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Hyper-variability in the Length of the Constitutive Heterochromatin Regions Associated With Cancer Susceptibility

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Cytogenetics

The field of cytogenetics is a relatively young area of study; its birth can be traced back to 1956 when Tijo and Levan made the discovery that the correct number of chromosomes in man is forty-six, not forty-seven as had been believed for the previous fifty years (Tijo and Levan, 1956). With simple technical innovations that permitted short-term culture of human cells and the preparation of metaphases with chromosomes suitable for detailed examination and enumeration, the field of cytogenetics blossomed. A number of cytogenetic banding techniques exist: each of which stain different regions of the chromosome and are used depending on specific clinical needs. Giemsa banding, which uses trypsin and Giemsa staining, is the most commonly used banding technique in clinical cytogenetic studies. R banding, developed by Dutrillaux and Lejeun (Dutrillaux and Lejeun, 1975), is a technique that stains chromosomes as the reverse of G-banding. Darkly stained bands are GC-rich and the light bands are AT-rich regions. Q-banding, developed by Caspersson, Zech and Johansson (1970), uses a quinicrine mustard solution to distinguish the satellites of some acrocentric chromosomes, the end of the long arm of the Y chromosome, and the centromeres of human chromosomes 3, 4, and 13. The C-banding technique, developed by Sumner (1972) based on the method of Arrighi and Hsu (1971), stains all centromeric regions and regions containing constitutive heterochromatin, primarily the secondary constrictions of human chromosomes 1, 9, 16, and the distal segment of the Y chromosome long arm.

The ability to visualize human chromosomes allowed scientists to discover the association of abnormal changes in the structure and number of chromosomes with

diseases such as cancer. In the early years of cytogenetics only conventional staining, which facilitated the measurement of chromosome length, centromere position and arm ratio was available and discrete banding patterns on the chromosomes could not be distinguished. Early cytogenetic studies showed that a gain or loss of specific chromosomes could lead to diseases, including Down syndrome, Turners syndrome and Klinefelter's syndrome. In 1960, culturing leukemic cells from patients with chronic myeloid leukemia (CML) allowed Nowell and Hungerford to discover a deletion of a portion of the long arm of one member of the G group of chromosomes (later determined to be chromosome 22) and a gain in the long arm of chromosome 9. This t(9:22)(q34:q11) was consistently found in different patients with CML (Goldman 1987) and proved to be critically important for understanding the mechanistic basis of the disease. Human cancers almost always possess major alterations in their chromosomes, including large deletions, duplications, and translocations of chromosomes and these can be used to characterize the disease and provide insights into the development of the disease. For example, many different alterations have been reported to occur in breast cancer cells; the most frequent chromosomal abnormality is the gain of the long arm of chromosome 1 (+1q) (Kallioniemi et al., 1994, Loo et al., 2004). The breakpoints are adjacent to the band 1q21, a band that contains the junction between the constitutive heterochromatin and euchromatin regions.

Heterochromatin, Epigenetics and RNA

The paracentromeric regions have been shown to be very important in maintaining genomic stability (Guenatri et al., 2004, Maison and Almounzi 2004,). In humans, the paracentromeric regions are formed by tandem repeats of DNA sequences, known as satellite DNA. Chromosome 1 contains classical satellite 2 DNA, a poorly conserved 5 bp GGAAT repeat and a small amount of classical satellite 3 DNA, comprised of a CAACCCGA(A/G)T(GGAAT)n repeat. Chromosome 9 heterochromatin contains only classical satellite 3 DNA.

Centromeres are sites of kinetochore assembly, a key structure that mediates the binding of the chromosome to microtubules during cellular division. The paracentromeric heterochromatin adjacent to the centromere is the site of sister chromatid cohesion which is important for the correct orientation of paired kinetochores during mitosis (Topp and Dawe, 2006). The paracentromeric heterochromatin is considered constitutive, remaining condensed throughout the cell cycle, and is generally considered to be transcriptionally silent. The histone modifications found in the paracentromeric heterochromatin include H3K9 $(me)_3$, hypoacetylation of histone proteins, hypermethylation of cytosine residues in DNA (Dimitri et al., 2009, Craig 2005) and non-histone proteins that bind to histones, the best known of which is HP1 (heterochromatin protein 1), which binds to H3K9 $(me)_3$. Studies in mice deficient in Suv39h, a histone methytransferase, indicate that without the presence of HP1 there is premature paracentromeric separation due to defects in sister chromatid cohesion (Guenatri 2004). Proper chromatid cohesion relies on the presence of functional HP1 in the paracentromeric heterochromatin (Serrano et al., 2009, Inoue et al.,

2008). The representations of rearrangements involving the paracentromeric heterochromatin and frequent aneuploidy in cancers may be the result of centromeric dysfunction at mitosis (Mertens et al., 1997, Mitelman et al., 1997).

As indicated above, centromeric and paracentromeric bands containing highly repetitive tandem repeats were historically considered to be transcriptionally silent under normal conditions, therefore variations in these bands were considered to be clinically insignificant (John 1998). In recent years, several studies have reported that these regions of the genome are not transcriptionally silent. In a review by Vourc'h and Biamonti 2011, transcription of the paracentromeric sequences has been shown to occur under normal physiological conditions, in normal and malignant cells and under conditions of cellular stress. Paracentromeric sequences have been shown to be transcribed into non-coding RNA (ncRNA) in normal adult testis (Jehan et al., 2007, Eymery et al., 2009b), in lung tumor cells (Eymery et al., 2009b), in HeLa cells (Valgardsdottir et al., 2007, Eymery et al., 2009b), during senescence and aging of progeria cells (Shumaker et al., 2006), during environmental stress (Jolly et al., 2004, Rizzi et al., 2004, Valgardsdottir et al., 2007), in cells where Dicer (Fukagawa et al., 2004) or KDM2A (Frescas et al., 2008) are knocked out and during treatment with azacytidine in HeLa cells (Eymery et al., 2009b). The exact function of the ncRNA is unknown: it has been hypothesized that this RNA may help ensure centromeric stability and inheritance. There are also reports that in certain pancreatic and epithelial cancers there is elevated expression of satellite transcripts (Ting et al., 2011), although the mechanism and consequence of the abnormal ncRNA is unknown. Another research group has recently shown that a loss of BRCA1 induces an

increase in the production of satellite ncRNAs from the classical satellite 2 region (Zhu et al., 2011). This increase in satellite ncRNAs is associated with centrosome amplification, increased DNA double strand breaks and lagging and bridged chromosomes. It has been proposed that non-coding RNA transcribed from paracentric heterochromatin may contribute to large-scale genome instability and consequently to chromosome evolution in the cancer phenotype (Zhu et al., 2011). Given the association between aberrant classical satellite ncRNA expression, chromosome instability and centromere dysfunction, it stands to reason that ncRNA transcribed from classical satellite DNA found in the paracentromeric heterochromatin may contribute to the various observed karyotypic rearrangements.

Chemical Sensitivity and DNA Repair

There are numerous reports of non-random spontaneous and chemically induced breaks, exchanges and pairings within the constitutive heterochromatin regions of chromosomes 1, 9 and 16 (Brogger 1977, Meyne et al., 1979, Funes-Craioto et al., 1974). Previously our lab investigated breaks in the constitutive heterochromatin in chromosome 1 using tandem labeled fluorescence *in situ* hybridization (FISH) and confirmed that the 1q12 region was hypersensitive to breakage caused by chemically induced agents such as mitomycin C (MMC), etoposide, and hydroquinone (Rupa et al., 1997). Other reports have shown that the constitutive heterochromatin is prone to breakage when exposed to busulphan (Honeycombe 1978), γ-rays (Dubos et al., 1978), various chemicals (Brogger 1977, Meyne et al., 1979), melphalan (MEL) (Mamuris et al., 1991), MMC (Cohen and

Shaw 1964, Simi 1985, Bourgeios 1974, Morad et al., 1973, Sontakke et al., 2009) or in the absence of xenobiotic exposure (i.e. spontaneous breaks) (Ayme et al., 1976). Treatment with MMC also induces exchanges and pairing between the constitutive heterochromatin regions of homologous chromosomes 1 and 9 (Abdel-Halim et al., 2005, Simi 1985). It has been reported that the constitutive heterochromatin region of chromosome 9 is particularly sensitive to breakage as compared to euchromatin or the constitutive heterochromatin regions of chromosomes 1 and 16. The most striking report of the unique susceptibility of 9q12 to breakage came from Meyne *et al.* (1979) who reported that when cells were treated with the chemical triethylenemelamine, 13% of all breaks throughout the genome occurred within the 9q12 band. In related study, Joseph *et al.* 1982, curious about the frequent exchanges that MMC caused in c-band regions of chromosomes 1 and 9, treated cell lines that were XYY with MMC. Their results showed that in the XYY cells MMC also induced exchanges within the C banded regions of the Y chromosomes, implicating a need for chromosome homology in order for exchanges and pairing to occur.

A common characteristic of the chemicals that target the constitutive heterochromatin regions of chromosome 1 and 9 is that they induce interstrand crosslinks (ICL) between the two strands of DNA that lead to breakage. ICLs are one of the most cytotoxic DNA lesions as only a small number (approximately 40) ICLs can kill a repair-deficient mammalian cell (Lawley and Philips 1996). Multiple repair pathways are involved in the removal of ICLs and the specific pathway involved depends on the availability of undamaged homologous sequences, cell cycle stages, and sequence

characteristics at the site of the lesion. Abdel-Halim et al. (2005) studied the effect of MMC-induced pairing on the constitutive heterochromatin of chromosome 9. They found that after treatment with MMC the inter-homologue distance decreased between the two chromosomes 9, indicating a repositioning of at least one of the homologs. Most models propose that in dividing cells the repair of ICL occurs primarily during S phase. An alternative model proposes that ICLs are recognized and incised by ERCC1/XPF during G1 and that repair occurs independent of DNA replication. The incised ICLs are then processed in S-phase where misrepair leads to chromosome exchange and breaks (Rothfuss and Grompe 2004). Abdel-Halim et al., (2005) treated XPF-deficient and XPA-deficient cells with MMC and observed that MMC-induced pairing was absent in the XPF-deficient cells but not the XPA-deficient cells. This indicates that XPF is required either to trigger pairing or required for disassociation once homologous regions have paired.

Polymorphisms

Among the general population, polymorphisms in the paracentromeric heterochromatin of chromosomes 1, 9 and 16 are common (Brothman et al., 2006). Polymorphisms refer to the variations in morphology of regions between homologous chromosomes, either in size or in location. Chromosomal abnormalities involving centromeric and pericentromeric heterochromatin regions have been reported to be present in various diseases such as the ICF syndrome (immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9 and 16 and facial anomalies) (Brown et

al 2995). C banding has made investigations into the occurrence of polymorphisms in normal populations and in patients with malignancies possible.

In 1977, Atkin first linked polymorphisms in the heterochromatin region on chromosome 1 and 9 with malignant diseases (Atkin 1977). Using C-banding to visualize the heterochromatic bands on chromosomes 1 and 9, he concluded that chromosomes in normal lymphocytes derived from patients with malignant disease have higher variations between homologous chromosomes. He suspected that this morphological alteration might be linked to an increased risk susceptibility of developing cancer. He and Baker (Atkin and Baker 1977a, Atkin and Baker 1977b) provided further evidence to indicate a possible association. In the 1980's and 1990's, studies were conducted by numerous researchers reporting increased or no increase in variations in the heterochromatin regions associated with various diseases (Kopf et al., 1990, Naujoks and Weil 1985) Aguilar et al., 1981) including breast cancer (Adhyaryu and Rawal 1991, Berger et al., 1985) Kivi et al., 1987), colorectal carcinomas (Heim et al., 1985), and in hematological disorders (Shabtai and Halbrecht 1979).

Berger *et al*. (Berger et al., 1985) reported increased size variability of the heterochromatin on chromosomes 1, 9, and 16 as well as a higher incidence of inversions in chromosomes 1 and 9 in lymphocytes taken from breast cancer patients. Similarly, a recent study (Roy et al., 1999) reported a high frequency of variability in heterochromatin in the lymphocytes of breast cancer patients and their healthy relatives as compared with healthy control subjects. In that study the observation that the size variability was also seen in the healthy relatives of the breast cancer patients suggested that this is a heritable

trait that would indicate an increased familial risk and could be associated with genomic instability affecting the whole family.

Previous studies researching the potential link between variations in heterochromatic bands of homologous chromosomes and increased risk of malignant disease have to be evaluated cautiously due to limitations in the methods used and variation among individuals and studies. Previous studies varied in how the length of the heterochromatin band was measured: some studies recorded the absolute length of the C-band, whereas others measured its relative length with reference to 21q, its relative length with reference to 16p, recorded 4 to 5 subjective levels defined visually, or evaluated the total areas of the C-bands. These earlier studies also varied in the types of controls and how the controls were selected. The controls included newborns and index patients to healthy same sex siblings, and the subjects were not always age-matched (Baskshi et al., 1997). These earlier studies were also conducted with relatively small numbers of lymphocytes per patient; for example most of the reports analyzed only 3-10 metaphases per subject. In these previous studies, the researcher typically photographed stained metaphase spreads, which were then projected onto a flat surface (i.e. a screen or blank wall) and the length of the heterochromatin was measured manually using a ruler. Another issue the previous studies have is that the boundaries between the dark stained heterochromatin regions and the light stained euchromatin regions are hard to discern using the previous studies techniques. Proper banding techniques and development of photographs were needed to insure that the edges of the heterochromatin bands are not "lost" when the lengths are measured.

In the 1980's a novel molecular technique called fluorescent *in situ* hybridization (FISH) was developed that allowed the visualization of specific targeted regions of DNA with fluorescently labeled probes (Gray and Pinkel 1992). This technique is widely used in clinical laboratories to screen cases for cancer and constitutional related abnormalities. In this study to more accurately measure the size of the heterochromatin, we developed an approach, that combines the use of fluorescent *in situ* hybridization (FISH) with probes that are specific to the heterochromatin regions on chromosome 1 and 9 with image analysis using normalized line profiles. This technique fluorescently paints the DNA sequence so the boundaries between the heterochromatin and euchromatin regions are clear and distinct and as a result accurate measurements of the length of the heterochromatin can be made. Our results indicate that this technique is highly accurate and reproducible, and allows more precise measurements of heterochromatin variability than the techniques used in the previous studies.

Specific Aims

There are two primary aims for this study; the first was to confirm and extend the earlier results from our laboratory using a newly developed FISH method technique. This aim was accomplished by reproducing earlier results using the same lymphoblastoid cell lines from breast cancer patients and matched controls.

The second aim was to investigate if the increased heterochromatin variability observed between homologous chromosomes 1 and 9 in the cell lines derived from the breast cancer patients could be due to inaccurate repair of DNA breaks caused by

endogenous or exogenous agents. To accomplish this six pairs of lymphoblastoid cell lines were treated multiple times over the course of three weeks with bifunctional alkylating agents that preferentially induce DNA breaks through the formation of ICL, and the percent difference between homologous chromosomes 1 and 9 were measured over time. Additional studies were performed with a structurally similar monofunctional alkylating agent that does not cause ICLs and is not known to preferentially cause breakage within the pericentromeric heterochromatin. Additionally, the micronucleus assay was performed to characterize the sensitivity of the lymphoblastoid cell lines to global DNA damage induced by the alkylating agents tested.

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Hyper-variability in the length of the constitutive heterochromatin regions associated with breast cancer susceptibility

Abstract

Breast cancer is the second leading cause of cancer deaths among women in the United States. Research has linked genetic and lifestyle factors to increased risks of developing this disease. However, in spite of recent advances, there continue to be significant gaps in our understanding of the causes, susceptibility factors and mechanisms underlying the etiology of breast cancer. Environmental factors also contribute and it is believed that unintentional exposures to various physical and chemical agents play a major role in the etiology of this and other types of cancer. Studies have reported that women with increased size variability of the constitutive heterochromatin regions of chromosomes 1 and 9 in normal peripheral blood cells are at increased risk of developing breast cancer. Previous work in our laboratory using a novel technique integrating fluorescence in-situ hybridization (FISH) with probes specific for the classical satellite regions located at the heterochromatin regions of chromosomes 1 and 9 was applied to lymphoblastoid cells lines derived from breast cancer patients and age-, ethnicity- and sex-matched controls. These results corroborated early studies reporting that the paracentromeric heterochromatin regions of chromosomes 1 and 9 were significantly more variable in size than those from the matched controls. The mechanisms underlying the increase in variability are unknown. The objectives of this research were to first, confirm and extend the earlier results from our laboratory, and secondly, to investigate the hypothesis that the increased variability observed is due to inaccurate DNA repair of DNA inter-strand crosslinks.

In comparing the results of the new and the earlier studies, similar results were seen, and as a result, the data were combined to enlarge the sample size and increase the accuracy of the results. The combined analyses confirmed that the size of the constitutive heterochromatin was significantly more variable in most cells obtained from the breast cancer patients when compared with the matched controls; the constitutive heterochromatin region was significantly more variable in 7 of the 10 breast cancer patient-derived cell lines when examining both chromosomes 1 and 9.

To assess the role of DNA damage, we exposed six breast cancer patient cell lines to the alkylating agents, mitomycin C, melphalan and 2-chloroethylamine to investigate changes in the heterochromatin regions that occurred following treatment. Two of the six cell lines showed variability in the heterochromatin region of chromosome 9 that was strikingly greater following treatment than that seen in the matched controls. Interestingly, a similar trend was not seen with chromosome 1. The increase in heterochromatin variability was seen following treatment with both bifunctional and monofunctional alkylating agents indicating that the increase was not dependent upon the formation of DNA crosslinks. Of note, the two particularly sensitive cell lines did not exhibit major increases in alkylating agent-induced chromosome breakage in the micronucleus assay indicating that variability is not likely to be due to inaccurate repair of genome-wide DNA breaks. Lastly, in a time-course experiment, the increases in heterochromatin variability in the two sensitive cell lines were seen at both 24 and 48 hr after treatment with mitomycin C, a potent cross linking agent. Interestingly, similar but only transient increases (at 24 hr. only) were also seen with DMSO treatment in the

sensitive cells, but not controls, suggesting that the observed increases are not due to changes in DNA sequence but are more likely due to epigenetic changes occurring in the sensitive cell lines.

Introduction

Breast cancer is the most common female malignancy and is the 2nd leading cause of cancer deaths among women in the United States (ACS, 2011). There are many factors that can lead to an increased susceptibility in developing breast cancer including menstrual and reproductive history, body mass index, smoking, alcohol intake, diet and genetic factors including mutations in genes such as BRCA1 and BRCA2 (Coyle 2009, Mayaddat et al., 2010). Only about 10% of breast cancer cases are the result of hereditary predisposition involving a germ line mutation in an identified cancer susceptibility gene. The remaining 90% of breast cancers cases are considered sporadic with unknown origins and with known risk factors explaining only a small portion of cases (Synowiec et al, 2010). Many authors have postulated that there is a contribution from environmental factors and that involuntary exposure to diverse physical, chemical and biological agents along with lifestyle and genetic factors all play a role in the occurrence of breast cancer (Irigrary et al., 2007). For example, some epidemiological studies have supported an association between breast cancer and exposure to polycyclic aromatic hydrocarbons, organic solvents and PCBs (Brody et al., 2007).

A recent study from our laboratory employed lymphoblastoid cell lines from breast cancer patients and age-, ethnicity-, and sex-matched controls to evaluate the

variability in the size of the heterochromatin regions between homologous chromosomes 1 and 9 using fluorescence *in situ* hybridization (FISH) in conjunction with image analysis with normalized line intensity plots to measure the size of the heterochromatic regions (Capsel et al., in preparation). Our results indicated that there was an increased variability in the size of the heterochromatin regions between homologous chromosomes 1 and 9 in lymphoblastoid cell lines derived from breast cancer patients as compared to the matched controls. The main purpose of the earlier study was to screen potential cell lines to use for follow-up studies to identify the mechanisms underlying the increased incidence of size variations in the cells from the breast cancer patients. A number of previous studies used primary lymphocytes from patients with malignant diseases and showed an association in variations in the size of the heterochromatin regions in homologous chromosomes 1, 9 and 16 with an increased risk of developing malignant diseases (Atkin NB 1977, Atkin et al., 1977a, Atkin et al., 1977b, Aguilar et al., 1981, Adhvaryu et al., 1991, Berger et al., 1985, Bakeshi et al., 1997, Roy et al., 1999, Heim et al., 1985, Shabtai et al., 1979, Kopf I et al .,1990, Naujoks et al., 1985). Currently the mechanism(s) underlying the increased variability in the cells from the cancer patients remains unknown.

There are also numerous reports indicating that the paracentromeric heterochromatin regions of chromosome 1, 9 and 16 are particularly sensitive to breakage induced by chemicals (Honeycombe 1978, Brogger 1977, Meyne et al., 1979, Mamuris et al., 1991, Cohen and Shaw 1964, Simi 1985, Bourgeios 1974, Morad et al., 1973, Sontakke et al., 2009), ionizing radiation (Dubos et al., 1978), or occurring to

spontaneously (Ayme et al., 1976). Previous studies from our laboratory investigating breaks in the paracentromeric heterochromatin region of chromosome 1 using tandem labeled FISH confirmed that the 1q12 and 9q12 regions here highly sensitive to breakage caused by chemically induced agents such as mitomycin C (MMC), etoposide and hydroquinone (Rupa et al., 1993). The paracentromeric heterochromatin region of chromosome 9 is particularly sensitive to breakage, pairing and exchanges with itself or with the constitutive heterochromatin regions of chromosomes 1 and 16. The agents that have been reported cause breakage, exchanges and pairing within the constitutive heterochromatin region are frequently chemicals that induce DNA interstrand crosslinks (ICL) (Dronkert and Kanaar, 2001, Muniandy et al., 2010). One hypothesis to explain the heterochromatin size differences is that cell lines derived from breast cancer patients have increased variability as compared to controls because these patients are unable to efficiently repair ICL in the heterochromatin regions caused by endogenous and exogenous agents, and that this damage leads to breakage and an associated expansion or contraction of the satellite DNA within these regions.

The first aim of this study was to assess the new cytogenetic image analysis technique that was recently developed in our laboratory. To accomplish this, a series of confirmatory experiments were conducted. In brief, metaphases from untreated lymphoblastoid cell lines were prepared using standard cytogenetic harvesting methods and hybridized with fluorescently labeled FISH probes to label the paracentromeric heterochromatin regions of chromosomes 1 and 9. The size of the heterochromatin was measured using the imaging software Image J using plots of normalized line intensity and

the results from the new analysis was initially compared, and then subsequently combined with those from the earlier study.

The second aim was to investigate whether the increased variability could be due to inaccurate DNA repair of interstrand crosslinks (ICL) and that this could account for the variability seen between homologous chromosomes 1 and 9 in cell lines derived from breast cancer patients. To accomplish this, six pairs of lymphoblastoid cell lines from breast cancer patients and age-, ethnicity-, and sex-matched controls were treated multiple times over a course of three weeks with the ICL-inducing agents MMC and melphalan (MEL). We also treated the cell lines with 2-chloroethylamine (CEA), a mono-functioning agent that does not induce ICL. Following treatment, the sizes of the heterochromatin regions were measured in the treated and untreated cell lines. The cytokinesis-block micronucleus assay was also used to assess the sensitivity of these cell lines to genotoxic insults (Eastmond and Tucker 1989). Micronuclei (MN) are small extranuclear bodies that arise in dividing cells from chromatid fragments or chromosomes that are lost during mitosis and not included in the daughter nuclei (Fenech and Morley 1985; Eastmond and Tucker, 1989). MN are commonly monitored as a biomarker of DNA alterations resulted from clastogenic (and aneugenic) insults (Fenech and Morley 1985, Eastmond and Tucker 1989).

Materials and Methods

Cell lines

Lymphoblastoid cell lines derived from female breast cancer survivors (BCS) and healthy matched controls (age- sex-, and ethnicity-matched) were obtained from NIGMS Human Genetic Cell Repository (Coriell Cell Repositories, USA). The cell lines selected for this study were from women with ages ranging from 32 to 51 years old and were selected from patients with familial breast cancer (FBC), and patients carrying mutations in the BRCA1 and BRCA2 genes. Table 1 lists the paired cell lines utilized to test robustness and confirm the techniques used in subsequent mechanistic studies.

The cell cultures were maintained in exponentially growing suspension cultures at concentrations up to $1x10^6$ cells/ml. Cultures were grown at 37°C in 95% air/5% CO_2 in RPMI 1640 media (Mediatech) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin-streptomycin (Mediatech, Cellgrow), and 10% iron-supplemented bovine calf serum (Hyclone Laboratories).

Metaphase Preparation and Fluorescence in situ Hybridization

To prepare slides for metaphase analysis, colcemid (Sigma) at a final concentration of 10 μg/ml was added 2.5 hours before harvest. At harvest, cell cultures were centrifuged, and the media aspirated. The cell pellets were re-suspended in 0.075 M KCl for 30 minutes at 25°C, fixed four times with methanol: acetic acid (3:1) and dropped onto pre-cleaned slides. The slides were stored at -20°C under N_2 until use.

For FISH, slides were pre-treated with 2xSSC for 30 minutes at 37°C, and dehydrated by incubation in a series of ethanol washes at room temperature (2 minutes each in 70%, 80%, and 95% ethanol). After drying, the chromosomes were denatured in 70% formamide/2×SSC (pH 7.0) at 68°C for 2 minutes and immediately dehydrated through an ethanol series at room temperature (2 minutes each in 70%, 80%, and 95% ethanol).

Classical satellite probes (Cs-1 and Cs-9) were prepared as described previously (Hasegawa et al. 1995, Capsel et al, in preparation). Briefly, the DNA probes were made by amplification of the classical satellite II (Csat2) sequences (5'-TCG AGT CCA TTC GAT GAT-3') and classical Satellite III (Csat 3) sequences (5'-TCC ACT CGG GTT GAT T-3') using PCR. The amplified product was directly labeled by nick translation with either Alexa-488 or Cy3 fluorescent labels. A 10 μl hybridization mixture containing 1 μl CY3-labeled classical satellite chromosome 1, 2 μl of Alexa-488 labeled-classical satellite chromosome 9 probe, 6 μl of master mix (MM2.1) composed of 55% formamide, 10% dextran sulfate and 1×SSC) and 1 μl of sheared herring sperm DNA was denatured at 68°C for 5 minutes and applied to the denatured slides, immediately covered with a glass cover slip, and sealed with rubber cement. After overnight hybridization in a pre-warmed humidified box at 37°C, the slides were washed three times in 2×SSC/50% formamide at 37°C for 5 minutes each, and then counterstained with 0.1 μg/ml 4', 6'-diamidino-2-phenylindole (DAPI) in a phenylenediamine antifade mounting medium (Rupa et al. 1997) and stored at 4°C until analysis.

Image Detection and Heterochromatin Size Measurement

The slides were coded and scored using an Olympus model BX-40 microscope with triple-band-pass filter (excitation at 360-370, 470-490, and 530-550 nm, and emission at 450-465, 505-535, and 580-620 nm) to visualize the green (Alexa-488), red (Cy3) and blue (DAPI) fluorescent signals. For each cell line, 40 TIFF images were taken with a CCD camera attached to the microscope using MetaVue (Molecular Devices, Sunnyvale, CA) imaging software. From these, 30 well-stained metaphase images were selected for heterochromatin size measurements using Image J software. To obtain the size variability between homologous chromosomes, the percent difference was calculated using the equation [((qh+) – (qh-))/ (qh-)] ×100% (Roy et al., 1999). The homolog with the larger sized heterochromatin band was denoted as qh+ and the homolog with the shorter sized heterochromatin band was designated as qh-. Since lymphocytes are heterogeneous in the size of the constitutive heterochromatin, the average percent difference was calculated per cell. Image and analysis were performed as previously described (Capsel et al., in preparation).

Confirmation of Technique

To confirm the reliability of the method developed previously in our laboratory, ten pairs of lymphoblastoid cell lines derived from breast cancer patients and matched controls were cultured, harvested, labeled and analyzed as described above. The percent difference between chromosome one and nine homologs were measured for each cell

line. The median percent difference and interquartile range was determined for each cell line as shown in Figure 1.

Time Course Chemical Treatment

The subset of cell lines used for the chemical treatments is shown in Table 1. Six pairs of matched cell lines were selected: BRCA1-1 and Control-1; BRCA1-2 and Control-2; BRCA2-4 and Control-4; BCS-5 and Control-5; BCS-8 and Control-8; BCS-10 and Control-10. These cell lines included paired cell lines that displayed high percent differences between the breast cancer patients and matched controls (BRCA1-1, BRCA2-4, BCS-5 and BCS-10) and paired cell lines that exhibited little or no percent difference between the breast cancer patients and matched controls (BRCA1-2 and BCS-8).

MMC, MEL and CEA were purchased from Sigma Chemical (St. Louis, MO). A 0.05 mM MMC working solution was freshly made prior to each treatment from a 100 mM stock solution (in DMSO) stored at -20°C. Figure 2 illustrates the protocol used for the time course experiments. Cells at a starting density of ~$2.5x10^5$ cells/ml were exposed to 0.05 mM MMC for 24 hours; the cultures were washed twice with MMC-free media at the 24-hr point and incubated in MMC- free media for 3 days. At 96 hr after the first MMC treatment, the cell lines were again treated with 0.05 mM MMC for 24 hr. The cells were then washed twice with MMC-free medium at the 24-hr point and incubated in MMC-free medium for 3 days. The cells were harvested 96 hr after the second treatment. Additionally, experiments were extended for a third and fourth treatment to assess a possible cumulative effect of MMC on heterochromatin size variations. For the three time

points, cells were harvested and hybridized with FISH probes to measure size variability in the heterochromatin between homologs.

A 50 mM stock solution of MEL dissolved in DMSO was stored at -20°C in a light resistant box. Prior to each treatment a freshly made working solution of 0.1 mM MEL was made from the 50 mM stock solution. Cell lines were treated with 0.1 mM MEL using a similar protocol as MMC, described above. A 100 mM stock solution of CEA dissolved in DMSO was freshly made prior to use. Cells were treated with 0.05 mM CEA in a protocol similar to that of MMC, as described above.

The doses used in the time course experiments were selected based on a preliminary dosing experiment in which a few cell lines where tested at different doses for two chemical treatments for a total seven days. Doses that did not induce a major inhibition of cell growth as measured by a cell counter were selected for the main time course experiments. For the time-course experiments, three replicate experiments were performed.

Micronucleus Assay

For each tested chemical, lymphoblastoid cell lines derived from the female breast cancer survivors (BCS) and their matched controls were cultivated for 24 hours in 10 ml cultures at a starting cell density of ~$2.5x10^5$ cells/ml and exposed to various concentrations of MMC, MEL, CEA and methyl methanesulfonate (MMS). As discussed previously MMC and MEL are agents that induce the formation of ICL. CEA is a agent that causes DNA adducts and MMS was chosen to see if an agent that methylates DNA

(i.e. forms small adducts) would induce an increase in DNA breaks in the hypersensitive cell lines. The doses chosen were those used in the time course experiments and to obtain a dose response curve, a dose that was half of that dose was used. Each chemical was brought up to a final DMSO concentration in the media of 0.1%.

For the *in vitro* cytokinesis-block micronucleus assay, cytochalasin B (4.5 μg/ml in DMSO) was added, to allow cells that had undergone a single cell division, to be scored. Cytochalasin B-treated cultures were harvested 24 hours after chemical (and cytochalasin B) treatment by cytocentrifugation directly onto cleaned slides at 600 r.p.m. for 5 min using a Shandon cytocentrifuge (Shandon Cytospin 2, Shandon Lipshaw Inc, Pittsburgh, PA). The slides were fixed in 100% methanol for 10 min at 25°C and stored under N_2 at -20°C until use. Coded slides were counterstained with 2.5 μg/ml of DAPI and scored microscopically. For each test chemical or corresponding control, a total of 1000 binucleated cells per test concentration per chemical were scored for each of 3 experiments as described previously (Eastmond and Tucker 1989).

Cytotoxicity

For the micronucleus assay, where cytochalasin B was used, the replicative index (RI) was calculated as a measure of cytotoxicity. RI was determined by scoring 200 interphase cells per control and test concentration of MMC, MEL, CEA and MMS for the presence of mononucleated, binucleated, trinucleated, and tetranucleated cells (Kirsch-Volders et al., 2003). RI was calculated based upon the equation published by Kirsch-Volders and colleagues (2003) where $RI = [(M2+2x(M3 + M4)/n)_T] /[(M2+2x(M3 +$

M4))/n)$_C$] x100. M2 to M4 represent the number of cells having two to four nuclei, respectively, and *n* was the total number of scored cells in each treatment (Kirsch-Volders et al., 2004).

Time Course Treatment to Determine Time Point for Induction of Variability

Six lymphoblastoid cell lines were used for the chemical treatments: BRCA1-1, Control-1 and Control-3; and BCS-5, Control-2 and Control-9). [See the Results section for an explanation on the use of two control cell lines per breast cancer patient cell line for this experiment.]

Cells were exposed to 0.05 mM MMC in 0.1% DMSO, 0.1% DMSO, or were untreated for 24 hours; the cultures were washed twice with MMC/DMSO-free media at the 24-hour point, and incubated in MMC/DMSO-free media for 24 hours before the 48 hour harvest. Cell cultures were aliquoted and harvested at 24 hours and 48 hours for FISH and image analysis as described above.

Statistical analysis

A number of statistical tests were used to analyze the experimental results. The comparisons of heterochromatin size variability between patient-derived cell lines and control cell lines were performed using a one-tailed Mann–Whitney U test (VassarStats website, http://vassarstats.net/index.html). For analysis of the time course and chemical treatment experiments, a Kruskal-Wallis test (VassarStats website, http://vassarstats.net/index.html) was used to determine differences between multiple

treatments across cell lines was used to find significant changes in variability between homologs. The Mann Whitney U test was used as a post-hoc test. For the micronucleus assay, the Cochran-Armitage test for trend in binomial properties was utilized to compare the micronuclei frequencies between control and treatment slides. The Fisher exact test was used as a post-hoc test.

For the primary comparisons such as the comparison of the individual cell lines, critical values were determined using a 0.05 probability of type I error. For follow-up studies such as the chemical treatment and time-course studies where multiple comparisons were performed, a 0.01 probability of type I error was used as a critical value.

Results

Assessment of Technique to Measure Size Variability in Constitutive Heterochromatin

To assess the newly developed FISH and imaging technique, a confirmatory experiment was conducted using the same ten matched pairs of cell lines, the same procedures and protocols as in the previous experiment. Similar trends values were obtained by the two scorers. However, due to concerns about variability, the heterochromatin size variation data for the two scorers was combined to increase the sample sizes and enhance the accuracy of the estimates. The results of the combined analyses are shown in Figure 1. In comparing the overall heterochromatin size variation of chromosomes 1 and 9, the variation in the breast cancer patient-derived cell lines was significantly greater than the size variation in the controls. ($p<0.01$, one-tailed Mann–

Whitney U test). Figure 1a and 1b show the pair-wise comparisons between patient cell lines with their matched controls for chromosome 1 and chromosome 9; 70% (7/10) and 80% (8/10) of the aged and ethnicity matched pairs showed a larger frequency of size variations on chromosome 1 and 9, respectively, in the breast cancer patient-derived lymphoblastoid cell lines**.** As illustrated in Tables 2 and 3, when comparing the differences between each cell line derived from a breast cancer patient and its matched control, only BRCA2-3, BCS-8 and BCS-9 for chromosome 1 and BRCA2-3, BCS-8 and BCS-10 were not statistically significantly increased as compared to their matched control cell lines (Mann-Whitney U test, $p<0.05$). Among the 20 comparisons performed, the cells from the breast cancer patients showed significantly greater variation than those of the matched controls in 14 cases – 7 for the chromosome 1 comparisons and 7 for chromosome 9 comparisons. In general, chromosome 1 showed a modestly higher variability between homologs than chromosome 9.

Induction of Variability in the Size of the Heterochromatin Between Homologs due to Chemical Treatments

Since environmental factors may contribute to the development of breast cancer, we decided to investigate if the size variations of the heterochromatin could be due to breakage and recombination caused by exposure to chemicals. We chose MMC and MEL, cross-linking agents that have been reported in the literature to target the heterochromatin regions of chromosomes 1 and 9 (Tomasz 1995, Povirk and Shuker 1994). In addition to determine if only chemicals that generate ICLs cause increased size

variability in the heterochromatin, we also tested CEA, a monofunctional alkylating agent that has a similar structure to MEL but can only form single DNA adducts, and is unable to form ICL (Wijen et al., 2000).

The effects of chemical treatment with MMC, MEL and CEA on the heterochromatin size variations of chromosomes 1 and 9 are shown in Figures 3 and 4, respectively. We hypothesized that MMC and MEL treatments would increase the size variability of the heterochromatin observed in breast cancer patient-derived cell lines and that treatment with CEA would have no effect.

As shown in Figure 3, a consistent increase in the size variability of the chromosome 1 homologs was not observed in the breast cancer patient-derived cell lines following treatment with the various alkylating agents. With treatment, significant increases in chromosome 1 heterochromatin variability were occasionally seen. However, these changes were relatively modest in magnitude and were not consistent across chemical treatments or by cell origin. In contrast, two of breast cancer patient-derived cell lines showed striking increases in chromosome 9 heterochromatin variability after chemical treatment with each of the chemicals, as seen in Figure 4 (see BRCA1-1, $p<0.01$ and BCS-5, $p<0.01$). This hyper-increase in variability for the chromosome 9 homologs was consistently observed in the two patient derived cell lines following treatment with both MMC and MEL was well as with CEA. Representative metaphase images following chemical treatment following hybridization with a CY3-labeled probe to the heterochromatin on chromosome 1 and FITC-labeled probe to the heterochromatin on chromosome 9 are shown in Figure 5.

Detection of micronucleated cells induced by mitomycin C, melphalan, 2-chloroethylamine, and methyl methanesulfonate

To determine whether the observed variability was due to an unusual sensitivity of the two responsive cell lines to DNA breakage affecting the entire genome, the micronucleus assay was used to detect breakage induced by MMC, MEL, CEA and another alkylating agent, MMS in the two sensitive cell lines, and the results were compared with those from two matched control cell lines per sensitive cell line. As shown in Figure 6, the two sensitive patient-derived cell lines exhibited similar, and not particularly sensitive responses to the chemical treatments as compared to the controls. Significant increases in micronuclei were detected for some of the cell lines but the increases were modest in magnitude and similar to those seen in the controls (with the exception of Control-5 as discussed below). For example, BRCA1-1 showed a statistically significant increase in the frequency of micronuclei following MEL and MMC treatments, both ICL-inducing agents, but not for treatments with CEA or MMS.

Unexpectedly, Control-5 exhibited a particularly noticeable increase in the frequency of micronuclei following treatment with each of the chemicals (Cochran-Armitage trend test, <0.001); the untreated micronucleus frequency for this cell line is also above the normal baseline frequency for micronuclei and suggests that this cell line may have an inherent sensitivity to DNA damage. We therefore concluded that it would not be an appropriate healthy control for future studies using BCS-5. As a result, for the later experiments, two other control cell lines were used for comparison for each of the sensitive cell lines derived from the breast cancer patients.

Measures of cytotoxicity

To characterize the cytotoxic effects of MMC, MEL, CEA and MMS on the different cell lines at the concentrations used in the various tests, the replicative index (RI) was calculated on the cells harvested for the micronucleus assay. The percent toxicity at each test concentration for the 4 chemicals is reported above each bar in Figure 6. Typically modest levels of cytotoxicity (<25%) were observed for the all four chemical treatments across the eight cell lines treated. The lowest RI observed was 62%, which was only seen at the highest dose of MEL for the Control-1 cell line (Figure 6b). The cytotoxicity results were otherwise similar for all other chemical treatments across the cell lines. These results indicate that the breast cancer patient cell lines and particularly, the two responsive cell lines, were not especially sensitive to the cytotoxicity effects of the test chemicals.

Time Point of Induction of Variability in the Size of the Heterochromatin Between Homologs due to Chemical Treatments

In the chemical experiments described above, the initial sample, taken to measure variability between homologous chromosomes, was collected at 192 hr after the 1st chemical treatment. To determine when the increased size variability first appeared in the two sensitive cell lines, a time course experiment was performed using MMC as the inducing agent. The results of the time course study on the chromosome 9 heterochromatin size variations are shown in Figure 7. Both the responsive breast cancer patient-derived cell lines exhibited a major and statistically significant induction of size

variability after a single treatment with MMC in 0.1% DMSO. Surprisingly, a similar effect was also seen with the DMSO only treatment at the 24 hr harvest time ($p < 0.01$). At 48 hr, the two patient-derived cell lines treated with MMC continued to exhibit the high size variability whereas for those that had been treated with DMSO only, the variability had returned to near basal levels. The only statistically significant response to MMC treatment in the control cell lines was seen in Con-2 at the 48 hr time point ($p<0.01$).

Discussion

Previous research using conventional cytogenetic techniques has frequently reported that the constitutive heterochromatin regions in normal lymphocytes isolated from breast cancer patients exhibited an increase in size variability when compared to those of control individuals; these constitutive heterochromatin regions on chromosome 1 and 9 are very susceptible to breaks, exchanges and pairing after treatment with ICL-inducing agents. Breakage induced by ICL-generating chemicals could lead to inaccurate DNA repair within the region and that this could underlie the increased variability seen between homologous chromosomes 1 and 9 in cell lines derived from the breast cancer patients. The main objectives of our study were to confirm the initial results seen in earlier studies, to assess a new FISH and imaging technique developed in our laboratory, and to test the DNA damage hypothesis by characterizing the response of lymphoblastoid cell lines to treatment with ICL inducing agents MMC and MEL and the monofunctional alkylating agent CEA.

One goal of our study was to evaluate the utility of using our new developed FISH plus image analysis approach. The earlier cytogenetic techniques used to measure heterochromatin size are not likely to be as accurate and precise as the newly developed technique we used in this series of studies. in the previous studies that linked increased size variability with an increase risk of developing malignant diseases, cytogenetically stained metaphase spreads were photographed, the developed slides were projected onto a flat surface (i.e. a screen or blank wall), and the length of the heterochromatin was measured using a ruler. Using this approach, the boundaries between the dark stained heterochromatin regions and the light stained euchromatin regions would likely be hard to discern. These earlier studies also evaluated small numbers of cells per subject evaluated. Fluorescence *in situ* hybridization (FISH) allows the regions of interest to be directly targeted using highly specific fluorescently labeled probes. The benefit of using FISH probes to detect the heterochromatin band is that metaphase images can be digitally captured using a camera attached to the fluorescent microscope and the size of the heterochromatin bands can be precisely measured using image software. This technique also allows a larger number of cells to be evaluated per subject.

We then assessed the technique developed in our laboratory. Generally, similar results were seen by the two scorers. Differences were seen approximately 25% of the time, which while not ideal, were considered to be acceptable since the evaluations by the two scorers were performed at different times, months to years apart. When differences were observed, it was not clear whether these were due to differences between the scorers, changes within the cell lines, or technical differences in how the measurements

were performed. Validation studies have indicated that the technique itself is quite reproducible (data not shown). Indeed, the heterochromatin size variation in the patient and control cell lines in the experiments shown in Figures 3, 4 and 7, which were conducted independently of those shown in Figure 1, confirm that the heterochromatin size variation in the patient-derived cell lines is greater than that seen in the control cell lines. Given the similarity in results, the data from the two scorers was combined in our analyses. As shown in Figure 1 and Tables 2 and 3 for the combined analysis, 7 out of 10 cell lines derived from breast cancer patients had greater variability for both chromosomes 1 and 9 than seen in the matched controls. Overall, the breast cancer patient-derived cell lines exhibit an increase in heterochromatin size variation when compared to the matched controls (Mann-Whitney, $p<0.01$).

The overall results of increased variability in heterochromatin regions of chromosome 1 and 9 in cells from breast cancer patients over matched controls confirms earlier studies that observed increased size variability in the heterochromatin regions of chromosomes 1, 9 and 16 in cells from patients with malignant diseases (Atkin NB 1977, Atkin et al., 1977a, Atkin et al., 1977b). The results from our studies as well as the earlier studies suggest that monitoring size variability in the heterochromatin regions of chromosome 1, and 9 may be useful as a biomarker to identify women with an increased susceptibility to breast cancer.

Our initial hypothesis was that hyper-variability seen in the heterochromatic regions between homologous chromosomes may due to inaccurate DNA repair of ICL. Low DNA repair capacity has been postulated to be a susceptibility factor for breast

carcinogenesis (Ramos 2004, Roy 2000). Interstrand crosslinking agents cause a wide variety of lesions and repair of the damage requires the coordination of nucleotide excision repair, homologous recombination, non-homologous end joining, and translesion synthesis repair pathways (Deans and West, 2011). Cells with low DNA repair efficiency will not be able to repair ICL-induced lesions appropriately and subsequent genotoxic damage could lead to carcinogenesis.

To test whether exposure to endogenous or exogenous crosslinking agents could induce increased heterochromatin variability, we treated the lymphoblastoid cell lines with a DNA crosslinking agents, some that specifically target the constitutive heterochromatin as well as an agent that only induces DNA adducts. As illustrated in Figures 3 and 4, MMC, MEL and CEA all have very similar patterns of inducing hyper-variability within the constitutive heterochromatin regions. No consistent effects were seen for chromosome 1 but two of the patient-derived cell lines showed a striking increase in heterochromatin variability following treatment with the 3 agents. These results demonstrate that DNA crosslinking is not required for the induction of the heterochromatin variation as effects were seen with CEA, a monofunctional alkylating agent that does not form DNA crosslinks. We postulated that some other DNA repair mechanism or another cellular mechanism is involved in increasing the size variability in the homologous chromosomes 9 in the two sensitive cell lines.

In recent years it has been discovered that in response to genotoxic lesions, a complex series of signaling cascades results in the relaxation of condensed heterochromatin to allow DNA repair proteins access to the damaged regions (Chiolo et

al., 2011, Murray et al., 2012, Greenberg 2011). Chiolo et al. (2011) who investigated the kinetics of DSB repair in heterochromatin, reported that within 40 minutes after genotoxic insult, the heterochromatin expands. This suggests that cells can rapidly respond to DNA damage by modifying the chromatin structure around DNA lesions in order to allow DNA repair proteins access to damaged sites. Because increased heterochromatin variability was observed within 24 hours after treatment with MMC, the observed variability may be a cellular response to DNA damage. However, because variability was measurable in only chromosome 9 and that this measurable effect continued to be seen 48 hours after treatment suggests that this is not a genome-wide and short-term effect.

As mentioned previously, approximately 10% of breast cancer cases are caused by mutations in genes such as BRCA1 and BRCA2. The corresponding BRCA1 and BRCA2 proteins help maintain genomic stability through an involvement in DNA double strand break repair and homologous recombination (Ciccia et al 2010). Researchers have proposed that repair of DNA lesions caused by ICL-inducing agents within the constitutive heterochromatin involves the homologous recombination pathway (Abdel-Halim et al 2005, Rothfuss and Grompe 2004). It seemed likely that mutations in BRCA1 or BRCA2 could have affected our results because of the roles of both BRCA1 and BRCA2 in repairing ICL induced lesions. However, our data suggest that following treatment with MEL, MMC or CEA, the heterochromatin expansion or deletion in repetitive sequences is independent of BRCA1 status as the two cell lines exhibiting hyper-variability between homologous chromosomes 9 have different BRCA1 status.

BRCA1-1 (GM13709) has a BRCA1 mutation in 2187delA in exon 11 leading to a frameshift at codon 690 and a truncation at codon 700. BRCA1 is 1863 amino acids long; a truncation at codon 700 will result in a loss of function in the BRCT region that is essential for maintaining genomic stability and DNA damage response (Yarden 2006). BCS-5 (GM13870), the other hyper-variable cell line is from a breast cancer survivor that has a family history of breast cancer; however, the BRCA1 and BRCA2 status is normal. Cell lines with BRCA-2 mutations (BRCA2-3 and BRCA2-4) did not exhibit increased heterochromatin variability in response to the chemical treatments. We currently do not understand why only two of the breast cancer patient-derived cell lines were hyper-variable and why the other breast cancer patient derived cell lines did not show similar effects.

One of the surprising results in the experiment is the difference in sensitivity between chromosome 1 and chromosome 9 to treatments with the alkylating agents. Band q12 of chromosome 1 contains classical satellite (Csat) II and a minor amount of Csat III whereas the band q12 of chromosome 9 is comprised of only Csat III. Similarly the FISH probes used to target chromosome 1 consisted of DNA complementary to both Csat II and Csat III sequences. In contrast, the FISH probes used to label chromosome 9 consist of DNA sequences from Csat III only. Our results suggest that MMC, MEL and CEA may specifically target Csat III DNA sequences that are present within the 9q12 band. A possible reason why we did not detect increased variability in the Csat III region of chromosome 1 is due to the lack of specificity of the FISH probe used. A change in variability may have occurred within the Csat III region of chromosome 1 but because of

the small size of the region and the non-specific nature of our probe, we were unable to detect the relatively small change during our analysis.

As mentioned above the Csat III sequences in chromosome 1 border the junction between the bands q12 and q21. The most frequent chromosomal abnormality seen in breast cancer cells is a gain of the long arm of chromosome 1 (+1q) (Kallioniemi et al., 1994, Loo et al., 2004) with breakpoints in the band q21. Increased size variability in the Csat III sequences on chromosome 1 may be a result of a change to the heterochromatin structure. A change to the chromatin structure at the 1q12, 1q21 boundary may alter the genomic stability of that region leading to chromosomal alterations. This could explain the origins of the subset of breast cancer cases that have the alterations to chromosome 1 with breakpoints adjacent to the q21 band.

Another possible explanation for the unique response of chromosome 9 as compared to chromosome 1 is that there may be a Csat III-specific process affected by these DNA damaging agents that does not occur in Csat II. It has recently been shown that under cellular stress cells induce transcription of Csat III repeats such as those present in the heterochromatin region of chromosome 9 (Valgardsdottir et al., 2008, Sengupta et al., 2009). Valgardsdottir et al. treated cells with a range of DNA damaging agents, methyl methanesulfonate, UVc and etoposide, along with other cellular stressors like heat shock, cadmium and aphidicolin. Their results indicate that an activation of transcription of Csat III sequences is a general phenomenon and can be triggered by a wide range of stressors. However, the similarity in cytotoxicity, an indicator of cellular stress, in the sensitive and non-sensitive cell lines measured in this study suggest that it is

unlikely that a cellular response to stress alone is responsible for the increased size variability seen between homologous chromosomes.

To characterize the sensitivity of the cell lines to DNA breakage, we measured the amount of global DNA breakage following chemical treatment using the micronucleus assay. Our results indicate that the two sensitive cell lines are not unusually sensitive to chemical treatment and that the concentrations used produced moderate levels of cytotoxicity. As illustrated in Figure 6, the two sensitive patient-derived cell lines exhibited similar patterns in the induction of micronuclei following treatment with the various chemicals as compared with the other cell lines. While the two sensitive cell lines from the breast cancer patients exhibited a significant increase in micronuclei when treated with the ICL-inducing agents MMC and MEL, the increases were modest in magnitude and were similar to those seen in the control cell lines. . No increase in micronuclei was seen when the two patient derived cell lines were treated with CEA, a monofunctional alkylating agent and with MMS, a methylating agent. These results could be due to the relative potency of the test agents as MMC and MEL are potent clastogens whereas CEA and MMS are less efficient chromosome-breaking agents. It should be noted that the agents and doses that did not induce breakage as measured by the micronucleus assay were sufficient to induce increases in hyper-variability between homologous chromosomes 9, as measured by the FISH and Image J method. This observation suggests that breakage within the constitutive heterochromatin may not be needed to induce the heterochromatin hyper-variability.

As seen in Figure 6, the cell line, Control-5 (GM01814), and its the age-, ethnicity- and sex-matched comparison cell line BCS-5 (GM13780) had a noticeably higher basal level of micronuclei. However, this elevated level of micronuclei did not result in an increase in variability between homologous chromosomes 1 or 9 again suggesting that DNA breakage may not be necessary for increased heterochromatin variability. The Control-5 cell line was highly sensitive to chemical treatments and also exhibited a high incidence of spontaneous breaks. Consequently, we decided that it would not be an appropriate control for subsequent studies with BCS-5.

The levels of cytotoxicity induced by the chemical treatments were assessed to determine if the observed increase in variability could have been a secondary effect of cytotoxicity. The concentrations used to induce heterochromatin variability between homologous chromosomes produced little to moderate levels of cytotoxicity as measured by the replicative index. In recent years, concerns have been raised about chromosome damage occurring as secondary effect due to nuclease activation in dying cells when excessive cytotoxicity occurs during an experiment (Galloway 2000). To address this issue, the Organisation for Economic Cooperation and Development recommends that the highest test concentration for the MN assay not exceed 55% toxicity (OECD 2010). Similarly, the most recent International Conference on Harmonisation (ICH) S2R1 draft guideline for *in vitro* genotoxicity studies recommends that inhibition of cell proliferation as measured by cell growth not be greater than about 50% (ICH, 2008). As shown in Figure 6, cytotoxicity in our study did not exceed 50% at the highest test concentrations for MMC, MEL, CEA or MMS. For the two cell lines that exhibited the hyper-variability

between homologous chromosomes 9, the % RI was low; for example the highest amount of cytotoxicity measured was 31% (RI of 69%; BCS-5, 0.05 μM MMC). At the highest concentration used in the micronucleus assay, the same concentration used for the time course experiments, significant induction of hyper-variability in the heterochromatin regions of chromosome 9 was seen in the two sensitive cell lines for all chemical treatments (Figure 4), yet only modest increases in cytotoxicity were seen, implying that these were not effects that occurred secondary to cytotoxicity.

To determine the time point at which variability was induced in the two hyper-sensitive cell lines from breast cancer patients a time course experiment was conducted. Six cell lines; the two sensitive cell lines from the breast cancer patients and two matched controls per breast cancer patient cell line were treated with MMC in 0.1% DMSO and with 0.1% DMSO. As illustrated in Figure 7, induction of hyper-variability occurred within 24 hr of treatment initiation and persisted until 48 hr. The surprising result of this experiment is that when the two sensitive cell lines from the breast cancer patients were treated with DMSO, the solvent used in time course experiments, heterochromatin variability was also induced at the 24 hours. After the wash out of DMSO from the cultures at 24 hr after treatment and following incubation for additional 24 hr in DMSO free media, the measured heterochromatin hyper-variability returned to near control values. These results indicate that the DMSO-induced variability was a transient event and did not involve a change in DNA sequence. DMSO is commonly used in genotoxicity studies as a solvent to carry chemicals across cell membranes and into cells, and is widely regarded as non-genotoxic. The unexpected response of the two hyper-

sensitive cell lines to the DMSO treatment implies that the two cell lines may have unusual properties and suggest that the variability may be due to epigenetic changes such as chromatin remodeling and is not the result of a change in DNA sequence.

A focus for future studies could be on characterizing the epigenetic differences between the cell lines with a goal to identify which cellular processes could be causing the increased variability between homologs of chromosome 1 and 9 and be responsible for the overall increased variability in cells derived from breast cancer patients compared with matched controls. The constitutive heterochromatin has many epigenetic markers that help to maintain a condensed heterochromatin state throughout the cell cycle. Constitutive heterochromatin is characterized by high amounts of DNA methylation, tri-methylation of serine 9 residue on histone 3 ($H3K9(me)_3$), binding of HP1, a non-histone protein that binds to $H3K9(me)_3$, and hypo-acetylation of histone proteins (Craig 2004). Future studies could also incorporate techniques to measure DNA methylation such as methylation-specific PCR, HPLC, microarray technologies or immunochemical approaches. Histone modifications in the cell lines could be measured using a ChIP assay or by an immunochemical assay. By studying these epigenetic markers and quantifying them across the different lymphoblastoid cell lines and across different treatments, it may be possible to identify the mechanism(s) underlying the increased heterochromatin size variability in the breast cancer patient-derived cells and the hyper-variability affecting chromosome 9 seen after chemical treatments in the two sensitive cell lines. As mentioned above, changes to the chromatin structure within the q12 band of chromosome

1 may alter the genomic stability of that region leading to chromosomal alterations and explain why a portion of breast cancer cases may arise.

Another approach that could be used for future studies would be to use microarray technology to investigate the potential mechanism behind the increased variability in cell lines derived from breast cancer compared to matched controls. Relatively little information is available on the lymphoblastoid cell lines that have been used in this study. A microarray approach could help identify differences in gene expression between the responding and non-responding cell lines and may help identify the cellular pathway(s) involved.

In summary, results from new and the earlier studies were compared and were found to be similar, confirming previous reports. The constitutive heterochromatin region was found to be significantly more variable in 7 of the 10 breast cancer patient-derived cell lines when examining both chromosomes 1 and 9. Two of the six cell lines from the breast cancer patients showed variability in the heterochromatin region of chromosome 9 that was notably greater than that seen in the matched controls following treatment with both bifunctional and monofunctional alkylating agents, indicating that the increase was not dependent upon the formation of DNA crosslinks. The two hyper-sensitive cell lines did not display a major increase in alkylating agent-induced chromosome breakage in the micronucleus assay indicating that variability is not likely to be due to inaccurate repair of genome-wide DNA breaks. The increases in heterochromatin variability in the two sensitive cell lines were seen at both 24 and 48 hr after treatment with mitomycin C, a potent cross-linking agent. Interestingly, similar but only a transient increase only at 24

hours were also seen with DMSO treatment in the sensitive cells lines, and not controls, suggesting that the observed increases are not due to a change in DNA sequence but possibly due to epigenetic changes occurring in the sensitive cell lines from breast cancer patients.

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Table 1. Cell lines derived from familial breast cancer survivors (BCS) or survivors diagnosed with BRCA1 or BRCA2 mutations, together with their age-, ethnicity, and sex-matched control cell lines. Ten pairs of cell lines were used.

Cell Line	Label/Mutation	Age	Race	Sex	Used for Chemical Treatments
GM13709	BRCA1-1	32	Caucasian	F	Yes
GM05380	Control-1	32	Caucasian	F	Yes
GM14091	BRCA1-2	46	Caucasian	F	Yes
GM14452	Control-2	46	Caucasian	F	Yes
GM14622	BRCA2-3	39	Caucasian	F	
GM10924	Control-3	39	Caucasian	F	
GM14626	BRCA2-4	37	Caucasian	F	Yes
GM14807	Control-4	35	Caucasian	F	Yes
GM13870	BCS-5	43	Caucasian	F	Yes
GM01814	Control-5	44	Caucasian	F	Yes
GM13806	BCS-6	45	Black	F	
GM14439	Control-6	45	Black	F	
GM13639	BCS-7	46	Caucasian	F	
GM14453	Control-7	46	Caucasian	F	
GM13643	BCS-8	42	Black	F	Yes
GM14508	Control-8	41	Black	F	Yes
GM13869	BCS-9	44	Caucasian	F	
GM01954	Control-9	44	Caucasian	F	
GM13790	BCS-10	51	Caucasian	F	Yes
GM01990	Control-10	51	Caucasian	F	Yes

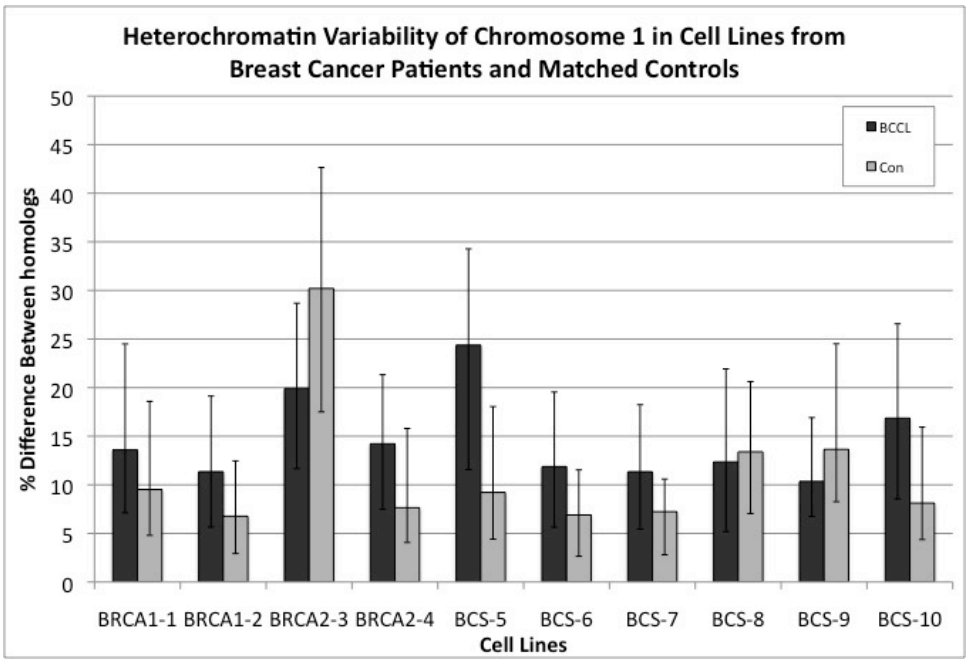
Table 2: Comparison of the heterochromatin variability results for chromosome 1 between cell lines derived from breast cancer patients and matched controls using a 1-tailed Mann-Whitney U test.

Cell Line	Abbrev.	Cell Line	Abbrev.	z-score	P-value
GM13709	BRCA1-1	GM05380	Control-1	3.26	0.0006
GM14091	BRCA1-2	GM14452	Control-2	4.73	<0.0001
GM14622	BRCA2-3	GM10924	Control-3	<0	NS
GM14626	BRCA2-4	GM14807	Control-4	4.64	<0.0001
GM13870	BCS-5	GM01814	Control-5	7.32	<0.0001
GM13806	BCS-6	GM14439	Control-6	4.27	<0.0001
GM13639	BCS-7	GM14453	Control-7	3.89	0.0001
GM13643	BCS-8	GM14508	Control-8	<0	NS
GM13869	BCS-9	GM01954	Control-9	<0	NS
GM13790	BCS-10	GM01990	Control-10	5.34	<0.0001

Table 3: Comparison of the heterochromatin variability results for chromosome 9 between cell lines derived from breast cancer patients and matched controls using a 1-tailed Mann-Whitney U test.

Cell Line	Abbrev.	Cell Line	Abbrev.	z-score	p-value
GM13709	BRCA1-1	GM05380	Control-1	4.16	<0.0001
GM14091	BRCA1-2	GM14452	Control-2	2.29	0.011
GM14622	BRCA2-3	GM10924	Control-3	<0	NS
GM14626	BRCA2-4	GM14807	Control-4	2.88	<0.0001
GM13870	BCS-5	GM01814	Control-5	4.62	<0.0001
GM13806	BCS-6	GM14439	Control-6	2.280	0.0113
GM13639	BCS-7	GM14453	Control-7	2.93	0.017
GM13643	BCS-8	GM14508	Control-8	<0	NS
GM13869	BCS-9	GM01954	Control-9	3.01	0.0013
GM13790	BCS-10	GM01990	Control-10	0.47	NS

(a)


Heterochromatin Variability of Chromosome 1 in Cell Lines from Breast Cancer Patients and Matched Controls
% Difference Between homologs
0
5
10
15
20
25
30
35
40
45
50
BCCL
Con
BRCA1-1
BRCA1-2
BRCA2-3
BRCA2-4
BCS-5
BCS-6
BCS-7
BCS-8
BCS-9
BCS-10
Cell Lines

(b)

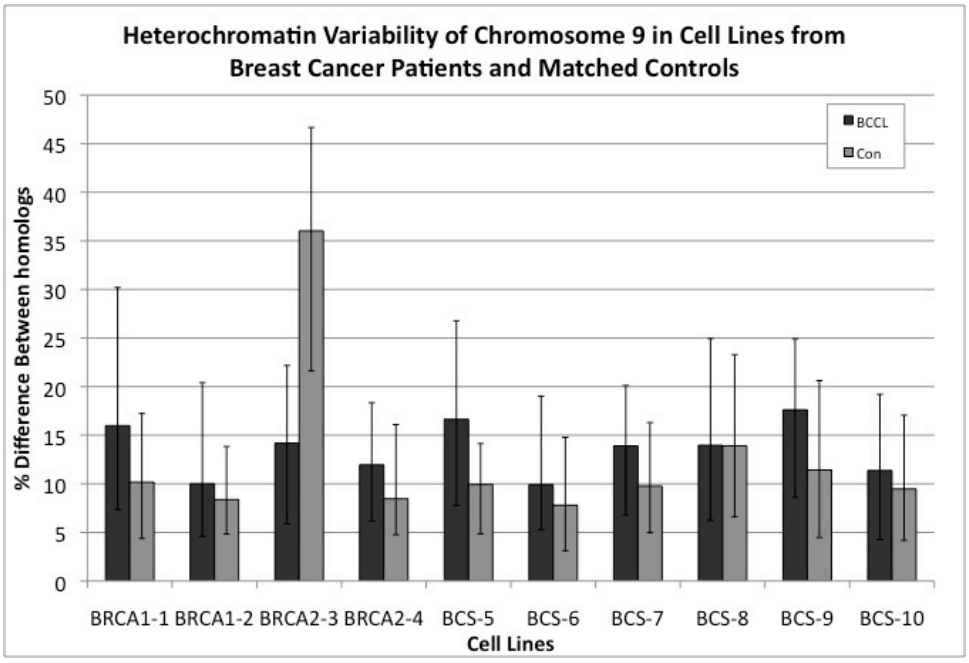

Heterochromatin Variability of Chromosome 9 in Cell Lines from Breast Cancer Patients and Matched Controls
% Difference Between homologs
0
5
10
15
20
25
30
35
40
45
50
BCCL
Con
BRCA1-1
BRCA1-2
BRCA2-3
BRCA2-4
BCS-5
BCS-6
BCS-7
BCS-8
BCS-9
BCS-10
Cell Lines

Figure 1. Histograms showing the percent difference in heterochromatin variability involving (a) chromosome 1 and (b) chromosome 9 for the ten pairs of breast cancer patient-derived cell lines (BCCL, labeled in black) and their matched controls (Con, labeled in white). BRCA1 – cell line from a breast cancer patient with a BRCA1 mutation; BRCA2 – cell line from a breast cancer patient with a BRCA2 mutation; BCS – cell line from a familial breast cancer patient. The medians and interquartile ranges are shown.

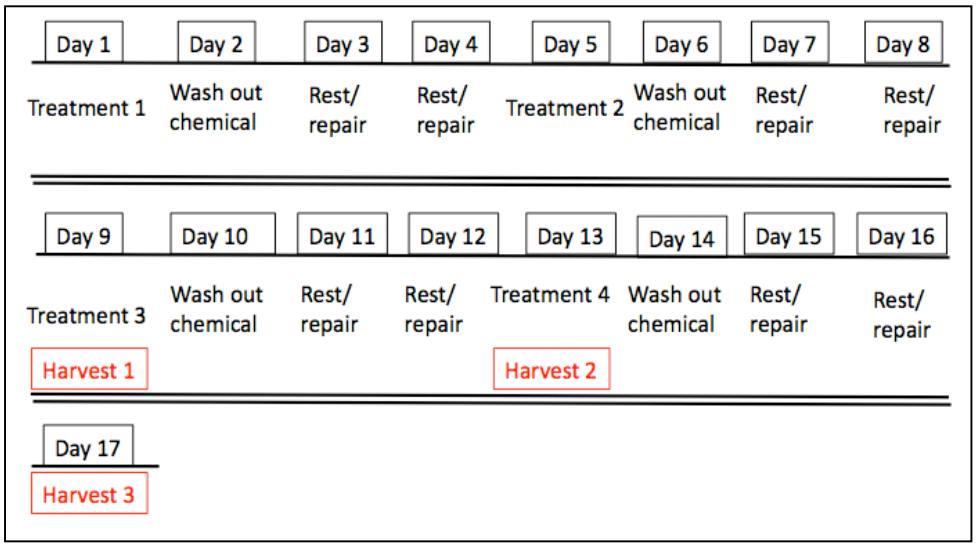
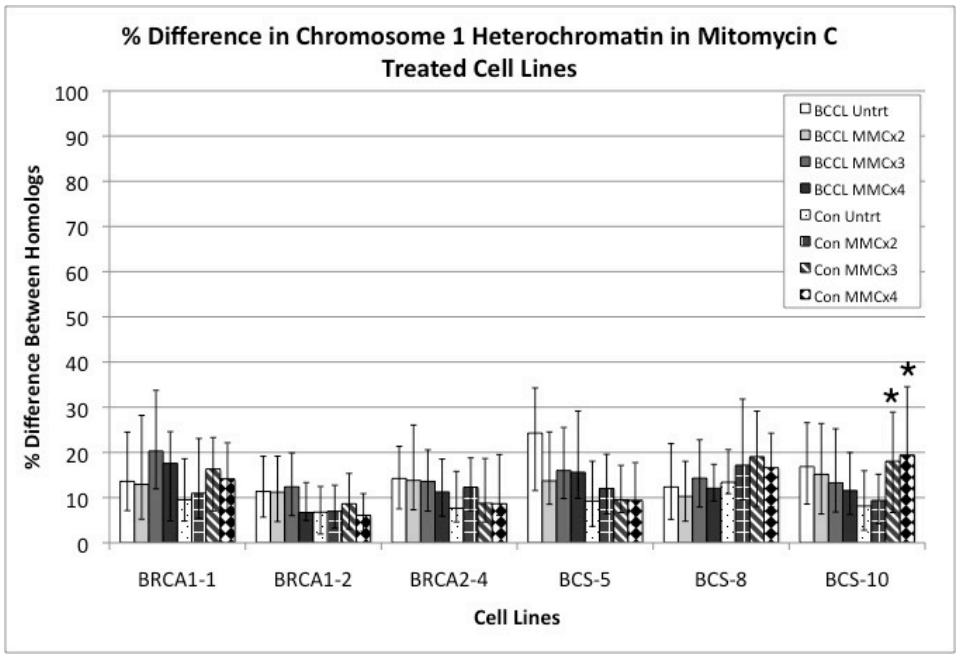
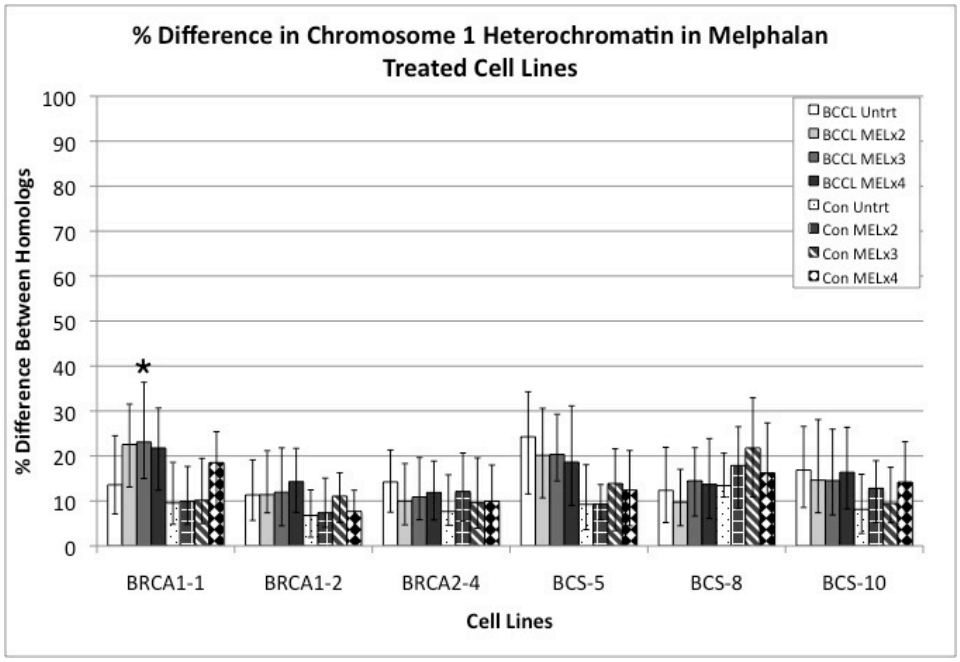


Figure 2. Protocol for chemical time course studies. See Materials and Methods for a more complete discussion. Briefly, cell lines were treated for 24 hours four times with the test chemical on day 1, day 5, day 9 and day 13. After 24 hours, the chemical was washed out of the cultures and left to rest/repair for two days before additional treatments. Cells were harvested on day 9, day 13 and day 17.

(a)


% Difference in Chromosome 1 Heterochromatin in Mitomycin C Treated Cell Lines
% Difference Between Homologs
100
90
80
70
60
50
40
30
20
10
0
BCCL Untrt
BCCL MMCx2
BCCL MMCx3
BCCL MMCx4
Con Untrt
Con MMCx2
Con MMCx3
Con MMCx4
*
*
BRCA1-1
BRCA1-2
BRCA2-4
BCS-5
BCS-8
BCS-10
Cell Lines

(b)


% Difference in Chromosome 1 Heterochromatin in Melphalan Treated Cell Lines
% Difference Between Homologs
100
90
80
70
60
50
40
30
20
10
0
BCCL Untrt
BCCL MELx2
BCCL MELx3
BCCL MELx4
Con Untrt
Con MELx2
Con MELx3
Con MELx4
*
BRCA1-1
BRCA1-2
BRCA2-4
BCS-5
BCS-8
BCS-10
Cell Lines

(c)

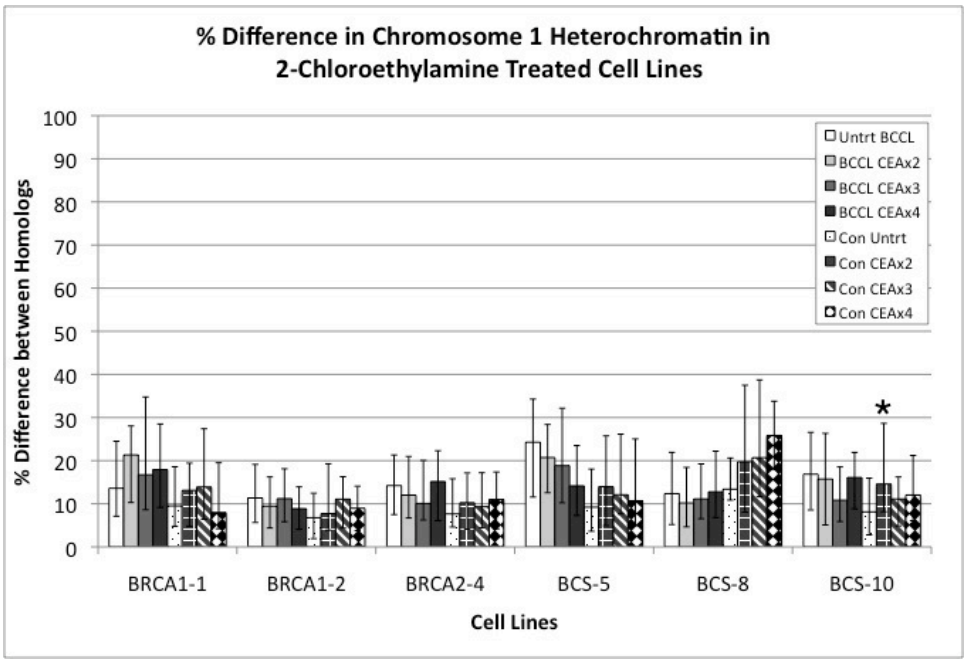
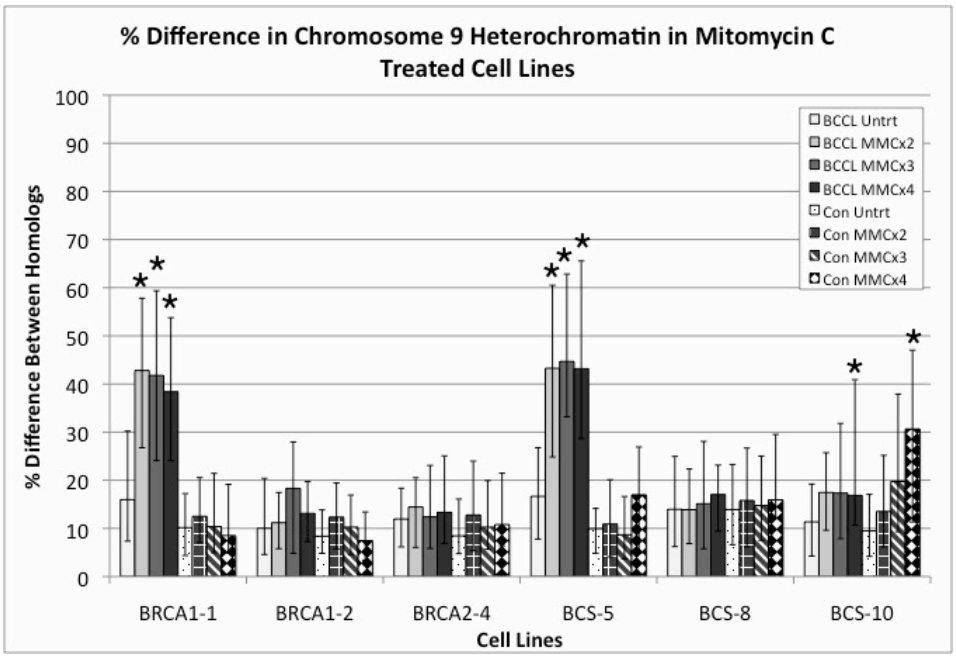
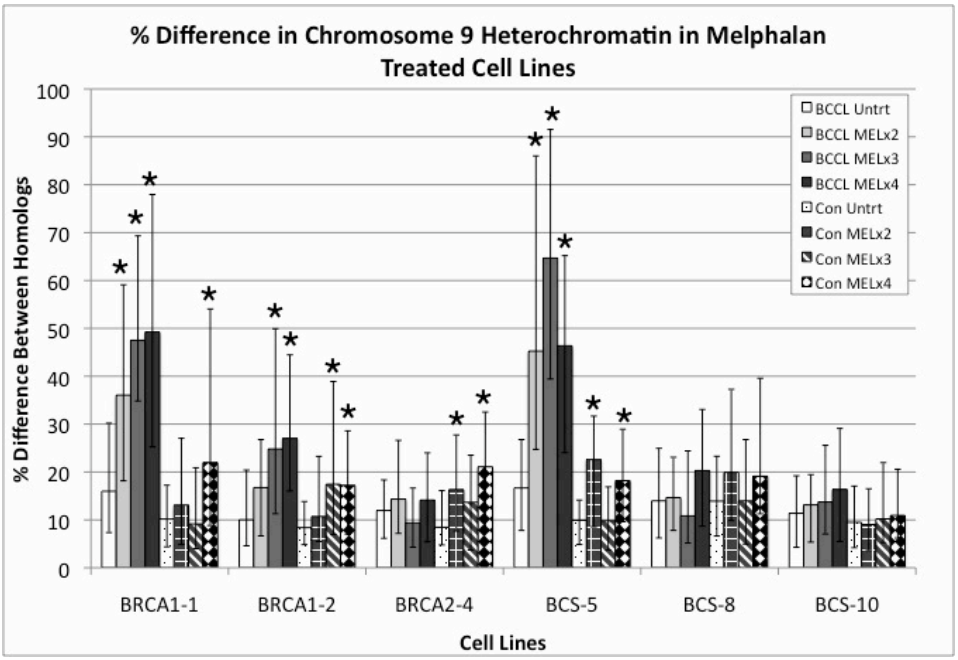


Figure 3: Comparisons of heterochromatin variations on chromosome 1 following treatments with (A) MMC (B) MEL and (C) CEA in six pairs of cell lines exhibiting a BRCA1 mutation, BRCA2 mutation, familial breast cancer with their respective controls. Histograms include breast cancer patient-derived cell lines before treatment (BCCL Untrt, labeled in white), after two rounds of chemical treatment (BCCL chemx2, labeled in light grey), after three rounds of chemical treatment (BCCL chemx3, labeled in dark grey), after four rounds of chemical treatment (BCCL chemx4, labeled in black), their matched controls before treatment (Con Untrt, labeled in dot pattern), after two rounds of chemical treatment (Con chemx2, labeled in plaid pattern), after three rounds of chemical treatment (Con chemx3, labeled in diagonal pattern), and after four rounds of chemical treatment (Con chemx4, labeled in checkered pattern). The medians and interquartile ranges are shown. Asterisks denote statistically significant responses ($p<0.01$)

(a)


% Difference in Chromosome 9 Heterochromatin in Mitomycin C Treated Cell Lines
% Difference Between Homologs
100
90
80
70
60
50
40
30
20
10
0
BCCL Untrt
BCCL MMCx2
BCCL MMCx3
BCCL MMCx4
Con Untrt
Con MMCx2
Con MMCx3
Con MMCx4
BRCA1-1
BRCA1-2
BRCA2-4
BCS-5
BCS-8
BCS-10
Cell Lines

(b)


% Difference in Chromosome 9 Heterochromatin in Melphalan Treated Cell Lines
% Difference Between Homologs
100
90
80
70
60
50
40
30
20
10
0
BCCL Untrt
BCCL MELx2
BCCL MELx3
BCCL MELx4
Con Untrt
Con MELx2
Con MELx3
Con MELx4
BRCA1-1
BRCA1-2
BRCA2-4
BCS-5
BCS-8
BCS-10
Cell Lines

(c)

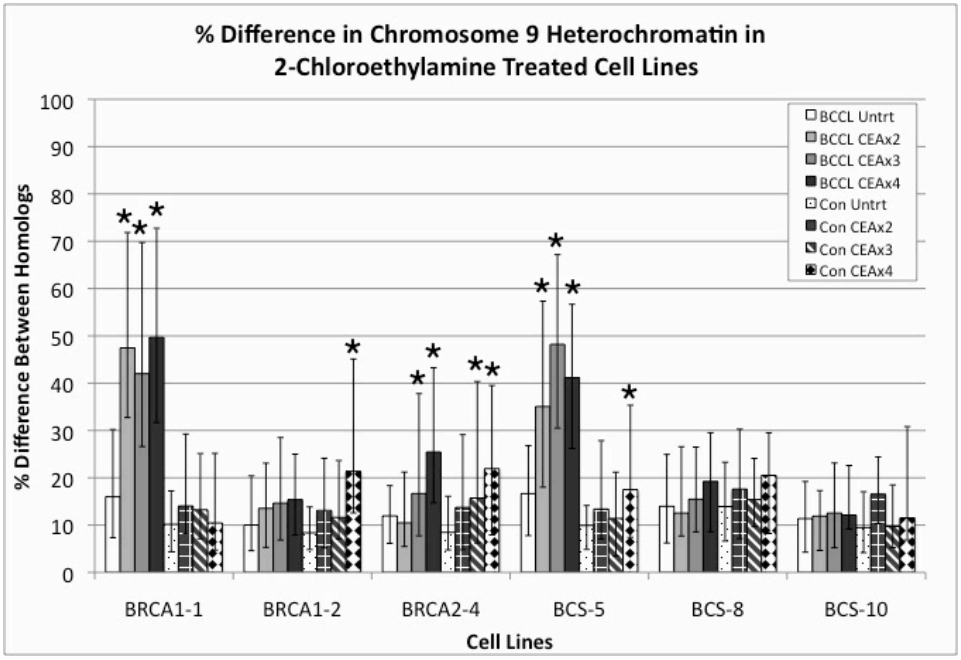
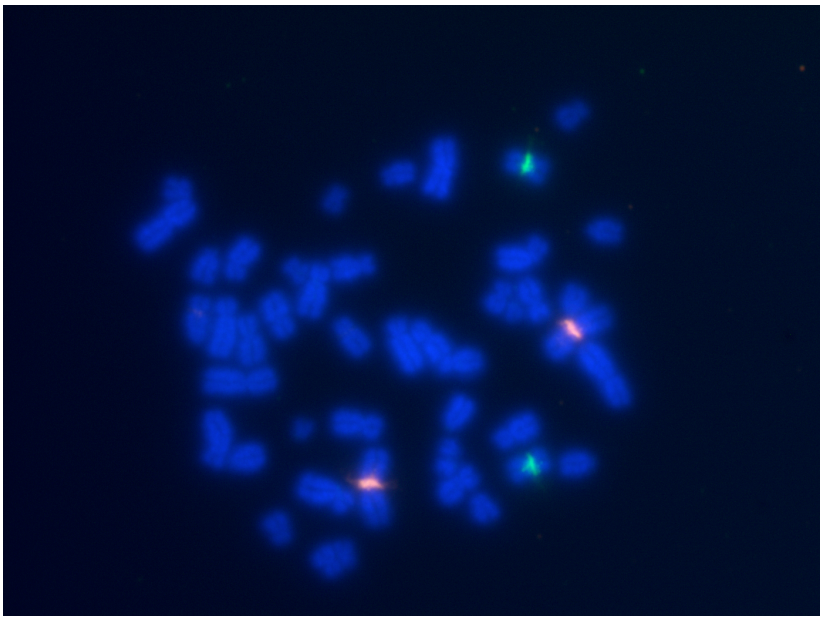
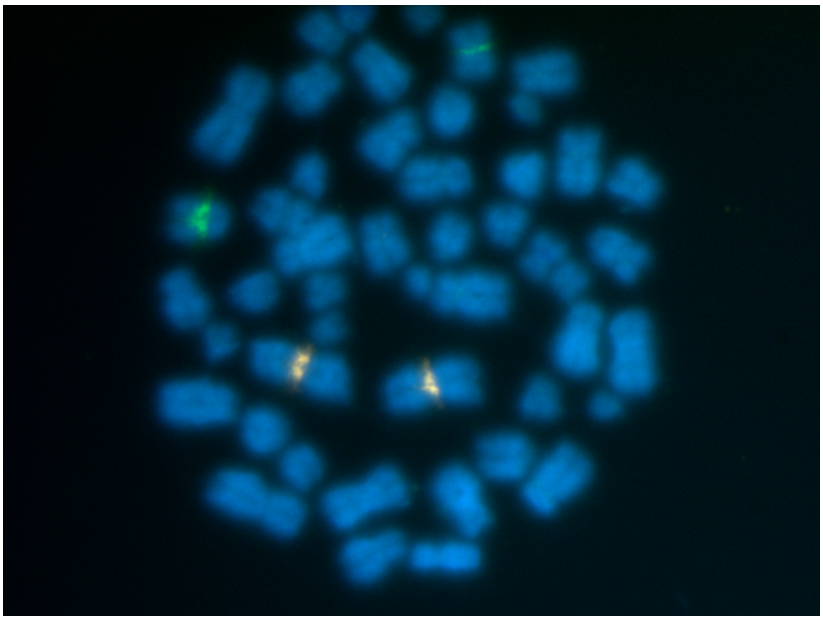


Figure 4. Comparisons of heterochromatin variations on chromosome 9 following treatments with (A) MMC (B) MEL and (C) CEA in six pairs of cell lines exhibiting a BRCA1 mutation, BRCA2 mutation, familial breast cancer with their respective controls. See the legend for Figure 3 for an explanation of the bar labeling. The median and interquartile ranges of the responses are shown. An asterisk denotes statistically significant response ($p<0.01$).

(a)



(b)



(c)

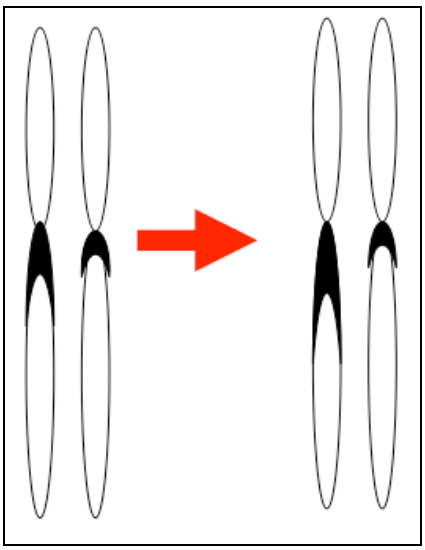
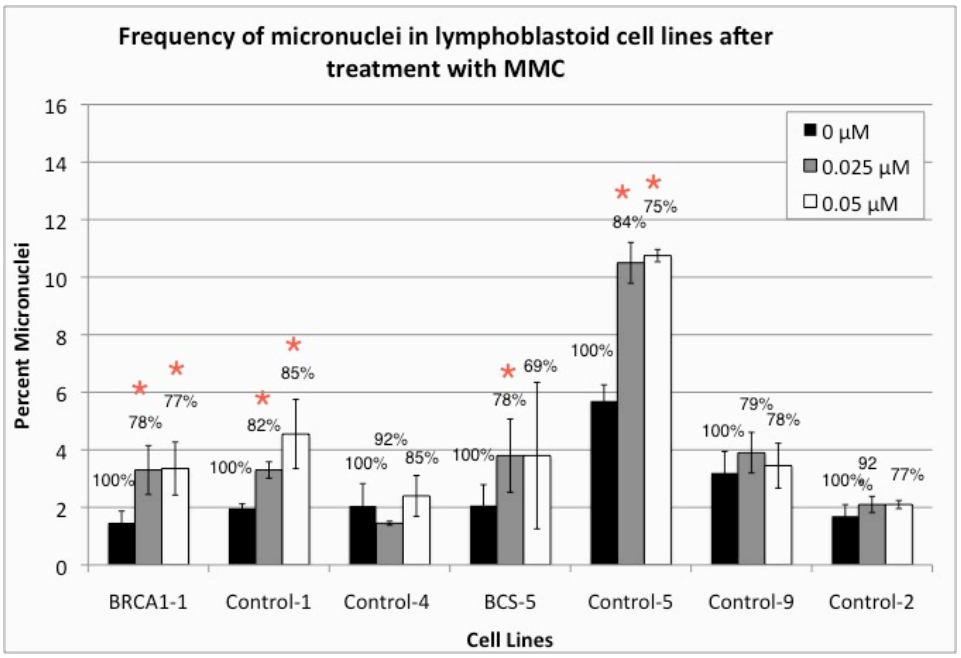
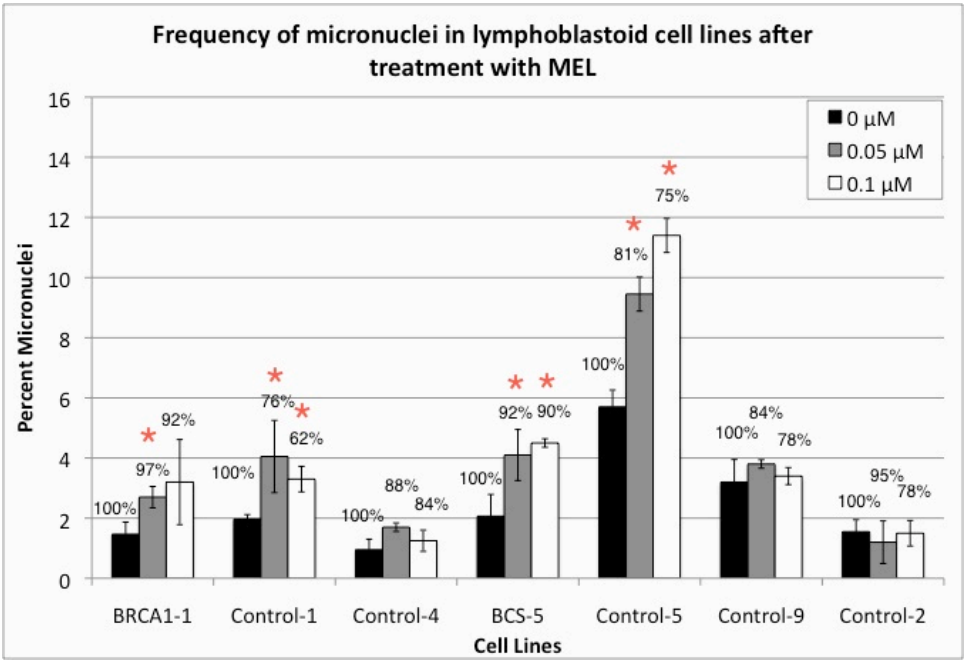


Figure 5. FISH analysis of classical satellite-containing heterochromatin region on chromosomes 1 and 9. Alexa-488-labeled classical satellite probe for chromosome 9 (green) and a CY3-labeled classical satellite probe for chromosome 1 (red) were used in the FISH analysis. Representative metaphases from an untreated breast cancer patient cell line (BRCA1, GM13709) (A) and a metaphase from the same patient-derived cell line treated twice with CEA (B). [C] An ideogram illustrating variability in size of the heterochromatin before and after treatments with the three alkylating agents. White areas represent the euchromatic regions of the chromosomes and the black areas represent the constitutive heterochromatin regions of the chromosome. The heterochromatin region on chromosome 9 shows a major difference in size between the chromosome homologs in the patient-derived cell line after treatment with CEA.

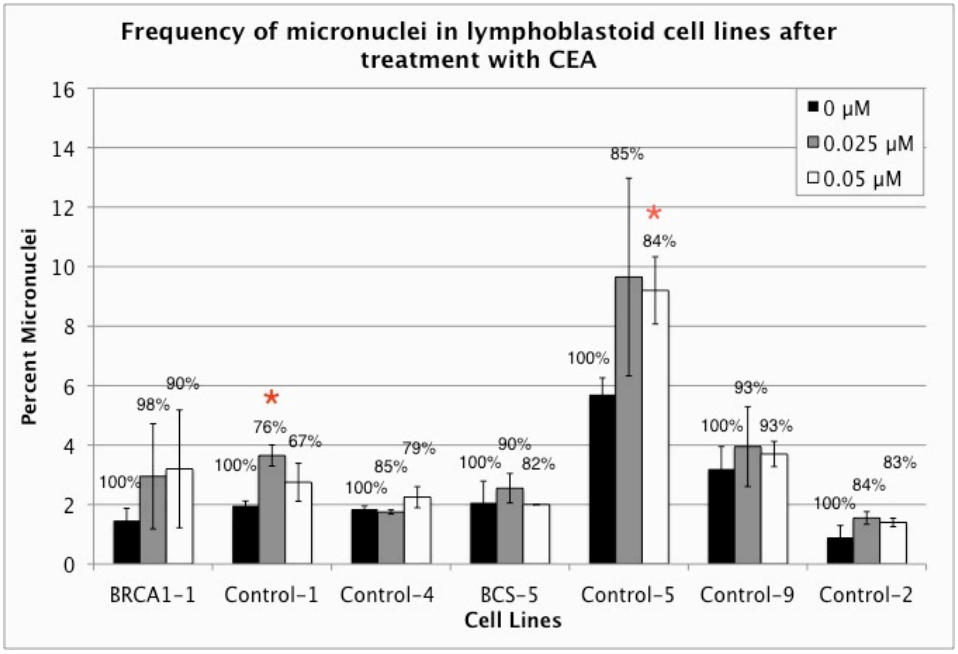
(a)


Frequency of micronuclei in lymphoblastoid cell lines after treatment with MMC
Percent Micronuclei
Cell Lines
0 µM
0.025 µM
0.05 µM
BRCA1-1
Control-1
Control-4
BCS-5
Control-5
Control-9
Control-2
100%
78%
77%
100%
82%
85%
100%
92%
85%
100%
78%
69%
100%
84%
75%
100%
79%
78%
100%
92
77%

(b)


Frequency of micronuclei in lymphoblastoid cell lines after treatment with MEL
Percent Micronuclei
Cell Lines
0 µM
0.05 µM
0.1 µM
BRCA1-1
Control-1
Control-4
BCS-5
Control-5
Control-9
Control-2
100%
97%
92%
100%
76%
62%
100%
88%
84%
100%
92%
90%
100%
81%
75%
100%
84%
78%
100%
95%
78%

(c)


Frequency of micronuclei in lymphoblastoid cell lines after treatment with CEA
Percent Micronuclei
0 µM
0.025 µM
0.05 µM
BRCA1-1
Control-1
Control-4
BCS-5
Control-5
Control-9
Control-2
Cell Lines

(d)

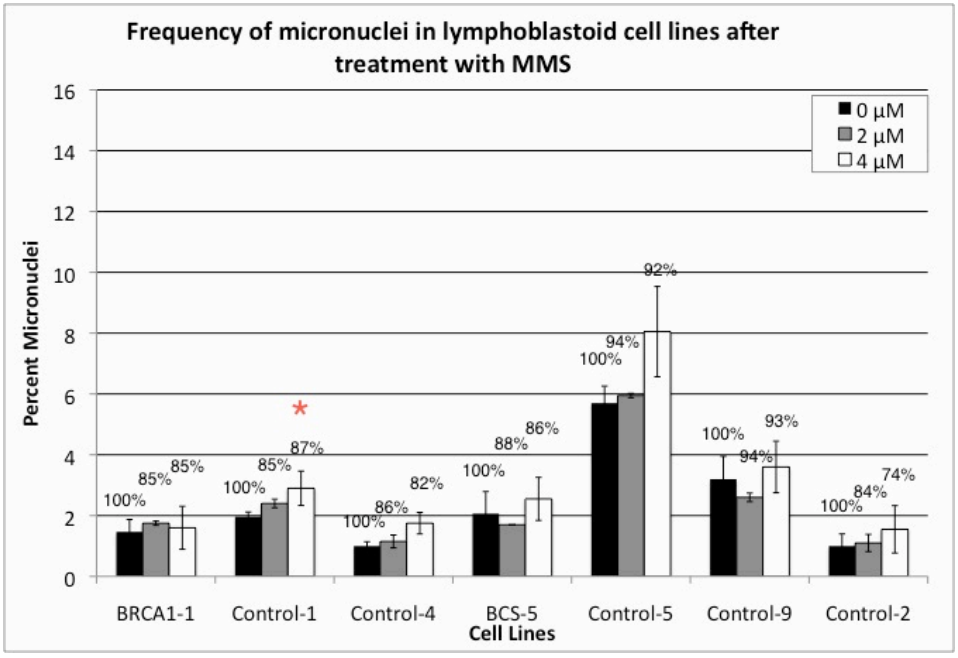

Frequency of micronuclei in lymphoblastoid cell lines after treatment with MMS
Percent Micronuclei
0 µM
2 µM
4 µM
BRCA1-1
Control-1
Control-4
BCS-5
Control-5
Control-9
Control-2
Cell Lines

Figure 6. Frequencies of micronuclei in lymphoblastoid cell lines treated with (a) mitomycin c, (b) melphalan, (c) 2-chloroethylamine or (d) methyl methanesulfonate for 24 hr. Results averaged from three independent experiments with standard deviations are shown. Asterisks represent statistically significant increases in the frequency of micronucleated cells as compared to untreated (black bars). (Fisher exact test; $p \leq 0.05$). The percent replicative index (RI) is presented as a percentage and is listed above each test concentration for each cell line.

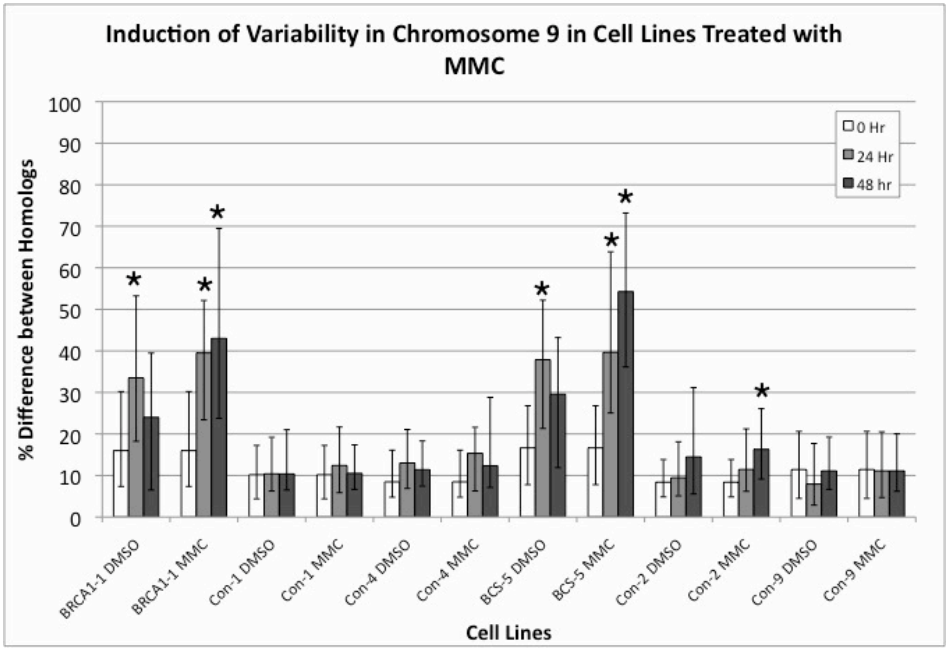


Figure 7. Comparisons of heterochromatin variations in chromosome 9 following treatment with MMC in six cell lines; two are sensitive cell lines derived from breast cancer patients (BRCA1-1 and BCS-5) with two control cell lines per BCCL (Con-1 and Con-4 for BRCA1-1 and Con-2 and Con-9 for BCS-5). Histograms show the median and interquartile ranges for heterochromatin variability for the cell lines before treatment (0 hr, labeled in white), after 24 hours of incubation with MMC (24 hr, labeled in light grey), at 48 hours or 24 hours after the MMC or DMSO wash out (48, labeled in dark grey). The medians and interquartile ranges of the responses are shown. Asterisks denote statistically significant responses ($p<0.01$).

Table 4: Median % difference and interquartile ranges for sizes of the heterochromatin of chromosome 9 homologs for MMC (in 0.1% DMSO) and 0.1% DMSO treated cell lines over a 48 hr time course. Each experiment was repeated at least 3 times. Note: the 0 hr samples were taken before treatment and are the same for the DMSO and MMC treatments. Asterisks denote statistically significantly values.

Cell Line and Treatment	**Median 24 Hr**	**Quartile 1**	**Quartile 3**	**Median 48 Hr**	**Quartile 1**	**Quartile 3**
BRCA1-1 DMSO	33.513*	18.234	53.240	23.998	6.493	39.463
BRCA1-1 MMC	39.501*	23.393	52.139	42.995*	23.765	69.495
Con-1 DMSO	10.395	6.876	21.071	11.400	7.436	18.303
Con-1 MMC	12.402	6.266	21.600	12.342	7.114	28.810
Con-4 DMSO	13.005	6.246	19.199	10.367	6.496	21.007
Con-4 MMC	15.329	5.863	21.665	10.516	6.660	17.366
BCS-5 DMSO	37.870*	21.310	52.205	29.554	11.880	43.198
BCS-5 MMC	39.638*	25.039	63.861	54.260*	36.125	73.195
Con-2 DMSO	9.458	5.088	18.114	14.472	5.541	31.168
Con-2 MMC	11.451	6.183	21.223	16.291*	9.120	26.075
Con-9 DMSO	7.920	2.852	17.700	11.096	6.659	19.229
Con-9 MMC	11.102	4.691	20.520	11.103	6.196	20.019