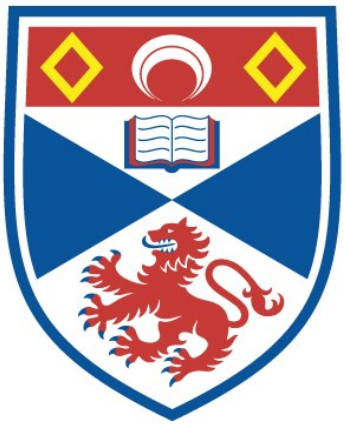
# MECHANISMS OF CHROMATID BREAKS

Faraia Mir

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1999

# MECHANISMS OF CHROMATID BREAKS

BY

FARAIA MIR

**Thesis submitted for the degree of M.Phil**
**to the Department of Biomedical Science**
**University of St. Andrews**
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# ABSTRACT

Individuals have widely differing radiosensitivities as shown in the G2 radiosensitivity experiments in blood lymphocytes from conditions such as breast cancer and ataxia telangiectasia (AT) (Sanford et al 1989, Scott et al 1994, 1996)). This study investigated whether there was a correlation in colour switch ratio (the number of colour-switch breaks/total number of breaks) and radiosensitivity in breast cancer and normal surgical controls and in an AT and normal lymphoblastoid cell line. Harlequin-stained cells were subjected to the G2 assay and the number of chromatid breaks in the light and dark strands and colour-switch breaks were scored in 100 cells per sample. The mean csr in breast cancer patients was 25.8% compared to a mean of 15.9% in control patients indicating a significant difference; P= 0.0024. The mean radiosensitivity (which was expressed as the frequency of chromatid breaks/100 cells) in breast cancer patients (56.2) was also significantly higher than the mean radiosensitivity in control patients (27.9); P= 0.0000. When csr was plotted against radiosensitivity, two separate cohorts were produced - one for breast cancer individuals and a second for control individuals suggesting a possible use in breast cancer screening. In the AT cell line, however, the mean csr was 29% compared to the mean of 26% in the normal control cell line. There was thus no significant difference in csr between the AT and normal cell line; P= 0.13. It might be expected that as AT individuals are hypersensitive to ionising radiation (as indicated by the higher number of chromatid damage), they might (if csr was related to radiosensitivity) have a significantly higher csr than normal or breast cancer patients. From the results presented, however, this appears not to be the case. The results indicate that csr and radiosensitivity are not always correlated. Thus the results show that although csr and radiosensitivity are not always correlated in the AT cell line investigated, when radiosensitivity was plotted against csr, the breast cancer cohort appeared to separate from the control patients. It was very interesting to observe that in this study, all of the breast cancer individuals were discriminated by this correlation of high csr and radiosensitivity in comparison to normal control individuals. If this correlation is confirmed in several more studies, this predictive test has the potential to be used

as an assay to detect individuals predisposed to breast cancer. If it discriminates almost all of the breast cancer cases as my experiments showed, then this could prove to be a more valuable and accurate assay than the G2 assay which has been shown to discriminate 42% radiosensitive breast cancer patients (Scott et al 1994).

Declaration

(i) I, Faraia Mir, hereby certificate that this thesis, which is approximately 22500 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date Signed

(ii) I was admitted as a research student in September, 1997 and as a candidate for the degree of M.Phil in September, 1997; the higher study for which this is a record was carried out in the University of St. Andrews between 1997 and 1998.

Date Signed

(iii) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of M.Phil in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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Dedication

For my dear mother.

Acknowledgements

I would like to express my sincere thanks to my supervisor, Dr. Peter Bryant, for his continuous and valued support, encouragement and help in completing this thesis within a year. I would also like to thank Mr. Angus Gleig for his expert technical assistance. I would like to express my appreciation to my dear mother, Dr. Maryam Hina Shah and my twin brother for there everlasting support (emotionally and financially), love and encouragement.

*List of tables*

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*Abbreviations*

| | |
|---|---|
| AT | Ataxia Telangiectasia |
| ATM | Ataxia Telangiectasia Mutated protein |
| BrdU | bromodeoxyuridine |
| BRC | breast cancer patients |
| CON | control patients |
| CO2 | carbon dioxide |
| csb | colour switch breaks |
| csr | colour switch ratio |
| D | frequency of chromatid breaks in the dark strand |
| DNA | deoxyribonucleic acid |
| DNA-PK | deoxyribonucleic acid protein kinase |
| DF | degree of freedom |
| dsb | double strand breaks |
| F | female control |
| H2O | water |
| irr | irradiated |
| I | incubator |
| Ig | immunoglobin |
| L | frequency of chromatid breaks in the light strand |
| M | male control |
| N | number of patients |
| r.p.m | rotations per minute |
| SD | standard deviation |
| S | shaker |
| unirr | unirradiated |

The aim of my research was to obtain a greater understanding of the underlying mechanisms involved in the variation of chromosomal radiosensitivity in humans. Apparently normal individuals vary to a considerable extent in chromosomal radiosensitivity, as indicated by the G2 assay.

*G2 assay*

The G2 assay gives a measure of the frequency of chromatid aberrations in the form of chromatid breaks and gaps present in metaphase cells usually 1.5h following irradiation of skin fibroblasts, peripheral blood lymphocytes or other cell types in the G2 phase of the cell cycle (Parshad et al 1984, Sanford et al 1989, Scott et al 1994, 1996, Mitchell and Scott 1997). The frequency of aberrations (almost exclusively breaks) resulting from the radiation treatment indicates the "radiosensitivity" (strictly speaking a parameter of a clonogenic survival curve) of an individual and how much this varies between individuals. About 9% of apparently normal individuals and 42% of breast cancer cases show elevated radiosensitivity i.e. in the range of Ataxia Telangiectasia (AT) heterozygotes, linking high radiosensitivity with predisposition to cancer (Scott et al 1994). Higurashi and Conen (1973) were one of the first to report the high levels of γ-ray induced chromosomal damage in lymphocytes of patients with AT, Fanconi's Anaemia and Bloom Syndrome. Also Parshad et al (1994) showed that when exposed to X-rays in the G2 phase, 13 cell lines derived from human tumors of diverse origin showed significantly more chromatid aberrations at all doses tested, in comparison to twelve lines of normal skin fibroblasts. The underlying mechanisms of G2 chromatid radiosensitivity, however, are unknown.

The work described here was aimed at understanding the reason for high chromatid radiosensitivity. The principle aim of my research was to investigate whether the relative frequency of chromatid breaks associated with exchanges (indicated by colour-switches of light and darkly stained harlequin chromosomes), is linked to radiosensitivity (determined by the G2 assay). This was achieved by investigating whether the colour-

switch ratio (the frequency of colour switch breaks/total breaks) in harlequin stained chromosomes was linked in any way to the chromatid radiosensitivity of human peripheral blood lymphocytes from breast cancer cases and normal surgical controls. My work involved scoring chromatid breaks and determining whether these involved a switch of colour or not.

*Suboptimal repair as a model for chromatid aberrations*

Sanford et al (1989) investigated the frequency of chromatid aberrations in G2 metaphase cells after X-irradiation in normal control individuals and individuals with various genetic disorders e.g. Ataxia Telangiectasia, (AT), Fanconi's anaemia, Bloom syndrome and familial polyposis to name but a few. Results showed that individuals with genetic conditions predisposing to cancer were 2-3 times more sensitive than normal control individuals and that this increase in sensitivity was suggestive of defects in DNA repair. There appears to be considerable discrepancy, however, in the results for the radiosensitivity of control individuals. One of Sanford's studies of the G2 assay was carried out between 1981 and 1984 and a second was performed between 1984 and 1987. The same procedure was therefore carried out on separate batches of control individuals over different years. Unexpectedly, the frequency of chromatid breaks and gaps varied considerably between the two batches of control individuals. Batch one of control individuals (1981-1984) showed an average of 1.6 breaks and 8.0 gaps scored in 100 cells, whereas the second batch (1984-1987) produced an average of 21.3 breaks and 21.3 gaps scored in 100 cells. One would have expected that the frequency of chromosomal aberrations in all control individuals would be similar, but they in fact showed greater variation (more significant than between cancer-prone individuals and control individuals). As there is considerable variation within normal control individuals from these results, confidence is reduced in the proposed relationship between radiosensitivity and cancer-disposition based solely on this work. However, from their work Sanford et al (1989) proposed that deficient DNA repair (elevated break scores in the G2 assays) was responsible for both the high G2 radiosensitivity (elevated frequency of chromatid breaks) and predisposition to cancer. They argued that reduced efficiency of DNA repair,

would reduce removal of carcinogenic lesions so predisposing individuals to cancer.

Helzlsouer et al (1996) further suggested that DNA repair deficiency was a potential susceptibility factor in predisposing women to breast cancer. They performed the G2 assay on several women who were at high risk of breast cancer (had no previous diagnosis of breast cancer and at least one first or two second degree relatives with cancer), control women and women with breast cancer, to measure the DNA repair deficiency which was measured as chromatid breaks and gaps. The value of greater than 60 breaks and gaps per 100 cells was indicative of suboptimal repair and 16-50 breaks and gaps for normal repair. The percentage of suboptimal repair demonstrated was 32% for control women, 71% for women at high risk and 100% for those with breast cancer. It was calculated that women at high risk were approximately 2.3 times more susceptible to breast cancer than control women. Suboptimal repair was detected in all breast cancer cases and only 32% of control women. This result therefore indicated to the authors that there was a correlation between suboptimal repair and a high risk of breast cancer.

Patel et al (1997) investigated DNA repair deficiency in breast cancer patients by also measuring the frequency of chromatid aberrations produced in the G2 assay in several breast cancer patients, their first-degree relatives and a group of control women. 93% of the breast cancer patients, 79% of the first-degree relatives and 35% of the control individuals showed sub-optimal DNA repair (based on high break frequencies in the G2 assay). The results indicate that there was a significant defect in DNA repair in breast cancer patients and first-degree relatives. The frequency of chromatid aberrations was measured at various different intervals (30, 90, 120 minutes) after X-irradiation to the time of sampling and it was found that there was a significant decrease in the frequency of aberrations observed in control individuals than in breast cancer patients and first-degree relatives. After carrying out various statistical tests, it was demonstrated that there was an association between DNA repair deficiency and the risk of breast cancer. First-degree relatives were found to be seven times more likely to have sub-optimal DNA repair than the control patients. They determined that breaks in chromosomes 1, 2, B, D and E groups occurred more frequently in the breast cancer patients than controls

whereas breaks in chromosomes 2, B, D and E groups were more frequent in first-degree relatives. It was proposed that these chromosomes were specifically targeted for damage following irradiation as they carry genes that are involved in DNA repair and cell proliferation control (Patel et al 1997). Thus these results support previous findings (Sanford et al 1989, Helzlsouer et al 1996) that DNA repair is defective in individuals predisposed to cancer and further proposed that there was a genetic basis to the predisposition of breast cancer.

In studies of human and rodent sensitive cell lines e.g. AT and CHO (Mozdarani and Bryant 1989, Macleod et al 1994, Pandita and Hittleman !992, Lui and Bryant 1994 and Bryant 1997) it was demonstrated that the rate of disappearance of chromatid breaks with time was found to be similar to that in normal cell lines, but that there was a higher frequency of chromosomal damage in the sensitive cell lines. They accounted for this higher sensitivity in sensitive cell lines by a "conversion" process that has recently been interpreted as an alteration in signaling of DNA damage (Bryant 1998, see below). As described above, Parshad et al (1983), Gantt et al (1986), Sanford et al (1989), Helzlsouer et al (1996) and Patel et al (1997), on the other hand, claim that the rate of DNA repair in radiosensitive cell lines is considerably slower than the rate in normal cell evidenced by a slower decrease in the number of chromatid breaks with time.

Scott et al (1996), repeated the same G2 assay protocol as Sanford et al (1989), on cells from AT patients, Xeroderma pigmentosum, Li-Fraumeni and familial polyposis and found no apparent discrimination (no major differences) in chromatid aberrations between control individuals and these cancer-prone individuals with the exception for AT homozygotes and heterozygous individuals. Scott et al (1996), found that all 7 AT homozygote individuals showed higher mean chromatid break frequencies than the control individuals (3 times more) but that only 2 out of the 10 AT heterozygotic individuals (20%) showed higher mean chromatid breaks than the mean for the controls. Discrimination was therefore present between AT homozygotes and normal control individuals whereas the chromatid aberration levels between AT heterozygotes and normal control individuals were not significantly different. In comparison, results from

Sanford's group (Sanford et al 1989), showed discrimination between all the AT heterozygotes and the control individuals (with the exception of 2 out of 34 control individuals). Therefore there was no overall significant difference between AT heterozygotes and controls in Scott's study in mean chromatid aberrations, whereas there were in Sanford's study. Scott et al (1996) used a modified G2 assay with a newer protocol that increased reproducibility of the results principally by harvesting cells in ice-cold hypotonic solution.

Using the modified G2 assay, Scott et al (1994) found that 21/50 breast cancer patients (42%) were radiosensitive i.e. fell within the range of radiosensitivity (110 breaks or more per 100 cells) for AT heterozygotes and found that 9% of control patients also showed high radiosensitivity within this range. A similar ongoing (but as yet unpublished) study of Tayside region breast cancer cases and normal surgical controls at St. Andrews University shows that about 50% of breast cancer patients examined show elevated chromatid radiosensitivity of peripheral blood lymphocytes (Bryant, personal communication).

To test the contention that chromatid radiosensitivity was linked with predisposition to cancer, additional experiments using a cell survival assay have been carried out on skin fibroblasts and peripheral blood lymphocytes from individuals with non-polyposis colon cancer (a cancer family syndrome) (Bender et al 1988). Results showed that despite using four different types of mutagen, affected family members showed indistinguishable results (i.e were not more radiosensitive) from unaffected members and control individuals i.e. there was no association with radiosensitivity and predisposition to cancer (Bender et al 1988). They proposed that affected individuals with the cancer family syndrome did not show elevated levels of sensitivity to irradiation and chemical mutagens. It may have been that this family was a particular exception or that the assay used did not provide suitable discrimination between affected and unaffected family members. However, these studies do not confirm Sanford's findings.

To account for elevated chromatid radiosensitivity, several G2 assay experiments were

performed indicating that the DNA of cancer-prone cells repair more slowly than that of normal individuals, or produced more breaks than that of normal cells (Gantt et al 1986). This supported the notion that metaphase cells that exhibit enhanced G2 chromatid radiosensitivity are deficient in DNA repair (Sanford et al 1989, Patel et al 1997).

Of the total percentage of breast cancer patients, only 5% can be accounted for by mutated BRCA1 and/or BRCA2 genes with a dominant predisposition to inheritance. It has however been suggested that a higher proportion of individuals may carry predisposing genes of lower penetrance (Scott et al 1994). Moreover these genes of low penetrance may be involved in the processing of damaged DNA and this might account for genetic predisposition to cancer association with high G2 radiosensitivity. It is quite difficult to assess directly how predisposed an individual is to breast cancer (in terms of chromatid radiosensitivity) in comparison to an apparently normal individual. The G2 assay, however, has been devised by Sanford and Prakash (1989) to detect how sensitive individuals are to radiation and whether a generalised association can be made between levels of radiosensitivity (in the form of chromatid breaks and gaps) and the genetic predisposition to cancer, i.e. individuals with a strong predisposition to cancer show a higher frequency of chromosomal aberrations, indicating greater radiosensitivity. According to more than fifty different types of cancer-prone conditions show high levels of radiation-induced chromosome damage (Parshad et al 1983, Sanford et al 1989).

The G2 assay experiments must be carried out on a larger number of cell lines with known genetic defects in repair before it can be determined whether or not it is the rate of DNA repair that is defective in sensitive cell lines or whether signaling is involved. The syndromes used in Sanford et al (1989) study do not show deficiencies in biochemical repair of dsbs and therefore it cannot be inferred from current data that the cause of high chromatid radiosensitivity results from a deficiency in DNA repair. It is therefore useful to study the G2 chromatid response of cell lines that are deficient in dsb repair such as the mutant Chinese hamster cell lines, xrs and murine scid cells.

*Micronucleus assay*

The micronucleus assay, an alternative assay, designed to measure the amount of damage caused by radiation to the chromosomes of a cell in G0 was found to give results which were different to that for the G2 metaphase assay - 31% (12/39) of breast cancer patients were identified by elevated micronucleus frequency and 5% (2/42) of healthy controls. These frequencies of radiosensitive individuals are lower than that obtained from the G2 assay (Scott et al 1998). Although the experiment was carried out on different patients it is likely that the mechanism accounting for G1 and G2 phase radiosensitivity occurs from different mechanisms.

*Breast Cancer*

To date there are two major genes predisposing women to breast cancer - BRCA1 and BRCA2. BRCA1 gene is on chromosome 17 and BRCA2 gene is on chromosome 13 (Bertwistle and Ashworth 1998). Mutations in these genes, however, does not account for all individuals that have breast cancer. Initially it was estimated that these genes; BRCA1 and BRCA2 contributed to over 90% of the cases of inherited breast cancer. Now it has been estimated that these genes account for less than 30% of the genetic contribution responsible for heritable breast cancer (Bertwistle and Ashworth 1998). The majority of the cases of breast cancer, however, are sporadic - they occur in individuals who do not have a family history of breast cancer or any other type of cancer (Taylor et al 1998). Another gene which predisposes individuals to breast cancer is p53. Mutations in this gene may lead to multiple cancers in families with Li-Fraumeni syndrome, particularly breast cancer. The presence of p53 mutations in sporadic forms of breast cancer suggest that it plays an important role in cell transformation (Ouchi et al 1998). Many environmental factors contribute to the development of breast cancer - such as exposure to ionising radiation, smoking, alcohol consumption, contraceptives and oestrogen replacement therapy (Osin et al 1998, Ansquer et al 1998).

There are two main types of breast cancer - non-invasive carcinomas and invasive

carcinomas. Non-invasive carcinomas are when the malignant cells are confined to the ducts or the acini of the lobules (Kumar and Clark 1996). As it is of the non-invasive type, there is no evidence of penetration of the tumour cells into the basement membrane surrounding these structures. Non-invasive carcinoma occurs when the lesions are confined within the spaces, where the originating cells were present. There are two forms of non-invasive carcinoma - ductal carcinoma in situ and lobular carcinoma in situ. Ductal carcinoma usually occurs in the 40-60 year age group and is usually detected by mammography screening or in surgical biopsies and treated by mastectomy (Anderson 1985). Ductal carcin main situ can spread along the duct system or into the lobules. Histological changes are usually found in the small and medium-sized ducts where some cells that are present may calcify. Lobular carcinoma in situ occurs predominantly in pre-menopausal women and accounts for 6% of all breast carcinomas (McGee, Isaacson and Wright 1992). Histological changes are found in the acini, where the normal cells are replaced by loose and non-cohesive cells. Usually one-third of the patients with lobular carcinoma in situ, will develop an invasive carcinoma (Anderson 1985, Kumar and Clark 1996). Invasive carcinoma occurs in pre- and post- menopausal women. In this type of tumor, the cells (intact or broken) pass through the basement membrane around the breast structure and spread into the surrounding structure. Breast carcinomas metastasise to more distant sites via the lymphatic system and the bloodstream (McGee, Isaacson and Wright 1992). Tumors can be graded for their degree of differentiation - where patients whose tumors are well differentiated are grade 1, while those tumors that are poorly differentiated are grade 3. If a women has a type of breast carcinoma, staging is undertaken, so as to assess whether or not it has spread locally and distantly and thus the extent of spread. The two main systems used are the Tumor, Node, Metastasis system (TMN) and the International Classification of staging (McGee, Isaacson and Wright 1992).

*Chromosome and chromatid-type aberrations*

There are two main types of aberrations: (i) chromosome and (ii) chromatid Chromosome aberrations are observed at metaphase after G0 or G1 irradiation whereas

chromatid aberrations, on the other hand, are observed after irradiation in the late G2 or S phase of the cell cycle. Both types consist of breaks, gaps and exchanges. The sequence of events over which chromosomal aberrations develop is still unclear.

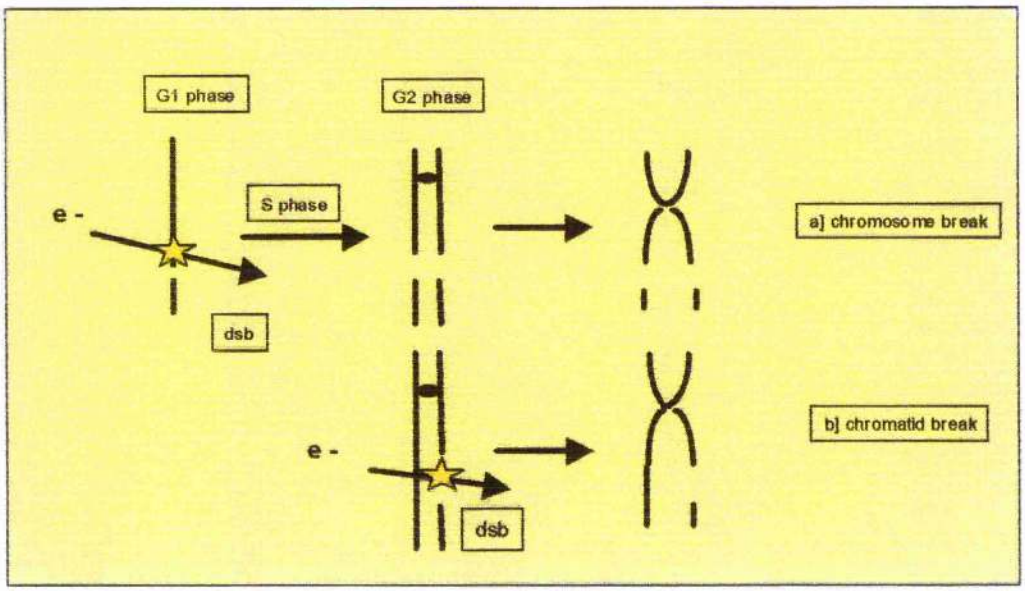



Figure I : Diagram of a chromosome and a chromatid break

After irradiation in the S phase, a dsb may form. After DNA synthesis the damage in the single strand of the chromatid has replicated and is observed in metaphase as 1 break in each chromatid strand i.e.a chromosome break as shown in (a). If a cell is irradiated in the G2 phase, a dsb may form in one of the chromatid strands and this is observed at metaphase as a chromatid break as shown in (b). As the cell has already passed the synthetic phase of the cycle, a dsb is present in only one strand of the chromatid.

*How are double-strand breaks converted into chromatid aberrations?*

In human cells, the presence of chromatid breaks can account for the loss of up to a third of a chromosome; 40Mbp of DNA - a considerable amount of genetic material. To date, it is still unclear, how such a large amount of DNA is lost (if it is indeed lost) as a result of a single passage of an ionising track.

There are three hypotheses (or models) that attempt to describe how chromatid aberrations may be produced from a double-strand break (dsb); namely the classical and

more accepted breakage and reunion theory or breakage-first hypothesis (Sax 1935), the exchange theory (Revell 1955) and the signal model (Bryant 1998).

*The Breakage-First or Breakage Reunion Hypothesis*

Perhaps the most influential hypothesis for the origin of chromatid aberrations was proposed by Sax (1935). He claimed that chromatid aberrations were produced as a result of single one hit interactions of ionising tracks in chromatid molecules. The outcomes of the damaged ends of one or two of chromatids falls into one of three categories as shown in figure II: either the damaged end will (1) reconstitute in the original configuration (rejoining), or (2) mis-join with the broken ends of another chromosome close in time and space to form an interchromosomal exchange or (3) remain unrejoined and become visible as an open break at metaphase. Sax's theory therefore predicts that chromatid breaks and exchanges are different outcomes of the same event - i.e. the chromatid break.

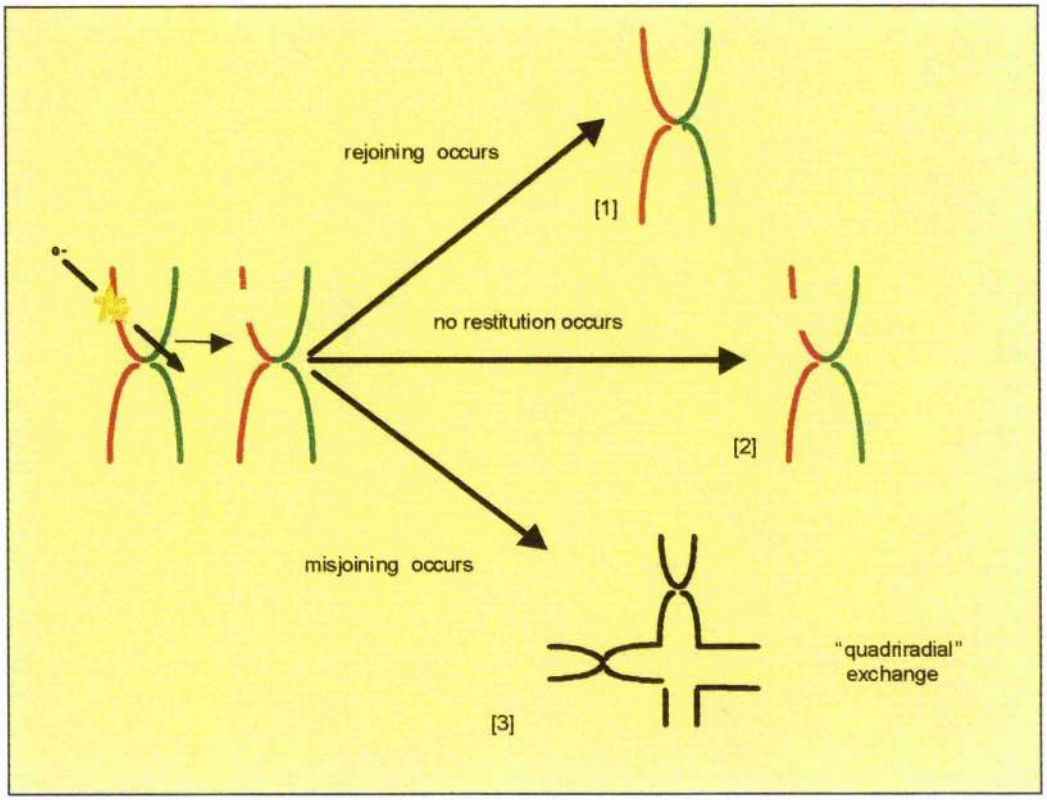



Figure II: The breakage-first hypothesis [Sax 1935]

Ionising radiation induces a dsb(s) (previously interpreted as a chromatin break in the chromatid strand). The outcome of the damaged ends of one or both of the chromatids will either rejoin into the original configuration as shown in (1), or remain unrejoined and become visible as a chromatid break in metaphase as shown in (2), or misjoin with the broken ends of another chromosome that is nearby in time and space as shown in (3).

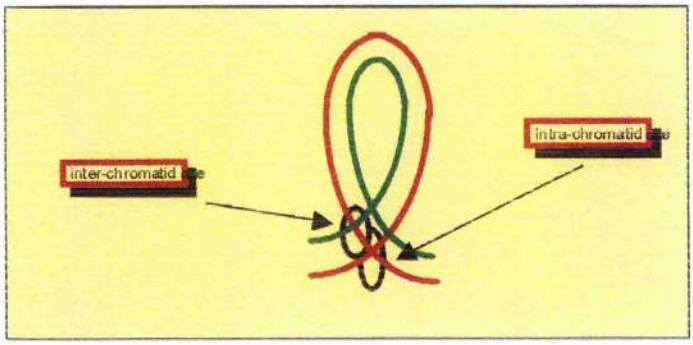
The relative frequency of the residual breaks observed, is interpreted by Sax (1935) as the remaining few of a much larger number of breaks that has undergone either (1) or (2), or both as indicated by the respective dose-response curves (Sax 1940). According to the breakage-first theory, chromatid breaks would be linearly related to dose, whereas exchanges would be quadratically related. This classical theory is based on the assumption that unrejoined chromatid breaks are the initial event induced at the point of irradiation and that these breaks undergo secondary rearrangements - this interchromosomal exchange process being a secondary event.

Sax was one of the first who investigated the relative frequencies of chromosome aberrations in mitosis following irradiation (Sax 1935, 1940). By carrying out a series of experiments of irradiating Tradescantia microspores with X-rays, Sax found that the frequency of chromatid breaks increased linearly with an increase in radiation dose - the radiation dose-response curve for chromatid breaks was linear (Sax 1935, 1940). This result provided evidence that one chromatin break is involved in the formation of a chromatid aberration, not two. There was no intensity effect observed with dose (i.e. variation of the dose-rate did not affect results), neither fractionating the dose by varying times. He found that the number of chromatid exchanges increased as the squared X-ray dose and when the dose was given over a longer period of time or fractionated the frequency of interchanges decreased. Thus as there appeared to be a linear relationship with chromatid breaks and a quadratic relationship for interchanges against dose, Sax (1935) proposed that the initial action of X-irradiation was the formation of breaks and then the possible reunion of these breaks, resulting in exchanges - the secondary action. Thus, for chromatid breaks to occur, only one hit is required but for exchanges to occur, two chromatid breaks "hits" are required and this is therefore called a "two-hit" event. However, evidence did indicate that a certain number of exchanges were one hit effects also (Sax 1940). This would occur if both "hits" in chromatids of two separate chromosomes were caused by the same ionising track. Thus, the observed chromatid breaks at metaphase under the breakage-first model are proposed to be a result of the primary action of X-rays and the presence of exchanges occurs due to the interaction of some of these initially formed breaks in two or more chromosomes (Sax 1935).

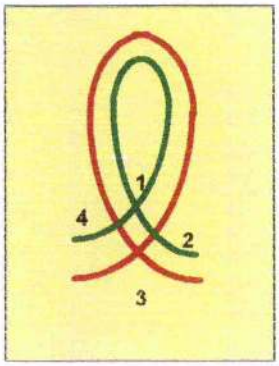
*The Exchange Hypothesis*

In 1955, Revell challenged the breakage-first hypothesis with his "exchange" model, which provided an alternative explanation to the Classic theory for the formation of chromatid breaks. His idea was that all chromatid breaks resulted from an exchange event occurring within or between the damaged chromatids. The basis of the exchange model was that chromatid breaks are essentially incomplete exchanges i.e. where complete

recombination between or within the chromatids had failed (Revell 1958). Revell (1959) postulated that this exchange process occurred sometime in the early stages of prophase, and thus may be regarded as a homologous process to meiosis. He proposed that at the time of irradiation, the initial events leading to the formation of a chromatid break were unstable lesions and not a frank break (as in Sax's hypothesis) and if left undisturbed, would naturally decay to a stable state or be repaired. Thus two ionisation events occurred such that the second unstable lesion was present near in time and space to the first lesion, they both could undergo a condition of "exchange initiation" (a secondary event in his model) resulting in an exchange event between the two or within one of the chromatids (therefore in Revell's model a chromatid break is a 2-hit event) (Revell 1959, 1963). This chromatid exchange occurs by the interaction of two unstable lesions (recombinational exchange), nearby in time and space, at the point of overlap of a chromosomal loop. Thus the dose-response relationship for chromatid breaks in the exchange model (Revell 1959, 1961) predicts a dose-squared relationship - that a two-hit component is involved in chromatid breaks and not one as proposed in the breakage-first hypothesis (Revell 1959, 1961).




The exchange site is where the two (or more chromatin threads lie within rejoining distance so that an intrachange or an interchange may result (as shown opposite). An intrachange site is an exchange site that is present within a chromatid. An interchange site is an exchange site that allows sister chromatid exchange.




Interaction at points 1-4 are equally likely according to the exchange hypothesis (Revell 1955). If a chromatid exchange occurs at points 1 or 3, an intrachange results. If a chromatid exchange occurs at points 2 or 4, an interchange results.

Figure IV: The Revell loop (Savage 1966)

The types of breaks predicted by Revell's model are shown in figure III. Revell predicted that the chance of any chromatid end undergoing an exchange event is equally likely. The predicted outcome for Revells model is that 2 interchromatid exchanges occur in the 5 types of chromatid breaks giving rise to the 40% value of colour-switches observed during the formation of chromatid breaks.

Revell's model thus predicts three types of single chromatid breaks: Type 1 where part of one chromatid is translocated to the other. As the looped chromatin ends up in one chromatid the two chromatids are of different lengths resulting in an apparent break (figure III).Type 2 results in a small deletion in one chromatid. The chromatid arrangement results in the formation of a minute ring. Type 3 results in an inversion in one chromatid. All single chromatid breaks are incomplete interchanges or intrachanges. All isochromatid breaks (of proximal or distal sister non-union)are complete intra-changes of type 4. As types 1a and b result in colour switches, 2/5 of the total breaks (40%) result in a colour switch (Revell, 1959). It is observed that in Chinese hamster cells 16% of breaks are colour switches. To explain this, Harvey and Savage (1991) suggested that type 2 and 3 intrachanges occurred more frequently than that predicted by the exchange model (i.e those involving no colour switches).

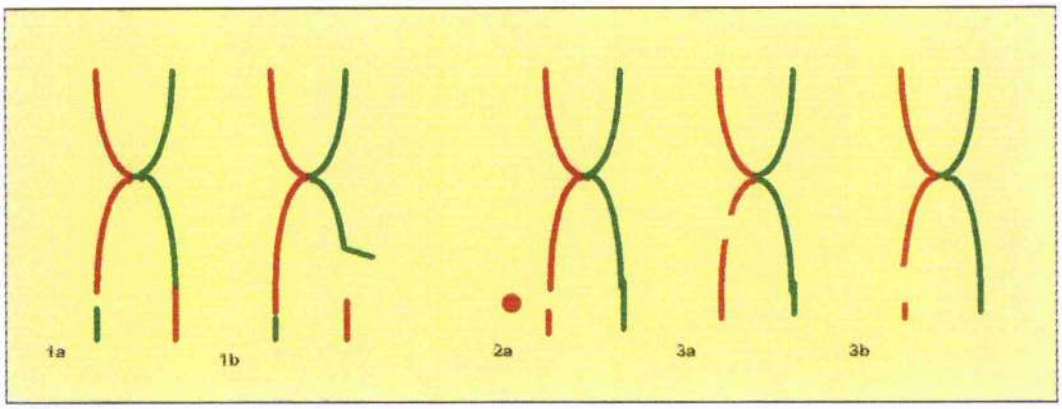



Figure III: Types of "colour-switch" and non - "colour switch" breaks
Type I occurs when part of one chromatid translocates to the other chromatid during an inter-chromatid exchange. Type 2 occurs when a small deletion in one chromatid forms a minute ring during an intra-chromatid exchange. Type 3 results in an inversion in one chromatid during an intra-chromatid exchange.

Bryant (1998) (reproduced with permission)

Thus in comparison to the breakage-first model, Revell proposed that the formation of a chromatid break was not due to the presence of residual unrejoined primary breaks but in fact a result of an incomplete exchange process between two lesions within an arm (intrachromatid exchange) or between chromatid arms (interchromatid exchange) as shown in figure IV. Thus chromatid breaks were a secondary result of two interacting lesions, not a primary break (Revell 1955). Sax's model on the other hand postulated that the residual chromatid breaks arose from a larger pool of primary breaks in which the majority underwent either restitution to its original configuration or illegitimate recombination.

*The Signal Model*

The signal model (Bryant 1998) provides a possible explanation for the conversion of dsbs into chromatid breaks. The essential feature of this model is that it associates principles of the Revell's exchange model (Revell 1959) with the idea that a single dsb is converted to a chromatid break by way of a signaling mechanism; which causes initiation

of a recombinational exchange event in the chromatid. If this process is incomplete a chromatid break is formed. Hence according to this model only one dsb is required to trigger off this recombinational event, whereas in Revell's exchange model, two independent lesions are required. It is assumed in the signal model that the initial dsb formed at the instant of irradiation may not necessarily itself be involved in the recombinational process, since a signaling pathway is involved. Unlike the exchange model, the signal model provides a possible explanation as to how a recombinational event results from a single dsb - whether it is an intra-change (an exchange within a chromatid) or an inter-change (an exchange between two chromatids), is achieved. Before further discussing the signal model, it is important to consider the differences in predictions of the dose-response relationships for each of the proposed models. The breakage-first model predicts a linear dose-effect relationship for chromatid breaks. However, because of the large size (5-40 Mbp) of chromatid breaks in relation to a dsb (1-3 bp), a chromatid break would require two interacting events to "chop' out" (delete) a section of chromatid. Thus, in terms of the breakage-first model, two interacting lesions are required and therefore two dsbs are required that would result in a dose-squared relationship for chromatid breaks. In Revell's exchange model (1959), two lesions (not defined) are required to produce a recombinational exchange and again, a dose-squared relationship for chromatid breaks is predicted. In the signal model (Bryant, 1998), however, as one dsb is predicted to result in a chromatid break following an incomplete recombinational exchange, a linear relationship for chromatid break frequency against dose is produced. The linear relationship observed rules out the possibility of two dsbs interacting (as this would result in a dose-squared relationship) therefore one dsb must be considered to be sufficient to produce a chromatid break (Bryant 1998). It was suggested that the chromatin loops proposed for the signaling domain might represent replication (or transcription) " factories".

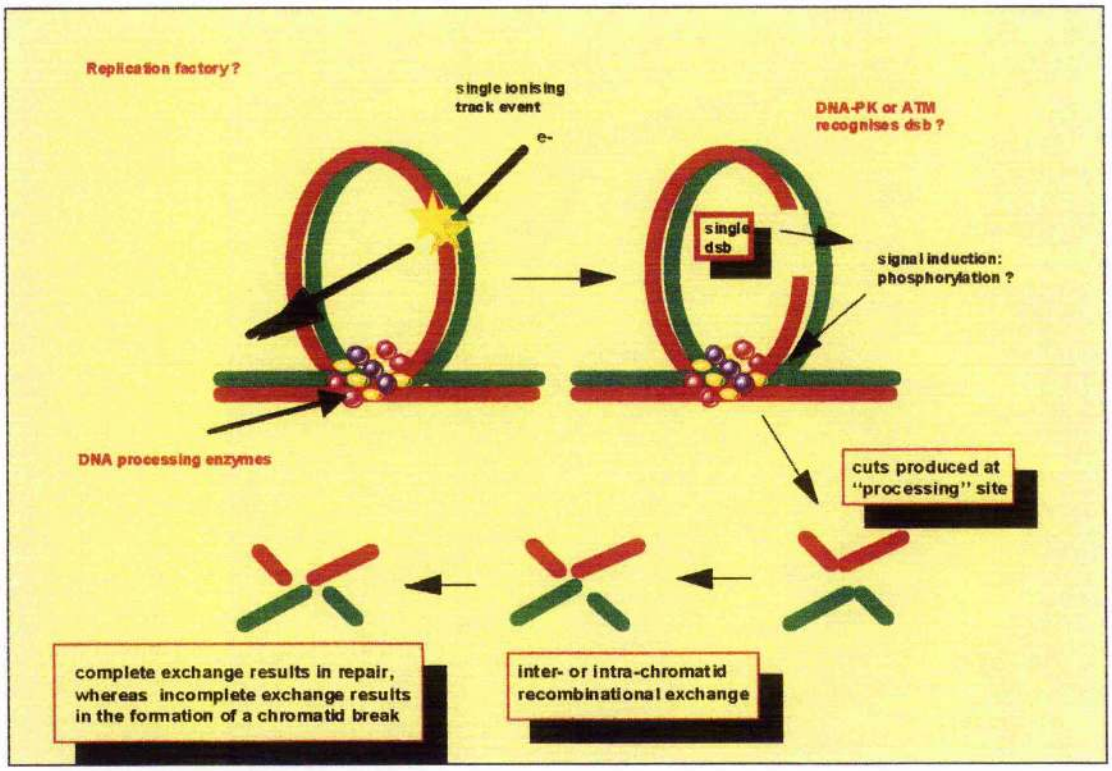



Figure V: The Signal model (Bryant 1998)

A single dsb is induced in the chromatin replication factory when the cell is exposed to ionising radiation. The damaged DNA is recognised by a signalling molecule e.g. the DNA-PK or ATM protein. The signalling molecule then mediates signal induction to the DNA processing enzymes, necessary for DNA repair possibly by a phosphorylation mechanism. This enables the cell to induce incisions (cuts) at the "processing" site enabling inter or intra recombinational exchange. If there is sufficient time for a recombinational exchange event to occur, repair is achieved. If there is insufficient time, a chromatid break is observed at the next mitosis.

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The signal model (Bryant 1998) is shown in figure V. A traversing electron or ionising particle induces a dsb(s) in the chromatin loop. The dsb is detected by a signaling protein, 'the signal molecule', that triggers off a recombinational exchange at the neck (cross-over points of the chromatids) of the chromatin loop. Bryant has suggested that the signaling protein may be the DNA-protein kinase or ATM protein that binds to the broken DNA ends and inducing a signal thereby activating the recombinational exchange. The

recombinational exchange might be mediated by a complex of DNA processing enzymes (e.g. exo/endo nuclease enzymes replicative enzymes, DNA helicases, DNA ligases and topoisomerases etc). The exchange would be initiated by controlled endo-nuclease mediated cuts at the neck of the loop possibly with the purpose of removing or rearranging the loop to negate the effect of the initial damage. This is a rather controversial proposal as it is difficult to understand why a cell would remove such a large loop of chromatin. A recombination event then occurs between the four resulting free chromatid ends. The recombination may occur within a chromatid (intra-chromatid exchange) or between sister chromatids (inter-chromatid exchange) and sequential joining then follows. Chromatid breaks are formed if the cell enters mitosis before the final joining step in the recombinational exchange has taken place; thus an incomplete exchange results in the formation of a chromatid break. Inter-chromatid exchanges are observed as colour-switches between harlequin-stained chromatids. When harlequin stained G2 CHO cells are exposed to ionising radiation about 17% of the breaks show colour-switches (e.g. Harvey and Savage 1996, Bryant 1998) which confirms that recombinational exchanges occur in the formation of breaks. As previously discussed, a linear relationship for chromatid break frequency against dose was found for human lymphocytes and more recently also for mouse fibroblasts (Bryant 1998). It was suggested that the disappearance of chromatid breaks with time does not represent dsb repair kinetics (Bryant 1998). It was suggested that this represents the last stage (i.e the final ligation) in the exchange process and not the rejoining of a subclass of dsb as suggested previously (Mozdarani and Bryant 1989).

Although insufficient evidence confirms this, strong candidates for signaling molecules are the DNA protein kinase (DNA-PK) and the ATM gene product. It is now apparent that DNA-PK is involved in dsb repair in mammalian cells (Boubnov et al 1995, Tacciolo et al 1994, Smider et al 1994). Evidence indicates that DNA-PK may play a structural and signal transduction role in this process.

*DNA-protein kinase*

The DNA protein kinase molecule, DNA-PK, is a trimer composed of a catalytic subunit, DNA-PKcs and the Ku 70/80 heterodimer which is thought to provide a DNA binding subunit. DNA-PK is a nuclear DNA-dependent serine-threonine protein kinase i.e that phosphorylates other proteins, so named because of its requirement of DNA for kinase activity (Anderson 1993). This requirement for DNA is fulfilled in the presence of Ku protein that binds to the free ends of DNA and therefore recruits DNA-PKcs. The DNA-PK will only be active subject to binding to linear DNA, not coiled or closed circular form DNA (Jeggo et al 1995). As DNA-PK is a serine/threonine protein kinase, it has been shown also to phosphorylate transcription factors that bind to DNA indicating a possible role in the regulation of transcription (Jackson and Jeggo 1995). This could either activate repair proteins or inactivate them once their function is completed so that dissociation from the complex is achieved (Jackson and Jeggo 1995).

A possible function of the DNA-PK complex is holding the correct alignment of DNA ends and providing a scaffold structure/arrangement so that the necessary assemblage of repair enzymes is made; this function is allowed by the high molecular mass of DNA-PK - 350KDa (Jackson and Jeggo 1995, Jeggo et al 1995, Blunt et al 1995). Enzymes such as DNA ligases exonucleases and endonucleases would be recruited to the site. Alternatively, DNA-PK may bring the two ends of the linear DNA together to which it is bound close together, in such an arrangement that base pairing occurs during the repair process. A further possibility is that DNA-PK complexes on either end of the DNA may be brought together (Jackson and Jeggo 1995).

The properties of a high molecular mass protein kinase, activated when bound to DNA (in the presence of KU), and deactivated by autophosphorylation, enables DNA-PK to be an ideal candidate for detecting dsb in chromosomes, (in response to ionising radiation), and initiating and recruiting a cascade of repair enzymes towards the site of DNA damage to allow repair. In addition, cell cycle checkpoints may also be induced by DNA-PK to inhibit DNA replication until the repair process is completed (Jackson and Jeggo 1995).

*KU*

Eukaryotic cells contain an abundant non-histone, DNA binding nuclear protein, KU autoantigen, that is a DNA-PK targeting subunit composed of two polypeptide chains of molecular mass 70KDa and 80KDa (Mimori et al 1981). The Ku heterodimer has sequence independent and sequence-specific DNA binding properties (Cary et al 1997 and Barnes and Rio 1997). As Ku is sequence non-specific, it will be able to recognise damaged DNA. Its sequence independent properties enables Ku to bind to free DNA ends, hairpin loops, single-stranded nicks and gaps and translocates along the DNA fragment in an ATP (and sequence) independent manner (Poullard and Strauss 1991). This indicates that Ku may play an essential role as a dsb repair protein (Troelstra and Jaspers 1994, Jackson and Jeggo 1995). Mimori and Hardin (1986) provided evidence from nitrocellulose filter-binding assays that the protein binds to free ends of linear double-stranded DNA. Proteins complexed with DNA was retained within the nitrocellulose filter assay, whereas free unbound DNA passed through. The family of XRCC5 mutants are deficient in Ku DNA-end binding in vitro . The gene encoding KU 80 was found to be present on chromosome 2 - the corresponding position of a fragment that complements xrs (Roth et al 1995 and Finnie et al 1995). This mounting evidence indicates that some radiosensitive cell lines have altered properties because of the function of Ku - in this circumstance, a DNA repair deficiency (Boulton and Jackson, 1996).

In the presence of Ku, DNA molecules were observed to be in the form of loops that were sequence independent. It is possible that after Ku has bound to linear DNA, DNA loop formation is initiated. DNA-PK is then recruited and complexes with the Ku heterodimer at the broken free ends of the looped DNA. This loop structure may be analagous to that of the signal model (Cary et al 1997). Consequently, this looping effect enables dsb repair by aiding in the holding down of broken DNA ends in place, and transcription regulation (as lampbrush chromosomes). When experiments were carried out with DNA-PKcs, DNA and Ku, it was determined that in the presence of ATP, the number of DNA-PK bound to DNA complexes rapidly declined - dissociation of DNA-PKcs occurred from

Ku. This provides evidence that autophoshorylation of DNA-PKcs inactivates itself and therefore dissociates from the Ku complex. The ability of Ku to function as an ATP-dependant DNA helicase (Jackson and Jeggo 1985) provides evidence for a potential role in DNA repair, for unwinding the DNA molecule, allowing either base-pairing to occur between the two ends of DNA, or the passage of repair enzymes to act. This shows the DNA-PK dependency for DNA by binding onto Ku bound DNA. The xrs-6 cell line is complemented by Ku80 cDNA and have mutant Ku 80 protein that has no DNA binding function; hence no DNA-PK activity (Taccioli et al 1993).

*Possible functions of DNA-PK*

The complexity in being able to monitor the activation of DNA-PK in vivo has prevented further advances in our knowledge with respect to the relationship between activation and function. As DNA-PK phosphorylates a variety of transcription factors and thereby inactivating them, its role may be of a general one rather than a specific one and this indicates a non-specific role in controlling gene expression (Jackson and Jeggo 1995). DNA-PK could be involved in the initiation step of transcription whereby it would phosphorylate a cascade of proteins in the transcription assembly for initiation and activation - it could recruit other proteins to the transcription factory. The ability of DNA-PK to be activated in the presence of DNA with gaps and nicks, however, debates a possible role in the recognition/detection of DNA damage and possibly act as a scaffold/backbone structure to hold the damaged DNA in position for the action of the repair enzymes. As previously mentioned in the signal model (Bryant 1998), DNA-PK could play a significant role in the repair of DNA by co-ordinating DNA segments to act as templates for different enzymes to act upon in the restoration of the DNA molecule (either to its original status or to another by recombination).

*The ATM protein*

As previously mentioned the ATM (Ataxia Telangiectasia Mutated) protein is another possible signalling protein for dsbs and may initiate a signal causing chromatid breaks via

recombinational exchange. The ATM gene product has a molecular mass of 370 KDa and is a member of the phosphatidylinositol (PI-3) kinase gene family (like DNA-PKcs) (Lehmann and Carr 1995, Savitsky et al 1995, Chen and Lee 1996, Brown et al 1997). Characteristics of this family is the presence of a highly conserved kinase domain near the C terminus. The C domain terminus has relatively high sequence similarity to the 110 kDa catalytic subunit of mammalian and yeast P13-kinase which mediates a diversity of signal transduction pathways and is also involved in meiotic recombination and cell cycle control (Chen and Lee 1996, Savitsky et al 1995, Jackson and Jeggo 1995). Members of the yeast P1-3 kinase family include the rad 3 gene of Schizosaccharomyces pombe. Thus rad3 and the ATM proteins are therefore structurally similar (Lehmann and Carr 1995).The function of these genes are in the participation of the cell cycle checkpoint response to damaged DNA and the mitotic dependency on completion of DNA replication. The substrate specificity for the ATM protein is still not known. The normal response of Schizosaccharomyces pombe cells to radiation-induced damage is to prevent entry into the G2 phase of the cell cycle (Carr 1994). Mutations in either of the rad genes results in the prevention of the cell-cycle block in the G2 phase and therefore subsequent cell death when damaged DNA proceeds in the cell cycle (Savitskyet al 1995). It was also postulated that the extreme sensitivity attributable to rad mutants is not simply due to their checkpoint defect (Lehmann and Carr 1985). Genetic analysis shows that a secondary function for rad genes as a mediator in a repair/recovery process. Structural similarity between these genes may indicate a similar functions also, but insufficient evidence is available to confirm this. Thus it appears that the ATM protein may play a functional role in monitoring DNA damage possibly via a signalling mechanism (Thacker 1994). A hypothetical model has been proposed by Meyn (1995) in the damage surveillance network model, where it is postulated that the ATM gene plays a vital role in may activate several signal transduction pathways in response to damaged DNA. Shefman et al (1997) showed that the ATM protein may interact with cAbl protein tyrosine kinase and activate it in response to damaged DNA resulting in G1 cell arrest. cAbl was shown to be constitutively expressed in control cells and not in AT cells. It is possible that in normal cells the ATM protein detects DNA damage and interacts with proteins such as cAbl (resulting in G1 cell arrest) and may initiate a cascade of

phosphorylation of other proteins to activate the appropriate repair enzymes in the transcription machinery. As the level of ATM protein is found to be constant throughout the cell cycle, this indicates that it may play a role in maintaining the fidelity of DNA repair and cell cycle regulation (Brown et al 1997).

As the DNA-PK complex and the ATM protein are both members of the phosphatidylinositol kinase-like proteins this may possibly confer signal transduction properties, involvement in meiotic recombination and control of the cell cycle. It is still unknown how this is achieved, but as mentioned above, there is considerable evidence that indicates that these proteins may be involved in signal transduction pathways due to the protein kinase property.

The elevated radiosensitivity of radiosensitive cell lines / individuals may be explained by a faster conversion rate of dsbs into chromatid breaks which has recently been interpreted as an alteration of signaling of DNA damage (Bryant 1998). It is possible that there are two signaling proteins - DNA-PK and ATM protein that detects damaged DNA. DNA-PK may be more efficient in responding to DNA damage than the ATM protein, thus in radiosensitive cell lines / individuals it is possible that the DNA-PK may be mutated and that the ATM protein is the only functional signalling molecule. As the ATM protein may be less efficient in detecting damaged DNA, a higher proportion of chromatid breaks may form. On the other hand, this may be true for the DNA-PK being a defective signaling molecule and the ATM protein, an efficient one.

*DNA double-strand breaks in the formation of chromatid aberrations*

When mammalian cells are X-irradiated, dsb are generated at about 40 dsb/Gy/diploid genome (Blocher 1982). Evidence indicates that dsbs are the critical lesions in the induction of chromosomal aberrations (Natarajan et al 1980, Bryant 1984,1985, Natarajan and Obe 1984). Cells exposed to X-irradiation, and then permeabilised using Sendai virus were subjected to endonuclease restriction derived from Neurospora. As ssbs were converted to dsbs the frequency of dsbs were doubled. The frequency of

chromosomal aberrations were observed to double as well (Bryant 1984, 1985).

Restriction endonuclease experiments are usually conducted when investigating the effect of dsbs on mammalian cell viability since they induce only pure DNA dsbs and no other type of lesion that may confuse the results. The dirty ends of dsbs are however not produced as when cells are irradiated and only clean breaks (3' OH and 5' PO4) are produced (Bryant 1985, Natarajan and Obe 1989). Furthermore, ionising radiation induces several other types of lesions such as single-strand breaks and various cross-links: DNA-protein, protein-protein. Restriction endonucleases are enzymes that recognise and cut specific DNA sequences at particular sites of the DNA molecule generating cohesive or blunt ends. Restriction endonucleases mimic the action of ionising radiation in so far as when introduced into cells they break DNA forming dsbs thereby providing a biological model to investigate the mechanisms of chromosomal aberrations. It is thought that cohesive ended dsbs are the main type of dsb that are generated by ionising radiation, which are known to be less clastogenic than blunt-ended dsbs (Bryant 1984, Natarajan and Obe 1989, Bryant and Johnston 1993, Liu and Bryant 1994). Experiments using restriction endonucleases to induce dsbs in DNA of various cell lines provided increasing evidence that the principle lesion in the formation of chromosome aberrations in irradiated cells are dsbs (Bryant 1984, Natarajan and Obe 1980,1984).

Further work supporting the notion that dsb are the critical lesion in chromosomal aberrations comes from work with radiosensitive mutant rodent cell lines deficient in dsb repair - particularly from the scid and the xrs (Jeggo and Kemp 1983) cell lines, which both showed an increased frequency of chromosomal aberrations compared to normal cell lines (Kemp and Jeggo 1986, Van Bunl 1997 and Bryant et al 1998). When X-ray sensitive (xrs) Chinese hamster cells were restriction endonuclease treated (Pvu II or EcoRV) the frequency of chromosomal aberrations induced were greater than that of the parental line, CHO (Bryant et al 1987, Natarajan, 1987, Natarajan and Obe 1989).

It was previously proposed that base lesions may be the principle lesion in the formation of chromosome aberrations (Preston 1980). However, Ahnstrom and Bryant (1982)

demonstrated that base lesions may produce dsbs from endogenous endonuclease cleavage of mammalian DNA, producing chromosome aberrations. This showed how difficult it was to identify one main lesion as the primary cause of chromosomal aberrations. Nevertheless it is now generally accepted that the dsb is the causative lesion for chromosomal aberrations.

*Ataxia Telangiectasia*

In studying a possible link between "colour-switch ratio" (csr) i.e the ratio of colour switch breaks to the total frequency of breaks, and radiosensitivity, a possible approach would be to investigate the csr in AT as a model system since as already explained the ATM protein may be involved in signaling. AT is a human genetic autosomal recessive childhood disorder, characterised by extreme cellular or chromosomal sensitivity to γ-rays and X-ray ionising radiation, dilation of the blood vessels (telangiectasia) over the bulbar conjuctiva of the eyes, ears and the parts of the face, severe immunodeficiency due to the abnormal synthesis of Ig A, E and G in serum and external secretions, and also progressive neuromuscular and vascular degeneration (Border and Sedgewick 1958, Harnden 1994, Jorgenssen and Shiloh 1996). Thus the nervous and reticuloendothelial system is affected, accounting for the variable effects of AT; i.e. high frequency of spontaneous chromosome damage, abnormal sensitivity to radiation and chemicals, specific cell degeneration (nerve cells) and an abnormal response of cellular and humoral immunity (Bundey 1994). The deficient synthesis of immunoglobins accounts for the increased susceptibility to infection, commonly respiratory diseases. It has, however, been reported that some AT patients have normal levels of Ig levels, therefore Ig deficiency may not be a direct effect of the mutant gene (Lehmann and Taylor 1978, Harnden 1994). Characteristics of this syndrome are defective cell-cycle checkpoints in the G2 phase of the cell cycle (Scott et al 1994, Meyn et al 1994), increased susceptibility to cancer and chromosomal instability (Easton 1994).

*Production and repair of double-strand breaks in AT cells*

Foray et al (1995), investigated the induction and rejoining of dsb, and found that the rate of rejoining dsbs was the same in AT cells as in normal cells and the induction of dsb was also similar rates. This result indicates that the enhanced radiosensitivity response of AT cellsin vitro may not be due to the insufficient rejoining of radiation-induced dsbs (Hamariharan et al 1981, Peacock et al 1989). AT cells are thus apparently proficient in dsb repair but extremely sensitive to ionising radiation. From such evidence it is now generally accepted that the principle biochemical defect in AT individuals is not a defect in the repair of induced DNA damage. Cornforth and Bedford (1985) used the, premature condensed chromosomes technique, PCC, and found that following radiation treatment, the disappearance of fragments in PCC in the G0 phase of the cell cycle in AT fibroblasts indicated normal repair (similar to the repair in normal cells). However there was a higher frequency of PCC fragments in the AT fibroblasts compared to normal fibroblasts. It was suggested by the author that there was a small fraction of unrepaired dsbs which accounted for the radiosensitivity of AT. Bryant and Slijepcevic 1993 had also suggested that a subclass of dsbs were unable to rejoin, even though the majority of breaks rejoined, accounting for the different yields of chromatid aberrations observed. The results from Cornforth and Bedford (1985) thus provided support for this hypothesis. The situation is thus complex. In G1 cells where there is more time for the repair of dsb the residual dsb may account for elevated chromosome breaks. On the other hand, G2 radiosensitivity cannot be accounted for in this way as there is insufficient time for residual breaks to accumulate. In AT, initial dsb repair is the same or faster than in control individuals, yet chromatid breaks are elevated.

In view of current reports, it is important to determine the relationship between the repair processes occurring at the G2 phase and the chromosomal damage induced by ionising radiation in AT cells. The significance of this radiosensitive response of AT individuals and the characteristics of this disease may classify the link to ionising radiation sensitivity.

*Colour switch ratio in mammalian cell lines*

The inter-chromatid exchange process leading to "type 1" chromatid breaks (see figure IV) can be detected using harlequin staining (where cells undergo two successive divisions in BrdU before irradiation) as a colour switch exchange between chromatids at the point of exchange (Heddle et al 1969), Heddle and Bodycote 1970, Savage and Harvey 1991). As mentioned above, Revell's model predicts that 40% of chromatid aberrations would be associated with a colour switch. Heddle et al (1969) tested this on a Potorous (rat kangaroo) cell line using tritiated thymidine to detect the colour switch exchanges in the chromatid molecules - 14/37 of such colour-switch breaks (38%) were recorded, close to Revell's prediction. A similar experiment was performed with Chinese Ovary cells using harlequin staining and 15-18% of colour switch breaks were recorded, (Heddle and Bodycott 1970). More recent studies have yielded similar values in Chinese hamster cells 15-20%, Harvey and Savage (1997), 16%, Bryant (1998). Wolff and Bodycote (1975) performed the Chinese Hamster cell experiment using harlequin staining and found only 8% of chromatid breaks had a colour jump which does not agree with other measurements.

The breakage-first hypothesis cannot account for the loss of the order of up to 40 Mbp of DNA in a chromosome, as viewed in some chromatid breaks (Bryant 1998). As the dose-response relationship for chromatid breaks is linear, two dsbs cannot be involved in removing such large amounts of DNA from the chromatids. In the exchange model, chromatid breaks are formed as a result of incomplete exchanges at the point of cross-over between two different points in one chromatid or sister chromatids in Revell loop (Savage 1986).

In this report, the csr has been measured in several human blood samples from breast cancer and from control patients, and in an ataxia telangiectasia lymphoblastoid cell line, AT-KS and normal lymphoblastoid cell line, CO-MR. Radiosensitivity was also measured in the form of the total number of chromatid breaks/100 cells observed at metaphase. The aim of this project was thus to investigate if there was a correlation between the csr and the radiosensitivity of human individuals (breast cancer and normal controls) and whether such a relationship might extend to extreme radiosensitivity, as

seen in AT cells.

# MATERIALS AND METHODS

*Culturing of Blood lymphocytes*

Fresh human heparinised blood lymphocytes (0.1ml lithium heparin; 100 /ml blood) from 11 apparently normal control individuals (patients from the Memorial hospital, St. Andrew) and 13 breast cancer individuals (patients from Ninewells hospital, Dundee) was diluted 1:10 in prewarmed, pregased RPM1 1640 medium (containing 10% foetal bovine serum and antibiotics with 15% FBS and 0.2ml L. glutamine). 0.2 ml phytohaemagglutinin was added to the blood lymphocyte culture and 33μM of BrdU was added (harlequin staining) to each flask in the dark and left in the 5% CO2 incubator at 37oC for 72 h. One set of flasks were placed horizontally in the 5% CO2 incubator, whereas the second set of flasks were placed horizontally on a shaker in the warm room. These different techniques were employed to determine whether a better technique for growing cells in BrdU could be devised but as shown in table I, both techniques produced similar results, so the CO2 incubator option was used.

*The G2 assay*

Flasks were irradiated after 72h of culturing at 0.4 Gy of γ-rays for 5s and then placed in the incubator. 0.2 ml [] colcemid (10 μg/ml) was added 30 minutes post-irradiation and left for 2h. The contents of each flask were split among 2 x 12.5 ml plastic centrifuge tubes and plunged into a bucket of ice and agitated to cool rapidly. The flasks were then spun at 1000 r.p.m for 10 minutes (0oC). Supernatant was then removed and 5ml pre-cooled 0.075 M KCl was added and left in the ice bucket for 20 minutes (harvesting of cells procedure). Cells were again spun at 1000 r.p.m for 10 minutes. Supernatant was removed to about 1ml above cell pellet, and freshly made fixative (methanol to acetic acid, 3:1) was added and mixed. Cells were immediately spun at 1000 r.p.m for 10 minutes, fixative changed and left in the fridge overnight

*Fluorescence plus Giemsa staining*

Slides were prepared after cells were exposed to the G2 assay. Slides soaked in distilled H2O were blotted; a solution of acetic acid and distilled water was spread on the slides and a drop of cells from the spun sample under investigation was dropped in the middle of the slide. The slides were then air-borne dried and dipped in 100 mg/ml Hoechst 33258 stain for 10 minutes in the dark; blotted and then placed in a deep tray containing 2 x 20 SSC under a UVA source for 6 h. Slides were then rinsed three times in distilled water and stained in 4% Giemsa for 10 minutes. Metaphase chromosomes were then viewed under the light microscope and breaks were scored in the light and dark strands or as colour switch breaks. The colour switch ratio was calculated as the percentage of the number of colour switch breaks (scored in 100 metaphases) of the total number of chromatid breaks (in 100 metaphase cells). Chromatid breaks and gaps (a continuation whose length is smaller than the width of a chromatid) are produced from the G2 assay. Only chromatids breaks were scored.

*AT lymphoid cell culture*

One AT lymphoblastoid cell line, AT-KS, and one control lymphoblastoid cell line, CO-MR, was used in these experiments. A similar protocol to the breast cancer patients was used in the AT and normal lymphoblastoid cell line, but cultured flasks were irradiated at 0.238 Gy of $\gamma$ - rays for 5s. One set of flasks for each line were unirradiated. All flasks were placed vertically in the incubator. Cells were harvested as in the G2 assay described above and slides were made and exposed to an UVA source for 6h (as described earlier) and stained slides were scored.

# RESULTS

**Table I**: Frequencies of chromatid breaks in breast cancer and normal subjects after 0.4 Gy irradiation

Blood samples from breast cancer and control patients were cultured and the number of colour- switch chromatid breaks and chromatid breaks in the light and dark strands were measured in the G2 assay. The number after BRC indicates the code number of the blood collected, not the age of the individual. F = blood from a normal female control patient at the Memorial hospital, St. Andrews. M = bloods from normal male control patients at the Memorial hospital, St. Andrews. I = flasks placed horizontally in a 37oC ($CO_2$) incubator, S = flasks placed horizontally on the shaker in a (37oC) warm room. D = dark (TB) strand of the harlequin stained chromatid and L= light (BB) strand of the harlequin stained chromatid. The G2 assay was carried out in parallel by a technician as part of another study that did not incorporate BrdU in the culturing stage (column 4).

| Sample code | Chromatid breaks | | | Total no of breaks | No of $G_2$ breaks – independent exp | Ratio of L/D | % csr |
|---|---|---|---|---|---|---|---|
| | D | L | CS | | | | |
| BRC 84S | 22 | 18 | 31 | 71 | 42 | 0.81 | 18 |
| BRC 84I | 20 | 23 | 26 | 69 | 42 | 1.15 | 15 |
| BRC 76S | 25 | 26 | 26 | 77 | | 1.04 | 18 |
| BRC 76I | 24 | 24 | 31 | 79 | | 1.6 | 22 |
| BRC 82S | 26 | 22 | 14 | 62 | 64 | 0.85 | 8 |
| BRC 82I | 28 | 21 | 17 | 66 | 64 | 0.75 | 10 |
| BRC 79S | 30 | 24 | 21 | 75 | 66 | 0.80 | 14 |
| BRC 79I | 26 | 20 | 18 | 64 | 66 | 0.78 | 10 |
| BRC 97I | 23 | 15 | 19 | 57 | | 0.65 | 9 |
| BRC 98I | 10 | 18 | 30 | 58 | | 1.8 | 12 |
| BRC 100I | 24 | 34 | 19 | 77 | | 1.41 | 14 |
| BRC 72I | 20 | 19 | 29 | 68 | 64 | 0.95 | 16 |
| BRC 73I | 16 | 17 | 37 | 70 | 58 | 1.06 | 19 |
| BRC 84I | 14 | 12 | 26 | 52 | 42 | 0.86 | 9 |
| BRC 86I | 23 | 20 | 30 | 73 | 74 | 0.87 | 18 |
| BRC 94I | 25 | 24 | 27 | 76 | 84 | 0.96 | 18 |
| BRC 107I | 12 | 8 | 38 | 58 | | 1.5 | 12 |
| F49S | 26 | 26 | 7 | 59 | 60 | 1.0 | 4 |
| F49S | 29 | 26 | 11 | 66 | 70 | 0.90 | 7 |
| M48S | 20 | 12 | 6 | 38 | 36 | 0.60 | 2 |
| M48I | 18 | 10 | 7 | 35 | 36 | 0.55 | 2 |
| M50I | 7 | 4 | 31 | 42 | | 0.59 | 5 |
| M49I | 7 | 7 | 7 | 21 | | 1.0 | 1 |
| F34I | 6 | 8 | 22 | 36 | | 1.3 | 4 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| M58I | 20 | 2 | 18 | 40 | | 0.75 | 4 |
| F20I | 11 | 10 | 16 | 37 | 50 | 0.9 | 4 |
| F45I | 10 | 13 | 23 | 46 | 68 | 1.3 | 7 |
| F30I | 11 | 8 | 17 | 36 | 32 | 0.73 | 4 |
| F58I | 12 | 9 | 13 | 34 | 40 | 0.75 | 3 |
| M45I | 6 | 4 | 29 | 39 | | 0.67 | 4 |

**Table II**: Frequencies of chromatid breaks in the normal unirradiated and irradiated control lymphoblastoid cell line

An AT cell line (AT-KS) and a normal cell line (CO-MR) were cultured and the number of colour switch chromatid breaks and chromatid breaks in the light and dark strands were measured in the G2 assay. Experiments were repeated four times using the same AT cell line (AT-KS) and normal cell line (CO-MR). Thus each table shows four results - one result indicates one experiment. (Table VI shows a summary of all the data for the four experiments carried out). normal unirr = unirradiated normal lymphoblastoid control cell line , normal irr = irradiated normal lymphoblastoid cell line, AT unirr = unirradiated AT lymphoblastoid cell line , AT irr = irradiated AT lymphoblastoid cell line.

| Sample code<br>normal cell line | Chromatid breaks<br>D | L | CS | Total no of breaks | Ratio L/D | % csr |
|---|---|---|---|---|---|---|
| unirr | 5 | 3 | 2 | 10 | 0.6 | 20 |
| unirr | 4 | 3 | 1 | 8 | 0.8 | 13 |
| unirr | 4 | 4 | 2 | 10 | 1.0 | 20 |
| unirr | 3 | 2 | 1 | 6 | 0.7 | 16 |
| Average | 4 | 3 | 1.5 | 8.5 | 0.78 | 17 |
| irr | 7 | 5 | 3 | 15 | 0.71 | 20 |
| irr | 6 | 5 | 4 | 15 | 0.83 | 27 |
| irr | 7 | 4 | 5 | 16 | 0.57 | 31 |
| irr | 5 | 6 | 3 | 14 | 1.20 | 21 |
| Average | 6 | 5 | 5 | 16 | 0.83 | 25 |

**Table III**: Frequencies of chromatid breaks in the unirradiated and irradiated AT cell line The number of colour switch breaks and breaks in the light and dark strands were scored in the G2 assay in the AT-KS and CO-MR lymphoblastoid cell lines.

| Sample code | Chromatid breaks D | L | CS | Total no of breaks | Ratio L/D | % csr |
|---|---|---|---|---|---|---|
| Unirr | 14 | 13 | 11 | 38 | 0.92 | 25 |
| unirr | 17 | 14 | 12 | 43 | 0.82 | 23 |
| unirr | 13 | 10 | 9 | 32 | 0.78 | 28 |
| unirr | 15 | 12 | 11 | 38 | 0.80 | 27 |
| average | 15 | 12 | 11 | 38 | 0.83 | 6 |
| irr | 28 | 30 | 19 | 77 | 1.10 | 29 |
| irr | 30 | 27 | 17 | 74 | 0.90 | 28 |
| irr | 25 | 24 | 19 | 68 | 0.96 | 28 |
| irr | 26 | 23 | 18 | 67 | 0.88 | 29 |
| average | 27 | 26 | 18 | 71 | 0.96 | 29 |

**Table IV**: Summary of the average results for the AT and normal cell line

The table below shows a summary of the number of chromatid breaks scored where each sample code result actually represents an average of four similar experiments.

| Sample code | Chromatid breaks D | L | CS | Total no of breaks | Ratio L/D | % csr |
|---|---|---|---|---|---|---|
| normal unirr | 4 | 3 | 2 | 9 | 0.78 | 17 |
| AT unirr | 15 | 12 | 11 | 38 | 0.83 | 26 |
| AT irr | 27 | 26 | 18 | 71 | 0.96 | 29 |
| norm irr | 6 | 5 | 5 | 16 | 0.83 | 25 |

**Table V**: Two sample t-test for colour switch ratio in breast cancer and control patients

A two sample t-test for csr was carried out for breast cancer and normal control patients to determine whether the two pairs of groups were significantly different. SD= standard deviation, N= number of patients, P=0.0024 DF= 22. From the results in table V, the P value is 0.0024 indicating that the csr between breast cancer and control patients are statistically significant. There is a significant difference in csr in breast cancer and control patients (when the P value is equal to or less than 0.005 the values become significant).

| Sample code | N | Mean csr | SD |
|---|---|---|---|
| BRC | 17 | 25.8 | 6.93 |
| CONTROL | 13 | 15.9 | 8.47 |

**Table VI**: Two sample t-test for the frequency of total chromatid breaks/100 cells (radiosensitivity) scored in breast cancer and control patients

A two sample t-test for radiosensitivity was carried out for breast cancer and normal control patients to determine whether the two pairs of groups were significantly different. P=0.0000 DF= 22. The P value (0.0000) indicates an extremely significant difference in the mean frequency of total chromatid breaks (i.e. radiosensitivity) between breast cancer and control patients .

| Sample code | N | Mean | SD |
|---|---|---|---|
| BRC | 17 | 56.2 | 12.0 |
| CONTROL | 13 | 27.9 | 14.8 |

**Table VII**: Two sample t-test for the ratio of the frequency of chromatid breaks in the light strand: the frequency of chromatid breaks in the dark strand, L/D, in breast cancer and control patients

A two sample t-test for L/D was carried out for breast cancer and normal control patients to determine whether the two pairs of groups were significantly different. P= 0.07 DF = 27. The P value (0.07) indicates that there is not a significant difference in L/D for breast cancer and control patients.

| Sample code | N | Mean | SD |
|---|---|---|---|
| BRC | 17 | 1.05 | 0.33 |
| CONTROL | 13 | 0.85 | 0.25 |

**Table VIII**: Two sample t-test for the ratio of colour switch ratio: the number of colour switch breaks in breast cancer and control patients (csr/csb)

A two sample t-test for csr/csb was carried out for breast cancer and normal control patients to determine whether the two pairs of groups were significantly different. P=0.0014 DF= 12. The P value (0.0014) indicates that there is a significant difference in the ratio of colour switch ratio: the frequency of colour switch breaks (per 100 cells) in breast cancer and control patients.

**Table IX**: Two sample t-test for the colour switch ratio in an Ataxia Telangiectasia and normal lymphoblastoid cell line

A two sample t-test for csr was carried out for the AT- KS and CO- MR to determine whether the two lymphoblastoid cell lines were significantly different. P=0.25 DF=3. The P value (0.25) for csr is not significantly different in the AT and control cell line.

| Sample code | N | Mean | SD |
|---|---|---|---|
| AT | 4 | 28.5 | 0.58 |
| CONTROL | 4 | 24.8 | 5.19 |

**Table X**: Two sample t-test for the frequency of total chromatid breaks/100 cells (radiosensitivity) in an ataxia telangiectasia and normal cell line

A two sample t-test for csr was carried out for the AT- KS and CO- MR to determine whether the two lymphoblastoid cell lines were significantly different. P=0.0002 DF= 3. The P value for radiosensitivity (0.0002) does show a very significant difference in the AT and control cell line.

| Sample code | N | Mean | SD |
|---|---|---|---|
| AT | 4 | 71.5 | 4.80 |
| CONTROL | 4 | 15.0 | 0.41 |

**Table XI**: Two sample t-test for the ratio of the frequency of chromatid breaks in the light strand: the frequency of chromatid breaks in the dark strand, L/D, in an Ataxia Telangiectasia and control cell line

A two sample t-test for L/D was carried out for the AT- KS and CO- MR to determine whether the two lymphoblastoid cell lines were significantly different. P=0.43 DF= 3. The P value for L/D (0.43) indicates that there is not a significant difference in L/D for

the AT and control cell line.

| Sample | N | Mean | SD |
|---|---|---|---|
| AT | 4 | 0.96 | 0.10 |
| CONTROL | 4 | 0.83 | 0.27 |

**Table XII**: Two t-test for the frequency of total chromatid breaks/100 cells in male and female control patients

A two sample t-test for radiosensitivity was carried out for the male and female controls to determine whether there were any significant differences. P=0.11 DF= 7. From the results, it is observed that there is not a significant difference in radiosensitivity between male and female controls.

| Sample | N | Mean | SD |
|---|---|---|---|
| male | 6 | 20.8 | 6.71 |
| female | 7 | 34.0 | 17.5 |

**Table XIII**: Two sample t-test for colour switch ratio in breast cancer patients and the Ataxia Telangiectasia cell line

A two sample t-test for csr was carried out for the breast cancer and AT- KS cell line to determine whether there were any significant differences. P=0.14 DF=16. From table XIII, it is observed that there is no significant difference in csr between breast cancer patients and the Ataxia Telangiectasia cell line as P= 0.14

| Sample | N | Mean | SD |
|---|---|---|---|
| BRC | 17 | 25.8 | 6.93 |
| AT | 4 | 28.5 | 0.58 |

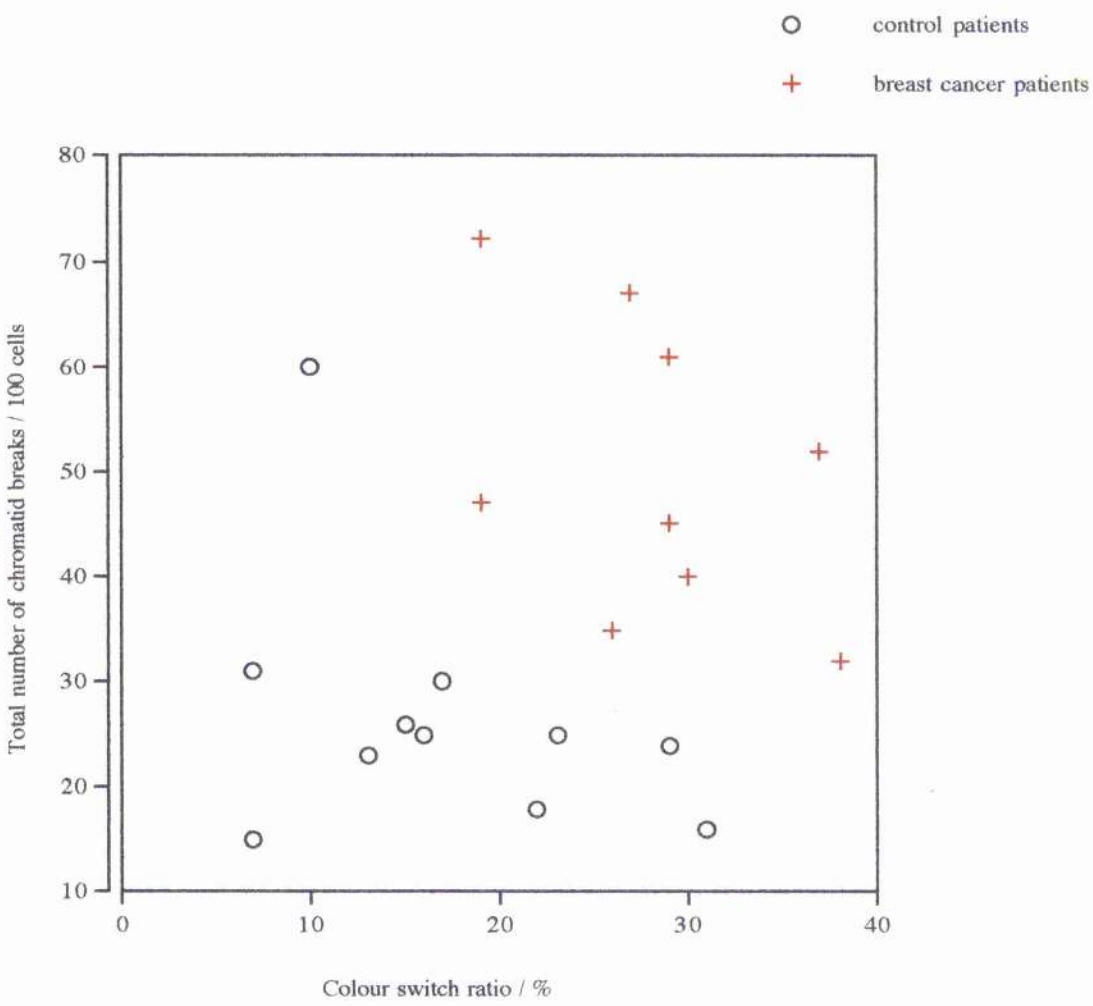


Figure VI : The relationship between colour switch ratio and the frequency of breaks per 100 metaphase cells in breast cancer and control patients

As shown in figure VI, the csr is variable in humans. There is a significantly higher mean csr in breast cancer patients (25.8% range 14-38%) in comparison to normal control individuals (15.9 range 6-29%); P=0.0024. When csr is plotted against radiosensitivity, two distinct groups are apparent - one for control individuals with a lower mean of csr and another for breast cancer patients with a higher mean of csr. The average mean of csr is approximately 1.5 times higher in breast cancer individuals than in normal control individuals. This figure demonstrates that two separate cohorts are produced; one for breast cancer patients and a second for control patients. It is interesting to note that one particular control individual (F49) lies in the breast cancer cohort. The two separate cohorts are spread out showing an obvious difference in csr between both groups (with the exception of F49). Thus, there appears to be a correlation when csr is plotted against radiosensitivity as shown in figure VI.

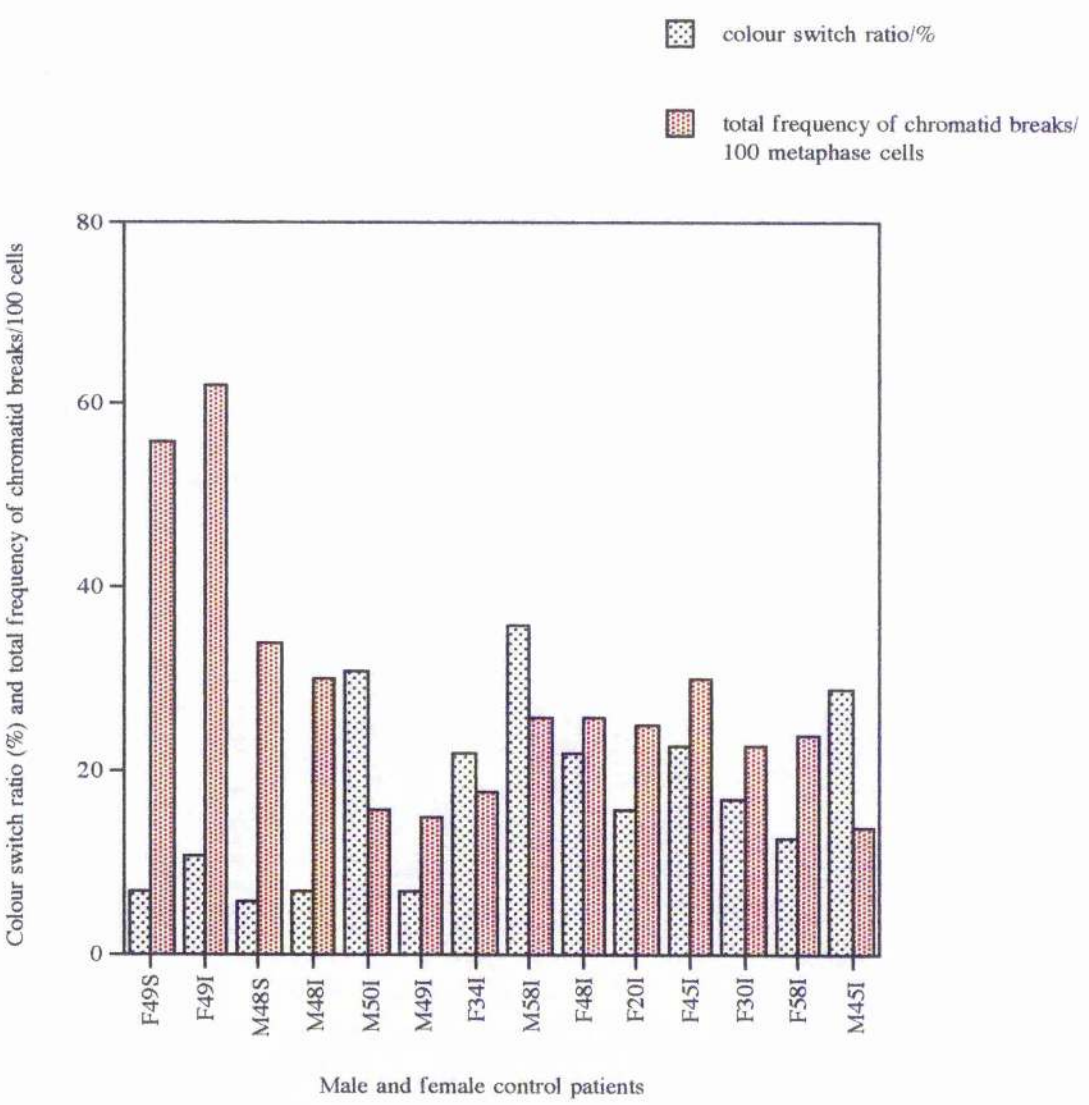


Figure VII: The relationship between colour switch ratio and the frequency of total chromatid breaks in male and female control patients

As shown in figure VII, there is no direct relationship between csr and the total frequency of chromatid breaks/100 cells (i.e. radiosensitivity) in control patients. Thus when radiosensitivity, or csr is plotted against the controls, no apparent correlation is observed. On the other hand, if csr was plotted against radiosensitivity as shown in figure VI, an apparent correlation is observed. It must be noted that control patient F49 has considerably higher radiosensitivity than the other patients.

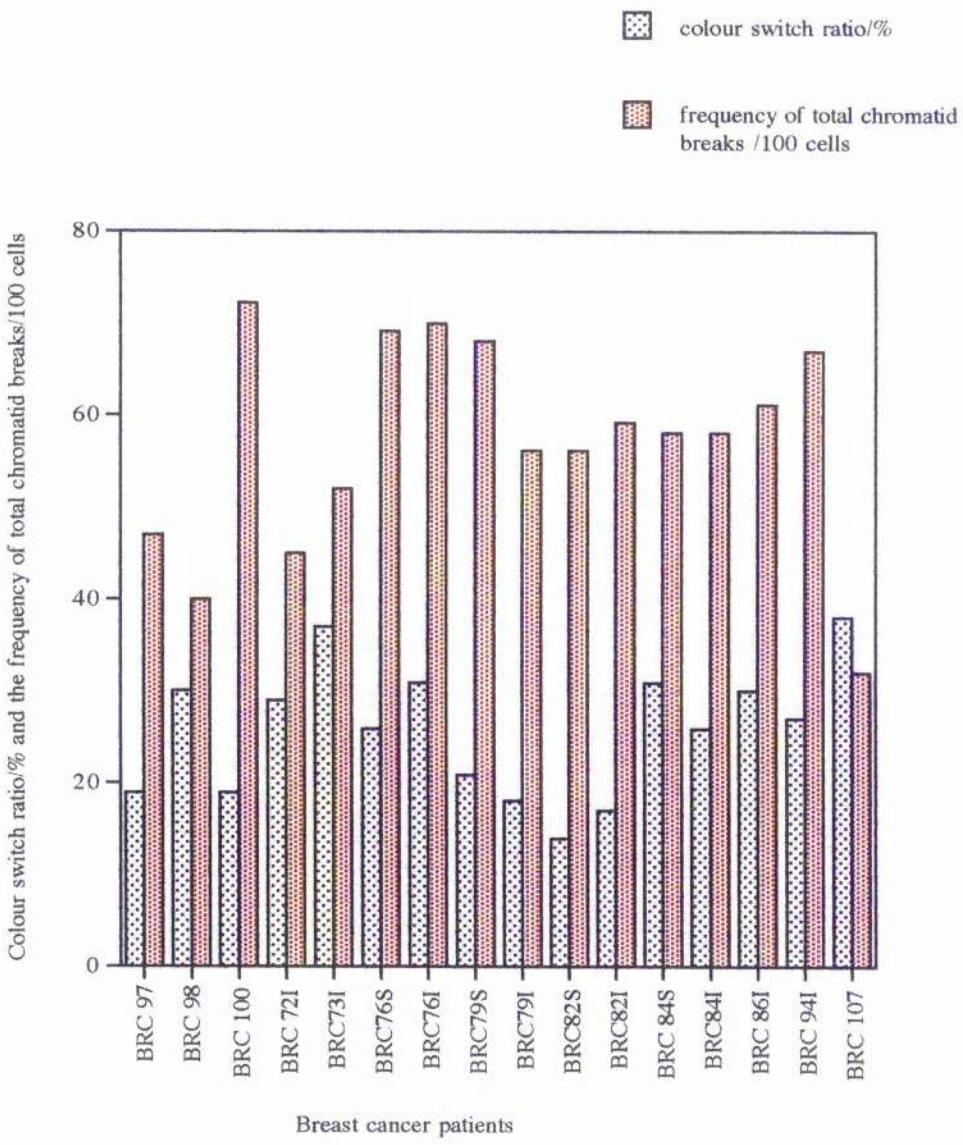


Figure VIII: The relationship between colour switch ratio and the frequency of total chromatid breaks in breast cancer patients

Figures VII and VIII show that there does not appear to be a particular correlation between the frequency in total chromatid breaks/100 cells (i.e. radiosensitivity) or csr in breast cancer or control patients. The mean csr and average frequency of total chromatid breaks/100 cells does, however, appear to be higher in breast cancer patients than controls (with the exception of F49 - see table V and VI). As previously mentioned, there does appear to be a correlation when csr is plotted against radiosensitivity for breast cancer and control patients though as shown in figure VI .

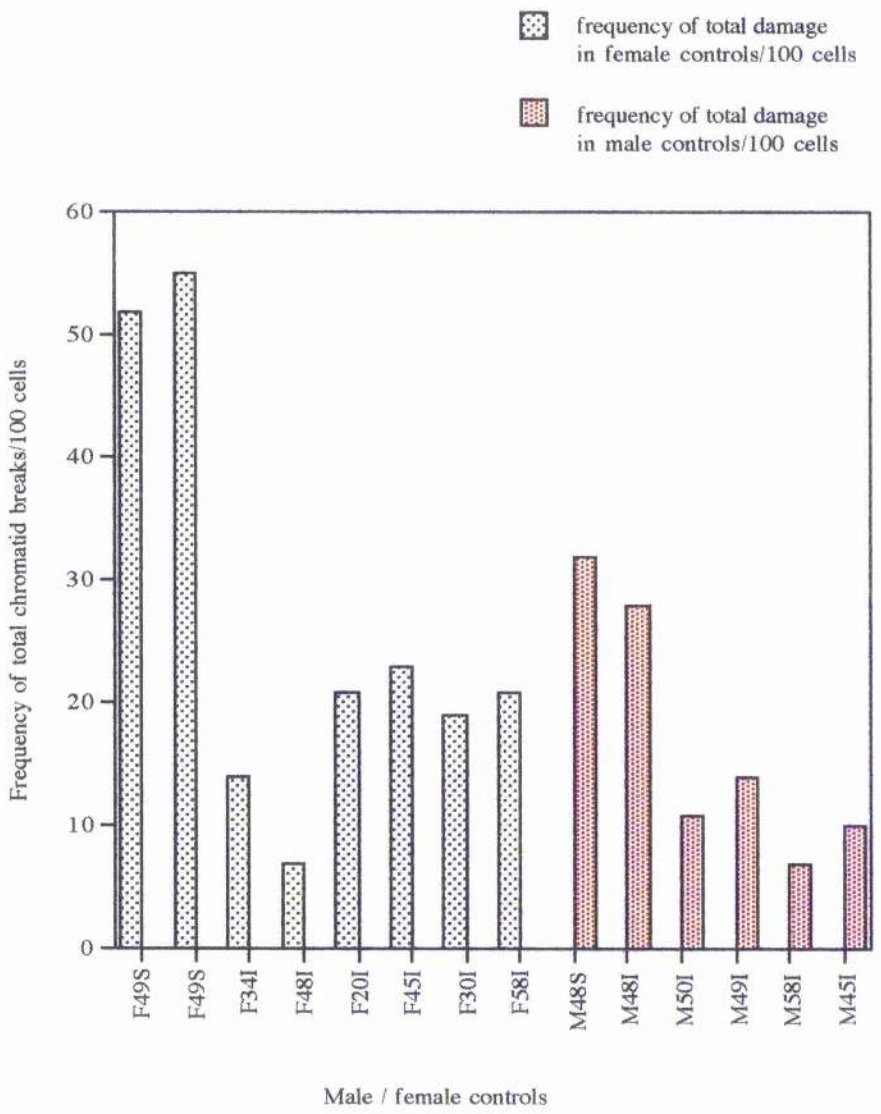


Figure IX: Frequency of total chromatid damage in male and female controls

It is observed in figure IX that there are no significant differences in radiosensitivity between sexes in control patients; P=0.11, but that there is a slightly higher mean frequency of chromatid breaks/100 cells observed in the female donors (34.0; range 23-62) than the male (20.8; range 15-30). The majority of the mean frequency of breaks in both sexes is in the range of 10-35 chromatid breaks/100 cells. There is an unusually high value, however, for the frequency of total chromatid damage/100 cells recorded for the female patient F49 (56 chromatid breaks and 62 chromatid breaks) and none as high in the male patients. This observation indicates the importance of scoring a large number of samples to deduce a possible relationship, if present. It would be of great interest to follow up patient F49 if she developed a form of cancer as this would provide support for this assay to be used as a predictive test for the predisposition ocer.

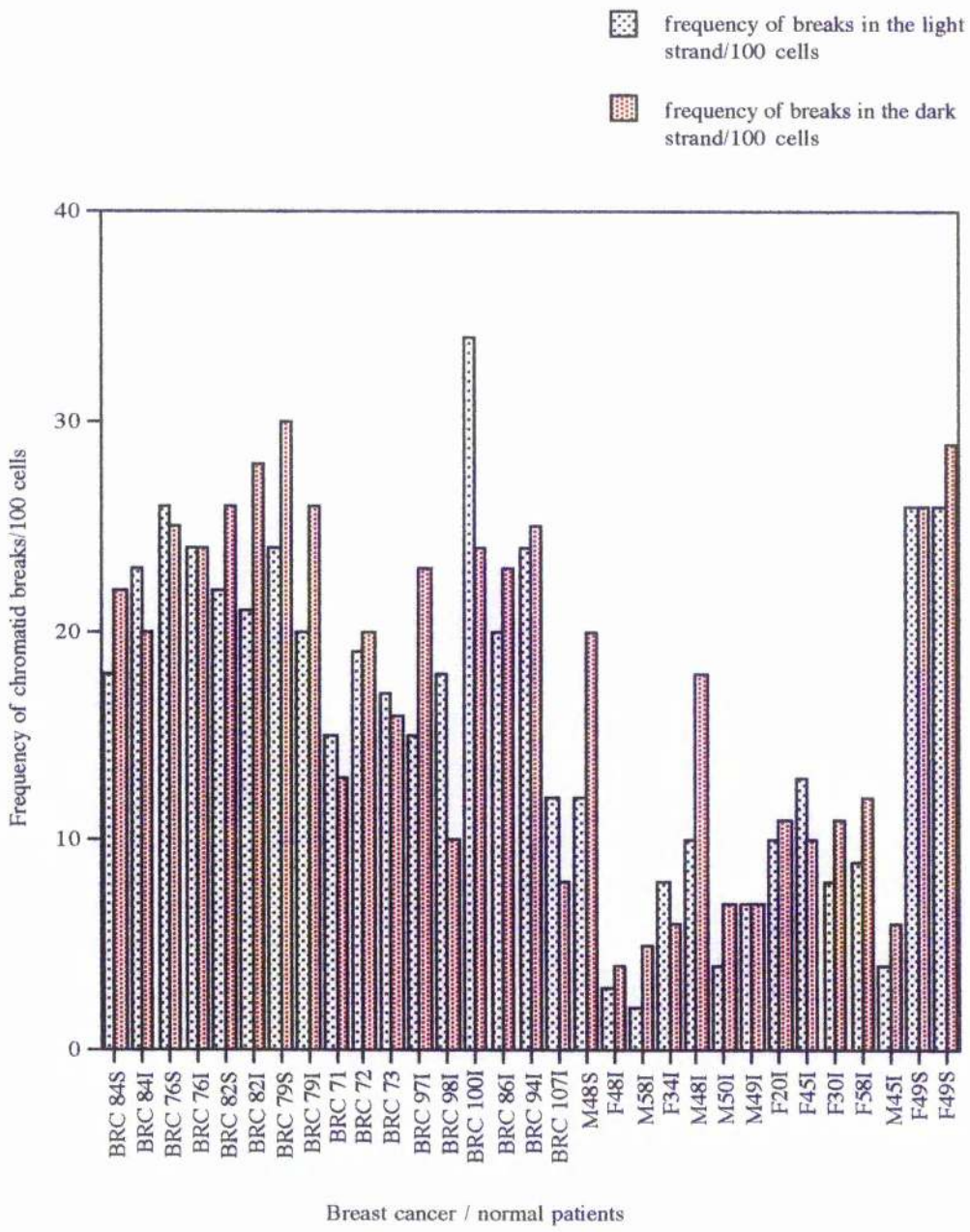


Figure X: A comparison of the frequency of chromatid breaks in the light and dark strands for breast cancer and control patients

There appears to be no trend indicating no significant difference between the frequencies of chromatid breaks in the light and dark strands in breast cancer (1.05; range 0.75-1.8) and control patients (0.85; range 0.6-1.3). The P value for L/D in control and breast cancer patients = 0.07. There is a higher frequency of breaks/100 cells in breast cancer patients than controls by an approximate factor of two (with the exception of F49 control donor). In BRC100, for example, it is interesting to observe a considerably higher number of breaks in the light strand as opposed to the dark strand (34 as opposed to 24) and a higher frequency of breaks in the dark strand as opposed to the light strand in control donors M48 and BRC97. There may be two explanations for this. The first being, that when BrdU is incorporated in the chromatid strands during the synthesis phase of the cell cycle (added while cell culturing) it renders the strands more radiosensitive - increasing the chances of chromatid degradation. This would explain the higher observed number of chromatid breaks in the light strands as both chromatids incorporate BrdU; but not the observed increase in numbers of breaks in the dark strands since only one strand incorporates BrdU. In the cases where the ratio of L/D is less than 1.0 (see figure XII), it appears that BrdU is somehow protecting the chromatids from breakage in the light strands (in the observed cases) where there are a higher number of breaks in the dark strands. The second and more likely explanation, is that as the dark chromatids are more easily visible under the light microscope when scoring, more breaks are recorded in comparison to the light breaks.

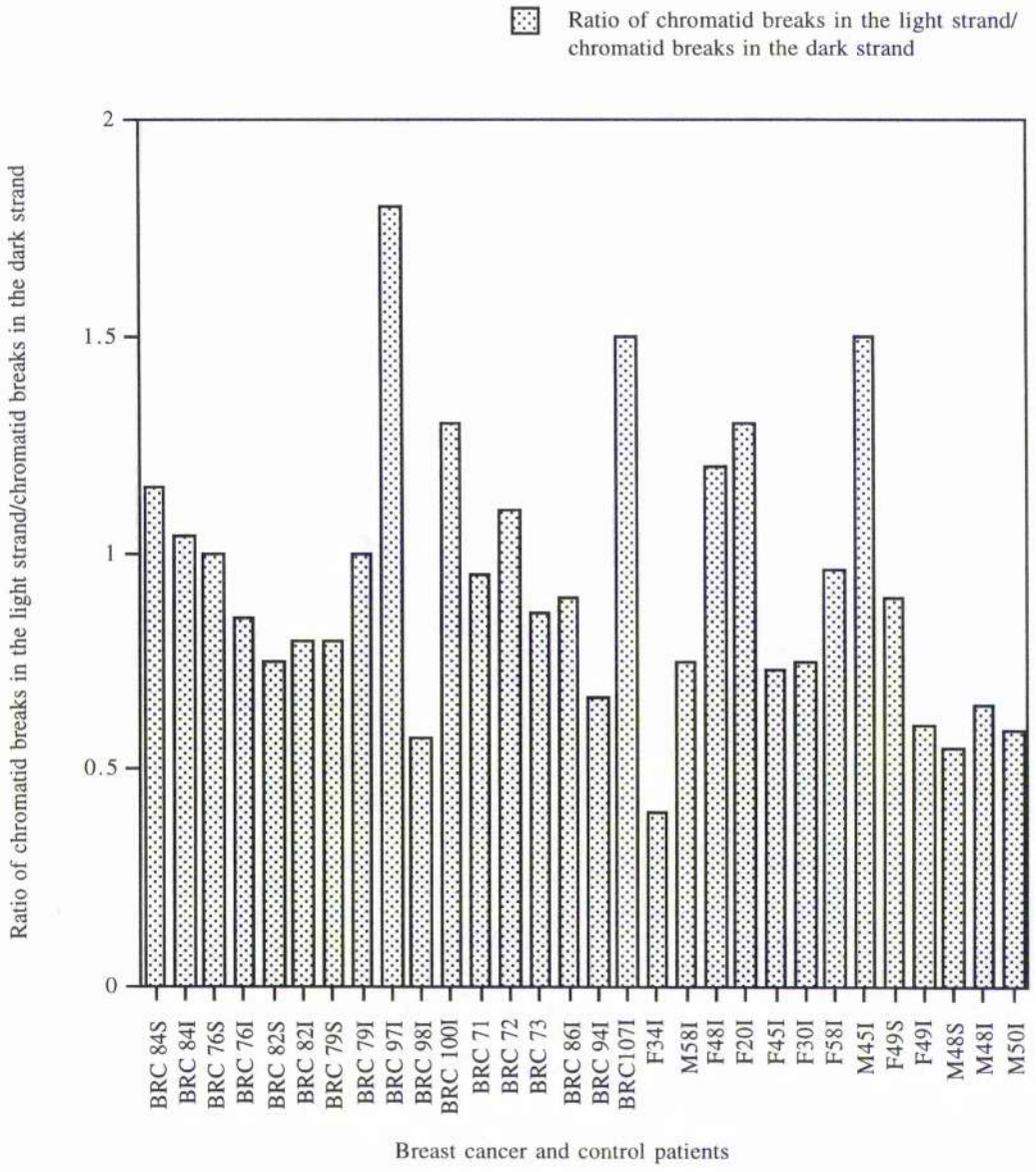


Figure XI: The ratio of chromatid breaks in the light strand: chromatidbreaks in the dark strand for breast cancer and control patients

As shown above in figure XI and table VI, the mean ratio of L/D for breast cancer patients (1.05) and control donors (0.85) is approximately 1.0. There is no significant difference in L/D between breast cancer and control patients; P= 0.07. There are a number of patients, however, that show values of L/D higher than 1.0, e.g. BRC 98 (1.8) and BRC 107 (1.8). This may be a result of the effects of harlequin staining (as discussed earlier) or inaccurate scoring. In comparing breast cancer and control patients, no significant differences in L/D is observed (P=0.07).

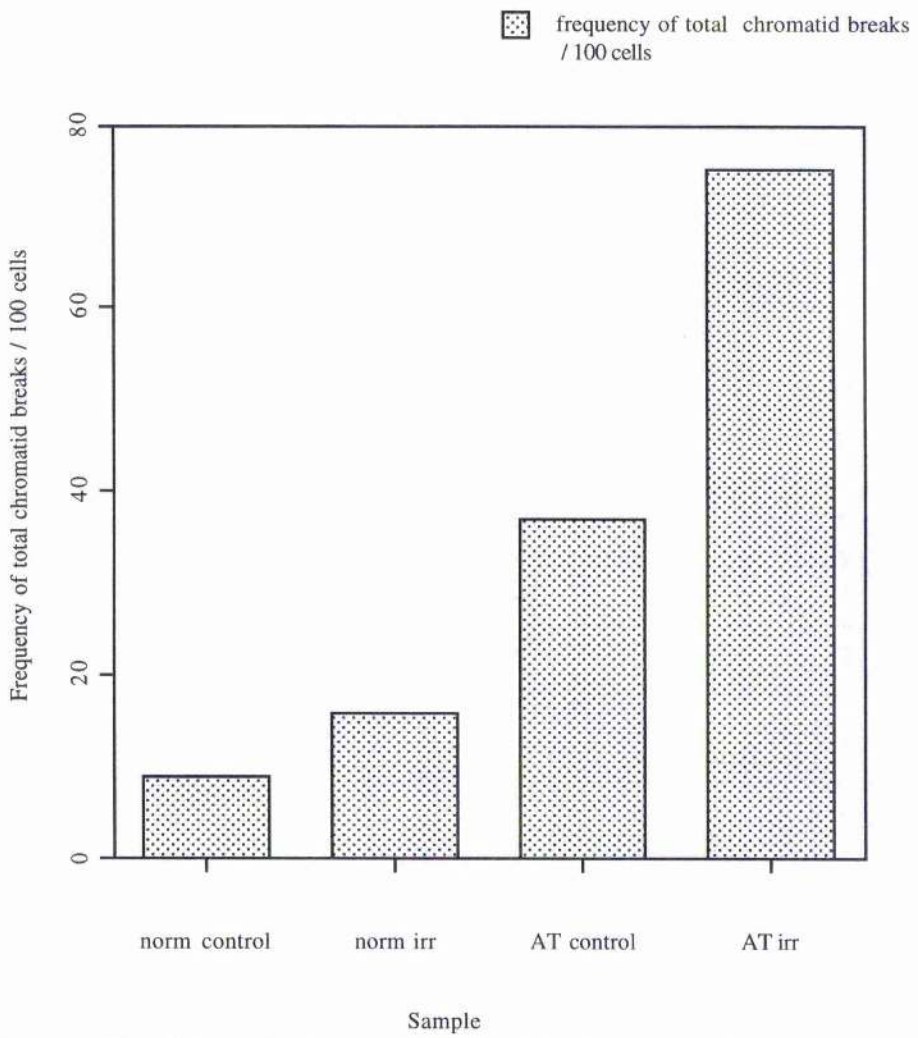


Figure XIII : The total amount of chromatid damage in irradiated and unirradiated normal and AT lymphoblastoid cell lines

From figure XIII, it is clearly observed that there is a significantly higher mean frequency of total chromatid breaks in the AT cell line/100 cells (71.5; range 67-77), in comparison to the normal cell line (15.0; 14-16). The P value for the mean frequency of total chromatid breaks in the AT and control cell line is 0.0002. The frequency of chromatid damage is approximately three times higher in the AT cell line than in the normal cell line after irradiation. This illustrates that AT individuals exhibit chromosomal hypersensitivity.

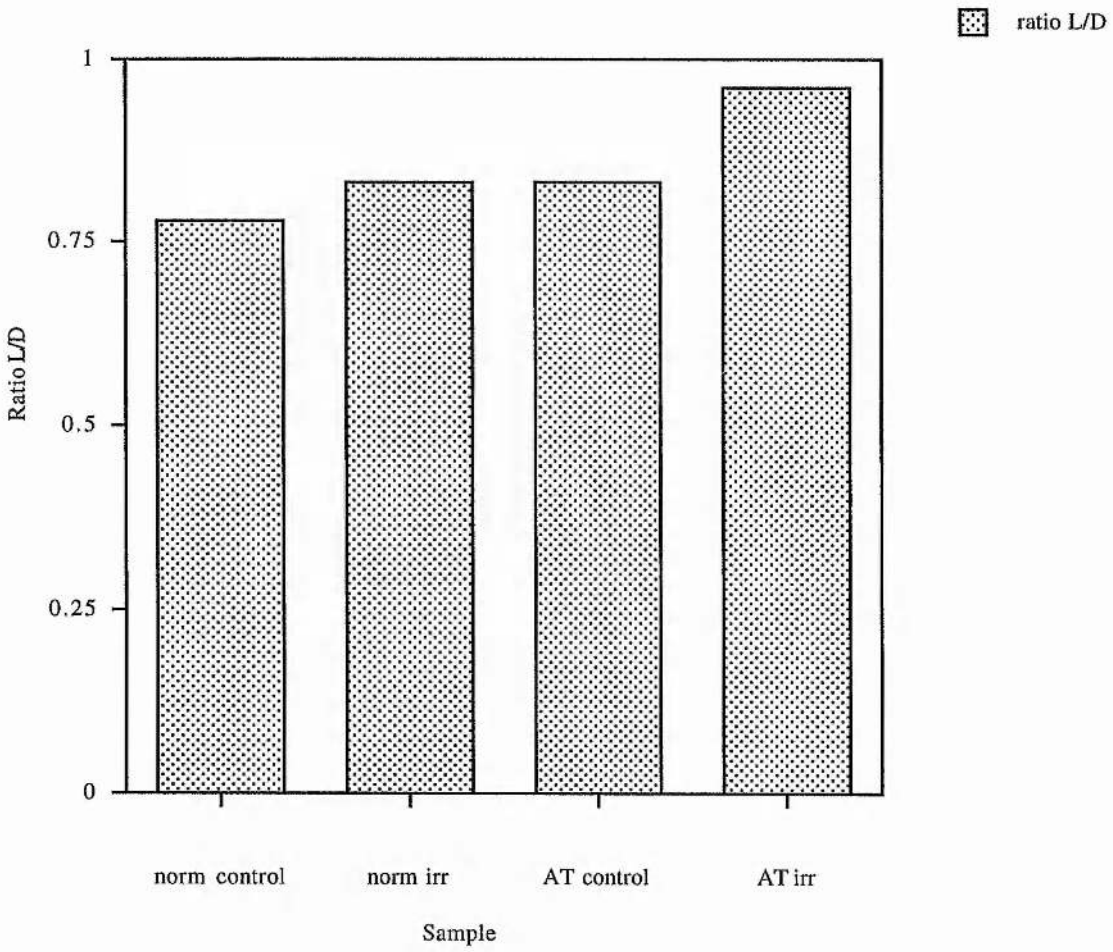


Figure XIV : The number of chromatid breaks in the light strand/ number of chromatid breaks in the dark strand for an irradiated and unirradiated normal and AT cell line

The mean L/D ratio for the normal cell line was 0.83 (range 0.57-1.20) and 0.96 (range 0.88-1.10) for the AT cell line. There was no significant difference between cell lines in the ratio of L/D as P= 0.43. The presence of a slightly higher number of observed breaks in the dark strand is unexpected as it should be more apparent in the lighter chromatid, if at all due to a higher incorporation of BrdU in the light strand.

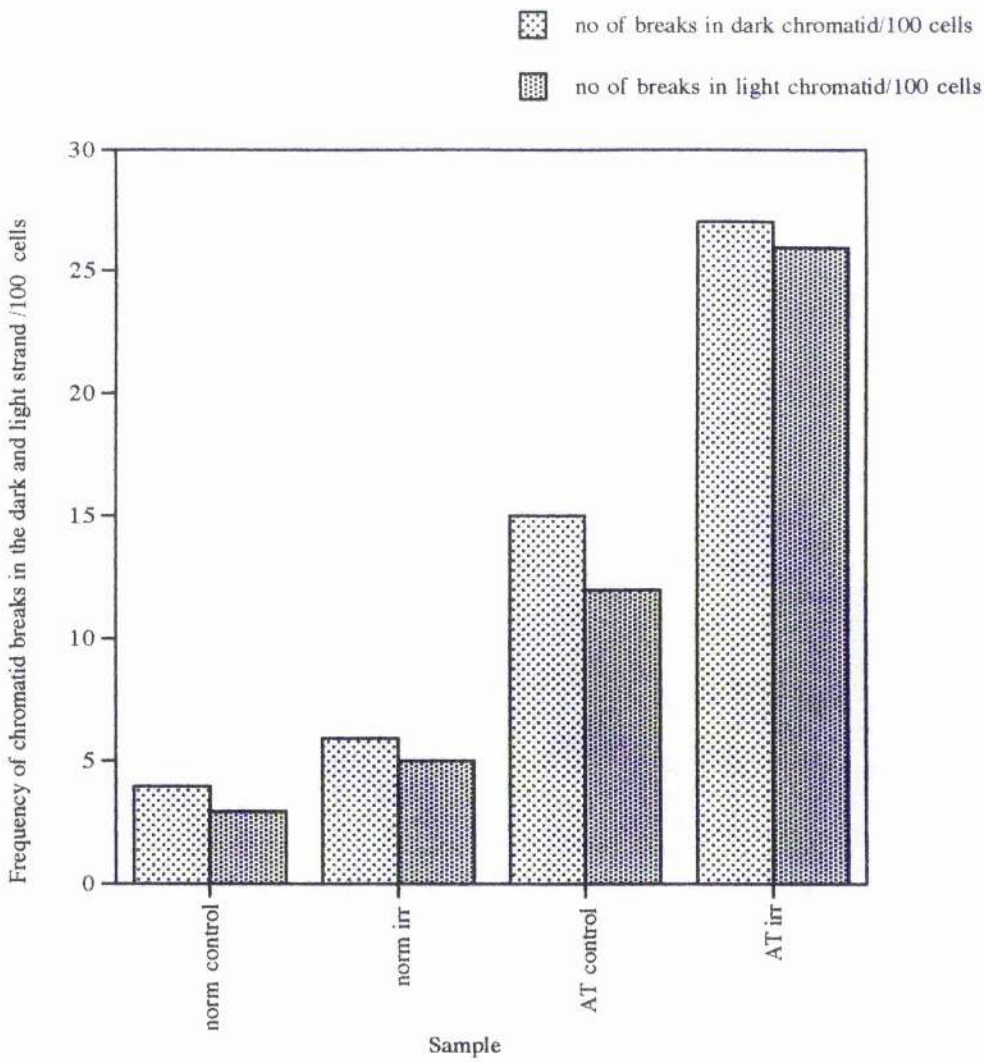


Figure XV : Frequencies of chromatid breaks in the light and dark strands in irradiated and unirradiated AT and control cell lines

Figure XV indicates that there is a slightly higher frequency of chromatid breaks in the dark strand (as opposed to the light strand) in both cell lines. The irradiated AT cell line is approximately five times more radiosensitive than in the normal cell line. This demonstrates that the AT cell line exhibits a higher mean frequency of total chromatid breaks and therefore higher radiosensitivity than the normal cell line; P= 0.0002 (see table X).

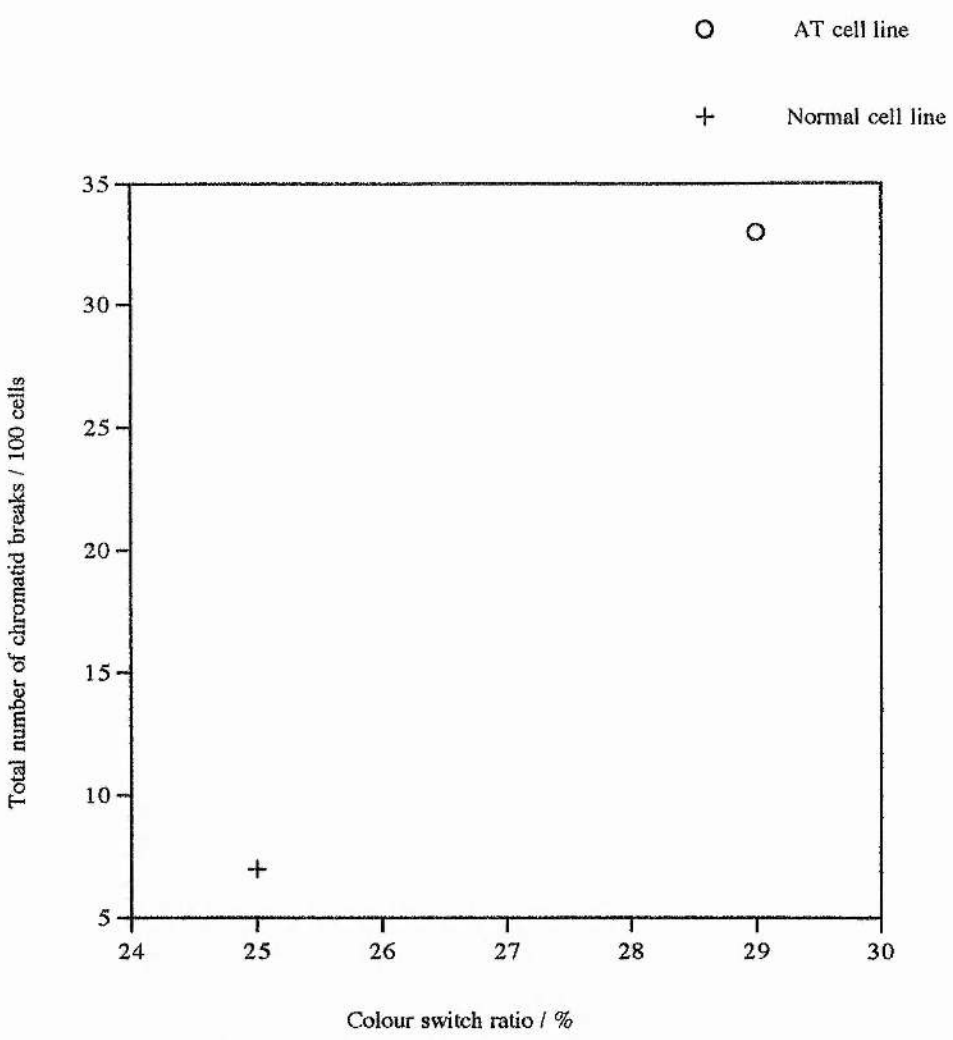


Figure XVI: The relationship between colour switch ratio and the frequency of chromatid break/100 cells in a normal and AT lymphoblastoid cell line

The AT cell line is more radiosensitive and has a higher mean csr than the normal cell line as shown in figure XVI. If this graph is compared to figure VI where the csr is plotted against the frequency of chromatid breaks/100 cells in breast cancer and control patients, then it is observed that there is a correlation between both graphs. The value in figure XVI for the AT cell line lies within the breast cancer patient cohort region and the value for the control cell line lies within the control cohort region in figure VI. The values of csr and radiosensitivity for the AT and control cell line are higher than the breast cancer and control patients are but a similar pattern in cohorts is observed in figure XVI.

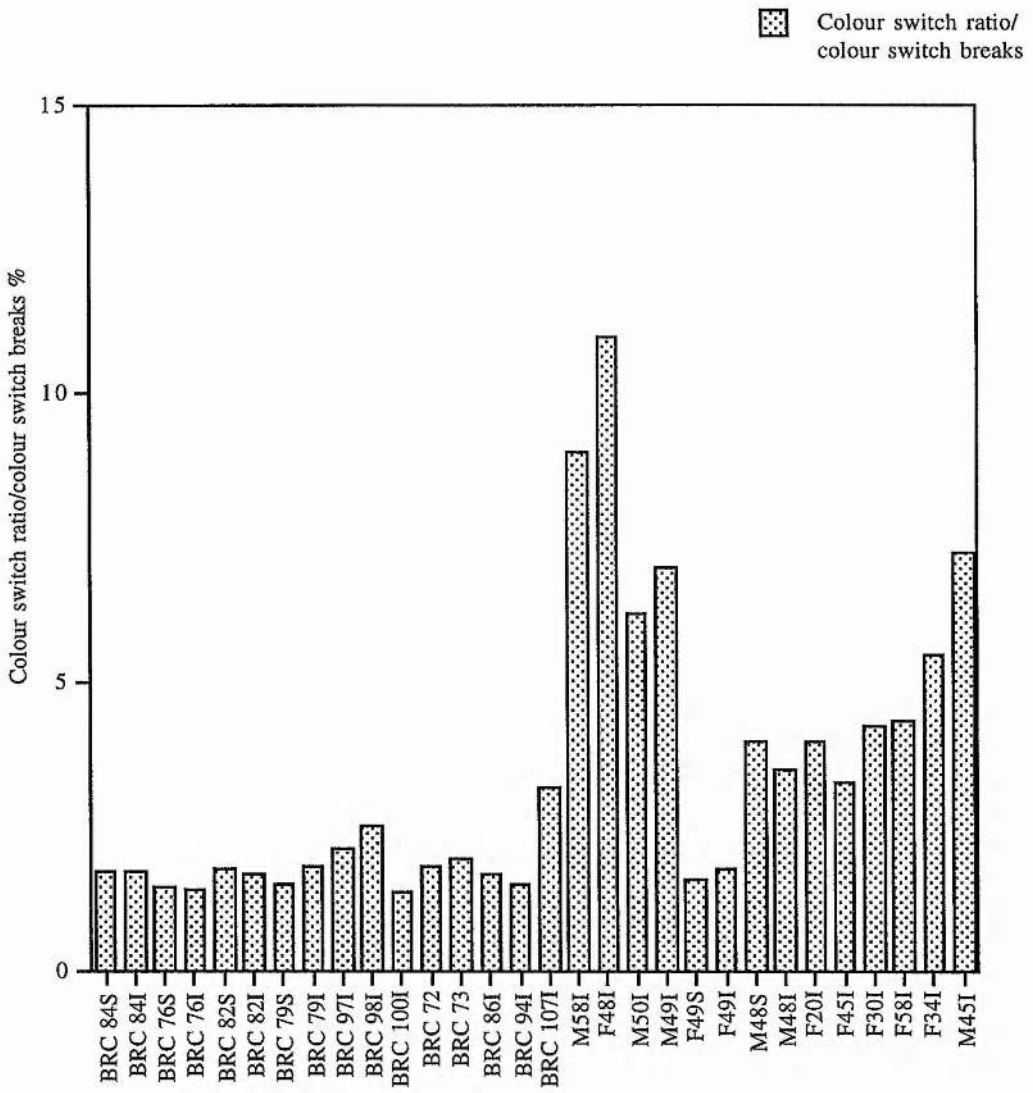


Figure XVIII: Colour switch ratio / frequency of colour switch breaks in breast cancer and control patients

As shown in figure XVIII, when colour switch ratio/colour-switch breaks (csb) is plotted for breast cancer and control patients, it is observed that the mean csr/csb is significantly higher in control patients (5.03, with the exception of F49) in comparison to the mean csr/csb in cancer patients (1.88). The P value is 0.0014 indicating that there is a significant difference in csr/csb between control and breast cancer patients.

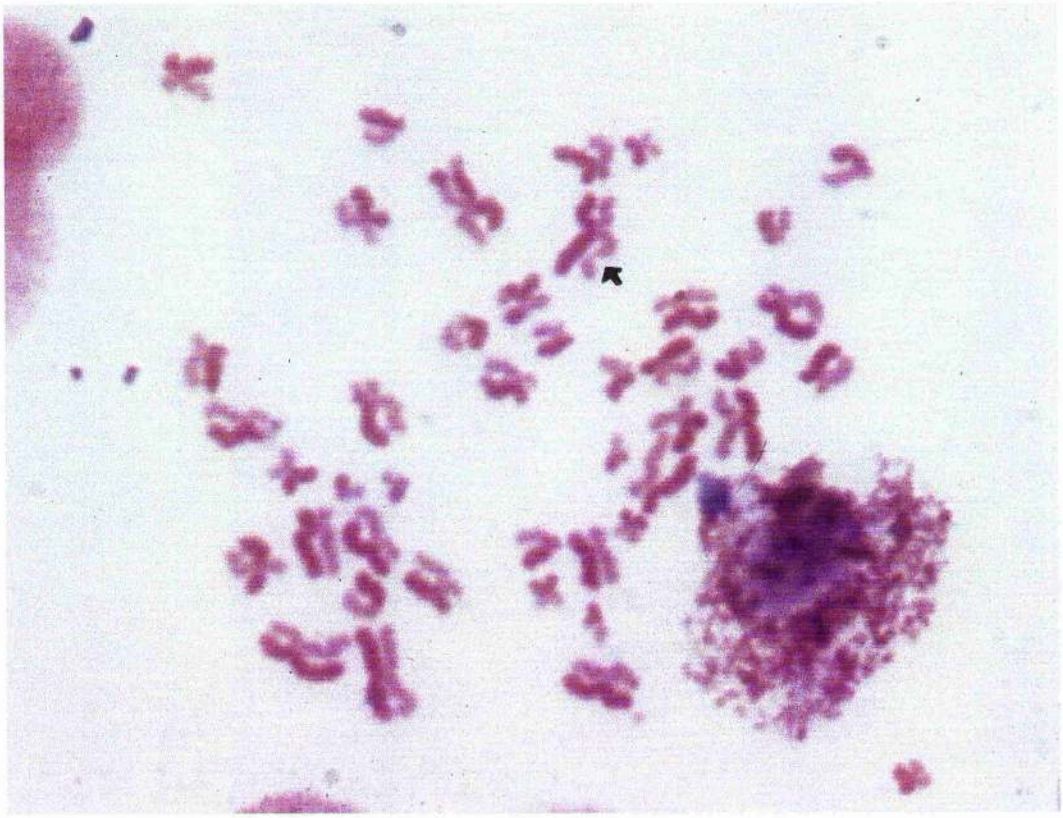

Figure XIX: An example of a harlequin stained Ataxia Telangiectasia metaphase cell.

Figure XIX is an example of a harlequin stained Ataxia Telangiectasia metaphase cell. Colour switches are observed by the exchange in light and dark chromatid strands. A colour switch break is a colour switch with a break in one of the chromatid strands. (dark or light). The arrow shows a simple break - this does not involve a colour switch though.

# DISCUSSION

*Optimisation of experimental protocol for harlequin staining of chromosomes of human lymphocytes*

Having tried without success to use a protocol reported in the literature for Chinese hamster cells (Perry and Wolff 1974), optimisation was clearly necessary. A number of preliminary experiments were performed to determine the optimal conditions for the G2 assay with BrdU labelling for the blood samples. Experiments were performed in which the concentration of BrdU was varied such that the concentration added to the cell culture would not inhibit the growth of the cells (as was assumed in preliminary experiments where labelling was poor), but in sufficient quantities to allow for Harlequin staining. The optimal concentration of BrdU for harlequin staining was found to be 33μM. Another idea to possibly increase the quality of harlequin staining was to place the culture flasks horizontally in the incubator so that more efficient mixing of cells with BrdU occurred. In the original protocol (Sanford et al 1989), as the culture flasks were vertically placed in the incubator it was thought that when the blood lymphocyte cells sedimented to the bottom of the flask, there would be poor mixing of the cells with BrdU. A second idea was to place flasks on a shaker in the warm room; so that cells were continually mixed. Although the latter procedure was carried out in the protocol, no significant differences were observed in the number of chromatid breaks scored for the same blood sample when cultured in the standard upright position. During these experiments, the mitotic index of the blood samples observed under the microscope when scored was low and to achieve an increase in the number of metaphases, the number of days of cell culture was increased by one extra day, i.e. four days incubation with BrdU instead of three days. However this did not result in an improvement of harlequin staining. As in the original G2 protocol (Sanford et al 1989), cells were harvested after 72 h of setting up the cell culturing of the blood so that light and dark strands of the chromatids were observed. The timing of the colcemid treatment was changed from the protocol of the G2 assay protocol, and mitotic cells were fixed for two hours in colcemid instead of the usual one to increase the mitotic index. Indeed this change in procedure did produce a slightly higher number of metaphases observed as indicated by the increase in the mitotic index and this new change was incorporated in the protocol. Thus after

experimenting with the concentration in BrdU, the positioning of the flasks, the number of days prior to harvesting and the number of hours of cellular exposure to colcemid, a specific protocol was finally decided upon for all the experiments performed with the blood samples. Thus following these preliminary optimisation experiments it was determined that 33μM BrdU, 2h in colcemid fixation and a culture time of 72h gave the best results.

*Colour-switch ratio and radiosensitivity in breast cancer patients*

From figure VI, it can be observed that there may be a correlation between the csr (number of colour-switch breaks/total number of breaks x 100) and radiosensitivity (i.e. when expressed as the frequency of total chromatid breaks/100 cells) of an individual highly susceptible to breast cancer. There was a significant difference in the csr value for breast cancer cases in comparison with normal' surgical control individuals. The csr values for breast cancer individuals gave a mean value of 25.8% (range 17-38%) compared to 15.9% (range 6-29%) for normal control individuals. The P value = 0.0024 indicated a significant difference in csr between breast cancer and control patients. The radiosensitivity for breast cancer individuals expressed as a mean of 59.7 chromatid breaks/100 cells (range 32-68) compared to 28.7 chromatid breaks/100 cells (range 15-60) was observed in normal control individuals. The results suggest that there does appear to be a relationship when csr is plotted against radiosensitivity. It is interesting to note that most of the breast cancer patients investigated were found to be approximately twice as radiosensitive than normal control individuals as shown in figure VI by the separating of patients into two cohorts.

Four other investigations of mammalian cell lines have been carried out using harlequin staining of chromatids to detect the presence of recombinational exchanges between chromatids. Bryant (1998) measured the csr in Chinese hamster cells at 2h and 4h after 0.77 Gy of γ-irradiation (at the later time it was expected that the majority of dsb would have been repaired). Results showed that 16% of colour switch breaks (12/75 colour switch breaks) were recorded at 2h after irradiation and 17.7% recorded after 4h. Thus,

there was no significant difference in csr between 2h and 4h post-irradiation. From my results, the frequency of colour-switch breaks was higher in human blood lymphocytes compared to the value in Chinese hamster cells, a mean value of 25.8% in lymphocytes (range 17-38%) from breast cancer cases and 15.9% (range 6-29%) in lymphocyte from control individuals. The mean csr value for breast cancer patients is significantly higher than the mean csr for control individuals $P = 0.0024$. This latter value is very similar to that obtained for CHO cells e.g. Harvey and Savage (1996) Bryant (1998). Using an earlier procedure with tritiated thymidine labelling, Heddle and Bodycote (1970) found a frequency of 15-18% for csr in the Chinese hamster cell line B14 FAF 15% when exposed to 0.6 Gy and 18% when exposed to 1.2 Gy). However, rather low numbers of breaks were scored - only 20 metaphase cells were scored and 3 colour-switch breaks recorded accounting for the 15% value, and 13 colour-switch breaks out of 71 metaphases were recorded for the 18% value which is perhaps more acceptable. Judging by the variability in scores obtained in my own studies, 20 metaphase cells seems an insufficient number to score while investigating csr. In order to achieve statistical power, at least 100 metaphases should be analysed and preferably where each metaphase contained breaks. It may be noted that Heddle and Bodycote's values are similar to the 16% value reported by Bryant, even though the dosage of radiation is different. Harvey and Savage (1997) viewed harlequin stained Chinese hamster metaphase cells irradiated (1.5 Gy) during the G2 phase of the cell cycle and found a range of 15-20% (12/75) colour-switch breaks. The studies described above agree that the csr in Chinese hamster cell lines are approximately 16%. When the csr was investigated using the tritiated thymidine technique in rat kangaroo cells, Potorous tridactylis, (Heddle et al, 1969), 14/37 colour switch breaks were recorded yielding a csr of 38%. This value supported the 40% prediction by Revell's exchange model and therefore suggested that the majority if not all chromatid breaks might be produced as a result of incomplete exchanges at the point of overlap in a recombinational loop. This was based on the assumption that exchanges between the four damaged ends of chromatids in a looped structure are equally likely. However, the exchange model, as already discussed is not tenable since it requires interaction of two independent lesions. The recent signal model (Bryant 1998) can accommodate the fact of a variable csr since it does not predict a specific value of csr.

Clearly, in hamster cells there is a preference for intra-chromatid exchanges.

*Colour switch ratio is not a constant value across mammalian species*

The results for human lymphocytes reported here indicate that the csr value is not a constant value across mammalian species. From my results, the csr range for humans was 15.9% - 25.8% in comparison to the csr range of 15%-18% for Chinese hamster cells (Harvey and Savage 1997, Bryant 1998, Heddle and Bodycote 1970) and 38% csr value for rat kangaroo cells, *Potorous tridactylis* (Heddle et al 1969). Harvey and Savage (1997) proposed that the csr was a constant under varying conditions and dosage within the Chinese hamster cell line. They previously investigated several Chinese hamster cell lines e.g. CHO-K1. Although they proposed a constant csr value in Chinese hamster cell lines, they implied that csr may be a constant value within a given cell line under different conditions. If this is indeed true, then following a number of similar experiments, it may be determined that the csr range for human individuals falls in the csr range of 16%-24%. The authors proposed that this constancy of csr was a result of a fixed proportion of lesions that were processed in a particular way that would result in a colour-switch break. It is feasible to suggest that a particular type of cross-over is favoured over the other types of recombinational exchange accounting for the constancy in csr. As hypothesized in the signal model (Bryant 1998), type 1 recombinational exchanges may be less common than types 2 and 3 accounting for the relatively low values of csr found compared to the 40% predicted if all types of exchanges are equally likely.

*Frequency of chromatid breaks in light/dark chromatids of human lymphocytes*

No significant differences were observed in the frequency of chromatid breaks in the light and dark strands in the lymphocyte experiments; The P value for the L/D ratio = 0.07. There was a mean average of 20 chromatid breaks/100 cells in the dark strand and 21 in the light strand in breast cancer patients, and 10 chromatid breaks/100 cells in the light strand and 12 in the dark strand in normal control individuals. The averaged value

for the frequency of chromatid breaks in the light strand/frequency of breaks in the dark chromatid strand, L/D, recorded for lymphocyte cells from the human cell line was approximately 1.0 (table I). This confirms previous reports (Bryant 1998,Wolff and Bodycote 1975), which concluded that there were no significant differences in the frequency of chromatid breaks in the light and dark strands. It might be expected, as indicated by Savage and Harvey (1991) that if the value was higher than 1.0, this would indicate sensitization of the light chromatids by BrdU. The light chromatid containing BrdU in both strands (light chromatid = BB) might thus be more sensitive to $\gamma$ - rays. BrdU incorporation in chromatids might be expected to sensitize them and increase the chances of radiation-induced breakage - particularly in the light strand as both strands contain BrdU. BrdU has been shown to sensitize mammalian cells to ionizing radiation (Elkind and Whitmore 1967) Following X-irradiation of Chinese hamster cells, Harvey and Savage (1997) determined that the ratio of the L/D was 1.5%. This investigation supported the hypothesis that BrdU sensitizes the light chromatid to damage. It was interesting to find that in some of the individual cases for breast cancer and control individuals, there were a number of patients with values of L/D higher than 1.0 e.g. 1.8 for breast cancer sample 98. In other cases, however, this value was slightly lower than 1.0 e.g. 0.4 for control sample M58. This latter result (and the others significantly below the 1.0 value) appears anomalous as it indicates that BrdU incorporation is somehow protecting the light chromatid strand from breakage. This would be expected, if at all, for the dark chromatids as they have incorporated BrdU in only one strand unlike the light strand that incorporates BrdU in both strands. The values higher than one can be explained, but not for those significantly less than 1.0. In contrast others, Wolff and Bodycote (1975) and Bryant (1998) both of whom investigated L/D ratios in Chinese hamster cell lines, found no significant difference between the frequencies of breaks in the light and dark strands after X or $\gamma$-irradiation.

*Colour-switch ratio and radiosensitivity in lymphoblastoid cell lines*

The experiments carried out with cells from AT individuals were from a lymphoblastoid cell line rather than blood lymphocyte cells as used in the breast cancer and normal

control patient studies. The csr value in the AT lymphoblastoid cell line (29%; range 28-29%) was slightly higher than that observed in the normal lymphoblastoid cell line (25%; range 20-25%), but not significantly higher; P= 0.13. The csr in the AT cell line tested (29%) was also higher than that recorded in breast cancer patients (26.8%) but again not significantly P=0.14. The average frequency of total chromatid breaks/100 cells in the AT cell line was 75 in comparison to 16 in the normal cell line. The P value for total damage between the AT and control cell line was 0.0002, indicating a significant difference in radiosensitivity between the two cell lines. It might be expected that as AT individuals are hypersensitive to ionizing radiation (as indicated by the higher number in total chromatid damage), they might (if csr was related to radiosensitivity) consequently have a significantly higher csr than normal or breast cancer patients might. From the results presented however, this appears not to be the case. The results indicate that csr and radiosensitivity are not directly correlated. This result may be due to the difference in cell type used - a lymphoblastoid cell line (in comparison to lymphocyte cells in the breast cancer experiments). It is not known whether the Epstein-Barr virus integrated as a provirus in the transformed lymphoblastoid cell might affect radiosensitivity or csr. If there was a correlation between csr and radiosensitivity, it would be expected that as AT individuals are highly radiosensitive, there would be a correspondingly high csr. The results indicate that this is not the case. A correlation is however shown in the breast cancer patients between csr and chromatid break frequency, so it is possible that the difference in cell type (as previously mentioned) - the lymphoblastoid cell line used in the AT experiments in comparison to lymphocyte cells used in the breast cancer experiments may have resulted in this difference. To determine whether this is so, the AT experiments should be repeated, but using AT lymphocyte cells. Thus the csr in AT lymphoblastoid cells is higher than that observed in the normal cell line, but not significantly high and is also similar to the csr determined in breast cancer patients. The csr value for AT thus appears to be within the range observed for normal and breast cancer lymphocytes.

*Frequency of chromatid breaks in L/D in AT*

In the irradiated AT lymphoblastoid cell line, the average frequency of chromatid

breaks/100 cells in the light strand was 26 and in the dark strand 27, producing a ratio L/D of 0.96. In the normal irradiated cell line the average frequency of chromatid breaks/100 cells in the light strand was 5 and 6 in the dark strand, producing a ratio L/D of 0.83. It is therefore observed that there are no significant differences between the frequency of chromatid breaks in the light and dark strands within the same cell line. There are, however a higher frequency of chromatid breaks in the AT cell line indicating a higher radiosensitivity than the normal cell line; P= 0.0002. From these results there does not appear to be a significant difference from the L/D ratio of 1.0, although it must be noted that the 0.83 value is lower than that expected (it would be expected to be higher due to BrdU sensitization). It is also interesting to observe that the L/D ratio for the normal cell line is slightly lower than that for the AT cell line. Thus the values for L/D are not significantly different but there is a slightly lower number of chromatid breaks in the light strand (compared to the dark) indicating that the BrdU labelling in the light chromatid is somehow protecting the chromatid from breakage - somewhat of an anomaly. It also can be suggested that the dark chromatid strands were more easily visible under the light microscope and therefore scored with greater accuracy.

# CONCLUSIONS AND FUTURE WORK

It is very interesting to observe that all of the breast cancer individuals in this study were discriminated by this correlation of high csr and high radiosensitivity in comparison to the normal control individuals. As shown in figure VI where csr is plotted against radiosensitivity (the frequency of total chromatid breaks/100 cells), there are two separate cohorts - one for control individuals and the other for breast cancer patients. It is exciting to observe that this test was able to identify all the breast cancer patients investigated. Although the maximum mean frequency of total chromatid breaks in breast cancer patients is approximately 60/100 cells; in an ongoing study at St. Andrews University (under Dr. Bryant's supervision) this value fits in the mean range of chromatid breaks in control patients (found to have a range of 0-80 breaks/100 cells). Thus the values for the mean frequency of chromatid breaks in breast cancer patients from my investigation lies within the range of the control patients in an ongoing investigation at St. Andrews. Due to a shortage of time only 13 breast cancer patients were investigated thus more experiments on a larger pool of individuals must be carried out to test this apparent correlation. Two separate cohorts for breast cancer and control patients are observed in my results, but in the ongoing study at St. Andrews, there is considerable overlap in radiosensitivity between breast cancer and control patients. Scott et al (1994) also found that there was overlap in radiosensitivity between breast cancer patients and control patients. He found that 42% of breast cancer patients were found to be highly radiosensitive as well as 9% of control patients. If this correlation is confirmed in several more studies, this predictive 'test' has the potential to be used as an assay to detect how much individuals are predisposed to breast cancer. If this test proves to discriminate almost all of breast cancer cases as my experiments showed, than this would be a more valuable and accurate assay than the G2 assay (Sanford et al 1989) which has been shown to be approximately 40% accurate (Scott et al 1994) in comparison. It would be exciting to determine an index of predisposition to breast cancer test, where a particular range of values for the ratio of csr/colour-switch breaks (csb) would signify whether the individual is more susceptible to breast cancer than normal control individuals. As shown in figure XVIII, the mean values for csr/csb in breast cancer patients 1.88 (range 1.41-3.17) is significantly lower that the mean for control patients 5.03 (1.57-7.25). The P value is

0.0014. Alternatively, to determine which cohort (breast cancer or control - see figure VI), the patient under test falls in their csr may be plotted against the radiosensitivity (both measured by the protocol used in material and methods) and then observed. If the value lies in the breast cancer cohort, then it may be important for that patient to undergo screening for breast cancer.

The results show that although csr and radiosensitivity was not always correlated in the AT cell line investigated, there was a separation of two cohorts in the breast cancer experiments. It was of interest to observe in figure XVI, when csr was plotted against radiosensitivity, the value for the AT cell line fell in the breast cancer cohort range and the value for the normal cell line fell in the control patients cohort range.

To account for the high csr, it is possible to suggest that in human cells, type 1 colour switch exchanges are more common than in Chinese hamster cell lines. The ratio of L/D in all the experiments was approximately one as previously determined by Wolff and Bodycotte (1975) Harvey and Savage (1991) and Bryant (1998).

Individuals who are more radiosensitive than others may be explained by considering the structure of the signal model (Bryant 1998, see figure V) Individuals that are highly radiosensitive may have a larger size of chromatin loops at the higher order level than individuals with 'normal' radiosensitivity. The greater the size in chromatin loop the higher the probability of exposure to dsbs when the cell is subjected to ionizing radiation. The cell is thus subjected to more dsbs and will therefore become more radiosensitive. The smaller the chromatin loop, the lower the frequency of dsb induction on exposure to ionizing radiation. It is also possible that after irradiation, a higher frequency of signal molecules are produced in response to the presence of a dsb, in radiosensitive cells, in comparison to normal cells. More dsbs will be converted to chromatid breaks due to an increase in incomplete exchanges. There may be mutations in the gene encoding for the signal molecule where dsbs are not effectively detected resulting in poor signal transduction which cannot therefore be processed efficiently and this might reduce the frequency of breaks. Alternatively the signal recognition pathway may be defective such

that the receptors detecting the signal molecule may be mutated in such a way so that the signal molecule is not detected. Thus a dsb results in an increase in recruitment of signal molecules, but due to a defective signal recognition pathway, the signal cannot be correctly interpreted. The recombinational enzymes proposed in the signal model to facilitate exchanges leading to breaks may be defective in more radiosensitive individuals - the enzymes may be mutated so that once a signal is generated in the transcription factory in the presence of a dsb, the mutated recombinational enzymes result in a higher frequency of incomplete chromatid breaks and therefore higher radiosensitivity. If the chromatin loops are more highly condensed in normal individuals this may produce a lower frequency of chromatid breaks and therefore cause lower radiosensitivity. Highly condensed chromatin loops signify the presence of inactively transcribed DNA i.e. transcriptionally silent DNA. A dsb in highly condensed chromatin may not be signaled and therefore not undergo recombinational exchange. The ideas described above to account for higher radiosensitivity in breast cancer and AT patients are hypothetical and have yet been proven. Experiments to determine whether these suggestions are true should be carried out in further AT and other mammalian cell lines with widely differing radiosensitivities before firm conclusions can be made.

The aim of this investigation was to determine whether csr and radiosensitivity are correlated in humans. A significant correlation was found in breast cancer individuals when csr was plotted against radiosensitivity, but not in the AT cell line. This correlation should not be ruled out and more similar experiments should be carried out in different mammalian cell lines with widely differing radiosensitivities. If a correlation does prove to be present between csr and radiosensitivity then this could be a predictive assay for individuals highly susceptible to breast cancer. As all the breast cancer patients were discriminated against normal control patients this provides evidence for the use of an accurate predictive assay.

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