassi et al., 2001). However, the study results of other investigators have shown an increased frequency of micronuclei with tobacco usage (Fenech 1993; Schneider et al., 2001), suggesting that the effect, if present, may vary with specific products consumed or be influenced by other factors (such as diet or other susceptibility factors).

The association of alcohol use on the micronuclei frequencies of the adults in this study was shown to co-vary with age. In the covariate model factoring age effects, alcohol use was shown to be associated with decreased micronuclei frequencies. While

the generalized effect of alcohol on micronuclei frequencies has been noted to provide varied results (Battershill 2008), researchers have consistently found an increased frequency of micronuclei with alcohol use in individuals having genetic variants in the alcohol metabolizing enzyme, alcohol dehydrogenase (Ishikawa et al., 2006; Kim et al., 2005). Teo and Fenech (2008) suggest an interaction between folic acid and alcohol use, where increased folic acid protects against the DNA damage caused by increased ethanol use. However, a factor confounding the interpretation of an association between alcohol consumption and micronuclei frequencies in this current study, as well as studies completed by other investigators, is the potential variation in the effect of moderate to low alcohol use (which may have no effect) compared to excessive alcohol usage, the latter of which has been associated with the presence of increased frequencies of micronuclei (Castelli et al., 1999; Maffei et al., 2002).

In summary, the frequency of spontaneously arising micronuclei in humans appears to be a complex trait that is influenced by unique environmental factors as well as other factors (such as additive genetic or common environmental effects). Recognition of the factors contributing to the baseline level of micronuclei is important for helping to better design studies that include the assessment of micronuclei frequency data for evaluating agents hypothesized to influence genomic instability.

Chapter 3.

The numerical contents of sex chromosome containing micronuclei and the corresponding binucleates

Introduction

DNA damage and chromosomal instability are commonly associated with disease; most notably cancer. The cytockinesis-blocked micronculeus (CBMN) assay has become one of the most frequently used tools for assessing chromosomal abnormalities that have been acquired in the somatic cells of an individual, particularly in response to environmental exposures (Fenech 2006; Battershill et al., 2008). The popularity of this assay stems, in part, from its ability to provide an accurate alternative to the time consuming process of identifying chromosomal damage through the analysis of individual metaphase spreads. Briefly, by scoring micronuclei (which are small chromatin-containing structures that were not incorporated into daughter nuclei following cell division), the CBMN assay provides information regarding the frequency of chromosomal aberrations present in somatic cells prior to the influences of selective growth pressures (Fenech and Morley, 1985). Thus, this technique is helpful in identifying genotoxic agents.

Studies from the Human Micronucleus (HUMN) consortium have shown advancing age to be consistently associated with an increased frequency of spontaneously

occurring micronuclei in adults (Bonassi et al., 2001). However, the underlying mechanisms leading to this age-related increase are not known. One approach that investigators have used to gain insight about the underlying chromosomal errors that result in the formation of micronuclei has been to characterize their chromatin content to determine if consistent patterns emerge. While one cannot directly visualize chromosomes in micronuclei, their chromatin content can be inferred using fluorescence in situ hybridization methodologies (Norppa and Falck, 2003). Through the use of pancentromeric and pantelomeric probes, investigators have inferred that the majority of spontaneously occurring micronuclei contain a single, intact chromosome (Leach and Jackson-Cook, 2001; Wojda and Witt, 2003; Lindberg et al., 2008). Furthermore, in adults the sex chromosomes (chromosomes X and Y) are most frequently excluded into micronuclei, with this frequency increasing with age (Norppa and Falck, 2003). While these studies have provided information for adults, very few of these investigations have included children, in part due to their low frequency of micronuclei. In one such study, in which the micronuclei present in the cells from 10 children (5 males and 5 females) were evaluated, Guttenbach et al. (1994) found 8% of micronuclei in females (ages 1-10 years) to contain X chromatin, with Y chromatin being present in 14% of the micronuclei in males (ages 0.5-4 years). Hando et al. (1994) and Nath et al. (1995) found no micronuclei containing sex chromosomes in the newborns they studied. Thus, little is known about the age at which the apparently preferential exclusion of sex chromosomes into micronuclei is initiated in humans.

Therefore, the primary aim of this study was to determine if there is a difference in the proportion of micronuclei that contain sex chromosomes in adults (19-81 years)

compared to children (7-15 years). This goal was achieved by scoring the number of centromeric signals present in micronuclei and their corresponding binucleates using probes specific for the pericentromeric regions of the X and Y chromosomes.

Materials and Methods

Sample Ascertainment

A total of 59 individuals (37 males and 22 females) were ascertained through the Mid-Atlantic Twin Registry, which is based at Virginia Commonwealth University. The individuals included in this study were selected from a larger, ongoing twin study, with their selection being: (1) random (n= 8); or (2) based on the individual having 5% or more of their micronuclei that contained chromatin from a sex chromosome [as ascertained from our studies using spectral karyotyping (n= 51) (see chapter 4)]. After providing informed consent (VCU IRB protocol 179), participants submitted blood samples. The peripheral blood specimens were drawn by the study participant's health care provider and shipped to our laboratory via an overnight delivery carrier.

Cell Culture

The cultures to assess micronuclei frequencies were established with lymphocytes [using Histopaque-1077 (Sigma)] according to the protocol of Fenech (1993). Briefly, cytochalasin B (3.0 μg/ml; Sigma, 14930-92-2) was added to the cells 44 hours after culture initiation. At 72 hours, binucleate interphase cells were harvested using standard techniques, which included a 10-minute incubation in hypotonic solution (0.075 M KCl), and serial fixation (three times using a 3:1 methanol: acetic acid solution). Slides were made following standard procedures (Leach and Jackson-Cook, 2001).

FISH Procedure

For males, the sex chromosomal contents of the micronuclei were determined through the simultaneous hybridization of 3 differentially colored probes according to standard procedures (Abbot Molecular). The pericentromeric probes used included ones specific for the X chromosome (DXZ1; Spectrum Aqua); the Y chromosome (DYZ3; Spectrum Orange); and chromosome 17 (D17Z1; Spectrum Green), with the latter probe being used as an autosomal control. For females, the same X (DXZ1) and autosomal (D17Z1) probes were used, but the Y chromosomal probe was not evaluated since previous GTG-banding studies confirmed that none of these females had a Y chromosome. The target (interphase chromatin) and probe DNA were denatured separately. The chromatin on the slides was denatured using a 70% formamide solution (pH 7.0) at 73°C for 3 minutes. Following denaturation, the slides were briefly rinsed in cold water and then dehydrated using an ethanol series (70%, 85%, and 100% for 2 minutes each). The probes (which were prepared using a 1:100 dilution in a C-Denhyb solution, Insitus Biotechnologies, Albuqueque, New Mexico) were denatured at 94°C for 10 minutes, and then cooled on ice for 2 minutes. Following the addition of the denatured probe to the denatured slide, the slides were placed in a humidified chamber and hybridized at 37°C overnight. Non-specific binding of probes was removed by washing in a 0.4xSSC/0.3%NP-40 solution at 73° for 2 minutes, followed by a one minute wash at room temperature in a 2xSSC/0.1%NP-40. To visualize the binucleates and micronuclei, the slides were counterstained with DAPI II (Abbott).

FISH scoring

Slides were scored on an Axioskop equipped with triple- and single-band pass filters (Zeiss). Only binucleates that contained one or more micronuclei, as visualized with DAPI staining, were scored. Following their identification, the number of fluorescent signals for each probe in the binucleates and micronuclei were scored (Figure 5). Representative images of the cells having micronuclei were captured using an image analysis system (Applied Spectral Imaging, Carlsbad, CA).

Statistics

The number of signals present in the micronuclei were evaluated for consistent patterns. Given that the frequencies of the micronuclei were not normally distributed, a square root transformation of the data was performed. Following transformation, the proportion of patterns present in the children and adults was determined using a general linear modeling (GLM) analysis [α =0.05; R (Version 2.9.1) software analysis system]. Gender and age were factors that were evaluated in the model.

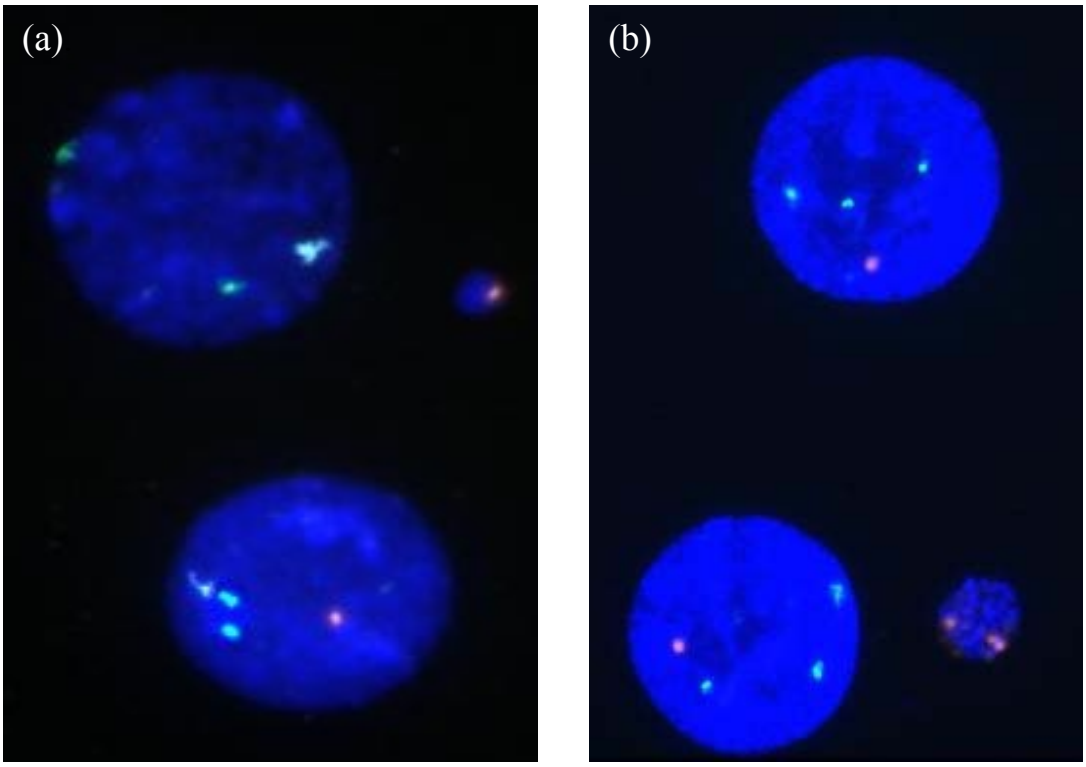

Figure 5. **Representative images of binucleates and their micronucleus following FISH.** The cells shown are from two males. (a) The micronucleus in this image contains chromatin from the Y chromosome (orange signal). The upper binucleate contains one aqua signal (X chromatin) and two green signals (17 chromatin), while the lower binucleate has one orange signal (Y chromatin), two green signals (17 chromatin), and one aqua signal (X chromatin). This binucleate was scored as having a hypodiploid cell pattern. (b) This micronucleus contains two orange signals (Y chromatin). The corresponding binucleate was scored as euploid because both cells have a normal complement for the chromosomes evaluated (1 aqua, 2 green, and 1 orange signal).

Results

Cellular distribution of sex and autosomal probe signals

Following hybridization, a total of 2,749 micronuclei were scored from the 37 male study participants, with 13.2% of the micronuclei containing Y chromatin, 5.7% containing X chromatin, and only 1.5% having chromatin from chromosome 17. For all three chromosomes scored (X, Y, and 17), the majority of micronuclei appeared to contain a single centromeric region (51.9%, 58.8%, and 92.9%, respectively). Similarly, of the 1,868 micronuclei scored from the 22 female study participants, 15.8% contained chromatin from the X chromosome, with only 2.1% having chromatin from chromosome 17. As in the males, the majority of micronuclei in the females appeared to contain a single centromeric region of the chromosome scored (54.4% for the X chromosome, and 92.1% for chromosome 17).

The probe signal patterns present in the total of 1,017 binucleated cells having at least one micronucleus with a signal for chromosomes X, Y, or 17 revealed the presence of three primary types of cells as shown in Figures 6, 7, 8, and 9. These consistently observed cell types included those having binucleates with: (1) a hyperdiploid complement [having one or more additional signals when compared to euploid expectations (X,Y,17,17 or X,X,17,17) in at least one binucleate], with the chromosomal region involved in the imbalance also being present in the micronucleus; (2) a hypodiploid complement in at least one binucleate (missing a signal for one of the chromosomal regions evaluated), with the missing signal being excluded into the micronucleus; or (3) a euploid binucleate complement, with an apparent chromosomal imbalance being “corrected” through its exclusion into the micronucleus.

Overall, the hypodiploid binucleate cell type was seen most frequently (63.2% of total), followed by the euploid binucleate pattern (28.7%) and the hyperdiploid cell type (8.1%). This pattern was found for each chromosome analyzed (Figure 6).

Binucleate FISH and age

To determine if the proportion of binucleate/micronuclei patterns varied with age or gender, a general linear model (GLM) analysis was completed. In the males, the percent of cells having the "corrected" euploid binucleate micronuclear pattern for the Y chromosome significantly decreased with age ($p=0.026$), while those having a hypodiploid binucleate pattern tended to increase with age (Figure 7). Similarly, in the females, the proportion of cells having a "corrected" euploid binucleate micronuclear pattern for the X chromosome was significantly decreased with age ($p=0.030$), while the proportion of cells having a hypodiploid binuclear complement tended to increase with advancing age (Figure 8). In contrast, for the males, no significant divergence in the proportion of binucleates having an unbalanced (hyperdiploid or hypodiploid) or balanced complement for the X chromosome was observed with age ($p=0.45$) (Figure 9). Moreover, no significant difference in the proportion of hyperdiploid binucleates was observed for the X (males or females) or Y chromosome (males) (Figures 7, 8, and 9). The number of micronuclei containing chromatin from chromosome 17 (total of 81) was too small to allow for a meaningful statistical analysis of age-related patterns for this autosome.

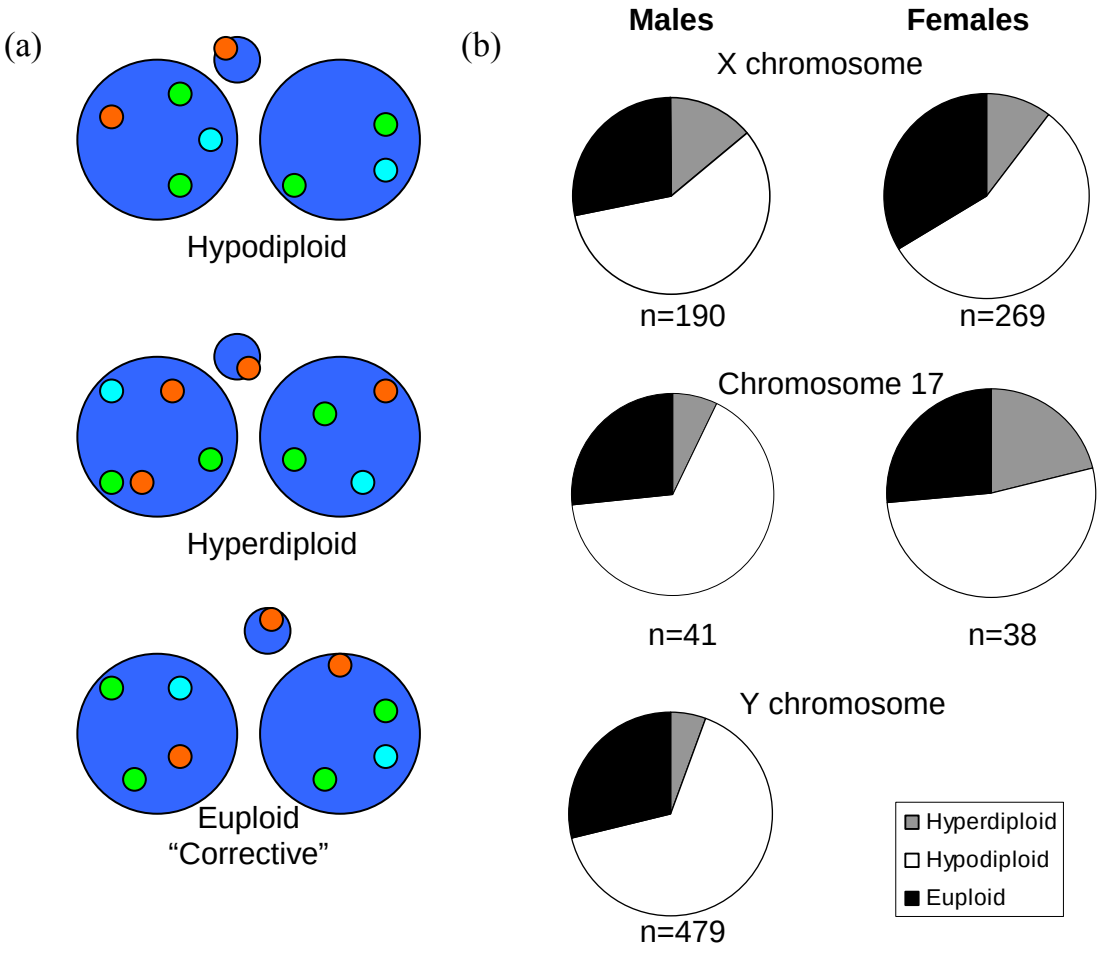

Figure 6. **Primary patterns observed in binucleates and micronuclei**. (a) The three generalized types of combinations of binucleates observed are shown as they appear for the Y chromosome (for the X chromosome see figures 8 and 9) (b) The proportion of total cells (all study subjects) having each of the three cellular patterns [hyperdiploid (gray); hypodiploid (white); and euploid (black)] in males and females are shown for each chromosome (n=number of binucleates scored for each chromosome/gender).

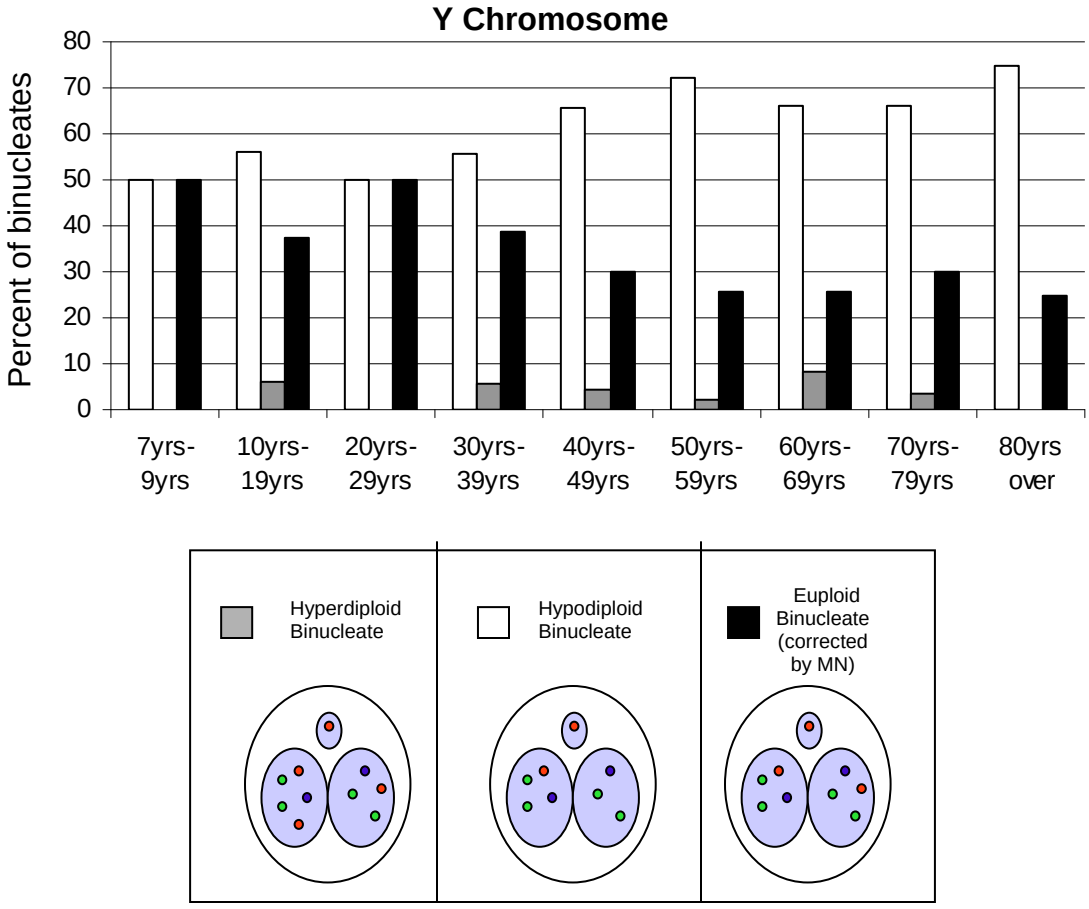

Figure 7. **Percent of each binucleate type for the Y chromosome by age**. The bar graph represents the percentage of binucleates that were hyperdiploid (gray), hypodiploid (white), and euploid (black). The age groups evaluated are shown on the X axis, with the percentage of binucleates being shown on the Y axis.

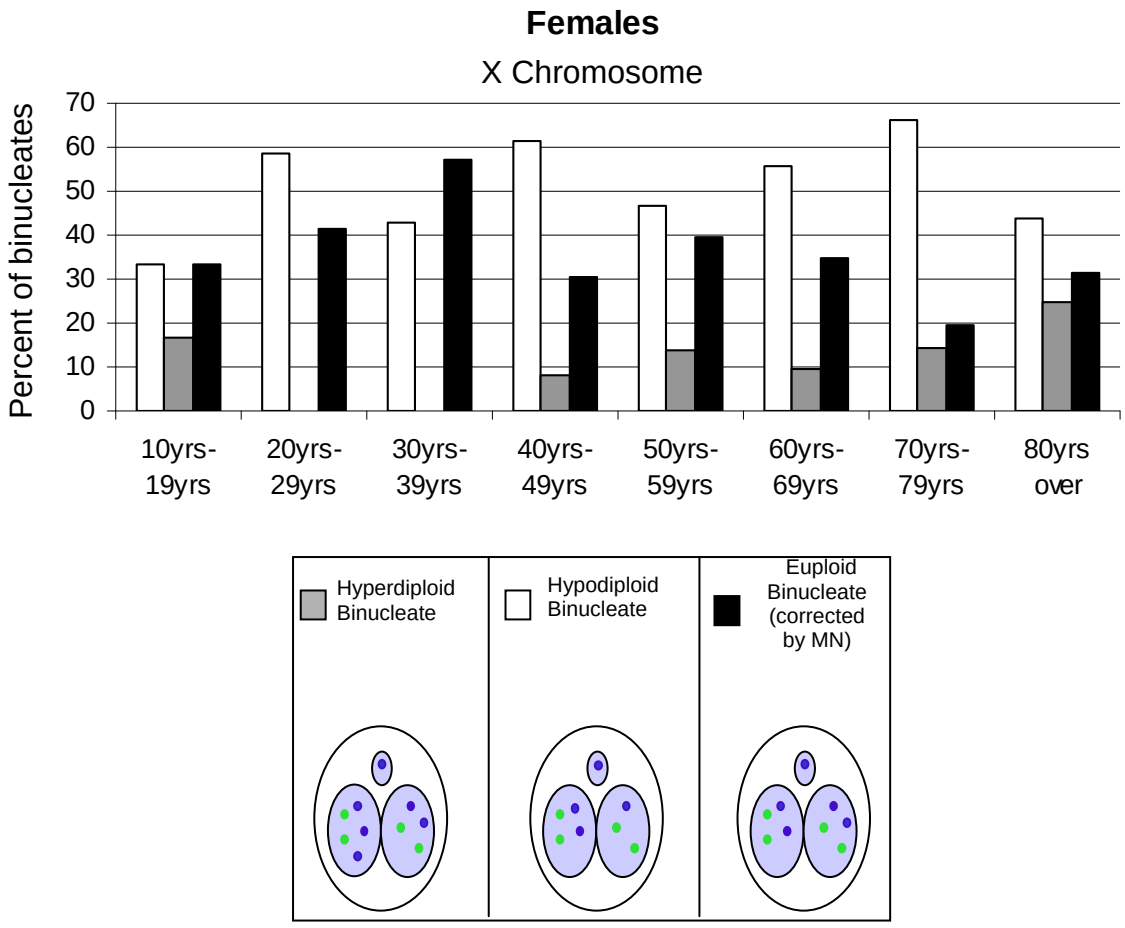

Figure 8. **Percent of each binucleate type for the X chromosome by age in females**. The bar graph represents the percentage of binucleates that were hyperdiploid (gray), hypodiploid (white), and euploid (black). The age groups evaluated are shown on the X axis, with the percentage of binucleates being shown on the Y axis.

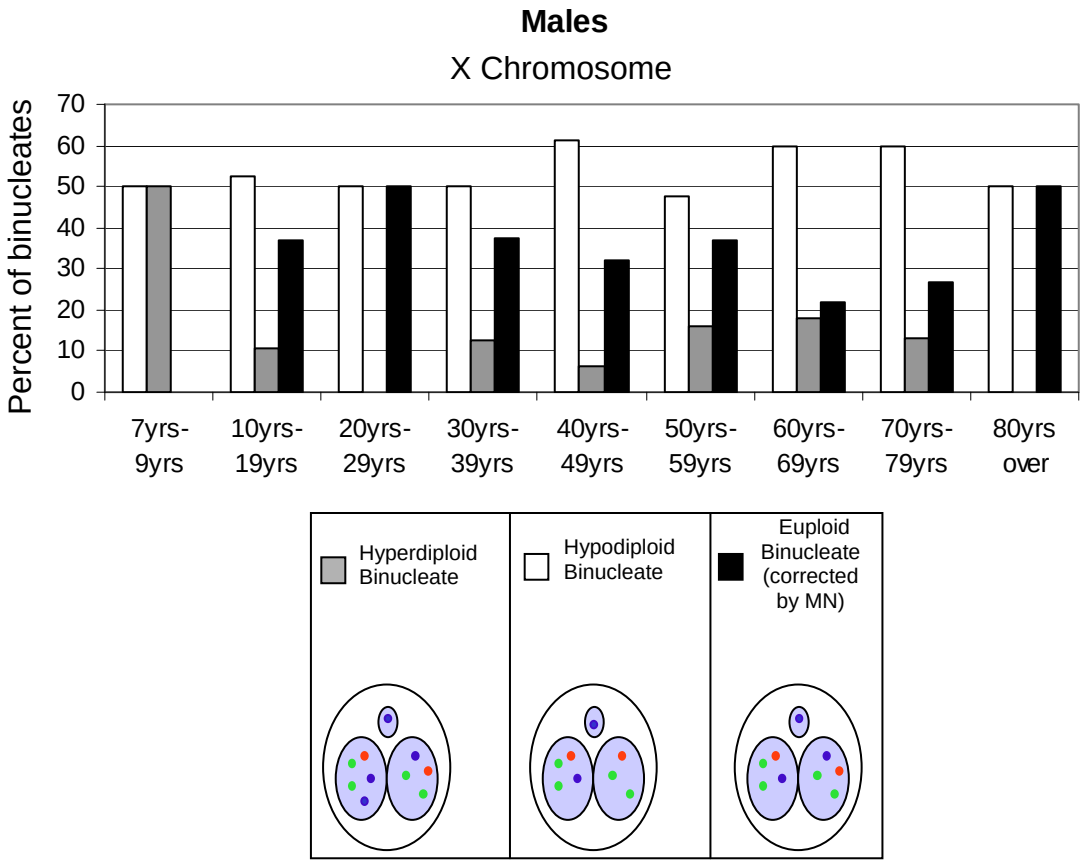

Figure 9. **Percent of each binucleate type for the X chromosome by age in males** The bar graph represents the percentage of binucleates that were hyperdiploid (gray), hypodiploid (white), and euploid (black). The age groups evaluated are shown on the X axis, with the percentage of binucleates being shown on the Y axis.

Discussion

In order to better understand the underlying basis for the observation of an age-related increase in sex-chromatin containing micronuclei, we evaluated micronuclei and their corresponding binucleates from males and females of varying ages. We expanded upon previously reported data by analyzing the number of signals that were present for the pericentromeric region of the sex chromosomes (as well as an autosome) in the binucleates, in addition to their corresponding micronuclei. The most frequently observed pattern that was detected in the study participants resulted in a hypodiploid sex chromosomal complement in a binucleate(s), as a result of the exclusion of that chromosome into a micronucleus. This finding is in agreement with the results of Hando, et al. (1994), who included a description of the binucleate signal patterns in their study of micronuclei containing X chromatin. In the adult females they studied, who ranged in age from 20 to 79 years, the only patterns reported were those resulting in a hypodiploid binucleates. While these investigators also obtained blood samples from 8 newborns, those samples were excluded from their X chromatin FISH studies due to the low frequency of micronuclei present. In contrast to their results, we also observed patterns resulting in hyperdiploid complements, as well as euploid complements in the binucleates.

Of particular interest was this latter category, in which the exclusion of a sex chromosome into a micronucleus resulted in the establishment of a balanced complement for the binucleate (possible trisomy rescue). The proportion of these "corrective" cells was significantly increased in the younger compared to older females and males for the X

and Y chromosomes, respectively. This age-related divergence in cell type pattern also included a trend toward an increase in the proportion of binucleates having loss for a sex chromosome (Y in males; X in females) in the older study subjects.

One can theorize that the "corrective" cells observed in this study might have arisen from a mitotic error, possibly caused by DNA damage and/or aneugenic environmental exposure, and that the formation of the micronucleus resulted from the ability of the cell cycle checkpoints (or alternative mechanisms) to correct this error. These processes may be less effective for detecting abnormalities resulting in chromosomal loss versus gain and/or may decrease in efficiency with age. Thus, the age-related changes that arise from loss mechanisms lead to an overall age-related increase in the frequency of micronuclei. Given the paucity of micronuclei containing chromosome 17 in this study, we were unable to determine if this phenomenon is true for autosomes, as well as sex chromosomes.

In summary, the pattern of sex chromosomal imbalance in binucleates containing a micronucleus varies with age. In addition to reflecting the presence of cell loss, which appears to be an age-related change, the presence of micronuclei may reflect a "trisomy rescue" event. While this information supports the conclusion that micronuclei frequencies serve as an indication of DNA damage and/or imbalance, in some cases, particularly when seen in children, their presence may also represent the cell's ability to correct for acquired damage. Therefore, when analyzing micronuclei frequencies, it may be informative to examine the contents of the binucleates, in addition to the micronuclei, to facilitate the recognition of varying cellular responses to environmental or genotoxic exposures.

Chapter 4.

Chromosomal contents of micronuclei determined using SKY

Introduction

Micronuclei frequencies are known to increase with age and have been associated with increased DNA damage. The cytokinesis-blocked micronucleus (CBMN) assay has become a useful tool for assessing micronuclei frequencies. While there is increasing information on the frequencies of micronuclei, there is minimal data regarding the chromosomal contents of the micronuclei. While one cannot directly visualize chromosomes in micronuclei, their chromatin content can be inferred using fluorescence in situ hybridization methodologies (Norppa and Falck, 2003). Through the use of pancentromeric and pantelomeric probes, investigators have determined that the majority of spontaneously occurring micronuclei contain a single, intact chromosome (Leach and Jackson-Cook, 2001; Wojda and Witt, 2003; Lindberg et al., 2008). In an attempt to determine the chromosomal contents of micronuclei, several investigators have used probes that are specific for the sex chromosomes (either centromeric probes or painting probes), with small subset of autosomes being evaluated, including chromosomes 1, 2, 9, 11, 16, 21, and 22 (reviewed by Norppa and Falck, 2003). Of the chromosomes evaluated, the sex chromosomes have been the most frequently observed chromosome present in the micronuclei, with their frequencies increasing with age (reviewed by Norppa and Falck, 2003). However, the vast majority of the existing data has been collected from assessments of micronuclei present in adults, and the few studies that have investigated the chromosomal contents of

micronuclei in children have limited sample sizes, with the number of children studied ranging from 8 to 18 (Guttenbach et al., 1994; Hando et al., 1994; Nath et al., 1995).

Technological problems that have been encountered by investigators striving to determine the chromosomal contents of micronuclei have included: (1) incomplete characterization of the chromatin present in the micronuclei since enumeration probes generally hybridize only to chromatin including the centromere region (thus providing no information for micronuclei containing acentric fragments); and (2) the number of chromosomes that can be evaluated using a whole chromosome painting approach (or a centromeric probe approach) is limited due to a small number of differentially labeled probes. Thus, the data pertaining to autosomal inclusion in micronuclei, which has been gained through these techniques, is inconclusive. Two groups (Tucker et al., 1996; Fauth et al., 1998 and 2000) reported that chromosome 9 was frequently excluded into micronuclei. However, Tucker, et al. (1996) based this conclusion on only the observation of two chromosome 9 positive micronuclei (from a total of 96 evaluated), and Fauth, et al. (1998, 2000) completed their study using only three individuals. Yet another investigative team reported that chromosome 2 was present in micronuclei with an increased frequency, but this conclusion was based on studies completed from a single individual (Peace et al., 1999).

To circumvent the methodological problems of determining the chromosomal contents of micronuclei arising from conventional FISH methodologies, Leach and Jackson-Cook (2001) developed a novel assay exploiting spectral karyotyping (SKY) technology for the detection of chromosome contents in micronuclei. This approach offered a huge advantage over the two previously used techniques since SKY allowed

for the simultaneous detection of all 24 chromosomes. However, like other investigations, this initial study was based on the assessment of only three females. Therefore, the primary aim of our study was to expand upon previous work and determine the baseline frequency with which sex chromosomes, as well as autosomes, are excluded into micronuclei by analyzing the chromosomal contents of micronuclei in males and females through the use of spectral karyotyping, while simultaneously determining if the pattern of chromosome involvement varied with age.

Materials and Methods

Sample Ascertainment

A total of 79 individuals (42 males and 37 females) were ascertained through the Mid-Atlantic Twin Registry, which is based at Virginia Commonwealth University. The frequency of micronuclei was determined for each of the 115 study participants according to the protocol of Fenech (2003). Spectral karyotyping was performed on those individuals having 0.4% or higher frequency of micronuclei. After providing informed consent (VCU IRB protocol 179), participants submitted blood samples. The peripheral blood specimens were drawn by the study participant's health care provider and shipped to our laboratory via an overnight delivery carrier.

Cell Culture

Lymphocytes were isolated using Histopaque-1077 (Sigma) and lymphocyte cultures were established according to standard procedures (Moorhead et al. 1960). Cytochalasin B (Sigma, 14930-92-2) was added to the cultures 44 hours after initiation at a concentration of 3.0 μg/ml. Cultures were harvested at 72 hours. Cells were treated with hypotonic solution (10 minutes with 0.075 M KCl), and fixed three times (3:1 methanol: acetic acid). Slides were made following standard procedures (Leach and Jackson-Cook, 2001), and aged at room temperature for four days before beginning the SKY procedure.

Spectral Karyotyping

SKY was performed according to the manufacturer's (Applied Spectral Imaging) protocol. Briefly, the chromatin on the slides was denatured using 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 using an ethanol series (70%, 85%, and 100% for 2 minutes each). The SKY probe (Applied Spectral Imaging) was denatured at 75°C for 10 minutes, followed by suppression hybridization at 37°C for 60 minutes. Following the addition of the denatured probe to the denatured chromatin, the slides were placed in a humidified chamber and hybridized at 37°C for approximately 44 hours. Non-specific binding of the probe was removed by washing in 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. To visualize the binucleates and micronuclei, the slides were counterstained with DAPI/Antifade (Applied Spectral Imaging) and viewed using a Zeiss Axioskop equipped with a DAPI filter and a custom triple-band pass filter (Chroma). For analysis, the micronuclei were captured using a SpectraCube system (Applied Spectral Imaging) and analyzed using the SKY software. At least one metaphase spread was analyzed per slide to ensure proper hybridization of the SKY probe cocktail. Once proper hybridization was confirmed, each micronucleus, along with the surrounding nuclei, was captured and analyzed. An example of the captured images is found in Figure 10.

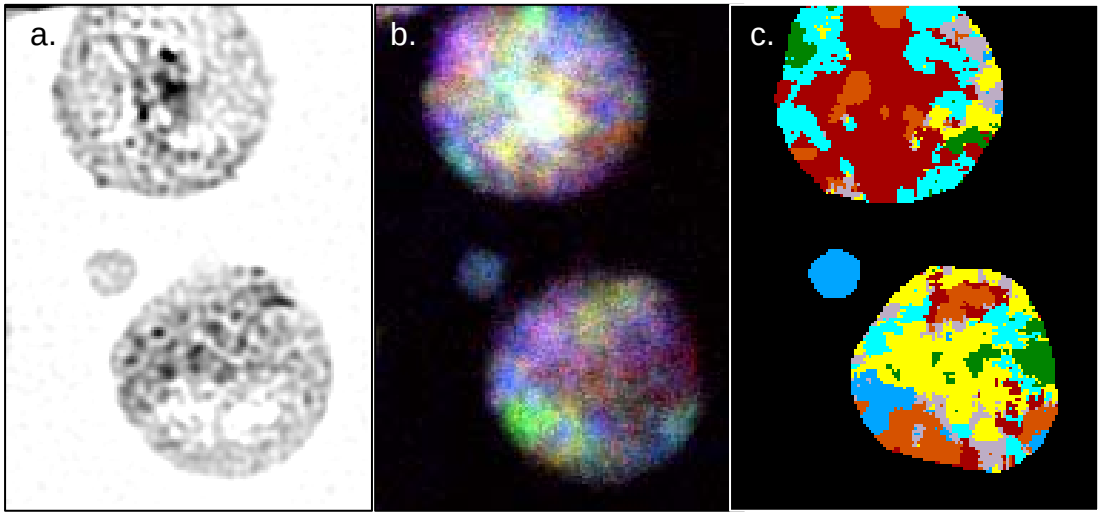

Figure 10. Representative binucleate and micronucleus following SKY. These structures are shown as they appear with reverse DAPI staining (a); the spectral image (b.) and the classified image (c.) The chromatin present in this micronucleus was determined to have originated from chromosome 11.

SKY assignment confirmation using FISH (WCP and CEP)

After analysis, slides were randomly chosen to assess the accuracy of the SKY classification. These slides were washed in 70% formamide/2xSSC solution (pH 7.0) at 70°C for 2 minutes to remove the SKY probe. Slides were then stained with DAPI and viewed to ensure complete removal of the probe. After a brief wash in 2xSSC, the slides were hybridized with either a whole chromosome painting probe (WCP) or centromere enumeration probe (CEP). The probes and slides were then co-denatured (75°C for 2 min for WCP or 73°C for 2 minutes for CEP). Following co-denaturation, the slides were placed in a humidified chamber and hybridized at 37°C overnight. Non-specific binding of the probe was removed by washing in 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. Slides were counterstained with DAPI II (Abbott Molecular) and viewed using a Zeiss Axioskop equipped with triple-band pass and single band pass filter sets (Abbott Molecular).

Statistical Analysis

Comparisons of the frequencies of chromosomal exclusion into micronuclei were performed using a contingency Chi-square test. In order to limit type I errors, an alpha level of $p<0.05$ was used for all statistical tests.

Results

Chromosomal contents in Micronuclei

Of the 3,611 micronuclei scored, the majority (89.6%) contained chromatin from a single chromosome. Micronuclei were most frequently found to contain chromatin from the sex chromosomes, with 27.3% of micronuclei in females containing X chromatin and 27.6% of micronuclei in males containing Y chromatin ($p<0.0001$). A non-random pattern of autosomal exclusion into micronuclei was also observed ($p<0.0001$), with chromosomes 4, 8, and 9 being present most frequently, and chromosomes 17 and 22 being present least frequently. No significant difference in these patterns of autosomes were observed between the males and females studied ($p=0.7$) (Figure 11).

Micronuclei contents with age

A significantly lower frequency in the proportion of micronuclei containing sex chromosomes was observed in the children (7-19 years) compared to adults (25-81 years), for both males ($p<0.0001$) and females ($p<0.0001$). However, no significant difference in autosomal patterns was observed with age ($p=0.9$) (Figures 12 and 13).

SKY accuracy

To determine the accuracy of the SKY methodology for evaluating the chromosomal contents of micronuclei, a total of 389 micronuclei were re-assessed using

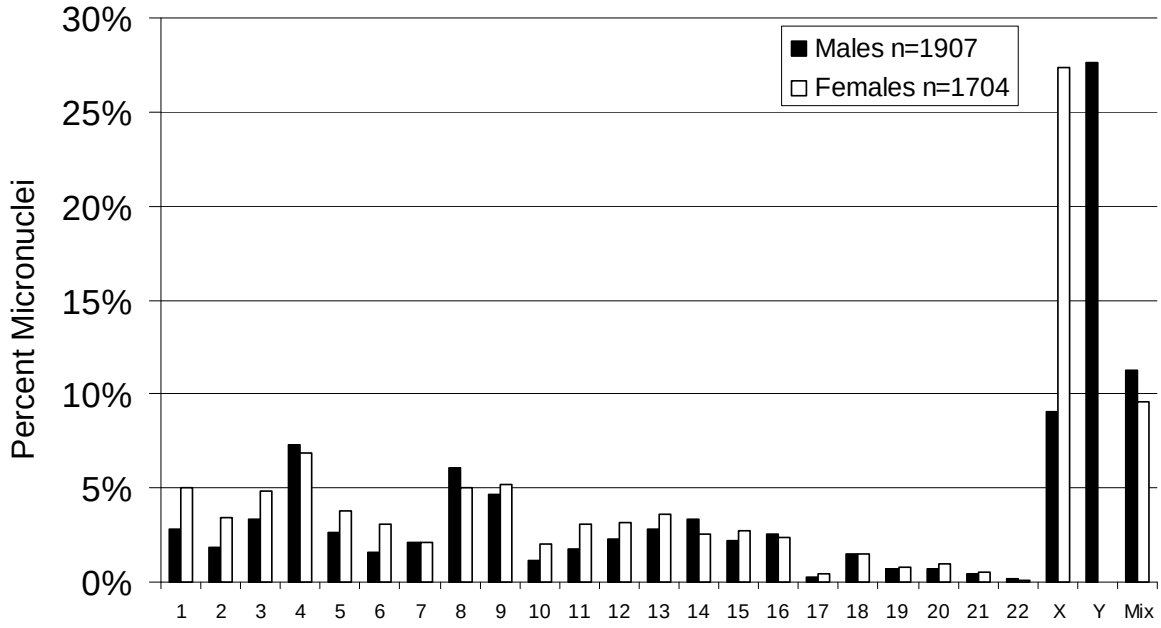

Figure 11. Distribution of chromosomes excluded into micronuclei in males (black) and females (white). The percentage of micronuclei (Y axis) that contained chromatin from each of the chromosomes (X axis) is shown.

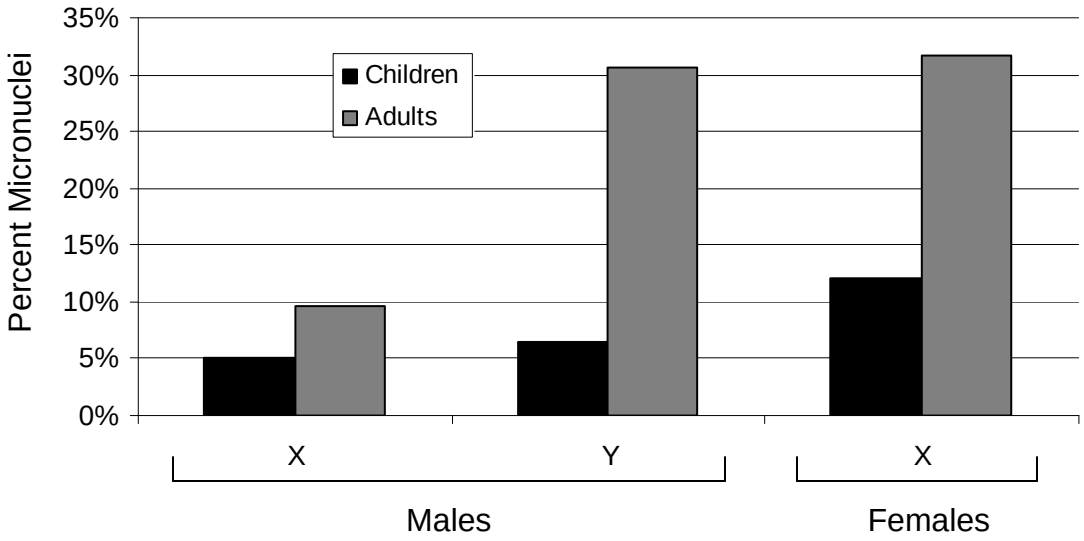

Figure 12. Distribution of sex chromosomes in micronuclei with age. The percentage (Y axis) of X and Y chromosomes present in micronuclei seen in children (black) and adults (gray) are shown. The distribution observed in males is shown for the X (left histograms) and Y (center histogram) chromosomes. The distribution observed for the X chromosome in females is also shown (right histogram).

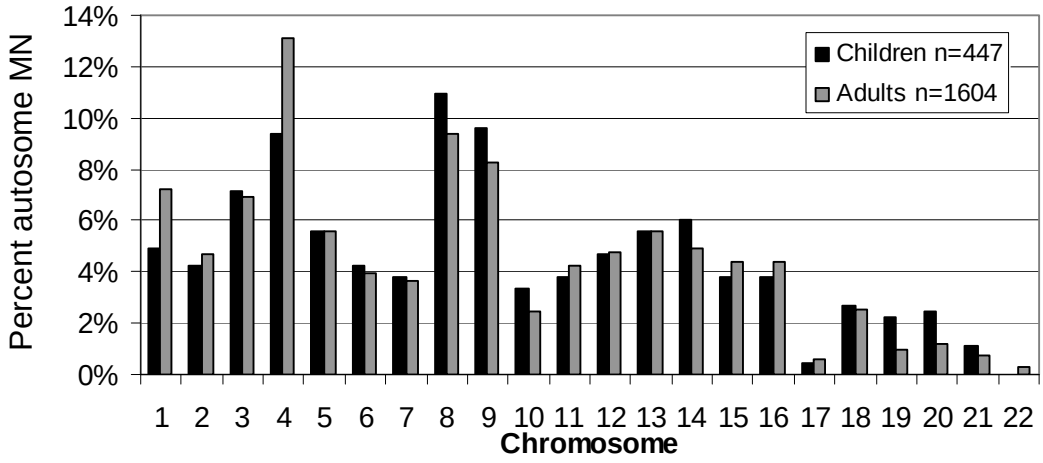

Figure 13. Distribution of autosomes excluded into micronuclei in children (black) and adults (white). The percentage of micronuclei (Y axis) that contained chromatin from each of the chromosomes (X axis) is shown.

single, chromosome-specific probes. Agreement in the chromosomal classification was present for 83% of the micronuclei scored using centromeric probes, with agreements being observed for 89% of the micronuclei evaluated using whole chromosome painting probes. An example of the image captured during these confirmation studies is found in Figure 14. All cases having a discrepancy failed to show a hybridization signal for the chromosome evaluated in the micronucleus but did demonstrate sufficient hybridization of the probes in the binucleate.

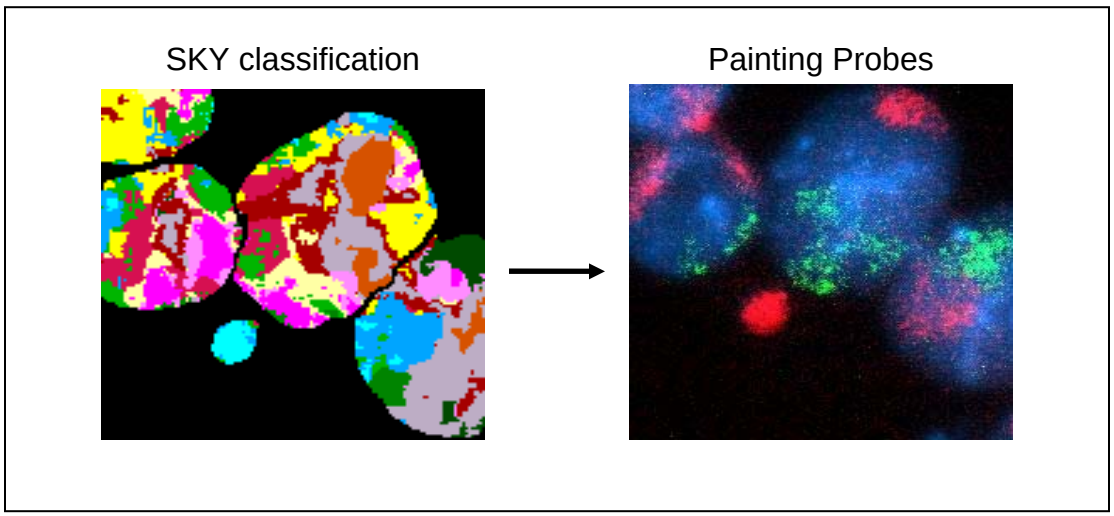

Figure 14. Representative images showing confirmation of SKY micronuclei assessment using a painting probe. The image on the left depicts a micronucleus classified by the SKY software as containing chromatin from chromosome 4. The image on the right depicts the same micronucleus after the SKY probe was stripped and the slide was re-hybridized with the chromosome painting probes 4 (orange) and 12 (green). The micronucleus hybridized orange, confirming the SKY classification.

Discussion

SKY analysis was performed to determine if there was a non-random pattern of chromosomes excluded into spontaneously occurring micronuclei in healthy individuals. This technique allows for the examination of all 24 chromosomes in a single hybridization. The majority of micronuclei were found to contain chromatin from a single chromosome. In both males and females, the sex chromosomes were most frequently excluded into micronuclei. Chromosomes 4, 8, and 9 were also found with a significantly higher frequency than expected by chance, while chromosomes 17 and 22 were found much less frequently than expected. This preferential exclusion of chromosome 9, which has been reported previously (reviewed by Norppa and Falck, 2003), is thought to be related to the large block of heterochromatin on this chromosome; however chromosomes 1 and 16 also contain large blocks of heterochromatin, and these chromosomes were not found to have an increased frequency. We found no difference in the pattern of autosomes present in the micronuclei studied from males compared to females. This observation is consistent with the results of studies reported by Wojda et al. (2007), who also did not find a gender difference in frequency of micronuclei containing the subset of autosomes they assessed using FISH techniques.

To determine if age influenced the distribution of specific chromosomes that were excluded into micronuclei, comparisons were made between adults (ages 25-81 years) and children (ages 7-19 years). Indeed, a significantly higher proportion of micronuclei containin sex chromosomes was seen in adults compared to children. This age-related

difference in sex chromosomal exclusion into micronuclei was observed in both the males and females. However, no significant differences in the proportion of micronuclei containing autosomes were detected with age or gender. Wojda et al. (2007) found a similar effect in adults, noting that the frequency of sex chromosome micronuclei increased with age, but the frequencies of the five autosomes that they tested (chromosomes 1,4, 6, 8, and 20) did not vary with age.

Given that the SKY methodology was developed for use on metaphase chromosomes, with software classification standards being established for a metaphase format, we performed confirmation studies, using chromosome-specific probes, for a subset of micronuclei. These studies allowed for an evaluation of the accuracy of this SKY methodology when used in this interphase cell format. These FISH confirmation studies, which primarily assessed sex chromosomal assignments, showed agreement between the FISH and SKY assays for 83% of micronuclei using centromeric enumeration probes (CEP), and 89% of the micronuclei using whole chromosome painting probes (WCP). The variation in this number most likely reflects the difference in the two methods used. The accuracy determined by using the CEP probes was most likely lower because this probe only allows for confirmation of micronuclei containing the centromeric region and would not allow for confirmation of micronuclei having fragments of that chromosome that did not contain the centromere. Therefore, these studies could produce false negative results (with the SKY software correctly identifying the chromatin, which originated from a portion of the chromosome without the centromeric region [thus the CEP probe would not bind]). The agreement in assignment for 89% of the micronuclei, as confirmed through the use of WCP probes, is expected to

yield a better estimate the true accuracy of the SKY method, since the whole chromosome probes will bind to any region of the chromatin. Collectively, these findings suggest that a fraction of the micronuclei may contain chromosomal fragments.

In conclusion, there is a non-random pattern of chromosome exclusion into micronuclei, with the sex chromosomes being most frequently involved. The frequency of autosomes excluded into micronuclei does not appear to vary with age or gender. This study further demonstrates the usefulness of the SKY method for the detection of micronuclei chromosomal contents. The frequencies of autosome involvement in micronuclei detected in this study can serve as normal, baseline data for other researchers.

Chapter 5.

Variation in global methylation profiles between identical twin pairs

Introduction

While acquired chromosomal changes in DNA (aneuploidy) were first noted nearly 50 years ago, it was only recently recognized that chromatin has the potential to undergo epigenetic changes with advancing age (Sedivy et al., 2008). Epigenetic modifications, such as DNA methylation, play an important role in the control of gene expression and chromosome structure (Frigola et al. 2002; Peaston and Whitelaw, 2006). Simplistically, methylation typically prevents gene expression, while loss of methylation allows transcription. However, the degree to which one's methylation profile changes with age is unresolved, with some researchers reporting that methylation increases with age while others report an overall decrease in methylation with age (Golbus et al., 1990; Cooney, 1993; Vertino et al., 1994; Toyota et al., 1999; Geigl et al., 2004; Clayton et al., 2005; Wojdacz and Hansen, 2006; Bollati et al., 2009). Furthermore, the cause(s) of these changes in methylation patterns with age, when present, are unknown. Some believe it is a function of diet and environment, while others believe it to be a random process (Cooney, 1993; Fenech 2002; Jaenisch et al., 2003; Bjornsson et al., 2004; Peaston and Whitelaw, 2006). It has been hypothesized that as humans age, they accumulate epigenetic and/or chromosomal changes over time (Wong et al, 2005; Petronis, 2006). Once multiple changes are acquired, these alterations may result in the development of an

age-related complex disease. In fact, many cancer cells have been shown to develop epigenetic changes, with some cancer types showing alterations that lead to hypomethylation of genes, while others have shown hypermethylation changes (reviewed by Iacobuzio-Donahue, 2009). The possible randomness and unpredictable nature of these modifications in DNA methylation has been proposed to explain the observation of monozygotic (MZ) twins who are discordant for certain diseases (schizophrenia at 50% discordance for MZ twins, 70% for rheumatoid arthritis, and 80% for breast cancer), despite their having no evident differences in environmental exposure (King et al., 1992; Petronis, 2001; Singh et al., 2002). The observation that there was no difference in the frequency of phenotypic discordance between MZ twins raised apart when compared to MZ twins raised together, led Wong et al. (2005) to suggest that the observed phenotypic divergence reflected a mechanism other than environment, implicating epigenetic alterations as a possible etiological factor. This hypothesis has gained supported from several investigators, including Petronis (2001), who suggested that a large number of epigenetic changes could accumulate during the numerous mitotic divisions that are completed in somatic cells as an individual ages and that these changes could lead to diversity between two genetically identical individuals. Indeed, in their paradigm shifting study, Fraga et al. (2005) found that patterns of epigenetic modifications in MZ twin pairs do diverge as they become older. Martin (2005) described this phenomenon as epigenetic drift. These alterations in an individual's epigenetic profile appear to be much more prevalent than DNA mutations (Bennett-Baker, 2003).

Since epigenetic mechanisms play a key role in establishing and maintaining chromosomal structure, specifically heterochromatin and euchromatin, it is possible that

epigenetic changes could be an underlying mechanism contributing to the acquisition of structural and numerical chromosomal abnormalities associated with aging. For example, methylation abnormalities have been linked to whole chromosome gains and losses in colon cancer cells, linking aneuploidy to changes in methylation (Lengauer et al., 1997; Reviewed by Richardson 2002). And more recently, hypomethylation of transposable elements (LINE and Alu sequences) has been associated with increased genome instability in non-small cell lung cancer (Daskalos et al., 2009).

Evidence that perturbations in methylation can result in increased frequencies of chromosomal abnormalities in humans has also been obtained through studies of people having immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome, which is a rare autosomal recessive condition resulting from a mutation in the methyltransferase gene (Kondo, et al. 2000). Individuals having ICF syndrome have an increased frequency of structural chromosomal abnormalities, with aneuploidy also being observed, albeit less frequently. Interestingly, the chromosomes of these patients show accelerated telomere shortening and telomeric dysfunction. However, the most striking observation regarding the preponderance of chromosomal abnormalities present in these individuals is that they have a non-random chromosomal distribution, with anomalies being seen most frequently for chromosomes 1 and 16, followed by chromosome 9, which are all chromosomes having large heterochromatic regions (Gisselsson et al., 2005; Ehrlich, et al., 2008).

To test the hypothesis that methylation alterations influence chromosomal aneuploidy in humans, Guttenbach and Schmid (1994) and Fauth et al. (1998) treated cells with the drug 5-azacytidine, which induces hypomethylation. Both groups observed

an increased frequency of micronuclei containing the chromosomes 1, 9, and 16, which led them to conclude that undermethylation of heterochromatin may be associated with the loss of specific chromosomes. In our own studies of spontaneously acquired chromosomal abnormalities in humans, we observed an increased frequency of losses for chromosomes 1, 9, and 16 in lymphocytes and buccal mucosa cells (Rehder, et al., 2009). One can speculate that the methylation status of these heterochromatic chromosomes may play a role in their increased propensity for age-related losses. While there does seem to be a consistent trend toward methylation differences playing a contributory role to the acquisition of chromosomal abnormalities, it is unclear if these alleged differences arise in response to environmental exposures.

One of the most straightforward means for detecting the presence of changes in methylation in humans is through the study of identical twins, who have identical genomic DNA. Assuming that monozygotic twins are born with the same methylation profile, any variations in the methylation profile observed in adult identical co-twins are thought to reflect acquired changes.

Thus, the primary aim of this small pilot study was to determine if acquired methylation differences could be detected between monozygotic twins having low compared to high micronuclei frequencies, as well as divergent environmental exposure histories. We hypothesize that the co-twins having increased variation in their micronuclei frequencies will also show more divergence in their methylation profiles.

Materials and Methods

Sample

Two female monozygotic twin pairs were chosen from a larger ongoing study on the basis of their frequency of micronuclei, as well as their environmental/lifestyle histories. One pair (age 85) was very similar in environmental exposures, health history, and micronuclei frequencies, while the other pair (69 years old) was very different for these variables (Table 7). After providing their informed consent (VCU IRB protocol #179), the participants submitted blood samples and a completed health history questionnaire. Blood samples were collected by the participants' health care providers. Following their collection, the samples were shipped to our laboratory via an overnight delivery carrier.

DNA Isolation and Zygosity Determination

Genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Qiagen). The zygosity status of the twins was confirmed using 13 highly polymorphic short tandem repeat sequences (AmpFlSTR Profiler Plus and Cofiler kits from Applied Biosystems, Foster City, CA) according to standard procedures.

MeDIP

A methylated DNA immunoprecipitation assay (MeDIP) was performed according to the protocol of Mohn, et al. (2009) as shown in Figure 15. Briefly, genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Qiagen). A 20µg aliquot of DNA was sonicated at 30% power for 10 seconds for a total of 4 cycles

Table 7. Participant information for the methylation study.

Individual	Age	Pair	Micronuclei observed/1000 binucleates	High blood pressure	Heart disease	Alcohol usage	Tobacco usage
1	85	1	13	Yes	Yes	No	No
2	85	1	12	Yes	Yes	No	No
3	69	2	14	Yes	No	No	No
4	69	2	35	Yes	No	No	Yes

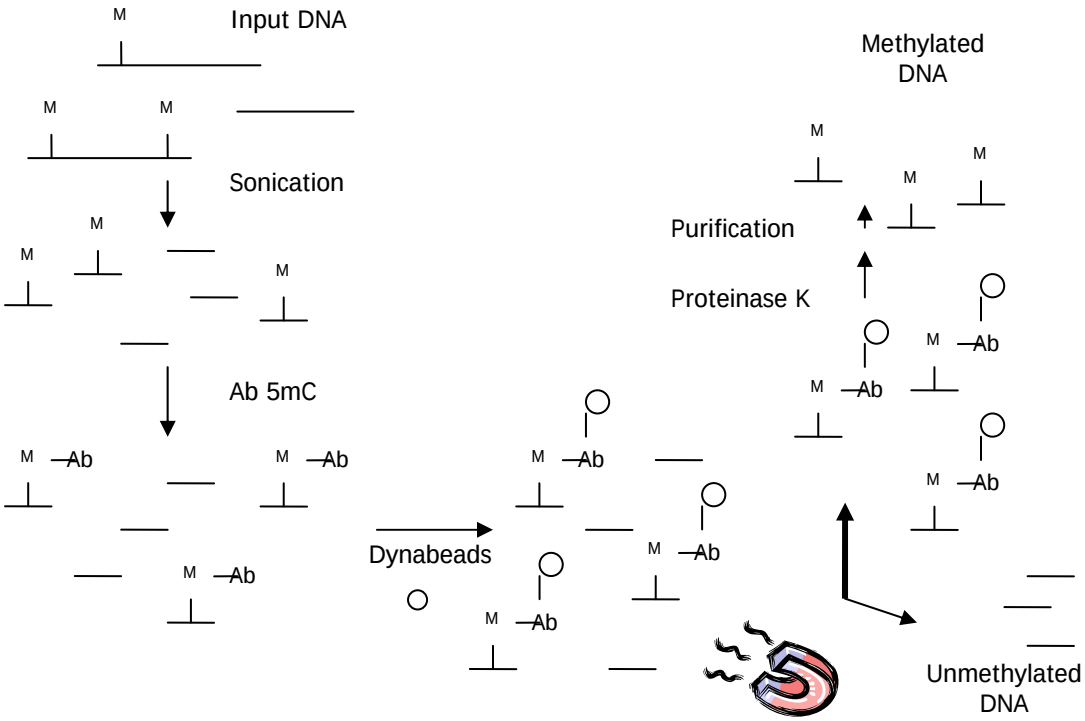

Figure 15. **Illustration of Methylated DNA Immunoprecipitation (MeDIP) procedure.** Whole genomic DNA (black lines) isolated from whole blood was sonicated. A 5-methylcytidine antibody was then added to the sonicated DNA, with the antibody (Ab) being bound to the methylated DNA (M). A secondary antibody (○), which was attached to magnetic beads (Dynabeads), was then bound to the primary antibody. Using a magnetic rack, the methylated DNA was separated from the unmethylated DNA. After a brief proteinase K digestion, the methylated DNA was purified.

(with 2 minutes "resting" intervals on ice between sonications), resulting in DNA fragments that were between 300-1000 base pairs in length. After DNA purification, a 5-methylcytidine mouse monoclonal antibody (Eurogentec) was added to the fragmented DNA and incubated overnight at 4°C with continuous shaking. A secondary antibody, Dynabeads M-280 sheep anti-mouse IgG (Invitrogen), was then added to the solution, which was incubated for 2 additional hours at 4°C. The magnetic beads were then collected using a magnetic rack (Invitrogen). After removal of the supernatant, the DNA was washed three times in a 1x IP buffer solution (Mohn et al., 2009), and then digested using proteinase K. The methylated DNA was then isolated by phenol:chloroform extraction.

Array CGH

To optimize this assay for the recognition of differences between identical co-twins, two experimental approaches were performed, as shown in Figure 16. In the first method, each individual's methylated DNA was differentially labeled and cohybridized to that same individual's sonicated genomic, or "input" DNA. In the second experimental approach, the methylated DNA fragments from the identical co-twins were differentially labeled (one twin labeled with Cy3; one with Cy5) and then competitively co-hybridized to the array chip. For both approaches, the array comparative genomic hybridization (CGH) technique was performed according to the manufacturer guidelines using a BAC array platform (over 5000 replicated BAC clones including 143 OMIM genes at 100 Kb resolution, subtelomeres at 250 Kb, and screening of genomic backbone at 565 Kb resolution.) (BlueGnome Ltd, Cambridge, UK).

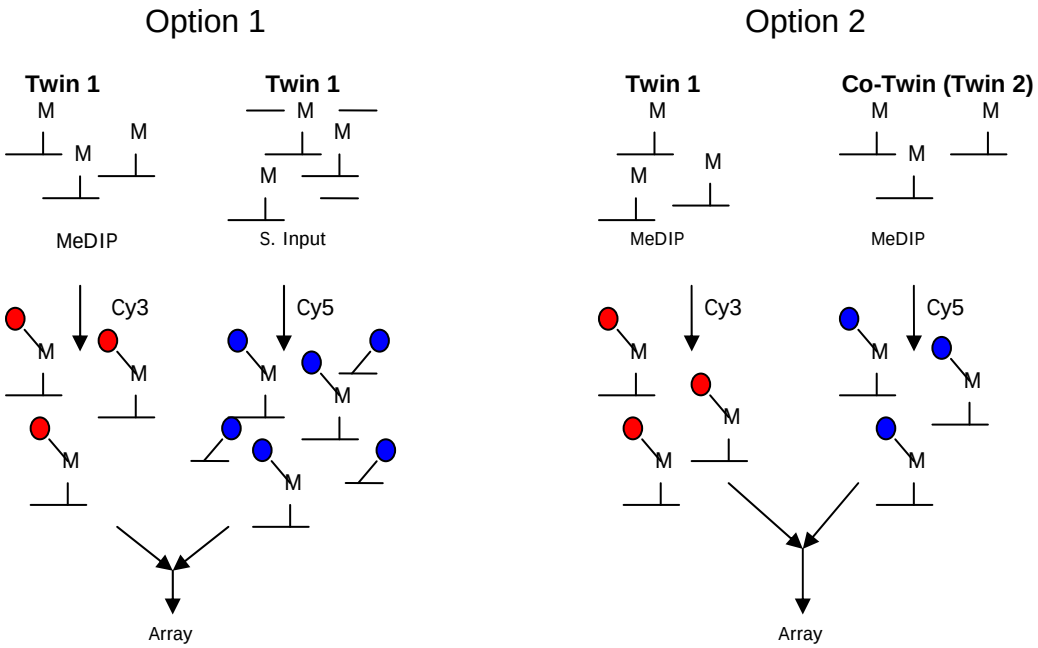

Figure 16. Illustration of the two different methods used for array CGH. In experimental approach 1, the methylated DNA was hybridized to that same individual's sonicated genomic DNA (S. Input). In experimental approach 2, the methylated DNA from twin 1 was hybridized to the methylated DNA of their identical co-twin (twin 2).

Array analysis

The arrays were scanned using a ScanArray Gx Plus scanner (Perkin Elmer). The images were analyzed using a proprietary software program that was developed by BlueGnome. Differences in methylated DNA amounts present were identified if the log2 ratio values exceeded 0.4 or were less than -0.4.

Results

Intra-individual MeDIP DNA hybridized to sonicated genomic DNA

The differential ratios of the methyl-enriched DNA compared to the sonicated whole genomic DNA within the 4 individuals studied resulted in a range of 26 to 103 areas of divergence. Overall, hypermethylated regions (ranging from 17 to 89 per individual) were detected more frequently than hypomethylated regions (ranging from 8 to 14). A comparison of the specific genomic information gained from these assays showed 12 loci to be consistently altered in all of the 4 individuals, with an additional 11 regions showing either consistent hypo- or hypermethylation in 3 of the 4 individuals (Table 8). While 17 of these common regions were consistently hypermethylated and 4 were consistently hypomethylated, 2 regions were differentially methylated (hypermethylated in 2 individuals and hypomethylated in 2 individuals). Overall, the methylation pattern in each individual was more similar to their co-twin's pattern than that of an unrelated individual (Table 9).

When comparing the co-twins' patterns, a total of 24 differences were observed between the co-twins of pair one, while a total of 22 differences were detected between the co-twins of pair two (Table 9). However, the standard deviations of these experiments, which were calculated by comparing the fluorochrome intensity values present on the replicate [2 for non-coding regions and 3 for genes] BACs on the arrays, were larger than those typically used for diagnostic interpretation, thereby underscoring the need for replication of these results. A list of the differentially methylated BAC clones and the genes involved are presented in Tables 10 and 11.

Co-twin comparison using MeDIP DNA (twin 1) hybridized with MeDIP DNA (twin 2)

The direct competitive hybridization of the methyl-enriched co-twins' DNA onto the microarray resulted in the detection of no (zero) differences in patterns between the co-twins of pair one. However, methylation differences were observed between the co-twins of pair two in 34 chromosome regions (Figure 17 and 18). Unlike the previous method, the standard deviations for this experiment (0.05 and 0.1) were within the values deemed acceptable for diagnostic use (0.1 or less). The genomic regions with differential methylation for the identical co-twins from pair 2 (40 BACs) included 142 protein coding genes, 10 processed transcripts, 3 RNA genes, and 14 pseudogenes (Table 12).

In this second experimental approach, one cannot clearly categorize the methylation change that occurred as hypo- or hypermethylation since a gain in twin 1 could reflect a hypermethylation from the "normal" state in twin 2, or it could represent a normal level, with twin 2 having hypomethylation.

Table 8. Regions having consistent methylation changes (approach 1).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-58A11	1p36.2	1MB backbone	PLEKHG5 NOL9 TAS1R1 ZBTB48 KLHL21		AL591866.1	AL591866.2
RP5-1033K19	1p31.2	1MB backbone	RPE65			AL139413.1 AL139413.2
RP11-290P14	1q44	1MB backbone	EFCAB2 KIF26B		U1	AL589763.1
RP11-480A4	3p14.2	1MB backbone	RP11-541C17.1			AC104164.2 AC104164.1 RP11-541C17.2
RP11-16M12	3p12.3	1MB backbone*		AC108752.1		
RP11-374I17	3q26.31	1MB backbone	NAALADL2		snoU13	
RP11-565F4	5q23.1	1MB backbone			AC119040.2	
RP11-420L4	5q35.1	1MB backbone	DOCK2			
RP11-417B10	7p11.1	1MB backbone				RP11-368M16.3 RP11-368M16.6 RP11-368M16.2 RP11-368M16.7 RP11-368M16.4 RP11-368M16.5 RP11-368M16.8 RP11-368M16.1 AC0648621.1
RP4-669I17	7q36.1	1MB backbone	MLL3			
RP11-19G1	9p23	1MB backbone	PTPRD			
RP13-438M3	10p11.21	1MB backbone				
RP11-357L5	10q22.1	1MB backbone	CDH23			
RP11-125F14	11p11.2	Known disease region	MADD MYBPC3 SPI1			

Table 8 (continued). Regions having consistent methylation changes (approach 1).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-176A16	11q14.1	1MB backbone		AC103881.1 AC062032.1		
RP11-75L1	12p13.31	1MB backbone	CLEC2D CLECL1 CD69 KLRF1 CLEC2B			
RP11-125G9	12p12.2	1MB backbone	SLCO1C1			
RP11-420C17	12q14.1	1MB backbone				
RP11-449024	12q24.21	Known disease region				
RP11-321F20	13q21.33	1MB backbone				
RP11-65B9	16p11.1	1MB backbone				
RP11-463D17	18q12.3	1MB backbone*				
RP11-97N8	19p12	1MB backbone	ZNF56 ZNF90 AC011477.4 AC007204.1			

Table 9. Differential Methylation Patterns Observed following inter-individual analysis

Individual (age)	# Hypermethylated regions	# Hypomethylated regions	# Differences with co-twin pattern
#73	73	7	2
#74	89	14	22
#C51	17	9	11
#C52	17	9	11

Table 10. Regions having differential methylation between co-twin pair 1 as assessed from a comparison of patterns from individual-based hybridizations (approach 1).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-24.23	8q24.23	1MB backbone				
RP11-145B4	Xp21.2	1MB backbone				
RP11-117D22	1p32.3	1MB backbone		AC119428.1 AC119428.2		
RP11-293B7	1q25.3	1MB backbone	RGL1			
RP11-389K20	2p16.3	1MB backbone		AC139712.4		AC0793041
RP11-575H3	2p11.1	Proposed disease region			U6	AC018696.2 AC018696.7 AC018696.6 AC0186964 AC018696.1 AC018696.3 AC018696.5 AC116050.1
RP11-554O1 RP11-120C2	3p14.3	1MB backbone	CACNA2D3 DNAH12			AC092057.1
RP11-45C13	4q35.2	1MB backbone	MTNR1A FAT1			
RP11-323M9	5p15.33	Proposed disease region	AC010443.2 PLEKHG4B AC021087.1 CCDC127 SDHA PDCD6		Y_RNA	
RP11-494C5	5q33.2	1MB backbone	GRIA1			
RP11-132E3	8q22.3	1MB backbone	RIMS2 AC107933.1			
RP11-328K22	10q22.3	1MB backbone	KCNMA1	AL731575.1		
RP11-349I16	11q21	1MB backbone	AMOTL1			

Table 10 (continued). Regions having differential methylation between co-twin pair 1 as assessed from a comparison of patterns from individual-based hybridizations (approach 1).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-320N7	12p13.32	1MB backbone				AC007207.1
RP11-480P7	12q21.33	1MB backbone				
RP11-38M15	13q11	1MB backbone	RP11-38M15.1	AL391382.6 AL391382.4		AL391382.9 AL391382.5 AL391382.2 AL391382.12 AL391382.3 AL391382.1 AL391382.11 RP11-38M15.3 RP11-38M15.2
RP11-183G17	15q11.2	Proposed disease region				
RP11-353B9	15q21.2	1MB Backbone	DTWD1 C15orf33			
RP11-143C19	15q26.2	1MB backbone	SPATA8		7SK	
RP11-342L3	16p13.3	Proposed disease region	KCTD5 PRSS27 PRSS33 PRSS21 TCEB2 SRRM2 AC141586.1 AC141586.2 AC092117.3 AC092117.1	AC141586.3	SNORA3 AC092117.2	AC092117.4

Table 10 (continued). Regions having differential methylation between co-twin pair 1 as assessed from a comparison of patterns from individual-based hybridizations (approach 1).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11.394B2	16q22.1	1MB backbone	IL34 MTSS1L VAC14 AC020763.2 AC020763.1			
RP11-46H21	17p25.3	1MB backbone	ZNF750 B3GNTL1 AC068584.1			
RP3-324O17	20q11.21	1MB backbone	HM13 ID1	AL110115.3 AL110115.1	U6	AL110115.2
RP1-128O17	20q12	1MB backbone				
RP11-124N4	Xq23	Known disease region	DCX			

Table 11. Regions having differential methylation between co-twin pair 2 as assessed from a comparison of patterns from individual-based hybridizations (approach 1).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-480A4	3p14.2	1MB backbone	RP11-641C17.1			AC104164.2 AC104164.1 RP11-641C17.2
RP11-239O20	4q31.22	1MB backbone	SLC10A7			
RP11-191M7	5q33.1	1MB backbone				
RP11-349C2	8q24.3	1MB backbone	ADCK5 CPSF1 AF205589.2 VPS28 CYHR1 NFKBIL2 KIFC2 FOXH1 LRRC24 LRRC14 PPP1R16A C8orf82 GPT MFSD3 RECQL4 AC084125.1	AC084125.2	has-mir-939 has-mir-1234	
RP13-438M3	10p11.21	1MB backbone				
RP11-125F14	11p11.2	1MB backbone	MADD MYBPC3 SPI1			
RP11-321F20	13q21.33	1MB backbone				
RP11-45G12	17p11.2	1MB backbone				
RP13-60P5	Xp21.3	1MB backbone	MAGEB18			RP13-60P5.1 MAGEB6B
RP5-914P14 RP11-549H3	Xq23	Known disease region	PAK3 CAPN6 DCX		U6	AL031117.1

Table 11 (continued). Regions having differential methylation between co-twin pair 2 as assessed from a comparison of patterns from individual-based hybridizations (approach 1).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-58A11	1p36.31	1MB backbone	PLEKHG5 NOL9 TAS1R1 ZBTB48 KLHL21		AL591866.1	AL591866.2
RP11-290P14	1q44	1MB backbone	EFCAB2 KIF26B		U1	AL589763.1
RP11-387A1	2q31.1	Known disease region	EVX2 HOXD13 HOXD12 HOXD10 HOXD11 HOXD9 HOXD3 HOXD8 HOXD4 HOXD1 AC009336.1	AC009336.5 AC009336.4 AC009336.2	has-mir-10b	
RP11-374I17	3q26.31		NAALADL2		snoU13 AC119040.2	
RP11-565F4	5q23.1		PRR16		U4	
RP11-140L4	11p15.4	Proposed disease region	MRGPRG MRGPRE	AC109309.1 C11orf36		AC109309.2 AC109309.3
RP11-179A16	11q14.1	1MB backbone				
RP11-75L1	12p13.31	1MB backbone	CLEC2D CLECL1 CD69 KLRF1 CLEC2B			
RP11-420C17	12q14.1	1MB backbone				

Table 11 (continued). Regions having differential methylation between co-twin pair 2 as assessed from a comparison of patterns from individual-based hybridizations (approach 1).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-38M15	13q11	1MB backbone	RP11-38M15.1	AL391382.6 AL391382.4	U6 AL391382.8	AL391382.9 AL391382.5 AL391382.2 AL391382.12 AL391382.3 AL391382.1 AL391382.11 RP11-38M15.3
RP11-183G17	15q11.2	Proposed disease region				

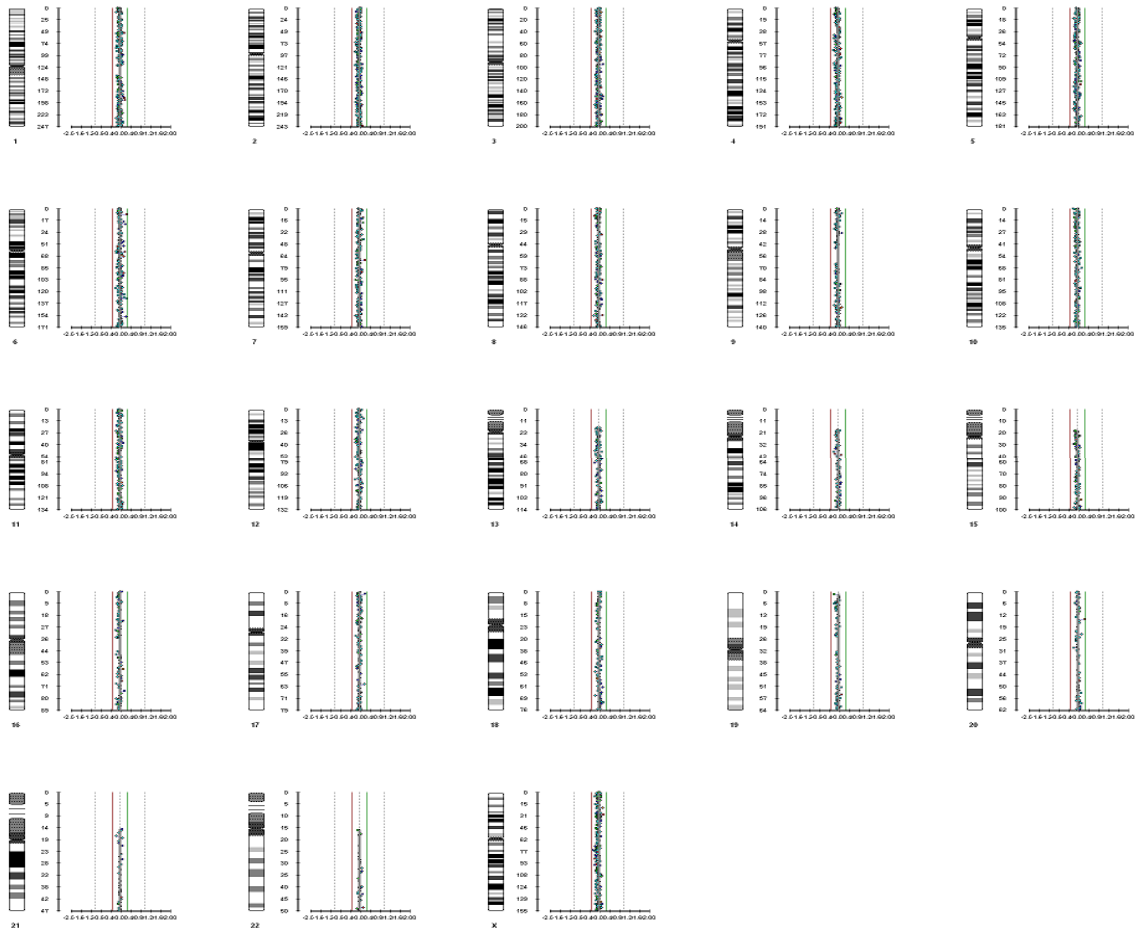

Figure 17. Whole genomic view from software analysis of MeDIP product from twin 1 hybridized to the MeDIP product from twin 2 (pair 1) No (zero) differences were observed for the pattern of methylated DNA present in these identical co-twins (s.d.= 0.05).

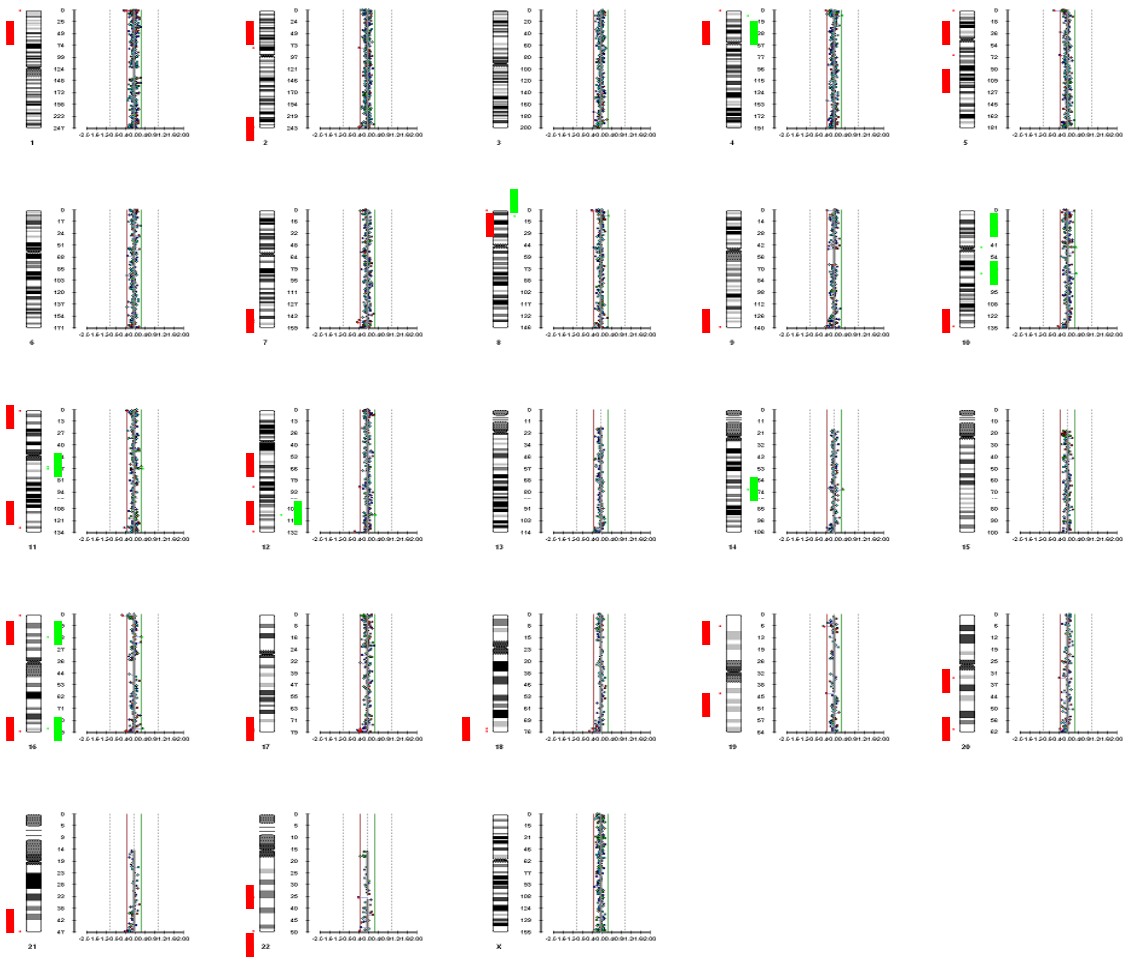

Figure 18. **Whole genomic view from software analysis of MeDIP product from twin 3 hybridized to the MeDIP product from twin 4 (pair 2).** A total of 33 differences in DNA methylation patterns were observed between these identical co-twins, who were discordant for high/low frequencies of micronuclei. These differences are depicted as red or green lines parallel to the ideogram at the chromosomal location of the divergent DNA (s.d.= 0.1).

Table 12. Regions having differential methylation between co-twin pair 2 as assessed from the competitive hybridization experimental approach (approach 2).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-181G12 RP5-832C2	1p36.33	Known disease region	PRKCZ C1orf86 AL590822.1 AL590822.3 SKI ATAD3B ATAD3A C1orf70 SSU72 AL645728.1	RP11-181G12.1 AL590822.2 AL590822.4		AL645728.3
RP11-9O10	2p12	1MB backbone				
RP11-118M12	2q37.3	1MB backbone	AQP12B AC011298.2 AC011298.3 AC011298.1 AQP12A KIF1A			
RP11-124522	4p16.3	1MB backbone				
RP11-180A12	4p16.1	1MB backbone	AC105916.1			
RP11-43F13	5p15.33	Known disease region	AC116351.2 NKD2 SLC12A7 AC116351.1 AC116351.5		AC1163513	AC1163514
RP11-138M1	5q13.2	Known polymorphic region	AC140134.2 AC138866.1			
RP4-800G7 RP4-669I17	7q36.1	1MB backbone	MLL3			

Table 12 (continued). Regions having differential methylation between co-twin pair 2 as assessed from the competitive hybridization experimental approach (approach 2).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-248K13 RP11-52B19	8p23.3	High GC content region	AC105233.6 FAM90A11 AC105233.4 AC105233.2 AC105233.3 AC105233.5 FAM90A24P		hsa-mir-548i-3	AC105233.1
RP11-417A4	9q34.3	1MB backbone	PNPLA7 MRPL41 WDR85 ZMYND19 ARRDC1 C9orf37 EHMT1			AL590627.1
RP13-244L6	10q11.21	Known disease region	CSGALNACT2 RASGEF1A	AC068707.3		
RP11-357L5	10q22.1	1MB backbone	CDH23			
RP13-449N13	10q26.3	1MB backbone	STK32C			
RP11-2B17	11p15.5	1MB backbone				
RP11-58D3	11p13.1	1MB backbone	KAT5 RNASEH2C OVOL1 SNX32 CFL1 MUS81			AP001266.2 AP001266.2
RP11-119D9	11q13.2	Known disease region	AP003716.2 AP003716.1			

Table 12 (continued). Regions having differential methylation between co-twin pair 2 as assessed from the competitive hybridization experimental approach (approach 2).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-567M21	11q24.3	1MB backbone	PRDM10 NCRNA00167 AP003041.3 APLP2		U4	AP003041.4 AP003041.1
RP11-429N9	12p21.32	1MB backbone	C12orf50 C12orf9 CEP290			AC090060.1
RP11-449O24	12q24.21	Known disease region				
RP11-503G7	12q24.33	1MB backbone	AC148477.3 MUC8 FBRSL1			
RP3-514A23	14q24.2	1MB backbone	RGS6 DPF3			
RP11-358F6	16p13.3	High GC content region	SOX8 SSTR5 C1QTNF8 AC009041.3 AC009041.7 AC009041.1 AC009041.6 AC009041.8 AC009041.4 AC009041.5 AL031713.1			AC009041.2
RP11-288I13	16p12.3	1MB backbone	XYLT1			
RP11-278A23	16q24.2	1MB backbone	JPH3 KLHDC4 AC010536.1 AC010536.2			

Table 12 (continued). Regions having differential methylation between co-twin pair 2 as assessed from the competitive hybridization experimental approach (approach 2).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-368I7 RP11-533D19	16q24.3	1MB backbone	CPNE7 DPEP1 CHMP1A C16orf55 CDK10 SPATA2L C16orf7 ZNF276 AC010538.1 SPIRE2 TCF25 TUBB3 DEF8 GAS8 DBNDD1 AC092143.1 AC092143.3 C16orf3 AC133919.7	AFG3L1		
RP11-475F12 RP11-45506	17q25.3	Proposed disease region	FASN CCDC57 SLC16A3 CSNK1D AC132872.4			AC132872.2
RP11-100K18 RP11-315M18	18q23	1MB backbone	AC068473.1			
RP11-330I7	19p13.3	1MB backbone	CD70 C3 TNFSF14 TRIP10 GPR108			AC008760.1

Table 12 (continued). Regions having differential methylation between co-twin pair 2 as assessed from the competitive hybridization experimental approach (approach 2).

BAC	Chromosomal location	Characterization	Protein coding	Processed transcript	RNA gene	Pseudogene
RP11-9B17	19p13.2	1MB backbone	ZNF781 ZNF607 ZNF573 AC093227.1			
RP11-353C18	20q11.22	1MB backbone	NFS1 ROMO1 RBM39 AL357374.3 PHF20			BXDC1P
RP11-93B14	20q13.33	Proposed disease region	RP11-93B14.1 SLCO4A1 C20orf90 NTSR1	RP11-93B14.2 AL357033.1		
RP11-135B17	21q22.3	1MB backbone	DIP2A S100B PRMT2			AP000339.1
RP5-1119A7	22q12.3	1MB backbone	TXN2 FOXRED2 EIF3D RP5-1119A7.1 CACNG2	CR383704.1 AL022313.1		
RP11-164E23	22q13.33	Known disease region	SAPS2 SBF1 ADM2 MIOX LMF2 NCAPH2 SCO2 TYMP ODF3B KLHDC7B	U62317.1		

Discussion

To achieve the primary goal of this study, the methylation profiles of 2 monozygotic twin pairs were compared using two different experimental approaches. For this preliminary assessment, these particular twin pairs were selected on the basis of: (1) their age (we selected older twins since it has been hypothesized that methylation changes accumulate with age); and (2) micronuclei frequencies (the co-twins from one pair had very similar micronuclei frequencies, while the co-twins from the other pair presented with significantly different micronuclei frequencies).

Each of the two whole genome experimental approaches used in this pilot study had apparent strengths and weaknesses. A strength of the first technique (Approach 1) was that it enabled one to directly assess if the observed changes in patterns resulted from hypermethylation or hypomethylation. Of interest was the observation of 23 consistent regions, for methylation, that were present between the study participants. Some common areas are expected since the sample DNA was isolated from the same tissue type (white blood cells). Interestingly, the standard deviations associated with these experiments exceeded those typically obtained from conventional genomic hybridization experiments. The cause for this increase is not clear, with additional experiments being required to discern if this finding reflects a consistent problem associated with the methodological approach. Despite the increased standard deviation values seen (which makes the detection of differences more difficult), variation in the identified regions was detected between the identical co-twins. However, the total number of differences between pairs

was very similar, with slightly more divergence being observed for the pair having the least difference in micronuclei frequencies and exposures.

In the second technique (approach 2), the methyl-enriched DNA from the identical co-twins were directly compared. As noted in the results, a shortcoming of this experimental approach is that one cannot directly classify the recognized alterations as being attributable to hyper- versus hypo-methylation, since the observed values are in relation to those seen in the methyl enriched portion of the co-twin's DNA rather than to the overall genome. However, a benefit of this method is that it is more cost effective (reduces cost by half) and may be more sensitive for detecting differences between co-twins, since it led to the recognition of 33 differences between the co-twins of pair 2 [but fewer (zero) differences in the co-twins of pair one]. Also, since the methyl-enriched DNAs that are co-hybridized in these experiments were collected using identical procedures, the standard deviations of this experiment were lower, falling within the clinically acceptable range.

When comparing the types of regions identified using each of the two techniques, it appears that approach 1 identified more RNA genes and pseudogenes, while approach 2 identified more protein coding regions. The biological relevance of this observation is unclear and warrants further evaluation using a second confirmatory approach (such as bisulfite sequencing methodology).

In summary, while the sample size used for this pilot study was small, the results support the hypothesis that changes in the global methylation profiles were present between these identical twins, who were 69 and 85 years old. Excitingly, based on approach two, these changes appear to be more prevalent in people having more diversity

for environmental/health histories, as well as biomarker (micronuclei) divergence. Our future studies, in which we will study epigenome patterns between identical co-twins ranging in age from 7 to 85, should enable us to determine at what age monozygotic twins begin to diverge epigenetically, if specific environmental exposures influence this divergence, and if this phenomenon can be consistently correlated with genetic indicators of aging, such as aneuploidy or micronuclei frequencies.

Chapter 6.

Conclusions

A. To determine the influence that genetic and/or environmental factors have on the frequency of spontaneously occurring micronuclei in children and adults, cells from identical and fraternal twins were evaluated. These studies led to the following primary conclusions:

1. No significant difference in micronuclei frequencies was seen for males (1.39% ± 0.11%) compared to females (1.22% ± 0.10%) ($p=0.87$).

2. Micronuclei frequencies significantly increased with age ($p<0.0001$, $r=0.446$), ranging from a low of 0.56% ± 0.11% in 7-9 year-olds, to a high of 2.25% ± 0.25% in 60-69 year-olds.

3. Although monozygotic twins ($r=0.5$, $p=0.003$) had a higher correlation coefficient than dizygotic twins ($r=0.42$, $p=0.118$), these values indicated a contribution of environmental factors. Model fitting, implemented in Mx, showed the best fit for the data to result from contributions due to either additive genetic and unique environment or common and unique environmental factors.

4. Environmental exposures and health conditions that showed a significantly increased frequency of micronuclei included a history of allergies ($p=0.00019$), and consumption of vitamin E supplements ($p=0.0251$). In contrast, significantly lower micronuclei frequencies were associated with eating fruit ($p=0.0162$), eating leafy green vegetables ($p= 0.0007$), having a history of arthritis ($p=0.0005$), and having a history of heart disease ($p=0.0381$). However, this latter effect may be confounded by medication usage since ACE inhibitors and diuretics were associated with significantly decreased ($p=0.0132$) or increased ($p=0.0023$) micronuclei frequencies, respectively.

B. For a subset of twins having 5% or more micronuclei containing sex chromosomes, the number of sex chromosomes (and chromosome 17) present in the binucleates having one or more micronuclei was determined. These studies led to the following primary conclusions:

1. In both genders, the frequency of micronuclei containing a sex chromosome was significantly higher than that observed for chromosome 17 (autosomal control).

2. The probe signal patterns noted in the total of 1,017 binucleated cells having at least one micronucleus with a signal for chromosomes X, Y, or 17 included three primary types of binucleate cells: those having a hyperdiploid complement; a hypodiploid complement; or a euploid complement, the latter of which had an apparent chromosomal imbalance that was “corrected” through its exclusion into the micronucleus (trisomy rescue).

3. In the males, the percentage of cells having a euploid binucleate with a "corrected" micronuclear pattern for the Y chromosome was significantly higher in the younger subjects ($p=0.026$), while those having a hypodiploid binucleate pattern tended to increase with age.

4. In the females, the percentage of cells having a euploid binucleate with a "corrected" micronuclear pattern for the X chromosome was significantly higher in the younger subjects ($p=0.030$), while those having a hypodiploid binucleate pattern tended to increase with age.

5. No significant age-related difference in the proportion of hyperdiploid binucleates was observed for the X (males or females) or Y chromosome (males).

C. The chromosomal contents of micronuclei were determined using Spectral Karyotyping (SKY) methodology. These age-related influences on chromosome-specific frequencies of micronuclei were also evaluated. These studies led to the following primary conclusions:

1. The majority of micronuclei (89.6%) contained chromatin from a single chromosome.

2. All 24 chromosomes were observed to be present in at least 5 micronuclei. However, the chromosome-specific frequencies varied, with the sex chromosomes being observed most frequently in the micronuclei evaluated from both males and females ($p<0.0001$).

3. Of the autosomes, chromosomes 4, 8, and 9 were found with a significantly higher frequency than expected by chance, while chromosomes 17 and 22 were found much less frequently than expected ($p<0.0001$).

4. No difference was observed in the pattern of autosomes present in the micronuclei studied from males compared to females ($p=0.7$).

5. There was no significant difference in the pattern of autosomal chromatin exclusion into micronuclei between adults (ages 25-81) and children (ages 7-19 years) ($p=0.9$).

6. A positive correlation was detected for the proportion of X chromatin-containing micronuclei in females with age ($p<0.0001$) and for the Y chromosome in males with age ($p<0.0001$).

7. The ability of the SKY software to accurately detect the chromosomal contents of a micronucleus was determined to be between 83% (when using centromeric probes) and 89% (when using painting probes). The discrepancy in this value suggests that some of the micronuclei may contain chromosomal fragments.

D. Global methylation profiles were determined (using a MeDIP Chip) for two monozygotic twin pairs by isolating methyl-enriched DNA and hybridizing it to either the co-twins methyl-enriched DNA or to the individual's sonicated total DNA (methylated and non-methylated). These studies led to the following primary conclusions:

1. Overall, hypermethylated regions (ranging from 17 to 89 per individual) were detected more frequently than hypomethylated regions (ranging from 8 to 14).

2. A comparison of the specific genomic information gained from hybridizing an individual's methyl-enriched DNA to their sonicated total DNA showed 12 loci to be consistently altered in all of the 4 individuals, with an additional 11 regions showing either consistent hypo- or hypermethylation in 3 of the 4 individuals.

3. Through direct competitive hybridization of the co-twins' methyl-enriched DNA onto a genome-wide microarray, an increased frequency of methylation differences was observed between the co-twins having different micronuclei frequencies and environmental exposures (33 differences) when compared to the divergence observed between the co-twin's having similar micronuclei frequencies and environmental exposures (0 methylation differences). This finding suggests there may be a link between acquired chromosomal instability and epigenetic changes.

In summary, genetic and epigenetic changes, as assessed through micronuclei and methylation, do occur with age and these acquired abnormalities appear to be complex

traits that are influenced by unique environmental factors as well as other factors (such as additive genetic or common environmental effects). We are the first to have discovered an *in vivo* link between blood pressure medications and micronuclei frequencies. We have also performed the most comprehensive study to date examining the chromosomal contents of micronuclei. In addition, we are the first to propose the possible "corrective" function of micronuclei. It is anticipated that the baseline micronuclei data we presented in this study will help future researchers develop preventative care, screening tests, and therapies for the various health conditions associated with micronuclei.

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Vita

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