Investigations of Genomic Changes Induced by the FAC Drug Combination

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Contents

Abbreviations

A - Adriamycin

ALL – **A**cute **L**ymphoblastoid **L**eukaemia

AML – **A**cute **M**yeloid leukaemia

CEPH - The **C**entre d'**E**tude du **P**olymorphisme **H**umain

C - Cyclophosphamide

DNA - Deoxyribo**n**ucleic **A**cid

D1S7 - The official HUGO number for MS1

EDTA - Ethylene**d**iamine**t**etra-**a**cetic acid disodium salt

ESTs - Expressed **S**equence **T**ags

FAC - Mixture of three anti-cancer drugs : 5-**F**luorouracil , **A**driamycin and **C**yclophosphamide

FCSB - Fetal **C**alf **S**erum **B**

F - 5-Fluorouracil

GMAX - Gluta*max*

MLL - Mixed **L**ineage **L**eukaemia or **M**yeloid/**L**ymphoid **L**eukaemia

MVR - Multi **V**ariant **R**epeat Unit

MVR-PCR - **M**ulti **V**ariant **R**epeat Unit **P**olymerase **C**hain **R**eaction

PCR - **P**olymerase **C**hain **R**eaction

SDS - **S**odium **D**odecyl **S**ulfate

SNPs - **S**ingle **N**ucleotide **P**olymorphisms

SP-PCR - **S**mall **P**ool **P**olymerase **C**hain **R**eaction

SSC - **S**aline **S**odium **C**itrate

t-AML - **T**reatment Related **A**cute **M**yeloid **L**eukaemia

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Abstract

Although therapeutic regimens that are currently in use to treat cancer have a high success rate, the cumulative toxicities of those treatments can present barriers to their long-term use. 5-Fluorouracil, adriamycin and cyclophosphamide (FAC) drug regime is widely used in breast cancer therapy. This combination of drugs is known to cause many side effects. One of the side effects that can be induced in small proportion of patients is acute myeloid leukaemia. At the present, researchers are not able to predict which individual patients might be susceptible to developing AML. Current research carried out in various laboratories is focused on identifying potential markers that would allow for identification of such patients. The analysis of gene expression profiles of cells treated with the FAC drug regime gave an insight on the impact that the chemotherapeutics have on non-cancerous cells. Exposure to the FAC drug regime caused many changes in gene expression profiles of treated cells. Experiments examining the impact of single doses treatments and combined FAC regime showed variations in gene expression responses to the xenobiotics. Gene expression profiles varied depending on the amount of time that the cells were exposed to the FAC drugs. Shorter exposure to FAC drugs generated more overexpressed genes. During the microarray analysis, synergistic interactions between FAC drugs were discovered. Such synergistic interactions could be very important for future drug discovery and population studies. Two candidate biomarkers were also investigated as potential indicators of susceptibility to developing secondary leukaemias. Minisatellite MS1 and MLL/AF4 translocations were analysed. Minisatellites are highly sensitive repeat regions in human genome that might be susceptible to changes induced by xenobiotics. MS1 has a highly variable internal structure, because of its variability and unstable nature, it was chosen to act as biomarker. An alternative method to detect DNA damage by identification of MLL/AF4 translocations was explored in this project. Chromosomal aberrations between those genes may cause secondary leukaemia in cancer patients. PCR based techniques were utilised to detect any MLL/AF4 translocations present both in cell lines and in patients' DNA.
Both minisatellite MS1 and MLL/AF4 translocations proved not to be suitable candidates as biomarkers.

1.0 Introduction

Overview

Research in the field of cancer management continues to make steady progress towards new approaches to combat this group of diseases. Cancer has been studied for decades and still there is much that is unknown. Many new anti-tumour treatments have been discovered and implemented with promising results (Chabner 1996). Although the improved anti-cancer regimes are more and more successful in treating the disease, there are still some unfortunate side effects (Bergerat *et al.* 1997) some patients suffer after the course of chemotherapy. In more severe cases, patients can develop treatment related cancers as a result of the treatment of their primary cancers. Treatment related cancers can develop years after the treatment of the primary tumour.

Breast cancer is one of the most common types of malignancy suffered by women (Chang *et al.* 1998). Many anti-cancer regimens are available to patients to combat the disease. One of the treatments used in breast cancer therapy is the FAC regime of drugs (Aman *et al.* 1989). This treatment is a combination of three drugs, cyclophosphamide (C), adriamycin (A) (also known as doxorubicin) and 5-fluorouracil (F) (Buzdar *et al.* 1979). Although this particular combination is highly effective, it causes many side effects such as sickness and hair loss and even in some cases treatment related leukaemia AML (acute myeloid leukaemia) (Felix 1998). The AML does not affect every patient treated with FAC regime since only a small number of patients might in later years develop this disease after their treatment for the primary breast

cancer ((Bergerat *et al.* 1997)). Still there is a great need to eradicate the treatment related cancers. In order to do that, a system of diagnosis needs to be established in order to determine potential patients who might be at risk of developing treatment related cancers. The anti-cancer drugs are very powerful chemicals (Chabner 1996). Although they are designed to eradicate cancer cells in the course of treatment, healthy cells are also affected. These agents affect the DNA structure in healthy cells, causing damage.

Recent development of the cDNA microarray analysis allows for the study of many thousands of gene expressions at one time. For the first time it has been possible to compare the gene expression profiles of different cancers (Martin *et al.* 2000). It has also become possible by using this technology to determine the impact that anti-cancer chemicals have on gene expressions of healthy tissue. Such studies might provide valuable insights into the mechanisms of anti-tumour activity of many chemicals.

In the human genome, there are regions which are highly sensitive to xenobiotics, such as minisatellites (Jeffreys *et al.* 1985). These characteristics may be used as early indicators of damage to the DNA structure that can possibly lead to development of treatment related leukaemia. The MS1 minisatellite could be such an indicator. It has been shown that it is highly sensitive to radiation (Dubrova *et al.* 1996). In response to radiation it mutates, changing its internal structure. These changes have been detected successfully (Jeffreys *et al.* 1990). This suggests that this minisatellite might be used as an early marker for patients whose DNA has been affected by the anti-cancer chemicals.

The FAC drug regime is a cause of acute myeloid leukaemia in a small number of patients (Sawyers *et al.*1991). AML leukaemia is a type of secondary cancer. The main cause of developing AML is chromosomal translocation. These translocations occur between chromosome 4 and 11. The alleles that take part in the translocation are MLL (chromosome 11) and AF4 (chromosome 4). By screening for the presence of these translocations, it might be possible to establish if some patients have already had such translocations even before the treatment with FAC. In such cases, careful monitoring of patients would establish if they were more susceptible to developing AML.

1.1 History of anti-cancer drug development

The discovery and development of anti-cancer drugs has been and still is a process of great difficulty. In their effort to discover better therapies, researchers all over the world have increased our knowledge of this group of different diseases (Clahsen *et al.* 1996). The foremost goal has always been to design therapies able to recognise diseased tissue from healthy ones and target it in order to eradicate cancer. What those investigators achieve is better understanding of the biological systems, their flexibility and resilience to xenobiotics. As the new therapeutic targets have been discovered and new drugs have been designed, tumour evasion of the therapeutic attacks has also been demonstrated (Tseng *et al.* 2002).

Historically, anti-cancer drugs have originated from many sources, including plants, microbes, fungi, synthetic chemicals and even chemical weapons. The systematic treatment of cancer began with the discovery of biological properties

of mustard gas. Physicians treating mustard gas victims during World War I observed that it had myleosuppresive and lymphocytolytic effects (Calabresi *et al.* 1975). The properties of sulphur mustard led scientists to examine subsequently the biological and chemical properties of nitrogen mustards since then. In 1942 the first patients with neoplastic disease were treated with nitrogen mustard (Calabresi *et al.*1975, Karnofsky *et al.* 1958). In 1946 some reports were published revealing clinical activity of nitrogen mustards in Hodgkin's disease, lymphoma, chronic lymphocytic and myleocytic leukaemia. The nitrogen mustard could combat those types of cancer, but was ineffective against solid tumours (Colvin *et al.* 1976). The anti-tumour properties of nitrogen mustards encouraged further efforts to find chemical agents with anti-tumour characteristics, leading to many of the anti-tumour agents in use today. At present, alkylating agents occupy a central position in cancer chemotherapy, in many cases as part of a combination of anti-cancer regimens. There are different types of alkylating agents used nowadays clinically. These include nitrogen mustards already mentioned here, aziridines, epoxides, alkyl alkane sulfonates and nitrosoureas. The first nitrogen mustard used clinically was mechlorethamine. In time it has been replaced with others of its analogues such as cyclophosphamide, melphalan and ifosfamine (Friedman *et al.* 1979, Chabner 1996).

After the alkylating agents had been established as good chemotherapeutic agents, attention turned to screening for other chemicals as anti-cancer drugs. During the clinical trials for alkylating agents, it was discovered that patients could develop resistance to the drugs that had been administered. In the 1950's extensive search for anti-cancer drugs led to the discovery of nitrosoureas

(Schepartz 1976). An important use of animal models led to the discovery of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Analogues of MNNG were also tested in attempts to find more active compounds (Greene *et al.* 1960). The research into nitrosoureas is still ongoing, but its history is very important for the discovery of anti-cancer drugs. It shows that by introducing extended screening and drug developing programs, it is possible to evaluate anti-tumour activity of potential anti-cancer agents (Skipper *et al.* 1961, Chabner, 1996).

Platinum complexes have also joined the ranks of anti-tumour therapeutics. The anti-tumour activities of this group of drugs were discovered in 1970's and led to the development of very powerful anti-cancer compounds, such as cisplatin (Rosenberg 1985). In spite of promising effects against testicular, ovarian, bladder, neck and cervical cancers, cisplatin suffers from two major drawbacks (Loehrer *et al.* 1984). Firstly, it is severely toxic to non-cancerous tissue and secondly, tumours develop resistance to it. Multidrug resistance develops when drug metabolism products induce the levels of glutathione S-transferase. The Glutathione S-Transferases (GST) are a superfamily of detoxification enzymes. These enzymes catalyse the nucleophilic addition of the tripeptide glutathione (GSH) to endogenous and xenobiotic electrophilic substrates. GSH forms soluble compounds with xenobiotics such as cancer drugs; any disruptions to those enzymes can possibly lead to drug resistance (Lomaestro *et al.* 1995). Despite those drawbacks, cisplatin is widely used in many therapies in combination with other anti-cancer drugs. Ongoing research into platinum drugs revealed more about mechanisms of drug resistance of tumour tissue (Muller *et al.*1994). Those findings will help to determine if the platinum-based compounds

currently used need to be modified in order to combat cancer more efficiently (Farrell *et al.* 1990).

In the search for potent anti-cancer drugs, antibiotic substances were investigated as potential chemicals in the treatment of malignancies. The first discoveries in mid 1950's led to experiments with anthracycline antibiotics. Extensive research led to the discovery of doxorubicin, which has a high activity against leukaemia, lymphomas, and a variety of solid tumours (Fogleson *et al.* 1992). One of the drawbacks of doxorubicin has been its high toxicity (Sunnenberg *et al*, 1985). As well as doxorubicin, a further compound from the anthracycline family showed high anti-tumour activity. That compound was daunorubicin, which is now widely used in combination therapy (List 1993). Since the discovery of doxorubicin, several other anthracycline analogues have been developed, but none of them shows such potent activity (Weiss 1992).

During an extensive search for natural anti-cancer compounds in plants, an alcoholic extract named camptothecin from the Chinese tree *Campothoteca acuminata* was found to demonstrate high anti-tumour activity (Wall *et al.* 1966, Wall *et al.* 1969). Unfortunately during clinical trials, it was discovered that when the compound was dissolved in sodium salts, it lost its anti-tumour activity (Giovanella *et al.* 1991). Research into mechanisms of action revealed that the compound inhibited topoisomerase I and production of active forms of the drug commenced. Topoisomerase I catalyzes the unlinking of DNA strands by making DNA breaks and allows a new DNA to traverse through those breaks. One of the topoisomerase I inhibitors already used clinically is topotecan (Kraut *et al.* 1995), which is easily soluble in water and therefore can be administered

by iv injection without loosing its activity. There are also many other compounds from this anti-cancer family of chemicals that are being investigated.

Research involving topoisomerase I inhibitors opened a pathway for the anti-cancer drug discovery in which chemicals are designed to inhibit particular enzymes in cancer tissue. This new approach enabled the development of anti-cancer drugs that inhibit or suppress topoisomerase II (Wang 1991). Both topoisomerases have some similar functions. They are both involved in relieving the torsional strain of DNA and can complement each other in this process. Topoisomerase II plays also an essential role in chromosomal condensation and segregation (Charvin *et al.*, 2003). Those properties make both enzymes prime targets for drug development. The principles of molecular interactions of those drugs are still being investigated. It was discovered that anti-cancer drugs originating from different families display topoisomerase II inhibitory characteristics, such as doxorubicin. Studies involving topoisomerase II inhibitors are still ongoing and may provide additional progress for cancer chemotherapy.

The discovery of anti-cancer therapeutics in recent years has focused towards specificity at the molecular level. The research carried out in the past 90 years allows for better understanding of cancer development. Recent discoveries in molecular biology have also enabled the use of more sophisticated methods of screening for potential anti-cancer drugs. New approaches have focused on targeting of particular genes involved in carcinogenesis. Gene therapy is a process in which new genetic material is transferred to cells of the individual resulting in beneficial change for that person (Mhashilkar *et al.* 2001). Gene therapy is still at its developing stage, but the experiments involving the use of

cell lines already show promising results (Asher et al. 1987). In gene therapy anti-tumour molecules have to be delivered directly to the tumour or to the tissue or organ, where the tumour is located. The delivery can be achieved in many ways, by direct injection or with help of liposomes, which surrender the DNA to be delivered at the site of action (Varga *et al.* 2001). Techniques using viral vectors have also been investigated (Mulligan 1993, Monohan *et al.* 2002)). Some clinical trials have also been conducted using gene therapy. Nabel (Nabel *et al.* 1993) carried out an experiment, in which the *HLA-B7* was injected into the tumour using DNA-liposome complexes. No toxicity was observed and in one patient increased levels of *HLA-B7* reactive *CTL* precursor was found. In an other patient a regression of lung metastasis occurred. Those results suggest that the gene therapy treatment administered to patients did enhance anti-tumour immune response (Nabel *et al.* 1993). New developments in molecular biology and genetic engineering have opened many possibilities for the specific delivery of anti-tumour agents without affecting the healthy cells.

Many thousands of compounds with different characteristics, but showing anti-tumour activities are screened yearly. The goal is to find an agent that will eradicate cancer without causing long lasting and damaging side effects. In our search we must remember the words of Paracelsus "All substances are poisons; there is none that is not a poison" Paracelsus (1493-1541).

1.2 FAC anti-cancer drug combination

In this project, attempts have been made to assess the impact of the FAC treatment on patient's DNA and on CEPH lymphoblastoid cells. The FAC

treatment usually consists of 6 cycles at intervals of three to six weeks. This drug combination reduces mortality from breast cancer, but may cause some side effects including developing treatment related cancer such as AML (Aman *et al.* 1989). Ongoing research is carried out to improve the identification of patients who are likely to develop secondary cancers as a result of the FAC treatment. FAC drug combination and individual drugs forming the regime will be discussed in this chapter.

1.2.1 FAC regimen

Adjuvant chemotherapy nowadays is a widely used treatment in patients with operable breast cancer. The treatment is used to prevent recurrence of the disease. Adjuvant chemotherapy refers to chemotherapy after the tumour has been removed by surgery. Chemotherapy can prevent cancer recurrence by destroying micrometastases that remain after the surgery. It prolongs survival of patients. The FAC combination (5-fluorouracil, adriamycin and cyclophosphamide) along with other various combinations is the most widely used adjuvant chemotherapy regime for breast cancer patients (Aman *et al.*1989). Clinical trials using FAC began in 1973 at the University of Texas (Buzdar *et al.* 1979). Adriamycin was included in the trials as the most effective chemotherapeutic agent against breast cancer at that time. Over the following years researchers still include adriamycin as part of adjuvant chemotherapy in combination with other anti-cancer drugs. During the trials the doses of adriamycin were altered (Malik *et al.*1982). Those alterations reduced the toxicity of the chemotherapy, but did not alter its effectiveness. Long term

studies showed that FAC regime was effective in improving disease free survival and overall survival compared to other treatments. In patients <50 years old treated with tamoxifen, a 7% decrease in mortality was observed. FAC treatment decreased the mortality by 55% (Aman *et al.* 1989). The clinical trials conducted evaluated the role of FAC as adjuvant chemotherapy treatment and found it superior to other regimens that did not contain adriamycin (Aman *et al.* 1989). Despite its effectiveness the FAC regime still causes side effects such as hair loss, nausea, vomiting and cardiac damage (Hortobagyi *et.al.* 1986). Some patients also develop treatment related cancers (Lue *et al.* 1995).

1.2.2 5-Fluorouracil

This chemical is an analogue of the pyrimidine uracil, belonging to a family of anti-cancer drugs known as 5-fluoropyrimidines (Heidelberger *et al.* 1957). The discovery of the pharmacological potential of 5-fluoropyrimidines was due to Rutman who determined that rat hepatomas utilise radiolabelled uracil more readily than non-malignant tissues (Rutman *et al.*1954). 5-Fluorouracil (5-Fu) is the most widely used anti-cancer drug from the fluoropyrimidine family. It is inactive as such in mammalian cells requiring to be bioactivated to its active form 5-fluorodeoxyuridine monophosphate. The activation of 5-Fu can occur through two pathways, see Figure 1.1. The first pathway involves direct transfer of ribose phosphate to 5-Fu from phosphoribosylpyrophosphate (PRPP). This pathway is catalysed by orotate phosphoribosyltransferase. This metabolic pathway seems to be of primary importance in activation of 5-Fu in tissues. Inhibition of orotate phosphoribosyltransferase protected against toxicity

supporting the proposal that activation in tissues is required (Shwartz *et al.* 1979). The second pathway involving 5-Fu tissue activation consists of two steps. The first step entails addition of ribose by uridine phosphorylase. Phosphorylation then takes place by the reaction with uridine kinase. Reaction with pyrimidine monophosphate kinase and pyrimidine diphosphate kinase result in the formation of fluorouridine diphosphate and fluorouridine triphosphate. The mechanisms of action involve inhibition of thymidylate synthase and incorporation into RNA. Thymidylate is a precursor of thymidine triphosphate, one of the deoxyribonucleotides essential for DNA synthesis and repair. Experiments carried out suggest that 5-Fu can produce alternations in DNA stability and even DNA fragmentation (Lonn *et al.* 1990). 5-Fu also decreases the surface charge and transmembrane potential of cancer cells and decreases protein synthesis. Incorporation of fluorouridine triphosphate into RNA by RNA polymerase may inhibit RNA synthesis. It can also affect mRNA processing and translation.

Those characteristics make 5-Fu a very potent anti-cancer agent (Lue *et.al.*1995). 5-Fu is usually administered orally, but oral administration reduces its bioavailability to 75% (Cohen *et al.* 1974). The main site of elimination is via the liver. The drug is used in treatments for a wide variety of carcinomas such as liver, colon, rectum, breast, lung, bladder, head and neck. Several side effects are associated with the administration of 5-Fu. Adverse effects to treatment include myelosuppression, mucositis, diarrhoea, dermatitis, cardiac toxicity, neurotoxicity and biliary sclerosis (Sullivan *et al.* 1960).

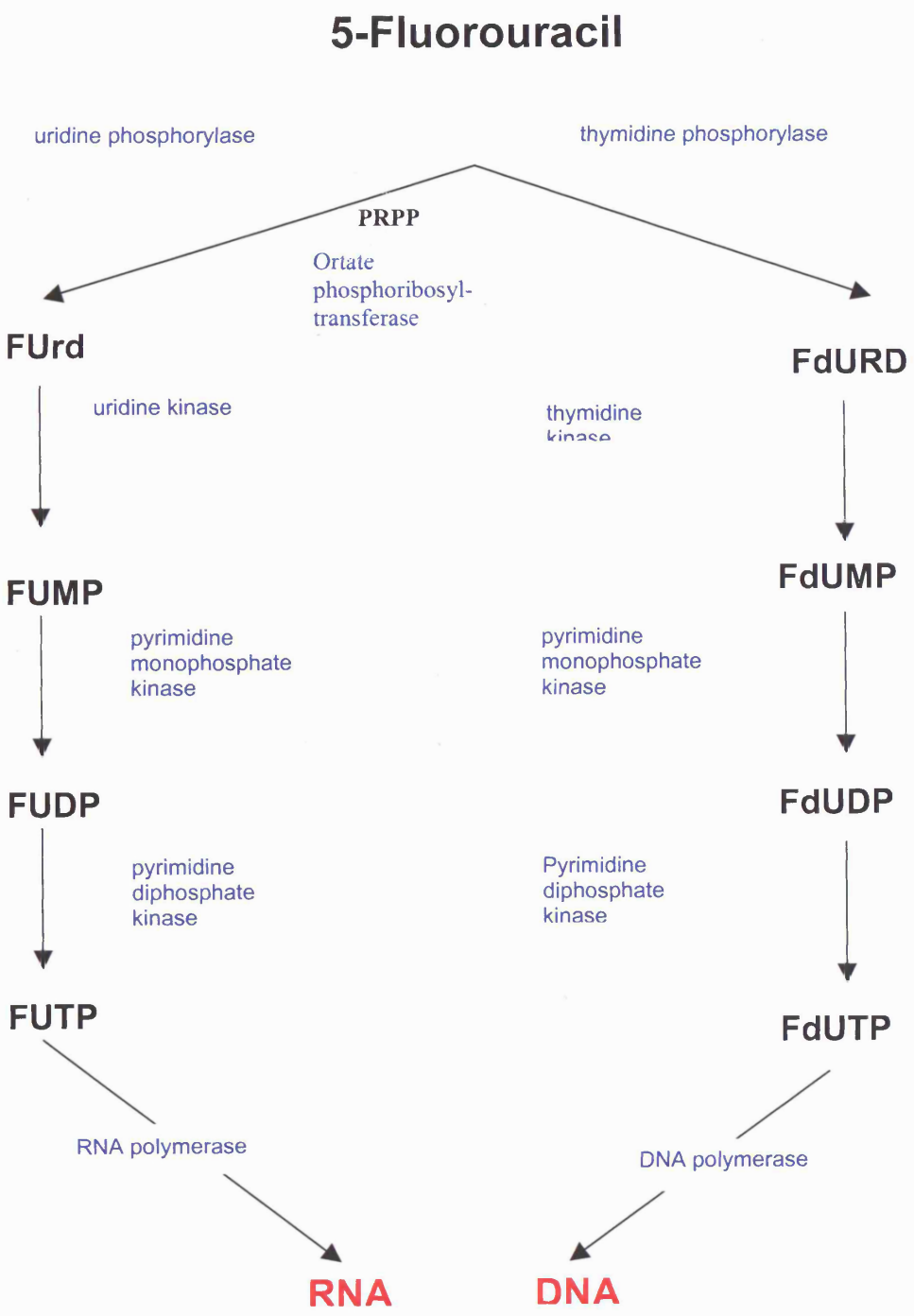


Figure 1.1 Outlines of metabolic pathway of the anti-cancer drug 5-fluorouracil.

1.2.3 Adriamycin (Doxorubicin)

Adriamycin belongs to a family of anthracycline anti-cancer drugs. The first research into anti-tumour activities of anthracyclines was carried out in 1950's independently by Grein and Dubost (Teicher 1997). The first anthracycline used in clinical trials was daunorubicin (Jackuallat *et al. 1966).* In 1969 DiMarco and colleagues reported that a new compound, a derivative of daunorubicin had been found. It was named doxorubicin (adriamycin). Their research revealed that adriamycin had higher preclinical activity that the parent compound (Teicher, 1997). Adriamycin became of use in many treatments not only as a single agent, but also in combination chemotherapy regimes (Booser *et al.* 1994). The chemistry of adriamycin and anthracyclines is very complex and is still not fully researched. All anthracyclines are able to undergo one and two electron reduction, which makes them highly reactive compounds capable of damaging intracellular macromolecules including lipid membranes, DNA bases and thiol containing proteins (Praet *et al.* 1986). One electron reduction of doxorubicin can occur in any intracellular compartments, such as nuclear membranes. The reaction can be catalysed by many enzymes including dehydrogenases, reductases, xanthine oxidase and cytochrome P450. These enzymes are available in almost all tissues and anthracycline mediated free radical formation has been reported in many tissues. The mechanism of action of adriamycin is still not clear. Research carried out revealed that the bulk of the drug in the cell can be found in the nucleus, intercalated in the DNA double helix (Chabner 1996). Subsequent studies revealed that adriamycin forms drug-

DNA-enzyme complexes (Trist *et al.*, 1989). The enzyme involved in formation of the complex is topoisomerase II. Adriamycin inhibits topoisomerase II by trapping DNA strand passage intermediates. These can be detected as protein-associated DNA breaks linked to the enzyme (Ernshaw *et al.* 1985). It has also been demonstrated that the drug produces topoisomerase related DNA cleavage in specific regions of the DNA, which might explain its gene specificity (Uemura *et al.* 1987). Adriamycin also affects helicases, leading to reduced DNA replication. It has to be noted that therapy using adriamycin and cyclophosphamide is associated with a higher risk of developing secondary tumours. Adriamycin also impinges on the cell surface, changing its fluidity and disrupting signal transduction pathways (Kartner *et al.*, 1983). The adriamycin drug regime causes many toxic side effects. The major complications are myelosuppression, mucositis, alopecia, cardiac toxicity and severe tissue damage if extravasation occurs during drug administration (Legha *et al.* 1982).

1.2.4 Cyclophosphamide

Cyclophosphamide is a highly reactive alkylating agent. It belongs to a group of chemicals first derived from nitrogen mustards. Historically alkylating agents were the first chemicals showing anti-tumour activity in clinical trials (Teicher 1997). The main mechanism of action involves the formation of covalent bonds between alkyl groups and cellular molecules. Cyclophosphamide replaced the nitrogen mustard in clinical trials due to its higher clinical activity. This widely used drug is activated to its active form by mixed function oxidase in hepatic microsomes (Cohen *et al.* 1970). The initial step involves oxidation of the

carbon ring and formation of 4-hydroxycyclophosphamide, which then can follow two pathways (see Figure 1.2). Its reaction with amino aldehyde will produce aldophosphamide. 4-Hydroxycyphosphocyclamide and aldophosphamide can be further oxidised and produce two compounds 4-ketocyclophosphamide and carboxyphosphamide. These compounds do not have high toxicity and are usually excreted via the renal route. Non oxidised aldophosphamide is able to enter tumour cells and by reacting with acrolein is reduced to phosphoramide mustard. Phosphoramide mustard is a highly active anti-tumour alkylating agent. The anti-tumour activities of the parent drug such as high selectivity and high therapeutic index are attributed to the 4-hydroxycyclophosphamide product, but probably phosphoramide mustard also plays an important role (Juma *et al.*, 1979). The major effects of tumour suppression and myelosuppression caused by cyclophosphamide are mediated by 4-hydroxycyclophosphamide and its metabolites (Cohen *et al.*, 1970). These metabolites are electrophilic, making them capable of "attacking" the patients DNA, altering its structure and giving rise to mutations. Recent data suggest that cyclophosphamide can induce microsomal enzymes responsible for its metabolism (Juma *et al.*, 1979). Cyclophosphamide inhibits DNA synthesis. One of its side effects is myelosuppression when it is combined with other anti-cancer drugs (Kolaric *et al.*1989).

The drug can also cause bladder damage in patients and even in extreme cases massive haemorrhage. The extents of the side effects are directly proportional to the dose of cyclophosphamide administered (Chabner 1996). The ability of the drug to bind to biological molecules makes it act as a hapten,

which can in some cases cause allergic reaction to the drug. Although the incidence of those allergies is increasing the overall frequency is low. Cyclophosphamide suppresses both humoral and cellular immunity this can lead to patients developing various infections. Continuous therapy can result in severe immunosuppression and lymphocyte depletion (Antman *et al.* 1987).

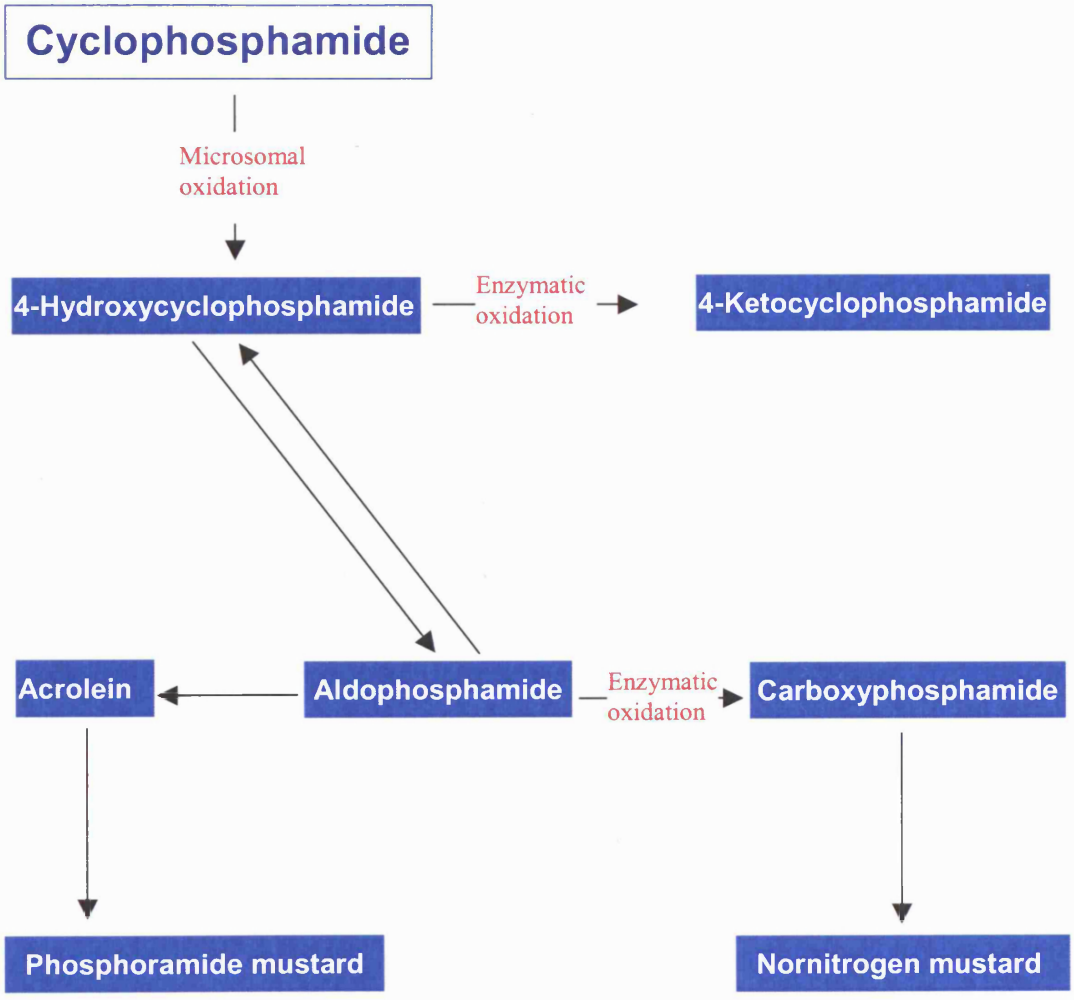


Figure 1.2 Metabolic pathway of cyclophosphamide.

1.3 Treatment Related cancer induced by the FAC drug combination

During the use of the FAC regime it become clear that this drug combination may cause many side effects (Alastair *et al.* 2001). Those treatment related side effects in some cases might lead to developing treatment related leukaemia in a small proportion of patients (Felix 1998). Treatment related leukaemias are very difficult to treat and in many cases patients do not respond to treatments at all. Acute lymphocytic leukaemia and acute myeloid leukaemia have been identified as major treatment related cancers in children and adults who had received treatments for various primary leukaemias and solid tumours (Pui *et al.* 1991). The incidence of AML - acute myeloid leukaemia will be discussed below.

1.3.1 Acute Myeloid Leukaemia

The major cause of developing of acute myeloid leukaemia in patients is cancer chemotherapy (Felix, 1998).This type of leukaemia has been reported in 10-20% of all cases of leukaemias developed by patients (Felix, 1998).The first time AML was suggested as a complication of chemotherapy by Crosby in 1969 (Crosby *et al.*1969).Treatment related leukaemias have been observed in patients receiving cancer treatments consisting of alkylating agents such as

cyclophosphamide and topoisomerase inhibitors such as adriamycin (Felix,1998).The above agents are powerful anti-cancer therapeutics capable of inflicting DNA damage on non malignant tissue. It has been suggested that those chemicals induce deletions in tumour suppressor genes such *RAS* and therefore contributing to developing treatment related leukaemias (Felix, 1998).The deregulation of the RAS pathway is one of the steps in development of cancers. Some of the genetic variations can predispose patients to developing AML. These characteristics include mutations in drug metabolising pathways such as deletion of glutathione S-transferase genes GSTM1and GSTT1. Also a deletion of p53 gene has been suggested as possible factor to susceptibility to chemotherapeutic agents. P Although genetic factors have been implicated in developing treatment related leukaemias, there is no developed system with which venerable patients could be identified.

The process, which is central to developing treatment related leukaemias, is translocation. AML is caused by translocations between MLL gene and one of the 30 candidate genes. Only some of the partner genes have been cloned to date (Felix, 1998). The partner genes encode protein products of different types such as: transcription factor (AF4), transcription factor (AF9), cell-cell junction protein (AF6) and transcriptional coactivator (p300) (Felix, 1998).In this project the translocation between MLL and AF4 genes will be studied. The MLL gene contains 36 exons and is 90 kb long. The gene products are involved in regulation of transcription. The AF4 gene is also a transcription factor. The MLL/AF4 translocations have been reported in many cases of acute myeloid leukaemias. Such cases are very difficult to treat and usually outcome for patients suffering from MLL/AF4 AML is very poor.

1.3.2 Acute lymphocytic leukaemia

Acute lymphocytic leukaemia (ALL) is a malignant disorder resulting from the clonal proliferation of lymphoid precursors with arrested maturation (Sawyers *et al.*1991). The disease can originate in lymphoid cells, giving rise to B- or T-cell leukaemias or sometimes mixed-lineage leukaemia. It was one of the first malignancies reported to respond to chemotherapy (Farber, *et al.* 1948) and was later among the first malignancies cured in children (Rivera *et al.* 1993). Since then, much progress has been made in treatment therapies. With more information about the disease better therapies have been designed. Because most cases are diagnosed in children (Pui *et al.* 1998), our current knowledge has originated from childhood disease. As differences between childhood and adult ALL become apparent, more research has been conducted and progress is being made in understanding ALL in adults. The etiology of ALL is not known, several studies have been conducted to identify risk factors for leukaemic development, but no conclusions were reached (Sandler *et al.* 1987). Detailed studies have identified families with multiple members affected by leukaemia (Gunz *et al.*, 1978). When an identical twin is diagnosed with ALL, the other twin has a higher risk of developing leukaemia (De Olivera *et al.*, 1986). Siblings of patients with leukaemia have also increased risk of developing leukaemia. (Gunz *et al.*, 1978). Several genetic syndromes have been associated with leukaemia, such as Down's syndrome which accounts for nearly 2% of all ALL cases in children (Robinson *et al.*, 1984). Other syndromes, such as Bloom syndrome, ataxia telangiectasia, Wiskott-Aldrich syndrome, and Fanconi's

anemia, are also associated with an increased risk. Exposure to radiation also may cause development of ALL (Curt-Brown *et al.* 1986). Exposure to low-dose radiation has not been proven to be leukemogenic. People exposed to radiation during nuclear disasters may have as much as a 10- to 20-fold higher risk of developing leukaemia. Exposure to various chemicals has also been associated with an increased risk of leukaemia. The best characterised association involves benzene, although more than two thirds of these cases are AML (Rinsky *et al.*1987). Philadelphia (Ph) chromosome abnormality is the result of a translocation between the long arms of chromosome 9 and 22, t(9;22)(q34;q11) is present in less than 5% of children with ALL but is found in 15% to 30% of adults with ALL (Gaynor *et al.* 1988). The incidence may be higher with more sensitive techniques. Molecular studies for Ph-related abnormalities are positive in up to 30% of adults with ALL. Ph-positive ALL is associated with older age and with a higher frequency of expression of *CD10* and *CD34* (Cortes *et al.* 1995). Nearly one half of all patients with Ph-positive ALL may have additional chromosomal abnormalities, particularly monosomy 7. At the molecular level, the Ph chromosome in ALL may be different from the one seen in patients with chronic myelogenous leukaemia (CML). In ALL, it involves band 34 of the long arm of chromosome 9, splicing the proto-oncogene *C-ABL* to band 11 of the long arm of chromosome 22 in the *BCR* gene (Greaves, 1993). In 50% to 80% of cases of ALL, the breakpoint in 22q11 falls between exons b1 and b2 of the major breakpoint cluster region, as opposed to between b2 and b3 or b3 and b4 in CML (Heistercamp *et al.* 1989). This translates into a different protein product of only 190 kDa (p190BCR/ABL) compared with that of CML (210 kDa, p210BCR/ABL) (Alexander, 1993). Both proteins have increased tyrosine

kinase activity. Protein p190BCR/ABL can induce acute leukaemia in transgenic mice and may have a comparatively higher transforming potential than p210BCR/ABL. 20% to 50% of adults with Ph-positive ALL express p210 rather than p190. The outcome of patients with Ph-positive ALL is poor, with significantly low complete remission rates (75% in children and 50% to 70% in adults) and long-term disease-free survival rates (less than 10%). Long-term prognosis following treatment is very good in children. More than 90% respond to treatments and 60% to 70% will be cured. In adults, the outcome is worse. When analysing the outcome in adults with ALL, it is important to consider studies that have a long follow-up. The initial studies from Memorial Sloan-Kettering Cancer Centre using the L2 to L10-M programs projected a long-term disease-free survival rate of more than 50%. (Linker *et al. 1991)* initially reported long-term complete response and survival rates of 50% to 60% in patients younger than 50 years. Both studies excluded patients with Ph-positive disease. The follow-up from Memorial Sloan-Kettering, with less stringent inclusion criteria, indicated a long-term complete response rate of approximately 25%, and the follow-up study (Linker *et al. 1991*) demonstrated a long-term disease-free survival rate of 35%. These and several other studies report a cure rate for adult ALL of 20% to 35%. Although a major improvement from results 3 decades ago, these findings pose a challenge for improving outcome toward what is now achievable in children with ALL.

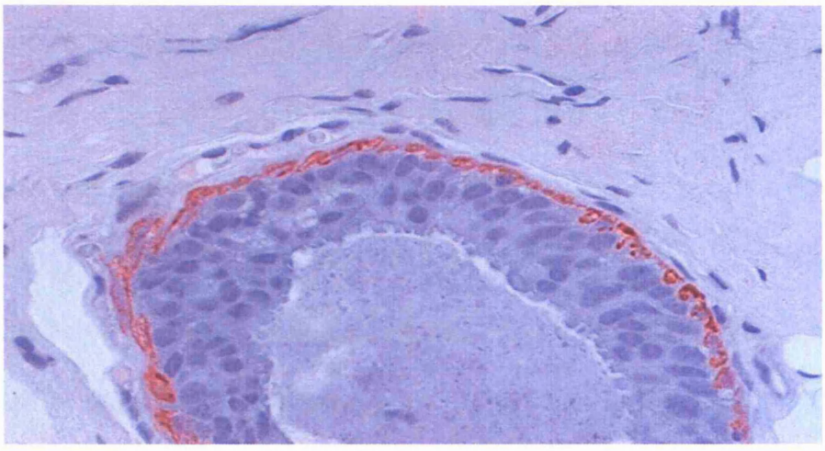
1.4 Breast cancer

Progress in the development of new anti-cancer drugs has improved the chances for survival of cancer victims, but the side effects that are caused by those drugs sometimes include treatment related cancers.

Breast cancer is one of the main types of cancers that affect women in affluent countries, affecting one in every ten women in Western Europe (Chang *et al.*1998). The disease mainly affects women in their middle to later years with highest incidence in women in their fifth decade (Lipman *et al.* 1992). After the menopause the incidence of breast cancer in women decreases, but only slightly. During recent years it was observed that the incidence of breast cancer cases was apparently on the increase. This was attributed to the screening programmes directed at women who were 40 years old and over (Lipman *et al.* 1992). It was also noted that that there were large variations in breast cancer incidence between various countries and within populations in each country (Lipman *et al.* 1992). This was attributed to the fact that many factors contribute to breast cancer development. Several such risk factors were detected, including family history of breast cancer (Arver *et al.* 2000), late onset of menopause, late childbirth (after the age of 35), benign breast disease and age at menarche.

The cause of breast cancers can be described as the result of accumulation of damage over many years to breast cells. Two main types of damage to cells can be described as responsible for breast cancer: DNA damage and cell proliferation (Weinstein, 1988). DNA mutations affect genes regulating cell

A)



B)

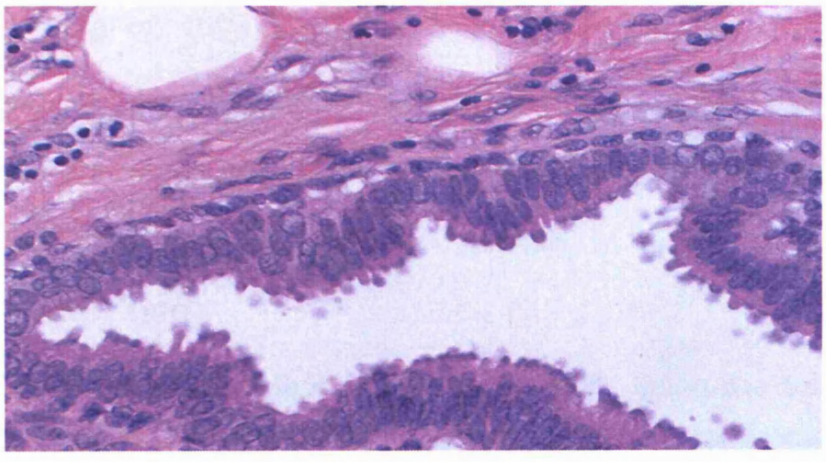


Figure 1.3 Pictures A and B demonstrate the appearance of a normal breast tissue. In picture A the tissue shows the myoepithelial cell layer around the breast acinus. The tissue was stained with immunoperoxidase. Picture B shows the appearance of normal breast acinus at higher power (www.cancerindex.org).

growth, apoptosis, death, proliferation and chromosomal replication (Ames *et al.*, 1995).Proliferation induces the development of cancer by promoting the expansion of cancerous cells (Cohen *at al.*, 1991). It is still unclear what agents lead to the genetic changes found in breast cancer. One of better defined causes of breast cancer development is inheritance of a mutated gene that increases an individual's risk of developing the disease. Such genes are BRCA1 and BRCA2 (Mikki *et al.*, 1994 and Wooster *at al.*, 1995).Both genes are involved in regulation of transcription, may slow down cellular proliferation and inhibit tumour growth *in vitro* (Holt 1996).These genes are not the only ones that may predispose to developing the breast cancer. Genes such as p53, *c-myc*, cyclin D1 and p16 have been found in a mutated form in breast cancer patients (Fraser *et al.*, 1996).It has also been identified that some of the breast cancers are steroid sex hormones dependent, such as estrogens and progestogens (Pike *et a*l., 1993).Both hormones can influence cell division rates and therefore may have an effect on genesis of breast cancer genesis (Butterworth *et al.*, 1991).

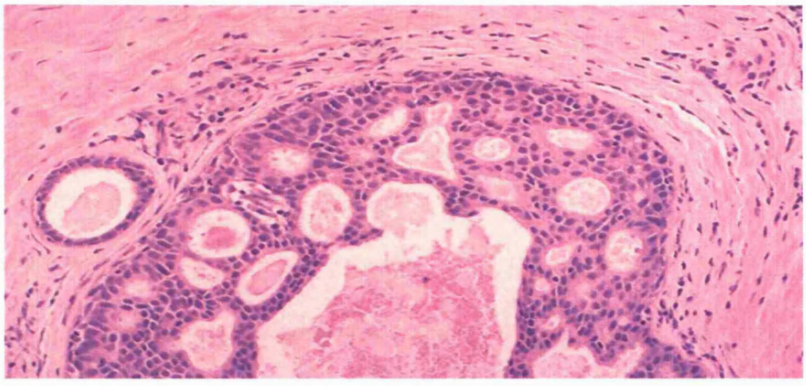
Breast cancers are derived from the epithelial cells lining the terminal duct lobular unit. There are different types of breast cancers. Patients are assessed according to laboratory, physical and radiological examinations. The information gathered helps doctors to devise the form of treatment most suitable for individual cases. The results show the size of the tumour, the involvement of the lymph nodes and presence or absence of metastases.

Most breast cancers are classified either as ductal or lobular, corresponding to the ducts and lobules in normal breast. Figure 1.3 shows tissue samples from the healthy breast.

1.4.1 Ductal carcinomas

These groups of cancers are characterised by presence of proliferating cancer cells within breast ducts with no invasion of stromal tissue around the cancer mass. Ductal carcinomas range from microscopic lesions to to those that can replace most or the entire breast (Roses 1999).The advancing edge of the tumour invades the tissue in irregular pattern, but it can be generally well defined. There are many subtypes of ductal carcinomas. The most common types of ductal cancers are: comedo, cribriform, micropapillary and solid (Lipman *et al.* 1992). It is also common to see mixtures of those types in patients. In most cases the ductal carcinoma *in situ* is broadly divided into comedo and non-comedo type. Comedo carcinoma is characterised by presence of pleiomorfic nuclei (large) and the presence of cellular debris in the centre of breast ducts. Figure 1.4 shows an example of this carcinoma. The non-comedo carcinoma is characterised by absence of cellular debris and presence of monomorphic nuclei. Ductal carcinomas *in situ* are also associated with nipple discharge and the presence of tumour mass. These types of cancers have been characterised recently, so there is not much data available to suggest the survival rate for patients with these types of cancer. The investigations carried out (Holland *et al.* 1990, Lagios *et al,* 1982) suggest that these types of cancers develop over a long period of time and sometimes are not primary causes of death.

A)



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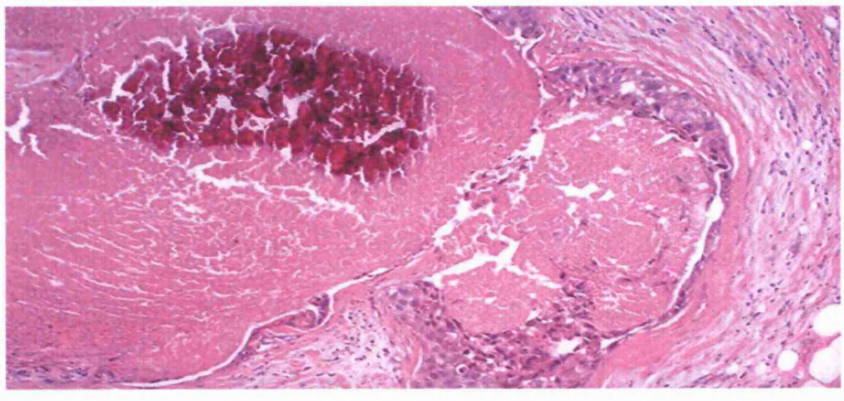


Figure 1.4 Picture A shows an example of intraductal carcinoma. Picture B shows comedo carcinoma. The neoplastic epithelial cells within the duct show pleomorphism. This type of cancer is characterised by characterised by the presence of rapidly proliferating, high-grade malignant cells. The cells in the centre of the ducts are often necrotic and calcify, as shown here (www.cancerindex.org).

1.4.2 Lobular carcinoma *in situ*

This type of lesion is localised within breast lobules. The cancer cells are usually small with round or oval nuclei. The borders of these cells are very clearly defined. Figure 1.5 shows an example of lobular carcinoma. Also the cancer cells in lobular carcinoma are highly proliferating (Lipman *et al.* 1992). The cancer is found in multiple areas of the breast unlike the case of ductal carcinomas where they are localised close to the nipple area. This type of cancer is found more commonly in pre-menopausal women (Lemanne *et al.* 1991) and presents a high risk of developing invasive cancer in patients (Page *et al.* 1991). This type of carcinoma needs to be closely monitored because of the risk of developing into invasive type of cancer, especially in women with history of family breast cancer (Roses, 1999).Lobular carcinoma in situ is now regarded as a marker that identifies a patient at increased risk of subsequent invasive disease, but is not treated as obligate precursor of invasive disease (Roses, 1999).As a marker lobular carcinoma does not specify which breast will develop invasive cancer (Page, 1987). The increase of this type of cancer has been noted in recent years, but this could be due to increased number of biopsies performed on pre-menopausal women (Lemanne *et al.* 1991).

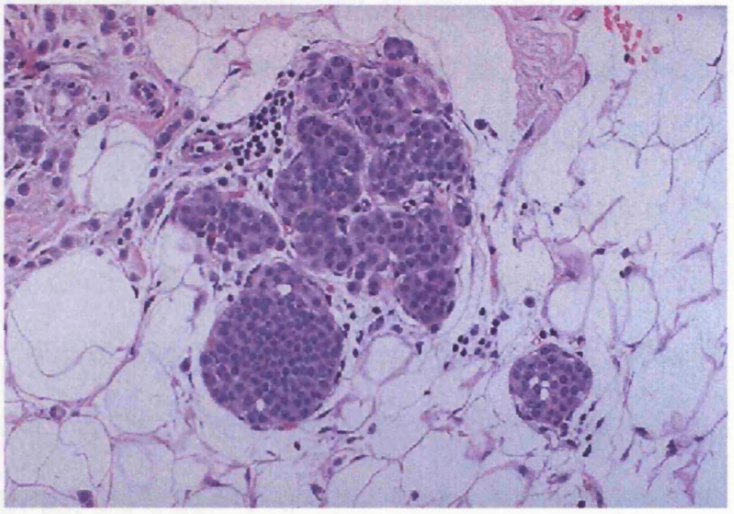


Figure 1.5 Lobular carcinoma *in situ* is characterised by neoplastic proliferation of cells in the terminal breast ducts .The cells are small and round. This type of cancer can lead to risk for development of invasive carcinoma in the same or the opposite breast (www.cancerindex.org).

1.4.3 Invasive breast cancer

There several types of invasive breast cancers and the rate of survival differ between types. Infiltrating ductal carcinoma is one of the invasive breast cancers. It does not posses any special histological characteristics, except for presence of ductal carcinoma *in situ* and various degrees of fibrotic response. These tumours have a poor prognosis for patient survival. It metastases to axiliary lymph nodes and progresses rapidly. Infiltrating lobular carcinoma is another invasive cancer and it is characterised by presence of small cells arranged in single files mostly around ducts and lobules. It metastasis to meningeal and serosal surfaces (Harris *et al.* 1984). In tubular cancer tubule formations are present but it is a not very common type of neoplasm (Lipman *et al.* 1992). The prognosis for tubular carcinoma is better than for patients with infiltrating ductal carcinoma. Medullary carcinoma is characterised by many histological features. It is infiltrated with lymphocytes and plasma cells. Nuclei in this type of cancer are poorly differentiated. The border of cancer cells is always well defined. When only some of the characteristics of this tumour are present the outcome for the patient is poor (Fisher *et al.* 1990). Mucinous carcinoma is characterised by a large amount of extracellular mucin surrounding cancer cells. It grows very slowly but has a very favourable outcome for patients.

The above types of breast cancers are just the examples, there are many other types, that can be classified according to their histological characteristics. One thing that they have got in common is that all of them if not treated can cause death to many women. Even with the treatment, the survival of the patients

varies. Some of the patients will develop treatment related cancers due to the nature of medicines that they have been given.

1.5 Chromosomal mutations

One of the human genome characteristics is the accumulation of mutations. Mutations allow for the evolution of organisms (Ohta 1974). A mutation is a change in genetic material of the individual organism. In regard to the study of population genetics, the inherited mutations are of the most importance, because they are passed down from generation to generation causing their accumulation (Crow 2000). Inherited mutations determine the genetic characteristics of population as well as the characteristics of the individual. There are many types of genetic mutations, which have different effects on the genomic makeup of individual or population.

Anti-cancer drugs are potent chemicals able to induce mutational changes in genetic makeup of the patient receiving a course of chemotherapy. These mutational changes usually involve inducing of chromosome mutations (Felix *et al.* 1998). Chromosome mutations are most likely to take place during crossing over at meiosis. There are a number of ways in which the chromosome structure can change. These changes can influence the genotype and phenotype of the organism (Crow 2000). However, if the chromosome mutation takes place in an essential part of the genome, it is possible that the mutation could cause death. There are several types of chromosomal mutations that can occur.

Chromosomal translocation involves the joining of two homologue genes. An example of such translocation is the MLL/AF4 fusion (Felix *et al.* 1998) which will be studied in this project. The translocation of MLL/AF4 chromosomes induces acute myeloid leukaemia. Major advances have occurred in understanding the process of molecular pathogenesis of human leukaemias. In some cases the translocations can cause severe side effects for the organism or even death. Analysis of patient karyotypes reveals that somatic mutations (induced by anti-cancer treatment) such as chromosome translocations occur in most acute myeloid leukaemias (Gilliland 2002). Those fusion oncogenes that have been identified utilise similar signal transduction pathways and transcriptional activation pathways to mediate their leukaemogeneic effect. It has also been discovered that fusion proteins that arise as a result of chromosomal translocations are central mediators of myeloid proliferation and transformation in these malignancies. Figure 1.5.1 illustrates the process of translocation.

Point mutation involves a change of a single base pair. Figure 1.5.1 shows an example of point mutation. The process can be caused during DNA replication when a wrong base is inserted during the course of new DNA synthesis. Another cause of point mutation can be during exposure to chemicals. An example of point mutations are the Burkitt lymphomas (Lindstrom *et al.* 2002). Incidences of Burkitt lymphomas arise from chromosomal translocations that activate the *C-MYC* oncogene through juxtaposition to one of the immunoglobulin (Ig) loci. It is not uncommon to find mutation in the *P53* tumor suppressor gene in these types of leukaemia. The evidence that point mutations

present at both the *PRb* and *P53* tumor suppressor pathways is critical for development of Burkitt lymphomas.

Loss of a significant part of a chromosome or even a whole chromosome is called a deletion. Genes or whole fragments of chromosome are permanently lost, as they become unattached to the centromere. Figure 1.5.1 illustrates the mechanism of chromosome deletion. This type of chromosomal mutation has been identified on chromosome 6 in cases of prostate cancer. Detailed analysis revealed that chromosome region 6q14-16 was deleted in approximately 50% of the prostate cancer samples. The analysis revealed the importance of the loss of genes in this part of chromosome 6 in prostate cancer pathology (Verhagen *et al.* 2002).

Inversion is another type of chromosomal mutations. In this mutation the order of chromosome is disrupted (see Figure 1.6); it can lead of total gene expression disruption. Inversions frequently occur in cases of acute myeloid leukaemias and are quite frequent. Chromosomal inversions occur at MLL gene at chromosome band 11q23 and are very common in infant acute myeloid leukaemias (AMLs). Another type of chromosomal inversion has been identified recently in infant patient with rapidly progressive AML. The newly discovered mutation involved an inversion of 11q [(11)(q14q23)] (Wechsler *et al.* 2002).

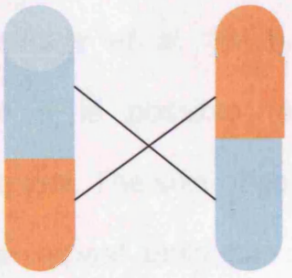
Chromosomal duplication involves the mutant genes being copied twice on the same chromosome. There are several sub types of duplication such as tandem duplication and reverse duplication (Nasheuer *et al.* 2002).

When the duplicated fragment is positioned next to original it is called tandem duplication. In case where both fragments are not adjacent displaced duplication takes place.

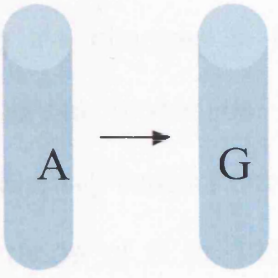
1.6 Minisatellite (D1S7) MS1

Minisatellites have been shown to be highly unstable genomic elements (Jeffreys *et al.* 1985). These characteristics have been used to detect changes (mutations) in humans that have occurred after exposure to radiation (Dubrova *et al.* 1996).The minisatellite MS1 has been shown to be highly sensitive in response to radiation. Those changes have been detected successfully (Jeffreys *et al.* 1990). Such sensitivity might indicate that this minisatellite might be used as an early marker for patients whose DNA has been affected by anti-cancer chemicals.

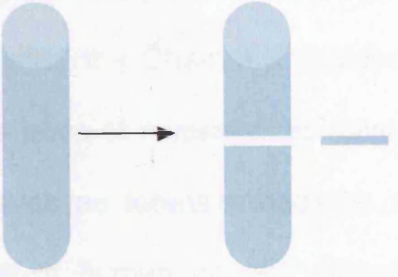
Minisatellites are arrays of short tandemly repeated DNA sequences scattered thorough the human genome. These structures are the most polymorphic regions present in chromosomes identified to date (Jeffreys *et al.* 1985). The polymorphism of the minisatellites is caused by a high rate of germline mutation, which causes loss or gain of the sequence lengths. The function of minisatellites has been an area of investigations for some time. It is thought that the minisatellites are the genomic regions where many mutations occur. The mechanisms of mutations believed to take place at minisatellites are unequal sister chromatid exchange, gene conversion, DNA polymerase slippage and deletion by intramolecular recombination (Jeffreys *et al.* 1985, Cederberg *et al.* 1993).



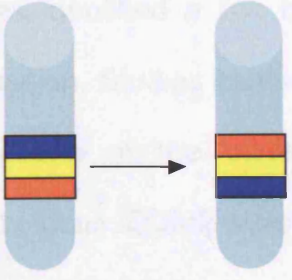
A) Translocation



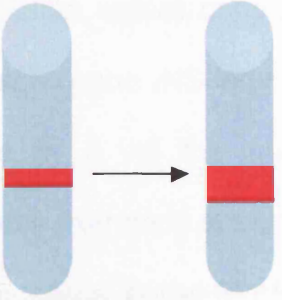
B) Point mutation



C) Deletion



D) Inversion



E) Duplication

Figure 1.6 Types of chromosomal mutations.

Minisatellites consists of many repeat units. The sequences of repeat units may vary, but the length of each repeat remains the same. The variations in repeat units create internal structure of minisatellites called MVR (minisatellite variant repeat) (Jeffreys *et al.* 1990). By studying the changes in MVR maps of minisatellite it is possible to study mutational processes that occur at minisatellite loci. The size of each minisatellite array varies from 0.7 to 20 kb in length. The repeat units can vary in the range of 6 to 100 bp. Because minisatellites are highly variable, they are very useful tools for investigating the processes of both spontaneous and mutagen induced human genome instability. The best example of this phenomenon is the elevation of minisatellite mutation rates observed after the Chernobyl accident (Dubrova *et al.* 1996). The polymorphic characteristics of minisatellites have been also used in many other areas of science such as forensic medicine and phylogenetic studies (Dijan 1996) and studies of human genetic diversity (Armour *et al* .1996, Chakraborty *et al.* 1991). Minisatellites were also used for positional cloning of genes associated with diseases. In recent years other studies carried out on minisatellites identified a link between minisatellite repeat copy number and gene expression. Studies carried out on the insulin gene INS indicated that the allelic variations of the minisatellite located in 5' of the human insulin susceptibility locus IDDM2 were linked to diabetes (Kennedy *et al.* 1995). It was also discovered that a presence of rare minisatellites in the 3' of human pro-oncogene H-ras-J caused increased chances of developing cancer for patients (Krontiris *et al.* 1993, Ding *et al.* 1998)). It was also discovered that minisatellite mutations could be used as prognostic markers in renal carcinoma (Uchida *et.al.* 1994). There are other diseases where variations in the minisatellite

regions have an effect such as in Myotonic Dystrophy and Fragile X chromosome (Richards *et.al*, 1992). It was also proposed that minisatellites could be used as markers for genotoxic chemicals. The PCR techniques used for such investigations have a capacity to detect minimal changes that can occur within minisatellite structures (Yauk 1998). Such studies have already been undertaken in the case of the Chernobyl incidence (Dubrova *et al.* 1996). Some studies have also been conducted on patients receiving chemotherapy. The rate of mutation of minisatellite at MS205 locus was reported to have higher germline mutation in patient after chemotherapy (Zheng *et al.* 2000).

Minisatellite studies have been carried out to assess germline and somatic mutations to a great extent (Jeffreys *et al.* 1995, Buard *et al.* 2000, Armour *et al.* 1999). The most variable human minisatellites studied up to date are MS32 (D1S8) (Appelgren *et al. 1997*) and MS1 (D1S7) (Maleki *et al.* 1997). The studies carried out on those minisatellites contributed to the understanding of the mutational processes that occur in minisatellites. It was discovered that the mutation processes do not occur in the flanking regions of the minisatellites (Jeffreys *et al.* 1994). Therefore the area of studies concentrated on the minisatellite variant repeats. It was also discovered that in germline minisatellite mutations the complex inter-allelic rearrangements occurred at one end of the minisatellites- cis (Jeffreys *et al.* 1997). The studies carried out on somatic mutations of minisatellites revealed that the mutational processes are not restricted to germline only (Armour *et al.* 1989).

In this project, the locus of interest has been minisatellite MS1 (D1S7) located on chromosome 1. MS1 is the second most variable, unstable minisatellite in human genome. It consists of tandem repeats 9bp long and can range from 0.7

to 20 kb in length. There are at least 19 variations of minisatellite repeat unit sequences at MS1. MS1 heterozygosity is estimated to be >99%. The average spontaneous germline mutation rate of this minisatellite has been estimated to be around 5.2% (Maleki *et al* 1997).

The tandem repeat loci, known as minisatellites, are highly informative markers of mutational processes occurring in both germline and somatic cells. The presence of sequence polymorphism within the tandem repeat array allows for the development of MVR-PCR (Multiple Variant Repeat unit-PCR) see figure in chapter 4. MVR-PCR is a tool by which the internal structures of minisatellite alleles can be studied. The MVR-PCR profile was first developed to study D1S8 (MS32), where it was revealed that the alleles even of the same length have different internal and even the diploid codes are highly individual specific (Jeffreys *et al.* 1990). In a case where the whole genomic DNA is analysed, the MVR-PCR profile detected is diploid in origin. Alternatively if the MVR-PCR profile is determined from a separated allele, it is a haploid in origin and MVR-PCR map of that allele can be generated. Not all of the minisatellites are suitable for the MVR-PCR studies. To detect any mutations the minisatellite has to be proven to be sensitive to genotoxic agents. In a case of the MS1 it has been proven that it is susceptible (Dubrowa *et al.* 1996). The allele has to have heterozygosity greater than 95% (Neil *et al.* 1993). Other characteristics include same length repeat units, not too many variations within repeat units and polymorphism. In some loci the map that is generated by the MVR system is of the diploid alleles but in some alleles single profiles can be obtained. Clearly it is easier to understand mutational events at MS1 locus with haploid data, rather than diploid. However, as they require no prior separation of alleles diploid MVR

profiles are easier to generate. This study has taken the approach of developing MVR-PCR in the diploid state (see Chapter 4,Figure 4.1), subsequently using diploid code analysis to show that mutational events at MS1 can potentially be characterised.

In order for MS1 to be a useful biomarker of chemically induced mutation events it is important to be able to detect rare mutational changes. The studies of the mutational changes at the MS1 locus in human colorectal carcinomas showed that MS1 could serve as a very good biomarker. It was found that MS1 showed high genetic instability in tumour cells (Hoff-Olsen *et al.* 1995). This demonstrates that MS1 might have potential as a biomarker to assess somatic mutations and shows that it is very sensitive and versatile. In this project many techniques will be applied to examine any potential mutational changes at the MS1 locus that might have occurred after the exposure of somatic cells to the chemotherapy agents FAC (5-fluorouracil, adriamycin and cyclophosphamide). The MS1 minisatellite was used to investigate to what extent if any the FAC anti-cancer drugs damage the DNA by causing somatic mutations at the MS1 locus.

1.7 MLL and AF4 –Chromosomes involved in t-AML treatment related acute lymphoblastic leukaemia

Secondary leukaemia developed by patients who received chemotherapy is a very serious draw back for patient's survival of cancer. There have been many studies investigating the problem (Bergerat *et al.* 1997, Sharma *et al.*1995, Lue *et al.* 1995). Treatment-related cancer in patients treated with anti-cancer drugs

can be caused by many factors. Some of those drugs include alkylating agents e.g. cyclophosphamide and topoisomerase II inhibitors, doxorubicin (Felix 1998). Patients with secondary leukaemia do not respond well to the treatment and their prognosis is very poor. They develop their secondary cancer 2-5 years after the treatment for the primary cancer (Felix 1998).

In this project the MLL and AF4 loci, which are involved in secondary leukaemia were investigated.

From the previous experiments conducted by other workers some information was already known about the MLL locus. The MLL gene is situated on chromosome 11 at 11q23 (Felix *et al.* 1998). It was found that in some cases of leukaemia (spontaneous or caused by anti-cancer treatment) the MLL gene is disrupted by chromosomal translocation (Felix *et al.* 1998). The MLL gene contains a breakpoint cluster region (bcr) located between exons 5 and 11. Most of the chromosomal MLL breaks are located in that region. The MLL gene has at least 30 translocation partners and has been termed a "promiscuous oncogene" (Stanulla *et al.* 1998). One of those partner genes is AF4, which will be discussed later in this chapter. The MLL locus is unusual in its transactional behaviour, usually the translocation occurs between two partner genes (Gregorini *et al.* 1998). The translocation between MLL and AF4 genes produces a chimeric fusion protein, which is believed to cause leukaemia. It is still unclear which of the products from the chromosomes cause secondary leukaemia, but it is believed that a product from the MLL gene is a culprit (Uckun *et al.* 1998). Chromosomal translocations are believed to arise from a single cell. The process involves firstly DNA damage in the case of treatment related leukaemia by anti-cancer drugs. Then the fusion proteins produced by

chromosomes fused together during translocation to take part in process of oncogenessis (Schnittger *et al.* 1998).

The AF4 gene is located on chromosome 4q21. Three breakpoint cluster regions are present at the AF4 locus where the chromosome breaks and forms translocations with the MLL 1(Nilson *et al.* 1997, Marschalek *et al.* 1995). In the breakpoint cluster regions of the AF4 locus there are several Alu elements present. Alu elements are repetitive fragments of DNA that are abundant throughout the human genome and may contribute to PCR artefacts being formed. It was discovered that the second cluster region of AF4 contained the least concentration of Alu elements. The other two cluster regions present in the AF4 locus contain multiple Alu elements. From the preliminary observations it was clear that the second cluster region offered an opportunity for better study of the MLL/AF4 translocations. The region would be much easier to study by the PCR techniques that the other two cluster areas. By using the PCR based techniques the workers were able to study the bcr regions of AF4 (Marschalek *et al.* 1999). The PCR is the principle technique by which the translocations are studied at the moment. Some of the studies employ RT-PCR or nested PCR to detect the presence of translocations, but like all of the techniques in some cases it does not detect translocations (Hunger *et al.* 1998). Nevertheless it is used for translocation studies and is considered to be the most available. It is also sensitive enough to detect rare translocation molecules that can be found in blood samples (Marcucci *et al.* 1998).

If rates of MLL translocation can be measured, this information may help to develop PCR-based techniques to distinguish individuals who are more prone to developing secondary leukaemia.

Whilst the primary mode of investigation of MLL/AF4 translocations in this project will be examination of FAC treated DNA samples obtained from the hospital patients, the possible MLL/AF4 translocations in CEPH cells treated with the FAC anti-cancer drugs will be studied. PCR-based systems used for the analysis of chemically induced mutations by FAC anti-cancer drugs are outlined in Chapter 5.

The tissue culture studies will involve lymphoblastoid cell lines from CEPH collection. The cells will be exposed to the FAC anti-cancer drugs and the investigations for the presence of possible translocations will be carried out. The purpose of these experiments is to establish the rate of somatic mutations that occur in lymphoblastoid cells after exposing them to FAC drugs. Again MLL/AF4 translocations will be used as potential biomarkers of DNA damage inflicted upon cellular DNA. Obviously, several aspects have to be taken into consideration, before conducting tissue culture studies. One of those aspects is that cyclophosphamide, which is a component of FAC needs to be metabolised to its active form before being able to act as an anti-cancer drug. There are several ways to solve this problem (see Chapter 3). Although there are some difficulties connected with tissue culture studies, there are also significant advantages. They provide ample DNA material for the PCR based studies. With tissue culture experiments it is also possible to create a model for investigations of DNA damage caused by FAC anti-cancer drugs and the techniques needed for studies of those cells are available SP-PCR, MVR-PCR. Considering all of the above points, tissue culture studies on lymphoblastoid cell lines from CEPH the panel are a good system to find out more about mutational events that take place in human DNA, after treatment with anti-cancer drugs.

1.8 Gene expression studies using microarray technology

Advances in molecular and computational biology have allowed for development of powerful methods for the analysis of differential gene expressions. The microarray analysis of mRNA is a novel technique, which is capable of examining gene expression changes in thousands of genes in one experiment. The data generated has a potential to give insights into processes of anti-cancer drug impact on human cells at the molecular level (Cole *et al.* 1999). The diversity of microarray technology might allow in future to monitor chromosome gains and losses, DNA resequencing and mutation detection (Cooper 2001). The principles of the technique involve printing of the arrays of discrete DNA sequences fabricated on glass or nylon membranes. The technique involves matching known and unknown DNA samples. The samples analysed are labelled with dyes. The ratio of the dyes indicates the level of expressions of the genes of interest (Brown *et al.* 1999). There are two types of microarray technology. Type one involves use of cDNA fragments immobilised on glass to which a mixture of labelled targets are hybridised. This method was developed in Stanford University (Ekins *et al.* 1999). Second type of DNA microarray technology involves use of oligonucleotides or peptide nucleic acids as probes immobilised on chips (glass or membrane) .The DNA sample is hybridised to immobilised probes. The expression of particular genes is determined by quantifying the abundance of complementary sequences (Lipshutz *et al.* 1999). This system was developed by Affymetrics Inc. and is

widely used alongside cDNA microarray. In this project cDNA technology was used to investigate gene expression changes. Figure 6.1 in Chapter 6 shows an example of the gene expression patterns that can be obtained.

The microarray analysis can be applied to studies of tumour classification, drug discovery and development, classification of organisms and many more applications. It has been employed to develop the profiles of tumour cells such as breast cancer cells (Martin *et al.* 2000) to give valuable information about the type of tumour analysed and its developmental stage. It has also been used to address such problems in cancer therapy as prediction of efficacy and toxicity of cancer regimes (Zembutus *et al.* 2002).

In this project studies conducted were designed to give overall information on the gene expression changes that may occur after the FAC anti cancer treatment on the lymphoblastoid cells. The microarray technology available allowed to investigate more than 6,000 of the gene expression profiles in one experiment. The abundance of the data that can be generated is one of the limitations of the technique. The analysis of obtained results requires the use of sophisticated software for collection and tracking of all data points. The recommended approach involves grouping of genes with similar expression patterns together (clustering). Such approach eliminates the genes that might be possibly of no interest. The data can be presented in a form of chart with different gene expressions represented by colours (Eisen *et al.* 1998). The use of different software programmes can make it difficult for different laboratories to compare and combine the data (Debouck *et al.* 2000). On the other hand the technique allows the study of the overall gene expression changes of many genes in one experiment. The cDNA microarray analysis is a very powerful tool

(Scherf *et al.* 2000), the precision of this technique means that the results obtained give a true picture of the events taking place. With the development of the microarray technology there is little limitation to the number to genes that can be studied at the same time so it is possible to presume that this technology could allow us in future to study the expression of whole genome of an organism. The versatility of this technique may enable for it to be used in future for population studies to discover inter individual differences in response to treatments with drugs.

The hypothesis in this project was, that the FAC drug combination may induce treatment related leukaemia through changes induced at the molecular level in patient's DNA.

The aims of this project were to identify the extent of molecular changes induced by the FAC drug combination. In order to carry out those investigations several techniques were applied. The DNA damage was assessed at the gene expression level, chromosomal and minisatellite structure level. Such investigations would give a clear picture of the possible mutations that may be induced by the FAC drug combination administered to the breast cancer patients.

2.0 Materials and methods

Common laboratory chemicals used in this project were of analytical quality and were purchased from the following companies:

Amersham-Pharmacia, Amersham, Buckinhamshire UK.

Cy3-dUTP

Cy5-dUTP

ECL (Chemiluminescence Reagent) Kit

Applied biosystems, UK.

Taq polymerase

CELLGRO, Herndon, USA

Dulbecco's minimal essential medium (DMEM)

Gibco BRL, Life Technologies Ltd, Paisley, UK.

Cot DNA

Foetal bovine serum

GMAX

Luria Broth medium

RPMI 1640 medium without L-glutamine

Superscript II Reverse Transcriptase

Hybaid, Teddington, Middlesex, UK.

Hybond N+ membrane

Pharmacia, Peapack, New Jersey, USA.

Low T dNTPs

Premier Beverages, Adbaston, Stafford

Dried skimmed milk (Marvel)

Promega, Madison, WI, USA.

RNAsin RNAse inhibitor

Santa-Cruz Biotechnology, Inc, USA

Bag-1 antibody, IgG

Bcl2 antibody, IgG

Cdk4 antibody, IgG

GADD 153, IgG

Mouse secondary anti-body

Rabbit secondary anti-body

Sigma Aldrich Chemical company, Poole, Dorset, UK.

Adriamycin

Agarose

Ampicillin

DMSO

Doxorubicin

5-Fluorouracil

Phenol/Chloroform

Saline solution (tissue culture grade)

Sodium Laurel Sulphate

Sonicated salmon sperm

TriReagent

Stratagene, La Jolla, California, USA.

Herculase enhanced polymerase (Taq)

Qiagen, Crawley, West Sussex, UK.

EndoFree Plasmid Maxi Kit

2.1 DNA samples

2.1.1 CEPH collection

A collection of human genomic DNA samples was investigated in this project. The samples came from the CEPH collection (The **C**entre d'**E**tude du **P**olymorphisme **H**umain). The collection contains extensive mixture of human DNA samples. The samples investigated come from the collection of French families:

02,12,21,23,28,35,37,45,66,102,104,884,1331,1332,1334,1340,1341,1344,1345,1346,1347,1349,1350,1362,1375,1408,1413,1416,1418,1420,1421,1423,1424,13291,13292,13293,13294 (individual sample numbers assigned by the **C**entre d'**E**tude du **P**olymorphisme **H**umain)

2.1.2 Cell lines

2.1.2.1 CEPH cell lines

In this project an *in vitro* tissue culture model was designed. For the tissue culture studies of the FAC drug impact on lymphoblastoid cells the following CEPH cell lines were ordered:

GM12565 - CEPH family 12, individual 06 MS1 allele size 2.739 kb and 3.776

GM12663 - CEPH family 23, individual 01 MS1 allele size 2.843 kb and 9.281

GM12547 - CEPH family 66, individual 01 MS1 allele size 1.703 kb and 2.538

GM12698 - CEPH family 45, individual 01 MS1 allele size 2.347 kb and 3.781

These cell lines were chosen, because those individuals contained small in size MS1 minisatellite alleles. The allele sizes were determined by A.Jeffreys and his co-workers (data not published (Department of Genetics, University of Leicester).

2.1.2.2 C450-13 cell line

To ensure bioactivation of cyclophosphamide in tissue culture studies the following rat cell line was ordered:

C-450-13- cell line expressing rat cytochrome P450 2B1 (obtained from A.

Chiocca, Massachusetts Hospital, Charlestown, USA) (Wei *et al.*1994).

2.1.2.3 SEM cell line

SEM – Cell line of cancer patient with a defined translocation between MLL and AF4 loci. The cell line is derived from lymphoblasts of a cancer patient with a defined MLL/AF4 translocation. This translocation links the 5' end of the MLL bcr (breakpoint at 2770 bp) to AF4 bcr region 2 (breakpoint at 32911 bp) this cell line was a gift from Greil (Greil *et al.* 1994). The DNA extracted from this cell line was used as a positive control in studies investigating the MLL/AF4 translocations.

2.1.2.4 Somatic cell hybrid DNA samples

The somatic cell hybrid samples used in this project came from the UK HGMP Resource Centre, Cambridge:

DNA from chromosomal somatic cell hybrid HHW416, containing human chromosome 4, (Carloc *et al.* 1986).

DNA from chromosomal somatic cell hybrid JICL4, containing human chromosome 11 (Kao *et al.* 1976).

2.1.3 Clinical samples

The blood samples were obtained from the Leicester Royal Infirmary, Department of Oncology from patients undergoing chemotherapy for breast

cancer, who agreed to donate their blood samples to this project (ethical approval was granted). Samples were taken before and after the FAC treatment that patients underwent (there was no information on reaction of individuals to the treatment). The patients did not previously receive any anti-cancer treatments. At the beginning of this project it was estimated that there would be around 50 samples available for analysis, but only 13 samples were received i.e. 6 full sets (before and after the treatment) and one sample, which was taken after treatment only. The samples were labelled PU and PT; samples labelled PU are those before the treatment, the marked PT were taken after the FAC treatment.

2.2 PCR to amplify MLL/AF4 translocations

The Polymerase Chain Reaction was applied in this project for selective amplification of genomic DNA, in this case MLL/AF4 translocations. Table 2.3 shows the sequences of MLL and AF4 primers designed. The following components were used:

PCR buffer - 5 μl (Applied Biosystems, made to order)

H_2O - 0.7 μl

Taq polymerase - 0.3 μl (Applied Biosystems Cat. no. AB-0192)

Primer A - 1 μl of 10 μM

Primer B - 1 μl of 10 μM

dNTPs - 2 μl of 25 mM

100 ng of human genomic DNA was added to each PCR reaction.

PCR conditions used for amplification were:

95°C - 1 min

62°C - 1 min

72°C - 2 min

---------- 25 - 40 cycles as appropriate.

After the amplification, PCR products were separated by agarose gel electrophoresis.

2.3 Primer preparation

The primers were designed to amplify the regions of interest that did not contain long regions of ALU elements. The sizes of primers were kept below 30 bp length and above 18 bp in length to ensure the amplification of the desired region. Short primers, below 18 bp in length might amplify more DNA fragments, that it is intended by annealing to other regions in the genome. Long primers might not anneal to the region of interest. The designed primers had G/C ratio of 50% to ensure that they annealed al lower temperatures and to desired DNA region. It was ensured that the number of long repeats of the same type of bases was not present at the primers; this would interfere with the primer's function. Before use primers required purification and their concentration needed to be determined and diluted to an appropriate volume. To each sample pure ethanol was added, three times as much as the sample volume. The sodium acetate was added to give a concentration of 0.3M. The samples were put on dry ice for 30 minutes in order for DNA to precipitate. The samples were centrifuged at 12000xg and the pellets were washed with 75%

ethanol. The washed pellets were resuspended in distilled water and concentration measured using a spectrometer Lambda 2S (Perkin Elmer) at wavelength of 260nm and 280nm.

2.4 Long range PCR

Amplification of long fragments of DNA was carried out by using Herculase Enhanced Polymerase (Stratagene, Cat.No. 600260). This polymerase is very powerful and is able to amplify fragments up to 48 kb in length. In this project this property was essential due to the possible large sizes of translocations (perhaps up to 25 kb) that may be present in investigated DNA samples.

The long range PCR reaction components – 20μl reactions

10 x Herculase polymerase reaction buffer - 2 μl (Stratagene Cat.no.600260)

H_2O - 12.6μl

Herculase polymerase (5U/μl) - 0.4 μl (Stratagene Cat.no.600260)

dNTP's mix (25mM of each dNTP) -2μl

Primer MLL X (10μM) - 1μl

Primer AF4 X (10μM) -1 μl

50 ng to 150 ng of human genomic DNA was added to each PCR reaction.

PCR conditions

94°C - 30 sec.

62°C - 1 min

70°C -1 5 min

---------- 35 cycles

The final PCR conditions can be found in the result section in this project.

2.5 Single Molecule PCR

The investigations carried out in this project required the use of amplifications of single DNA molecules. In single molecule PCR reactions, DNA was diluted to 6 pg per microliter. The PCR conditions were not altered, but the number of PCR cycles was increased from 35 to 42. This technique is a very sensitive tool, which enables investigations at single molecule level of translocation events in DNA samples. The use of Herculase polymerase in this method allowed for amplification of large (more than 5 kb) fragments of DNA.

Primer	Sequence 5' to 3'	Region
MLL 1A	GGATCCTGCCCCAAAGAAAA	MLL BCR
MLL 8A1	GTGTGACATCACAATGTCAG	MLL BCR
AF4 A1	AGTCTTCACATGTGGCAGGCAGT	AF4 -second BCR
AF4 A2	AGCTGTACTTCGAGAACAGGCT	AF4 -second BCR
AF4 A3	AGTAAGGGAGCATCAGGGATGT	AF4 -second BCR
AF4 A4	TGAGCCAGCTCAGCTGCTTCAGA	AF4 -second BCR
AF4 A5	AGAGCTGGCCAGGACCAATTG	AF4 -second BCR
AF4 B1	ACTGCCTGGGTGTGCCTACTTCA	AF4 -second BCR
AF4 B2	AGCACTGCCTCCTACATGTACA	AF4 -second BCR
AF4 B3	TCTGCTGAAGACTGGCGCTGAA	AF4 -second BCR
AF4 B4	CTGGCTTCCTGTATGTAGACCA	AF4 -second BCR
AF4 B6	AGCCAGTAGGAGGATTTCCCACA	AF4 -second BCR
AF41 A1	GCTTAATTGGTCCTAAGGCAAGT	AF4 -first BCR
AF41 A2	ACATGCGTCAGTCATGCACAGCT	AF4 -first BCR
AF41 A3	CTCCTAAGAGAGGATCCTGGTT	AF4 -first BCR
AF41 B1	GCTAGACATCTTCCAGTTGACGT	AF4 -first BCR
AF41 B2	TCTTAGGTACACATGCTCGGT	AF4 -first BCR
AF41 B3	AGTTCCGTATTACCTTGCTCA	AF4 -first BCR

Table 2.3 MLL and AF4 primers.

2.6 Agarose gel electrophoresis and Southern blotting

To detect the products of PCR, DNA samples were separated on agarose gels. In all cases the gel concentration was 0.8% *i.e.* 0.8g of agarose per 100 ml of TBE buffer. The voltage used was 120 V with eight hours electrophoresis for good separation. The buffer used was TBE.

DNA samples were amplified to a level visible by ethidium bromide staining (5 μl per 100 ml of agarose gel) and so Southern blotting was performed in order to verify true PCR products. Southern blotting steps include:

1) The gel with samples was washed in 0.4 M NaOH (15 min.) and blotted, using Hybond N + membrane (Amersham).

2) The membrane was blotted for 6 hours in order for DNA to bind tightly to the membrane.

3) After the blotting the membrane was washed in 6 x SSC to remove excess DNA. The membrane was then exposed to UV light in order to bind the DNA fragments more strongly. The prepared membrane was then hybridised with a MLL probe (see below). Figure 2.1 shows the diagram of Southern blot analysis.

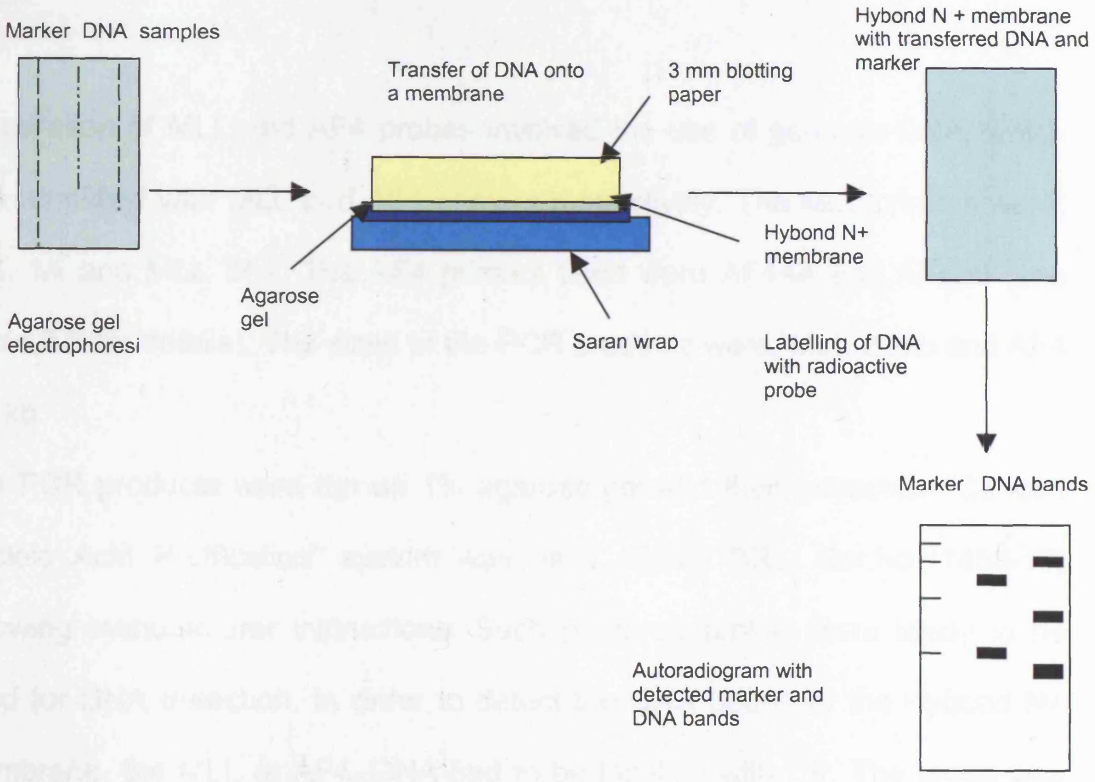


Figure 2.1 Diagram of DNA detection by Southern blot analysis.

2.7 MLL and AF4 probe preparation

Preparation of MLL and AF4 probes involved the use of genomic DNA, which was amplified with MLL and AF4 primers respectively. The MLL primers were: MLL 1A and MLL 8A1. The AF4 primers used were AF4A4 and AF4B6 (see Table 2.3 for details). The sizes of the PCR products were, MLL 2.9kb and AF4 1.0 kb.

The PCR products were run on 1% agarose gel and then extracted. "Concert Nucleic Acid Purification" system was used (Gibco BRL, Cat.No.11456-19) following manufacturer instructions. Such prepared probes were ready to be used for DNA detection. In order to detect the DNA bound to the Hybond N+ membrane, the MLL or AF4. DNA had to be labelled with ^{32}P. The probe was labelled with the α dCTP ^{32}P isotope using the "Random Primed DNA Labelling Kit" (Boehringer Mannheim 1004 780), following the manufacturers protocol. The prepared probe was then precipitated with the following reagents: 20 µl of 0.5 M EDTA, 70 µl of 2M Sodium acetate (7.0 pH), 70 µl of herring sperm DNA (3 mg/ml), 140 µl of water and 1 ml of 100 % ethanol. The precipitated probe was then resuspended in 1 ml.

2.8 PCR to amplify the MS1 alleles

Polymerase Chain Reaction was used in this project for selective amplification of genomic DNA, in this case minisatellite locus MS1. Table 2.3 shows the

sequences of primers MS1 A and MS1 B designed for the amplification of minisatellite MS1.

The following components were used:

PCR buffer - 5 µl (Applied Biosystems, Cat.no. Q9517)

Dist.water - 0.7µl

Taq polymerase - 0.3 µl (Applied Biosystems Cat. no. AB-0192)

Primer A - 1 µl of 10 µM

Primer B - 1 µl of 10 µM

dNTPs -2 µl of 25 mM

100 ng of human genomic DNA was added to each PCR reaction.

PCR conditions used for amplification were:

96 C - 1 min

65 C - 1 min

70 C - 5 min

---------- 35 cycles

After the amplification, PCR products were separated by agarose gel electrophoresis in 1 x TBE buffer.

2.9 MVR-PCR

For MVR-PCR, the basic PCR protocol was followed (see above), with the exception that primer MS1 A was replaced by the internally priming primers AB,

BB and CB. To prevent collapse of amplified products during the PCR reaction AB, BB and CB primers were used at higher concentrations (10μM) and PCR driven by the TAG primer (1μM) continued within each of AB, BB and CB repeat dimers. The designed primers annealed only to "B" type repeats, which are rare in the MS1 allele. Table 2.3 contains the sequences of designed primers.

The MVR-PCR reaction components:

PCR buffer	- 5 μl (Applied Biosystems, Cat.no. Q9517)
H_2O	- 0.7 μl
Taq polymerase	- 0.3 UL (Applied Biosystems Cat. no. AB-0192)
Primer AB or BB or CB	- 1 μl of 10 μM
Primer B	-1 μl of 1 μM
TAG	-1 μl. of 1 μM
dNTPs	-2 μl of 25 mM

100 ng of human genomic DNA was added to each PCR reaction.

The cycle test was performed first to determine the best conditions for MVR-PCR

i.e. cycle temperatures, time, in order to achieve the best results.

PCR conditions used for cycle test were:

96°C - 1 min

55 – 68°C - 1 min

70°C - 5 min

---------- 18-40 cycles

The final PCR conditions can be found in the Results chapter.

2.10 MS1 probe preparation

E.coli containing the MS1 1.1b clone was grown overnight at 37^0 C in Luria Broth medium with added ampicillin (100 μg/ml). The DNA was extracted using the Qiagen EndoFree Plasmid Maxi Kit. The extracted DNA was then precipitated, using ethanol. Plasmid DNA concentration was measured, using the spectrometer Lambda 2S (Perkin Elmer).

MS1 plasmid DNA was then digested with the enzyme *Hae* III. 50 μl of plasmid DNA at a concentration of 200 μg/μl was digested with 25 uμl of the enzyme (Boehringer Mannheim), in a reaction containing 125 μl of water, 25 μl of BSA and 25 μl of buffer M. The digested DNA was then run on agarose gel and the DNA fragment containing MS1 probe was isolated using a "Quick Pick Electroelution capsule "(Stratagene, Cat. No.400855), following the manufacturers instructions.

In order to detect the DNA bound to the Hybond N+ membrane, the MS1 DNA had to be labelled with ^{32}P. The probe was labelled with ^{32}P isotope, using the "Random Primed DNA Labelling Kit"(Boehringer Mannheim 1004 780), following the manufacturers instructions. The prepared probe was then precipitated with following reagents: 20 μ of EDTA, 70 μl of 2M sodium acetate (7.0 pH), 70 μl of herring sperm DNA (3 mg/ml), 140 μl of water and 1 ml of 100 % ethanol. The precipitated probe was then resuspended in 1 ml of water.

2.11 Hybridisation and autoradiography

The hybridisation buffer used contained 1 M $NaPO_4$ and 15% SDS in proportions of 1:1. The membrane containing bound DNA was firstly pre-hybridised (30 min) at 65^{0}C and then the boiled probe (3 min) was added. After overnight hybridisation, the membrane was washed in a solution of SSC and SDS in water, in proportions of 10 ml of 20 x SSC, 2 ml of 10% SDS made up to 2 L with water. The hybridisation and washing of the membrane was done at 65^{0}C. Next, the washed membrane was exposed to autoradiographic film in order to obtain a picture of the any translocations present. The exposure time was usually 16 hours, at –80^{0}C. The exposed film was then developed in an X-Ograph processor. The obtained autoradiograph was then analysed.

2.12 Enzyme Digestion

DNA digestion was carried out using following mixture of enzyme and buffers

800 ng of DNA

1 µl of 20 µl of enzyme

0.2 µl of BSA

15.8 µl of H_2O

---------- 20 µl reaction mix

The samples were incubated at 37^{0}C for 2 hours.

2.13 Tissue culture

2.13.1 CEPH cell lines

Cells were seeded at 2x 10^6 per 1 ml of medium.Maintenance of lymphoblastoid cells required preparation of appropriate medium, containing RPMI 1640 medium without L-glutamine. To the medium foetal calf serum and GMAX were supplemented in a proportion of 75 ml of foetal calf serum(15%) and 15 ml of GMAX(10%) in 500 ml of medium. The cell culture was maintained at the concentration of 2 million cells per 1 ml of medium at 37^0C, 5% CO_2.

2.13.2 C450-13 cell line

These cells were propagated in appropriate growing medium consisting of Dulbecco's minimal essential medium (DMEM) with high glucose (CELLGRO, Cat.no.10-013-LM). To the basic medium 10% foetal calf serum was supplemented with 1000,000 U/L of penicillin and 100 mg of streptomycin. Cells were maintained at 200,000 cells per 10 mm diameter Petri dish at 37^0C.

2.13.3 C450-13 and CEPH cell lines co-culture

Both C450-13 and CEPH cell lines were co-cultured in the medium used for growth of CEPH cell lines. The C450-13 cells were grown until confluent and then medium was taken off and CEPH cells with their medium were added. The

C450-13 cells for a layer of cells which adhere to the bottom of the cell culture flask. CEPH cell lines do adhere and form free moving clusters in the flask. Those properties of these cells made it possible for easy separation of those two cell lines. The activity of the rat P450 2B1 gene cloned into C450-13 cell line was not assessed. The possible assessment could have been carried out by the use of MTT assay, in which cells would be exposed to various concentrations of cyclophosphamide and their viability assessed. Such test would asses to what degree C450-13 cell line was bioactivating cyclophosphamide.

2.13.4 SEM cell line

SEM cells were cultured in Iscove's MOD, DMEM medium supplemented with Glutamax 1 and 10% foetal calf serum. The cells were seeded at 1 million per ml of medium at 37^0C. The cells were cultivated for the purpose of extraction of DNA in order to study MLL/AF4 translocations.

2.14 FAC concentrations

In *in vitro* investigations of the DNA damage caused by FAC treatment regime, the doses of drugs administered needed to be established. It was found out, that therapeutic concentrations of those drugs used in combinations are

5-Fluorouracil -0.5 mM /ml

Cyclophosphamide - 0.5 mM /ml

Adriamycin - 0.05 mM /ml

As well as therapeutic concentrations, other concentrations of drugs were also administered to asses a potential impact of higher and lower concentrations. It was decided to treat the cells with the concentrations of 1.0 μM of C, 1.0 μM of F and 0.1 μM of A as well as 0.1 C, 0.1 F and 0.01 A in order to asses the degree of the toxicity of the FAC drugs. The extraction of DNA was performed in a Class I tissue culture hood in order to avoid any contamination that can arise by handling human blood samples.

2.15 DNA extraction

The method was both used for DNA extraction from blood and culture cells. 0.5 ml of blood of collected cells were put into 1.5 ml eppendorff tubes and around 1 ml of 1 x SSC was added. The samples were spun for 2 minutes and the supernatant was discarded. Next 1.4 ml of 1 x SSC was added and samples spun as previously and supernatant discarded. Obtained pellets were resuspended in 270 μl of 0.2 M NaAC (pH 7.0) and 30 μl of 10% SDS were added. Samples were left for 1 hour and later 200 μl of phenol/chloroform was added to extract DNA. The mixed samples were spun for 10 minutes and supernatant was placed in fresh tubes. Extracted DNA was precipitated with 1.0 ml of 100% ethanol, by 5 minutes spin and then washed with 80% ethanol. Such obtained DNA pellets were redissolved in milliQ water and stored at 4^0C.

2.16 RNA extraction using phenol/chloroform

CEPH 1206 cells treated with FAC drugs were spun and the pellet was resuspended in TriReagent (1ml). To 1ml of that solution 0.2 ml of phenol/chloroform was then added. Samples were shaken and left for 15 min to stand at room temperature. The samples were then spun at 14000xg at 4^0C. The aqueous phase was then transferred to a fresh eppendorf tube, 0.5 ml of isopropanol was added and samples were left to stand for 10min at room temperature. Samples were then spun for 10 min at 14000xg at 4^0C.The RNA forms a pellet at the bottom of eppendorf. The supernatant was removed and the pellet washed with 75% ethanol. Samples were vortexed and spun at 7000 x g. The pellets were then dried and resuspended in distilled water. For microarray experiments the RNA needed to be ultra clean, so the extraction procedure was performed twice on each sample. The final pellets were each resuspended in 10μl of water.

Finally the samples concentrations were measured, by using the absorbance readings at 260nm and 280nm using plastic cuvettes.

2.17 Determination of DNA and RNA concentrations

The concentrations of DNA and RNA were determined using absorbance values measured for diluted DNA of RNA samples at 260nm and 280nm. An absorption reading of 1 corresponds to 50μg/ml of double stranded DNA, 40μg/ml of RNA or single stranded DNA and 20μg/ml of oligonucleotide. The absorbance readings between 0.1 and 1.0 were taken as accurate. The more

concentrated samples were further diluted. Distilled water was used as a blank. Calculations to obtain the concentration values were as follows:

OD_{260nm} x dilution factor x 50 = μg/ml of double stranded nucleic acid

The purity of the samples was determined by calculating the ratio of two wavelengths 260nm/280nm. The pure DNA or RNA had a ratio between 1.8 and 2.

2.18 Protein extraction for Western blotting

Treated lymphoblastoid cells were collected and resuspended in lysis buffer. The lysis buffer for 10 ml mix was set up as follows:

Tris	- 50mM pH 7.4
EGTA	- 5 mM
NaCl	-150 mM
Benzamidine	-25 mM
Pepstatin	-10 mg/ml
Aprotonin	-2 mg/ml
Leupeptin	-10 mg/ml
100 mM Na Vanadate	-100 μl
Triton x 100	-400 μl
DDT 500 mM	-10 μl

2.19 Bradford Assay

To measure the concentration of protein in each sample the Bradford assay was used. The calibration curve was first plotted from the known protein concentrations. A 1 μg/ml solution of BSA was used to plot the calibration curve. The concentrations used ranged from 0.5μg/ml to 20μg/ml. The BSA was diluted with water. Aliquots (0.8ml) of each BSA sample was taken and 0.2 ml of Biorad reagent was added to form a coloured solution. The absorbance readings were measured at 595 nm. The absorbance values were plotted against the concentration values. Treated samples were prepared in the same manner i.e. 0.8 ml of samples were mixed with 0.2 ml of Biorad solution. The absorbances of the samples were measured and the sample concentrations were calculated from the calibration curve.

2.20 Western blot analysis

The presence of particular proteins in treated samples was determined by Western blot analysis. The total protein amount used for detection were 30 ug for each sample. The samples were mixed with the sample buffer(12% SDS,36% glycerol,50 mM Tris Base,25 DTT and 0.01% bromophenol blue) 1:1 and denatured before loading on to the gel. The samples were run on a gel to separate them and detected using appropriate antibodies. The separation gel consisted of two layers. The separating gel layer and stacking gel layer. The separating gel consisted of 30% acrylamide 37.5:1(4.0 ml), 0.75 M Tris/HCl pH

8.8(7.5 ml), 2.64 ml of H_2O, 2% SDS (750 µl), 10% (w/v) ammonium persulfate (105 µl) and Temed (4.5 µl). Stacking gel consisted of 30% acrylamide/bis 37.5:1(1.5), 0.5 M Tris/HCl pH 6.8 (2.5 ml), H_2O (6.2 ml), 10%(w/v) ammonium persulphate (30 µl) and Temed(7.5 µl). The separating gel was poured first and when set the stacking gel was added. The samples were then loaded onto the gel and run for 1 hour at 150 Volts. The running buffer used consisted of the following, 250ml of 1.5 M Tris base, 1.92 M glycine and 1% SDS. The gel was transferred onto a transfer membrane and the proteins were transferred (2 hours) with electric voltage (100 V) onto the membrane. The transfer buffer used at this stage consisted of 50 mM Tris base, 40 mM glycine, 20% (v/v) methanol. The membrane with transferred proteins was then blocked with Marvel or BSA (1h) and incubated with primary antibodies (1h) and with secondary antibodies (1h). The membrane was treated with ECL for 1 minute and then exposed to autoradiographic film for 1 min to detect proteins.

The following antibodies were used in this project:

Bag-1 –rabbit affinity-purified polyclonal antibody, reacts with human BAG-1 (Santa Cruz Ltd sc939), 1/500 dil. In 10% Marvel

GADD 153 - mouse monoclonal antibody IgG, reacts with human GADD 153 (Santa Cruz Ltd sc579) 1/100 – 1/2000 dil. In Marvel 10% and BSA 5%

Bcl-2 - mouse monoclonal antibody IgG, reacts with human Bcl-2 (Santa Cruz Ltd sc 7382), 1/1000 dil. In 10% Marvel

CDK4 - rabbit affinity-purified polyclonal antibody, reacts with human CDK4 (Santa Cruz Ltd sc431), 1/1000 dil. In 5% Marvel

2.21 Gene expression detection using microarray technology

The cDNA microarrays can measure gene expression patterns of thousand of genes simultaneously. The expression patterns can provide indirect information about function, interactions and response to xenobiotics of individual genes or cluster of genes. In this technique many gene-specific polynucleotides (ESTs) derived from the 3' end of RNA transcripts were arrayed on glass slides coated with poly-L-lysine and crosslinked with UV prior to use. Expressed sequence Tags are small pieces of DNA sequence (usually 200 to 500 nucleotides long) that are generated by sequencing either one or both ends of an expressed gene. The majority of clones were obtained from Research Genetics (set of known human genes, http://www.res-gen.com), some clones were obtained from GeneBank. All of the clones were sequence verified (Turton *et al.*2001). The total cDNA from control and treatment samples, which was fluorescently tagged, was hybridized onto those slides. The cDNA sequences representing each individual transcript hybridized specifically with the corresponding gene sequence in the array. The fluorescent signals detected allowed the determination of the abundance of transcripts present in examined samples. Generating microarray gene expression profiles in this project involved following many steps. Figure 2.2 shows the diagram of steps involved in gene expression detection using microarray hybridization.

2.21.1 Labelling of RNA

Extracted RNA obtained in tissue culture studies was labelled with fluorescent dyes CYP-3 and CYP-5. This involved setting up reactions where control samples and treatment samples were labelled with different dyes which help to determine the extent of changes in gene expression levels in treatment samples, when compared to controls. These dyes are fluorescent and each is of a different colour (red and green).The use of differentially labelled samples prevents mistakes with assessing the hybridisation; the ratio of two dyes is always measured. During the scanning of the arrayed samples, fluorescence of each dye is measured separately (Brown *et al.*, 1999). Each reaction contained a 50μg of RNA in 10μl volume of water. Firstly, the 4μg of dT25 oligo was annealed to RNA, the incubation started at 70^0C for 8 min and then the temperature was reduced to 42 ^{0}C over 30 minutes. Next the RNA was labelled with Cy5 (control) and Cy3 (treated sample). The labelling reaction consisted of 0.2 mM of Cy3-dUTP or CY5-dUTP, 2x first strand buffer, 0.02 M DDt, 1.0 mM dGTP, 1.0 mM dATP, 1.0 mM dCTP, 1.0 mM dTTP, 10 U/μl RNAsin and 1000 U/μl of II Reverse Transcriptase. The mixture was incubated for 1 hour at 42 °C, and then more II Reverse Transcriptase was added and the incubation continued for 1 hour.

2.21.2 RNA Hydrolysis

To remove unlabelled RNA a hydrolysis reaction was performed. To RNA samples hydrolysis mix was added, the mix consisted of the following: 1 μl of

0.5 M EDTA, 1 µl of 10% SDS and 3 M NaOH. The mixture was incubated for 30 minutes at 68°C and then for 15 minutes at 25 in order to hydrolyse any remaining RNA. NaOH was included in this reaction in order to prevent the precipitation of SDS.

2.21.3 Labelled RNA purification

The hydrolysed RNA was purified using Centrisep columns. Samples were spun at 2000 x g for 5 minutes and collected in the fresh tubes. To control samples 10 µg of Human Cot-1 DNA was added and samples were dried. The dried pellets were resuspended in 15 µl (usually control sample) of buffer and mixed together (control with treated sample). The samples were then denatured for 2 minutes at 100°C and then incubated for 30 minutes at 42°C. Such prepared RNA was ready for hybridisation onto arrayed slides.

2.21.4 Hybridisation onto arrayed slides

The cover slip was placed onto the arrayed slide and the prepared sample was gently pipetted next to the cover slip. Great care was taken to avoid formation of the air bubbles, which would prevent the hybridisation of the samples in the areas taken up by air. The solution was then sucked in by capillary action under the cover slip. The slide was placed in a water tight hybridisation chamber and incubated at 42°C in water bath overnight.

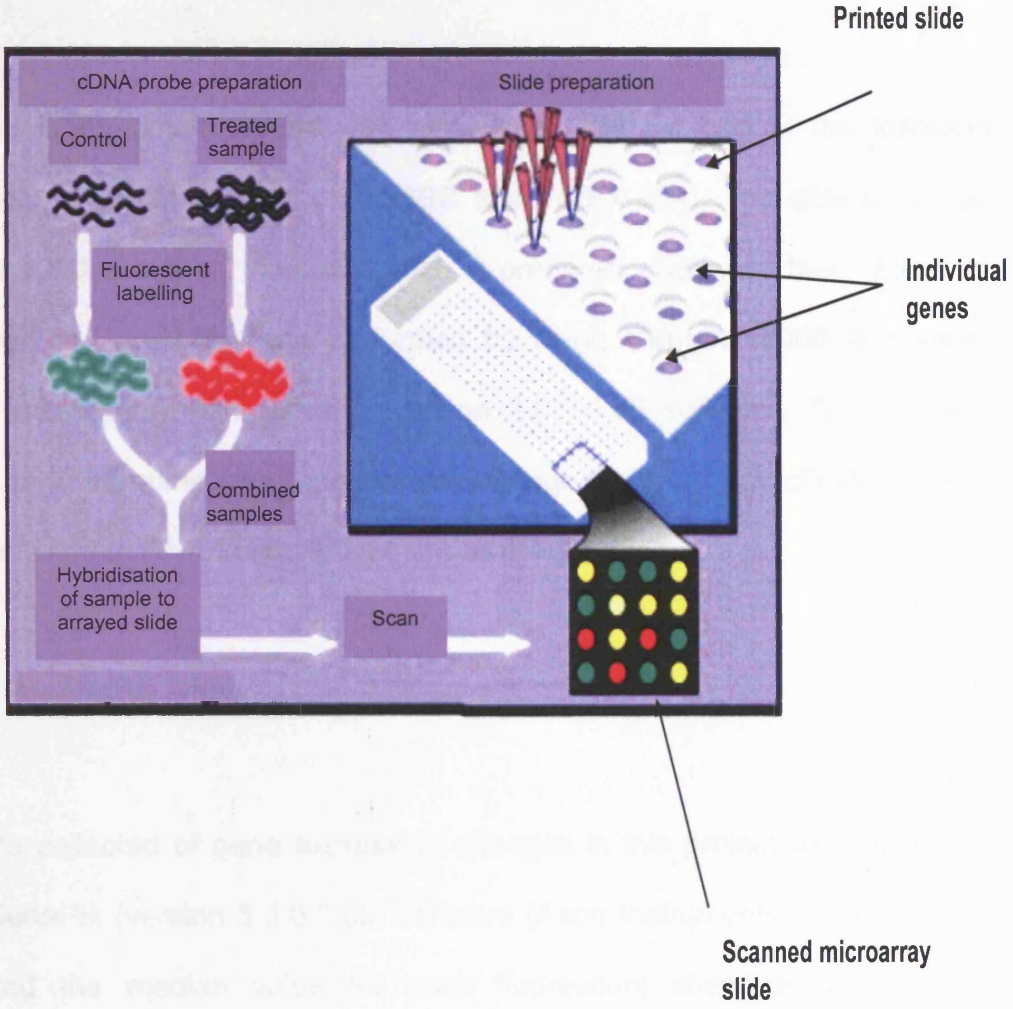


Figure 2.3 Diagram of microarrayer gene expression analysis. Diagram was adapted from htpp://www.accessexcellencc.org

2.21.5 Scanning of microarrays

After the incubation the slide with microarray was washed in the following solutions: 1 x SSC/3% SDS, 0.2 x SSC and 0.05 x SSC. The slide was then spun at 2000 x g for 2 minutes. Such a prepared slide was then ready for scanning. The scanning was performed by using GenePix 4000 A scanner (Axon Instruments) with GenePix version 3.0.0.0.85 software. The program allowed the determination of the fluorescent signals of labelled cDNA samples that hybridised to RNA transcripts on the arrayed slide.

2.21.6 Analysis of data

The data collected of gene expression changes in this project were analysed using GenePix (version 3.0.0.0.85) software (Axon Instruments). The software calculated the median value for each fluorescent chemical (control and treatment). A computer programme was used to normalise the array data after the subtraction of background values. ConvertData version 3.2.3a (latest version available at http://www.le.ac.uk/cnht/twgl/array-fp.html). These values were then used to calculate the ratio of fluorescence to determine differential gene expressions. The over expressed genes were shown in red, under expressed genes were shown in green and non-differentially expressed genes were shown in yellow. The generated gene expression profiles from all of the experiments were also analysed using Tree View programme. Tree View is a software programme designed to establish statistically the patterns of correlation between examined gene expressions (http://www.rana.lbl.gov.) It is

capable of clustering genes that show similar patterns of expression at the RNA level i.e. groups of genes which were overexpressed or suppressed in a particular experiment. The program also allowed the examination of data from several experiments at the same time.

3.0 *In vitro* model for studies of genome changes caused by FAC drug combination

Summary

The tissue cultures studies were performed in order to create an *in vitro* model to asses the impact of the FAC drug combination on the genome integrity and gene expression profiles. These studies involved developing optimal conditions for the cell growth of different cell lines used in this project. Investigations carried out assessed the FAC drugs impact on cell viability. The tissue culture studies provided genetic material for studies of the somatic mutations and chromosomal translocations (Felix and Jones 1998) that might arise after the exposure of cells to the FAC drugs (Chabner 1996). The gene expression profiles and protein expression profiles were also established by using RNA and protein lysates derived from the cells treated with the anti-cancer drugs.

The *in vitro* system designed utilised the C450-13 cell line, containing human cytochrome P450 gene 2B1. It revealed that it indeed did bioactivate cyclophosphamide to its active form.

3.1 Introduction

Investigations of the effects of the anti-cancer drug combination FAC on human genome integrity and gene expression profiles *in vitro* required designing of a suitable cell culture model. The FAC drugs have strong lymphosuppressive

actions (Chabner 1996) and so the cells chosen for the tissue culture studies were lymphoblastoid cells from the CEPH collection. Cell lines chosen for these studies contained sizes of MS1 alleles suitable for the PCR based assays (small in size).

Preliminary studies carried out on lymphoblastoid cells concentrated on establishing the optimal conditions for culturing them such as, nutrient concentration and cell density. The establishing of optimal growth conditions would ensure data validity, so that the DNA changes observed subsequently could be correctly interpreted. In addition to establishing optimal conditions for the cell growth, it was necessary to determine whether 5- fluorouracil, cyclophosphamide and adriamycin did affect the cell growth.

Cyclophosphamide needs to be bioactivated (Cohen *et al.* 1970) in order to act as anti-cancer drug. Therefore an additional cell line was used to establish the conditions under which cyclophosphamide could be bioactivated to its active form. Such a cell line was C450-13 (obtained from A. Chiocca, Massachusetts Hospital, Charlestown, USA). It contained human cytochrome P450 gene 2B1, expressing the enzyme needed for the bioactivation of cyclophosphamide (Wei *et al.* 1995).

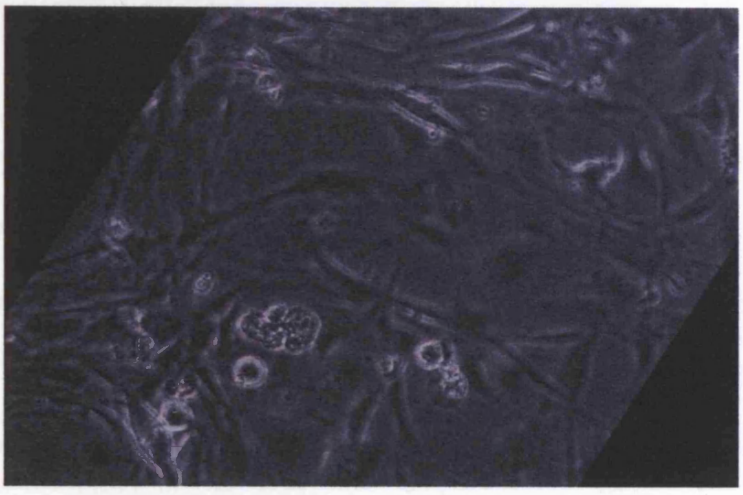
3.2 Results

3.2.1 C450 –13 cell line

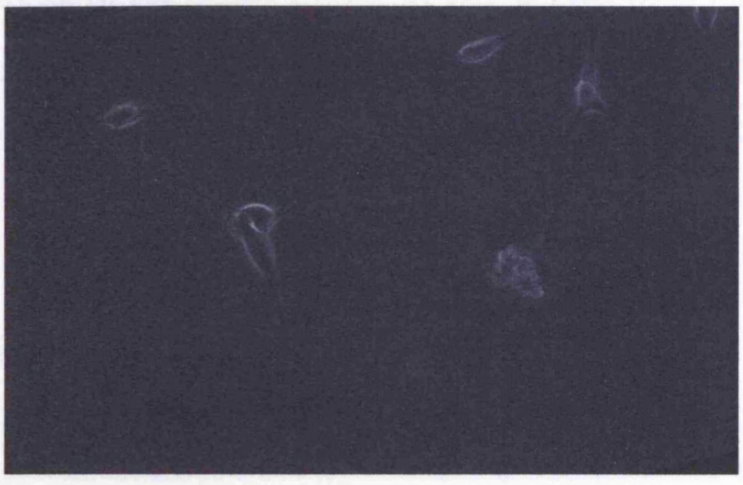
The C450 – 13 cell line containing rat cytochrome P450 gene 2B1 was treated with cyclophosphamide, the anti-cancer drug that needs to be bioactivated in order to be effective. A control experiment performed showed that the CP450–13 cell line was actively metabolising the cyclophosphamide. The viability count of cells showed that with higher concentration of the drug the cell survival was lower, due to the bioactivation which killed the cells. Table 3.1 shows the results of an experiment in which C450-13 cells were exposed to cyclophosphamide. Viability counts increased with decreasing cyclophosphamide concentrations administered. Figure 3.1 shows the photographs of C450-13 cells after 48 h of incubation with 1.0 mM of cyclophosphamide or the respective control (no drug added). This experiment demonstrated the importance of co-culture of CEPH cell lines with C450-13 cells, because the latter activates the cyclophosphamide, making the drug a metabolically active component of the FAC regime. To confirm the data, MTT assay might have been applied, which would give clearer view on cyclophosphamide bioactivation.

Well No.	Live	Total	%Viability	Treatment
1, C450-13	6	26	23	1.0mM Cyclophosphamide
2, C450-13	9	31	29	0.5 mM Cyclophosphamide
3, C450-13	36	80	45	0.1 mM Cyclophosphamide
4, C450-13	68	80	85	Negative

Table 3.1 Results of C450-13 cell line treated with cyclophosphamide. Mean results of duplicate experiments are presented in the table.



Photograph A



Photograph B

Figure 3.1 Photograph A, shows the C450-13 cells, which had no drug added. The cells look healthy and there are many of them. Photograph B, shows C450-13 cells to which 1.0 mM of cyclophosphamide was added. The drug had caused cell death and very few live cells are present. This experiment illustrates that C450-13 cells metabolised cyclophosphamide to its active form. Both photographs were taken after 48 hours incubation time.

3.2.2 CEPH 1206 cell line

This cell line was treated with cyclophosphamide to see, if the drug would be cytotoxic in the absence of C450-13 cells. The cells were treated for 48 hours. Treatment was applied when cells were 70% confluent. Cell death was assessed by counting cells exposed to triptan blue. It was very clear from the results obtained (see Table 3.2), that cyclophosphamide did not have effect on the viability of lymphoblastoid cells which suggests that no cells were killed by cyclophosphamide. This experiment proved that in order to create the similar conditions to those of the patient's body, in *vitro* studies must be carried out using the co-culture of CEPH cells and C450-13 cells. Figure 3.2 shows the photograph of CEPH 1206 cells and co-culture of CEPH 2 cells and C450-13 cells.

Well No.	No. of Live Cells	Total No. of Cells	% Viability	Treatment
1, CEPH2	91	95	96	1.0 mM Cyclophosphamide
2, CEPH2	140	145	97	1.0 mM Cyclophosphamide
3, CEPH2	107	114	94	0.5 mM Cyclophosphamide
4, CEPH2	124	130	95	0.5 mM Cyclophosphamide
5, CEPH2	92	97	97	0.1 mM Cyclophosphamide
6, CEPH2	85	87	98	0.1 mM Cyclophosphamide
7, CEPH2	92	94	98	Negative
8, CEPH2	79	80	98	Negative

Table 3.2 Results of CEPH 1206 cell line treated with cyclophosphamide for 48 hours. There is no effect of cyclophosphamide on CEPH cells; the drug needs to be bioactivated in order to affect the cells. Values are the mean of duplicate samples.

3.2.3 Co-culture of cell lines and FAC administration

The co-cultured cell lines were treated with FAC drug combinations as well as with single drug doses. The results of single drug exposure are presented in Figure 3.3.The CEPH cells were seeded at 2 x 10^6 per 2 ml of medium in each well. The C450 –13 cells were seeded at 5 x 10^4 per 2 ml and grown until 70% confluent and then 2 x 10^6 of CEPH cells were added and the FAC drugs were administered. The co-cultured cells were incubated with FAC drugs overnight (17 hours). After the incubation period, cells were counted and collected and the DNA was extracted using a method described in the Materials and Methods section.

The single drug exposure experiment indicated that each drug had a different impact on CEPH cells. The cell response measured by cell viability count showed that adriamycin had the greatest effect on cells. Cyclophosphamide and 5-fluorouracil cause less cell death. From the graphs it can be seen that cyclophosphamide has the smallest effect on cell survival of all the drugs administered to the cells. The previous studies performed on CEPH cells proved that C-450-13 cells bioactivate cyclophosphamide. Therefore the effect of cyclophosphamide on CEPH cells is caused by activation of the drug. Investigations of the effect of FAC drug combination on selected CEPH cell lines were performed in order to determine if different drug concentrations yielded different responses from cells. Figure 3.4 presents the results. The effect caused by the FAC drugs seemed to be the same as above. The highest dose of the FAC drug 1.0 mM causes the cell viability in all of the cell lines to

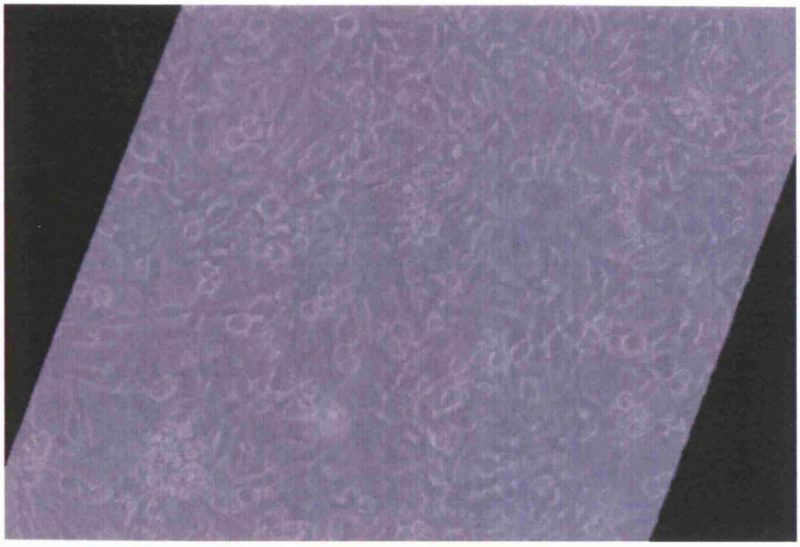
drop below 40%. The concentration of 0.1 mM had a small effect on the viability of the cells, the viability values being around 60-70%.

In order to assess the results obtained from the combined FAC treatment experiments the data was examined statistically by Analysis of Variance. This test assessed whether there were differences between cell lines used. It also allowed evaluation whether cell lines responded differently to a range of FAC drug concentrations. Table 3 .3 shows the results of analysis.

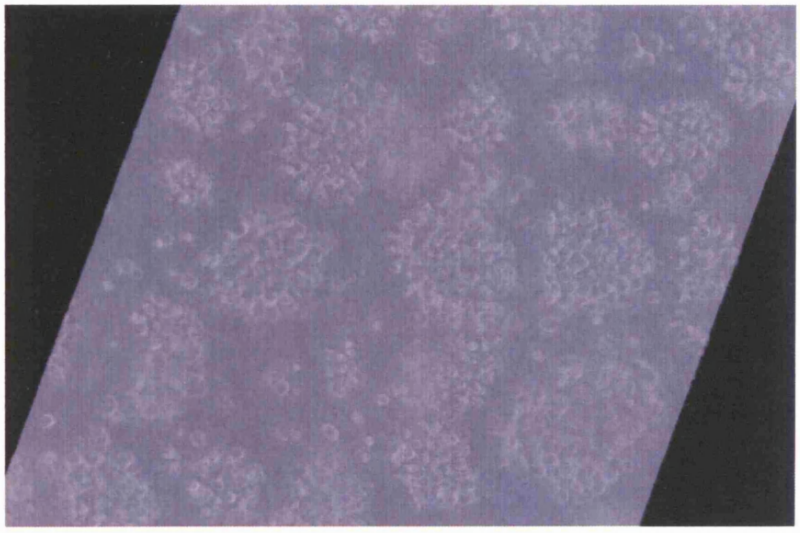
Source	**DF-degrees of freedom**	**SS-sum of squares**	**MS-mean square**	**F-distribution**	**P-probability**
Treatment	3	20373.2	6791.1	91.58	0.001
Cell line	3	273.4	91.1	1.23	0.332
Treatment and cell line	9	1043.7	116.0	1.56	0.209
Error	16	1186.5	74.2		
total	31	22876.8			

Table 3.3 Analysis of Variance.

From the results of the Analysis of Variance it is clearly visible, that there is no statistical difference in the response to FAC treatment between individual CEPH cell lines. As the P = 0.209 all CEPH cell lines used in this project react to FAC drug concentrations in the same fashion. It is also clear that the responses of cell lines to different FAC concentrations differ statistically, the P value being 0.001.This means, that the higher concentration of drugs the lower the cell's viability. The Analysis of Variance test also shows, that there was no significant difference between each cell line (P= 0.332).



Photograph A



Photograph B

Figure 3.2 Photograph A shows a co-culture of untreated CEPH 1206 cells and C450-13 cells. The CEPH 1206 cells form spherical clumps on the layer of C450-13 cells. Photograph B shows untreated CEPH 1206 growing on their own. CEPH cells grow in suspension culture, whereas C450-13 cells are adhesive.

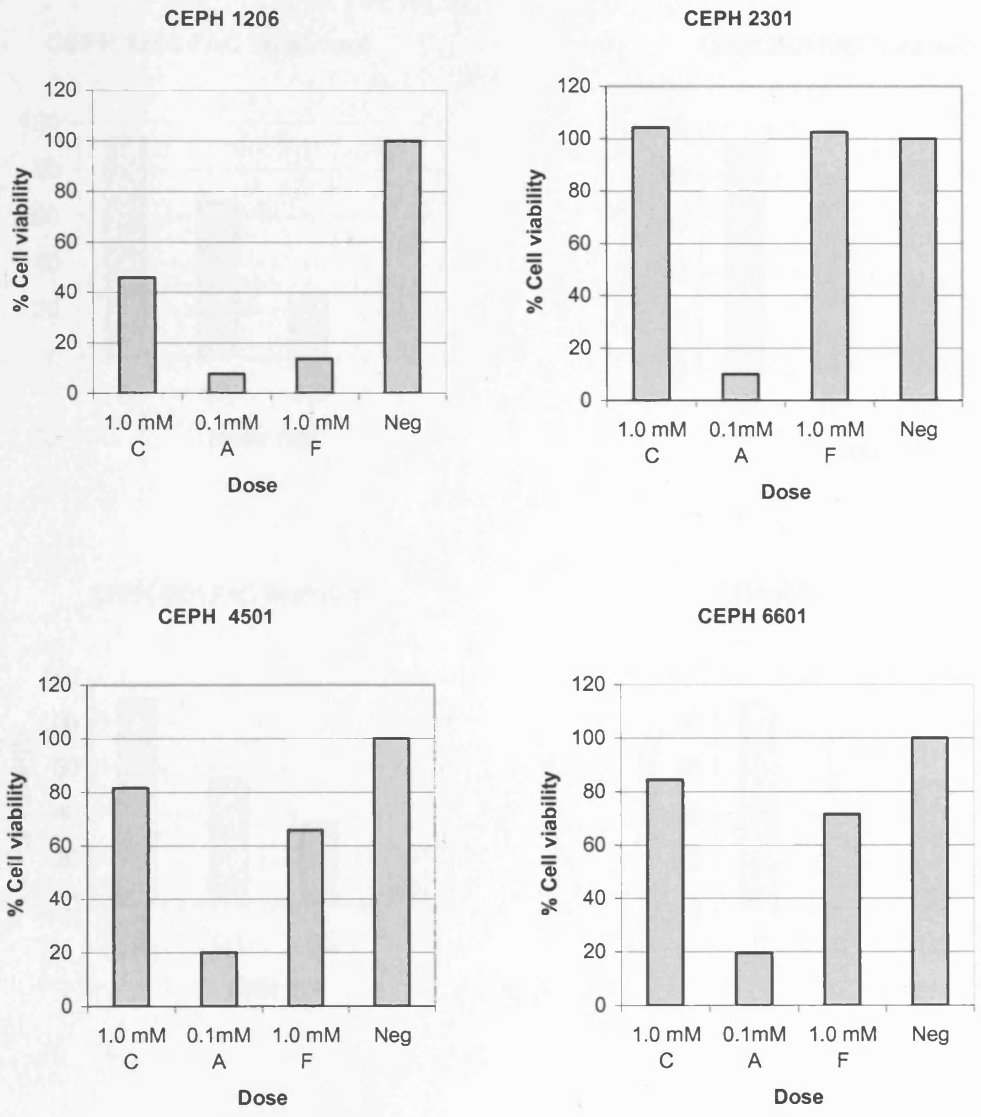


Figure 3.3 Graphs representing the CEPH1206,2301,4501 and 6601 cell viability in response to single drug administration of F, A and C drugs (17h). It is clearly visible that there was a difference in cell response to different drugs. Adriamycin was the most potent drug, and caused the lowest cell viability whereas cyclophosphamide caused the least change in CEPH 1206 cells viability. The experiments were performed in duplicate and their mean was presented in the above graphs.

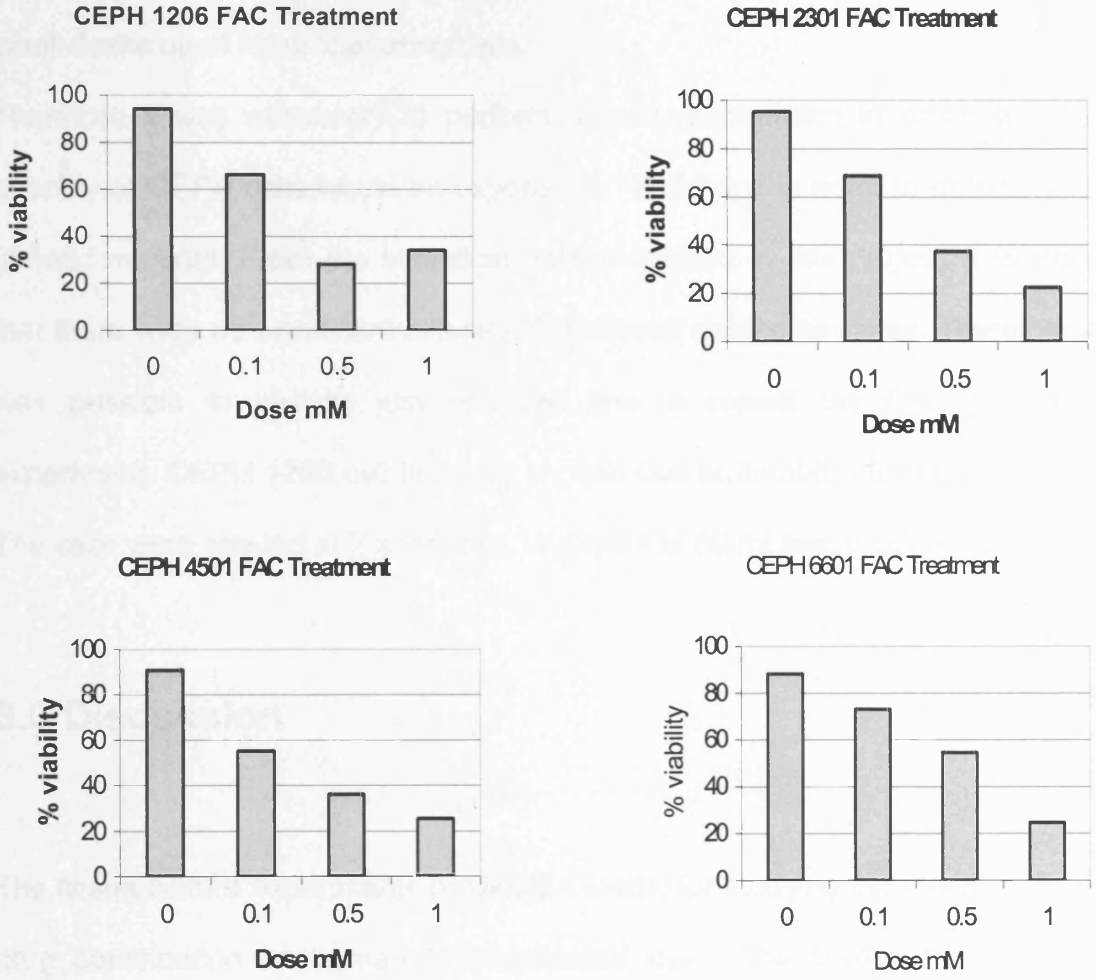


Figure 3.4 Graphs representing the viability studies of lymphoblastoid cells treated with the range of the FAC drug concentrations (17h). The data represent means of duplicate experiments.

The DNA concentration extracted from the above experiments was low, due to small flasks used for propagating cells.

Therefore it was necessary to perform another experiment in which a large quantity of CEPH cells would be exposed to FAC drugs in order to obtain more genetic material. From the statistical data presented in this project, it is clear that there were no significant differences between treated cell lines. Therefore it was possible to choose just one cell line to repeat the FAC exposure experiment. CEPH 1206 cell line was chosen due to its ability for rapid growth. The cells were seeded at 2 x 10^6 per 10 ml, but in 50 ml tissue culture flasks.

3.3 Discussion

The tissue culture experiments provided a model for studying the effects of FAC drug combination on human lymphoblastoid cells. The DNA extracted from treated cells has been used in various experiments including MS1 mutation detection and MLL/AF4 translocation detection (Felix *et al.* 1998). The experiments were also set up to establish gene expressions (Cole *et al.* 1999) and protein expression changes after FAC drug exposure. The cells chosen were lymphoblastoid CEPH cell lines transformed with the Epstein-Barr virus and C450-13 rat cell line containing human cytochrome P450 gene 2B1. After the preliminary investigations it was found that all of the CEPH cell lines react to the FAC exposure in the same manner (viability counts). The CEPH 1206 cell line was chosen as the cell line on which all the *in vitro* experiments were performed. The cell line was very easy to maintain and the cells grew in abundance providing easy source of genetic material for various investigations.

The CEPH lymphoblastoid cells with co-culture of C450-13 cells made a robust cell culture system that was used to asses the extent of the DNA damage (Gu *et al.* 1994) and gene expression changes caused by FAC anti-cancer drugs (Chamber 1996). The system developed also has the potential to be used for a long term exposure of cells to FAC drugs. Such studies could give light to effects of chronic exposure of FAC drugs on lymphoblastoid cells. The model could also be used on different combination of the anti-cancer drugs and its effects on the genome integrity and gene expression. The studies performed using genetic materials obtained in tissue culture studies can be found in the next chapters. The *in vitro* system designed proved to be very robust and can be possibly used in investigations of other xenobiotics that might have an impact on the human genome.

4.0 Gene expression changes in CEPH 1206 cells treated with FAC drug combination.

Summary

In order to study the changes that FAC drug combination inflicts upon human lymphoblastoid cells at the expression level, microarray gene profile analysis was used. The technique allowed for investigation of thousands of genes, the expression of which could be altered by the FAC drugs. Investigations revealed that administration of the FAC anti-cancer drugs changed gene expressions of non-cancerous lymphoblastoid CEPH 1206 cells. Experiments undertaken revealed that the particular gene expressions differed where the cells were treated with single doses of 5-fluorouracil, adriamycin and cyclophosphamide compared to the combined FAC treatment. Moreover with some genes synergistic responses were also observed. The gene expression studies were followed by protein expression studies using Western blot analysis. Those studies were to determine whether elevated mRNA levels of particular genes were reflected in changes in protein expressions in the cells treated with the FAC drugs. The experiments carried out on the treated cells gave encouraging results, they showed that the FAC treatment not only had an effect on the mRNA expressions of some genes, but indeed studies also proved that some protein concentrations were similarly altered. The time course experiments showed that the changes in gene and protein expressions were greater with longer exposure to FAC drugs. The studies showed that the FAC anti-cancer

drug combination induces changes in the gene expression and protein expression of some of the studied genes as well as their direct interaction with DNA.

4.1 Introduction

The microarray analysis of mRNA is a novel technique, which is capable of producing large quantities of gene expression in short space of time. These data have a potential to give insights into processes of anti-cancer drug impact on human cells at the molecular level (Cole *et al.* 1999). The information generated allows us to examine the changes in expression of thousands of genes in one experiment. The principles of the technique involves printing of the arrays of discrete DNA sequences on microscope glass. The samples to be analysed are labelled with dyes. The ratio of the dyes indicates, whether the genes of interest are overexpressed, did not change or are suppressed (Brown *et al.* 1999). In this project the criteria used for the overexpressed genes was two fold. This meant that if the gene dye ratio was less than two fold, the gene was not considered to be significantly overexpressed. Figure 4.1 shows an example of the gene expression patterns that can be obtained.

The microarray analysis is extremely versatile. It can be applied to studies of many different organisms (Kittler *et al.* 2000) as well as diseases (Cooper *et al.* 2001). Many scientists used this technique in studies of cancer. This technique for instance has been employed to develop the profiles of tumour cells such as breast cancer cells (Martin *et al.* 2000). Data obtained can give valuable

information about the type of tumour analysed and its development stage (Bertucci *et al.* 1999). It also gives some preliminary information about possible adverse effects to treatment (Zembutus *et al.* 2002).

In this project the impact of the FAC anti-cancer drug combination on human CEPH 1206 lymphoblastoid cells was to be determined. Apart from studies of combined FAC drugs, single drug treatments were also performed. The studies conducted were designed to give overall information on the gene expression changes that may occur after the anti cancer treatment on the lymphoblastoid cells. It was predicted that the impact of xenobiotics would cause some of the treated cells to become apoptotic.

In this project the main focus of interest was on overexpressed genes. The reason for this being that those genes might possibly compensate for the impact of the anti-cancer drugs on cells.

The investigations of the gene expression profiles also concentrated on synergistic interactions between FAC drugs. Such drug interactions are found where combined doses have more effect than the additive effects of single doses of the same drugs. Not only were mRNA levels investigated but also protein expressions of those genes susceptible to synergistic effects of FAC drugs were examined.

During this project it was possible to examine many thousands of the gene expression profiles in one experiment. The abundance of the data made it difficult to examine all of the genes at the protein levels. Therefore it was decided to concentrate on a few genes. Microarray technology made it possible to study the overall gene expression changes in all approximately 6,000, proving to be a robust technique.

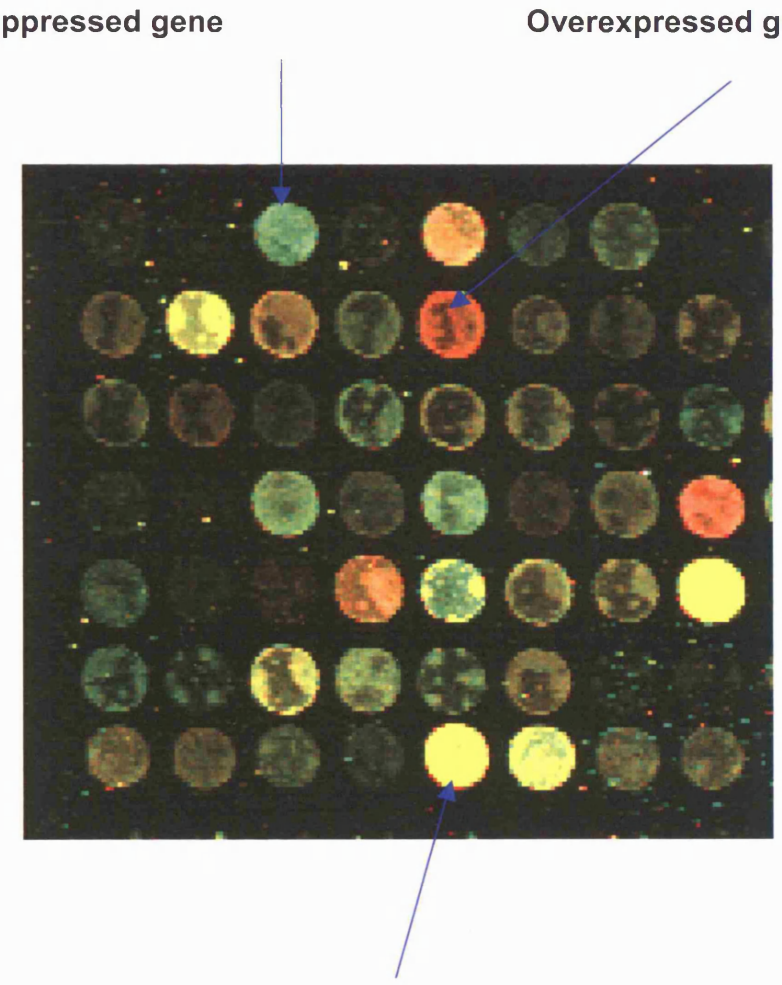


Figure 4.1 An example of the gene expression data obtained by microarray analysis. The different colour circles represent individual genes. The yellow coloured circles represent the genes, which gene expression did not change. The green circles represent genes, where the gene expression was suppressed. The red circles represent overexpressed genes.

As was mentioned earlier microarray analysis is a very powerful tool with which it is possible to analyse biological events such as tumour development at a molecular level (Bertucci *et al.* 1999). The precision of the technique means that the results obtained give a true picture of the events taking place. With the development of the microarray technology there is little limitation to the number of genes that can be studied at the same time, so it is possible to presume that this technology could allow us to study the whole genomes of the organisms. Such a powerful technique is a very valuable tool, which can be used to understand more the mechanisms of the diseases and also the response of organisms (at the molecular level) to xenobiotics, such as anti-cancer FAC drug combinations. The technique could be also used in population studies to discover individual to individual differences in response to treatments.

4.2 Results

4.2.1 Gene expression studies of the CEPH 1206 lymphoblastoid cells treated with 0.5mM of 5-fluorouracil.

Studies of the gene expression changes in CEPH1206 human cells were performed by the administration of a single dose of 0.5mM of 5-fluorouracil. The CEPH 1206 cells were co-cultured with C450-13 cells. The cell culture was incubated for 17 hours at 37°C. After the incubation the cells were harvested and RNA extracted. RNA was then labelled with fluorescent dyes Cy3 and Cy5 (negative control was labelled with different colour of dye to that of treated

RNA). The mixture of labelled treated and untreated mRNA was amplified and hybridised to a glass slide containing the array of human genes. The hybridised slide was scanned and data was generated. The scanning was performed by using GenePix 4000 A scanner (Axon Instruments) with GenePix version 3.0.0.0.85 software. The data generated is presented in the form of graph shown in Figure 4.2. Each experiment was performed twice and data was presented as means. The overexpressed genes were taken into consideration if the gene expression in the treated sample was increased by 2 fold compared with the untreated sample. Table 4.1 shows the examples of the genes that were overexpressed after the treatment of CEPH 1206 cells with 0.5 mM of 5-fluorouracil. In total around 37 different genes became overexpressed. In some cases, where the signal was the same as a background signal the genes were discarded. The same applied to the genes, whose expression was not changed significantly (0.5-1.5).

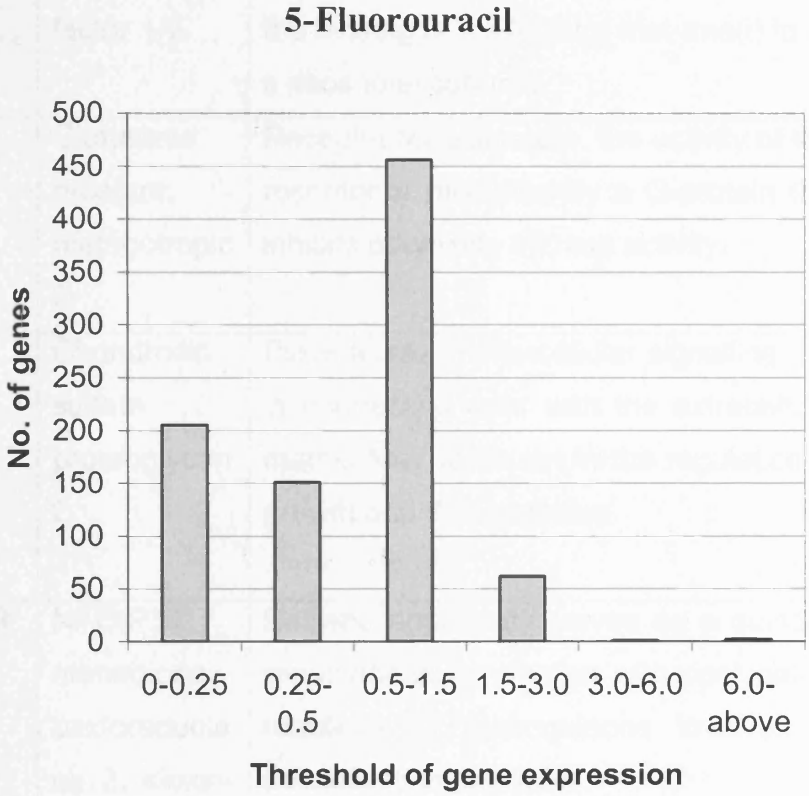


Figure 4.2. Graph representing gene expression data obtained after the treatment of CEPH 1206 cells with 0.5 mM of 5-fluorouracil for 17 hours (mean of two experiments).

Symbol	Full name	Function	Mean ratio
EIF1AY	Eukaryotic translation initiation factor 1A	Required for maximal rate of protein biosynthesis, enhances ribosome dissociation into subunits and stabilises the binding of the initiator met-trna(i) to 40 s ribosomal subunits	3.3
GRM3	Glutamate receptor, metabotropic 3	Receptor for glutamate, the activity of this receptor is mediated by a G-protein that inhibits adenylate cyclase activity.	3.3
CSPG2	Chondroitin sulfate proteoglycan 2	Plays a role in intercellular signalling and in connecting cells with the extracellular matrix. May take part in the regulation of growth and differentiation	3.3
NMOR2	NAD(P)H menadione oxidoreductase 2, dioxin-inducible	Enzyme apparently serves as a quinone reductase in connection with conjugation reactions of hydroquinons involved in detoxification pathways.	3.1
WNT2	MMTV integration site family, member 2	Probable developmental protein. Possibly signalling molecule which affects the development of discrete regions of tissues	2.8
WEE1	Wee1+ (S. pombe) homolog	Possibly acts as a negative regulator of entry into mitosis (G2 to M transition) by protecting the nucleus from activated cyclin b1-complexed cdc2 before the onset of mitosis.	2.8
AZGP1	Alpha-2-glycoprotein 1, zinc	Stimulates lipid degradation in adipocytes and causes the extensive fat losses associated with some advanced cancers.	2.7

Symbol	Full name	Function	Mean ratio
TXN	Thioredoxin	Thioredoxin participates in various redox reactions	2.6
MUT	MutL (E. coli) homolog 3	Involved in the repair of mismatches in DNA	2.5
ECGF1	Endothelial cell growth factor 1 (platelet-derived)	Possibly involved in maintaining the integrity of the blood vessels. It has growth promoting activity on endothelial cells,	2.4
GSTT2	Glutathione S-transferase theta 2	Conjugation reactions of reduced glutathione produce to a wide number of exogenous and endogenous hydrophobic electrophiles	2.3
FKBP5	FK506-binding protein 5 (51kD)	It interacts with functionally mature hetero-oligomeric progesterone receptor complexes along with hsp90 and p23.	2.3
CCNG2	Cyclin G2	Possibly involved in growth regulation and in negative regulation of cell cycle progesssion.	2.2
GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	This enzyme is necessary for target cell lysis in cell- mediated immune responses. It may also be involved in apoptosis.	2.2

Symbol	Full name	Function	Mean ratio
BIRC3	Apoptosis inhibitor 2	Apoptotic suppressor.	2.2
ZNF43	Zinc finger protein 43 (HTF6)	Possibly involved in transcriptional regulation	2.1
NMOR1	NAD(P)H menadione oxidoreductase 1, dioxin-inducible	This enzyme apparently serves as a quinone reductase in connection with conjugation reactions of hydroquinons involved in detoxification pathways	2.0
GSTA2	Glutathione S-transferase A2	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles.	2.0
API3	Apoptosis inhibitor 3	Apoptotic suppressor. Inhibitor of caspase-3 and caspase-7.	2.0

Table 4.1. Examples of some of the upregulated genes in CEPH 1206 lymphoblastoid cells treated with 0.5 mM of 5-fluorouracil.

4.2.2 Gene expression studies of the CEPH 1206 lymphoblastoid cells treated with 0.05mM of adriamycin.

Human CEPH 1206 lymphoblastoid cells were co-cultured with C450-13 cells. To the co-culture 0.05 mM of adriamycin was added and the cells were incubated for 17 hours at 37°C. The data was generated using procedures described in Materials and Methods chapter. The experiment was conducted twice. Generated results can be seen in Figure 4.3. The analysis of data indicated that more genes were overexpressed after the cells were treated with adriamycin than those treated with 5-fluorouracil. There are also twice more genes overexpressed greater than 3 fold, where compared with 5-fluorouracil experiment. This might suggest that adriamycin had a greater effect on the gene expression profiles. Table 4.2 contains some of the examples of genes that were upregulated by adriamycin. There are some differences in the suppressed gene population in both experiments. Those differences can be due to the fact that some genes gave a weak fluorescent signal, which was the same as the background signal. Those genes might have been possibly discarded, therefore there are some differences present.

There are around 169 genes upregulated by adriamycin, that is almost five times as many as the genes upregulated by 5-fluorouracil.

In adriamycin experiment A and B the number of the genes with no change in gene expression is similar. It can be concluded that the number of the unchanged genes is the largest, around 1400 if the mean is taken from both experiments. This experiment indicated that there are differences between the way individual drugs from the FAC combination affect the RNA expressions. It

highlights the importance of the single dose studies of gene expressions when the combination doses are used as the main treatment.

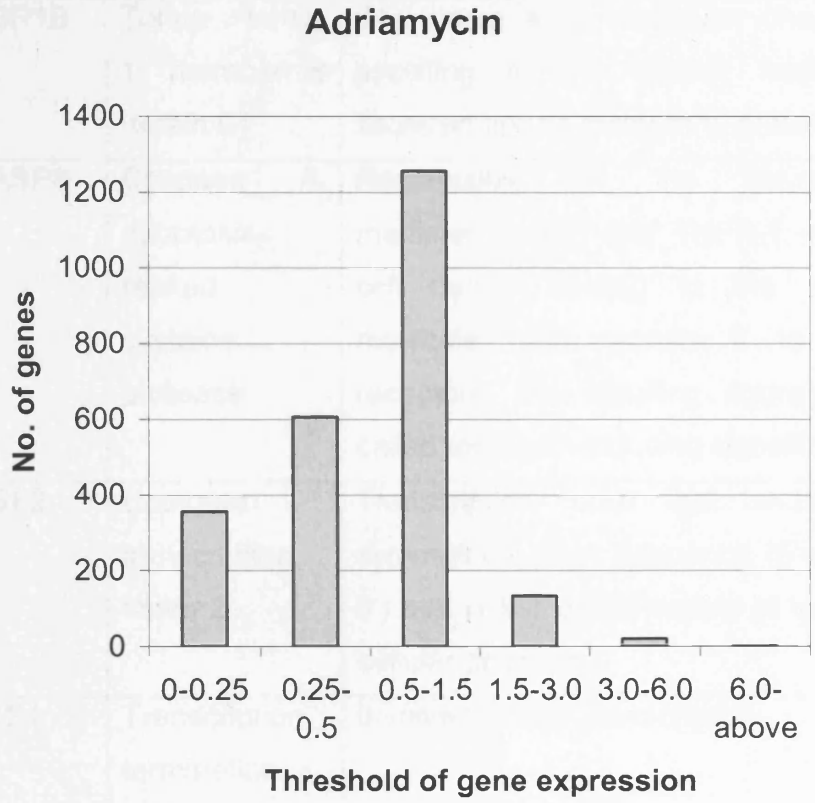


Figure 4.3. Graph representing mean of gene expression data obtained after the treatment of CEPH 1206 cells with 0.5 mM of Adriamycin (Doxorubicin) for 17hours.

Symbol	Full name	Function	Mean ratio
TOR1B	Torsin family 1, member B (torsin B)	Can serve as a molecular chaperone assisting in the proper folding of secreted and/or membrane proteins	3.9
CASP8	Caspase 8, apoptosis-related cysteine protease	Responsible for the fas-receptor mediated (cd95) and TNFR-1 induced cell death. Binding to the adaptor molecule fadd recruits it to either receptors, the resulting aggregate is called the death-inducing signalling	3.6
USF2	Upstream transcription factor 2	Transcription factor that binds to a symmetrical DNA sequence (5'-cacgtg-3') that is found in a variety of viral and cellular promoters.	3.6
TTF1	Transcription termination factor, RNA polymerase I	Involved in RNA transcription	3.5
CLCN6	Chloride channel 6	Voltage-gated chloride channel involved in the regulation of cell volume; membrane potential stabilisation and signal transduction	3.5
PCTK3	PCTAIRE protein kinase 3	Plays role in signal transduction cascades in terminally differentiated cells.	3.4
UGT2B15	UDP glycosyltransferase 2 family, polypeptide B15	Udpgt is of major importance in the conjugation and subsequent elimination of potentially toxic xenobiotics and endogenous compounds.	3.3

Symbol	Full name	Function	Mean ratio
YY1	YY1 transcription factor	Transcription factor that exhibits positive and negative control on a large number of cellular and viral genes by binding to sites overlapping the transcription start site. Plays an important role in development and differentiation	3.2
FADD	Fas-associating protein with death domain	Apoptotic adaptor molecule that recruits caspase-8 or caspase-10 to the activated fas (CD95) or TNFR-1 receptors. The resulting aggregate called the death-inducing signalling complex (disc) activates caspase-8.	3.0
CHEK1	CHK1 (checkpoint, S.pombe) homolog	Involved in cell cycle arrest when DNA damage has occurred or when unligated DNA is present.	2.9
TFDP2	Transcription factor Dp-2 (E2F dimerization partner 2)	It can stimulate E2F-dependent transcription. It binds DNA with e2f family members through the E2F recognition site, It can be found in the promoter region of a number of genes whose products are involved in cell cycle regulation or in DNA replication. Complexes formed with the protein appear to mediate both cell proliferation and apoptosis.	2.9
BLK	B lymphoid tyrosine kinase	Blk can function in a signal transduction pathway that is restricted to B lymphoid cells.	2.8

Symbol	Full name	Function	Mean ratio
GFI1	Growth factor independent 1	Transcription factor involved in regulating the expression of genes active in the s phase during cell cycle progression in T cells. It can also be involved in tumour progression.	2.7
GTF2F1	General transcription factor IIF, polypeptide 1 (74kD subunit)	General transcription initiation factor that binds to RNA polymerase, it promotes transcription elongation.	2.7
TOP1	Topoisomerase (DNA) I	Reaction catalysed by topoisomerases leads to the conversion of one topological isomer of DNA to another.	2.7
NUCB1	Nucleobindin 1	It has DNA-binding and calcium-binding properties	2.5
MCL 1	Myeloid cell leukemia sequence 1 (BCL2-related	Involved in programming of differentiation and maintenance of viability but not of proliferation (probable). It is also an inhibitor of apoptosis.	2.1
POU2F1	POU domain, class 2, transcription factor 1	Protein is a transcription factor for small nuclear RNA and histone h2b genes. It recognises and binds to the DNA sequence 5'-atgcaaat-3'.	2.0

Table 4.2 Examples of upregulated genes by more than 2 fold in CEPH lymphoblastoid cells treated with 0.05 mM of adriamycin.

4.2.3 Gene expression studies of the CEPH 1206 lymphoblastoid cells treated with 0.5mM of cyclophosphamide.

Single dose studies of the impact of cyclophosphamide on CEPH 1206 cells utilised microarray analysis. In order to obtain RNA the CEPH lymphoblastoid cells were co-cultured with C450-13 cells. Cyclophosphamide needed to be bioactivated to its active form as a anti-cancer agent, by those cells, which contained human cytochrome P450. The co-culture was incubated for 17 hours at 37^0C with 0.5 mM of cyclophosphamide. The RNA was extracted and examined using microarrays chip. Figure 4.4 contains mean data generated during the investigations. Results revealed that the drug had very little effect on the overall gene expressions. From the previous tissue culture experiments it was known, that the model system used for bioactivation of cyclophosphamide worked. Therefore it can be concluded that cyclophosphamide had very little effect on the gene expression of the treated CEPH 1206 cells. Table 4.3 shows 3 of the 5 genes, which were upregulated by cyclophosphamide.

It can be concluded that the adriamycin out of the three drugs had the greatest effect on gene expression of the treated samples although its concentration used in the experiments was the smallest. This indicates that adriamycin appears to be the most potent drug out of the FAC anti-cancer drug combination. Figure 4.5 shows the diagram of the upregulated genes of the F, A and C. It shows that none of the FAC drugs induced the overexpression of the same genes and that adriamycin upregulated the most genes and cyclophosphamide the least.

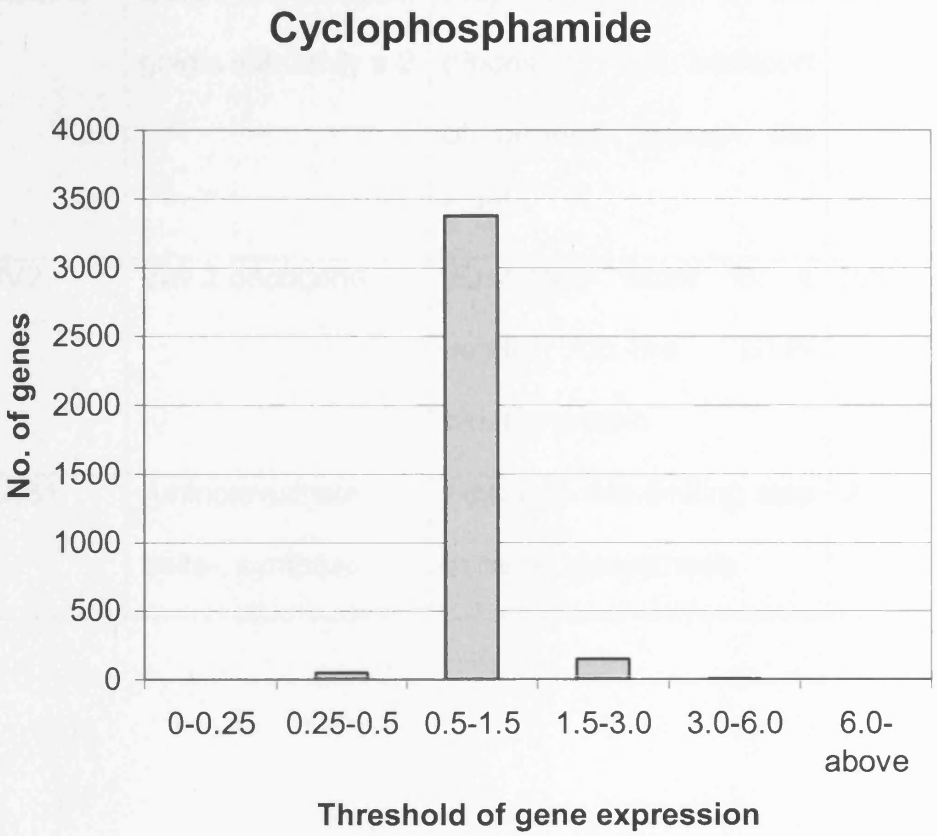


Figure 4.4 Graph representing gene expression data obtained after the treatment of CEPH 1206 cells with 0.5 mM of cyclophosphamide for 17 hours.

Symbol	Full name	Function	Mean ratio
GOLGA2	Golgi autoantigen, golgin subfamily a 2	Plays a function in the processing and transport of proteins through the golgi	3.1
VAV2	Vav 2 oncogene	Exchange factor for a small ras-like GTP-binding protein	2.8
ALAS1	Aminolevulinate, delta-, synthase 1	First and rate-limiting step in heme biosynthesis	2.7

Table 4.3. Upregulated genes by more than 2 fold in CEPH 1206 lymphoblastoid cells treated with 0.5 mM of cyclophosphamide.

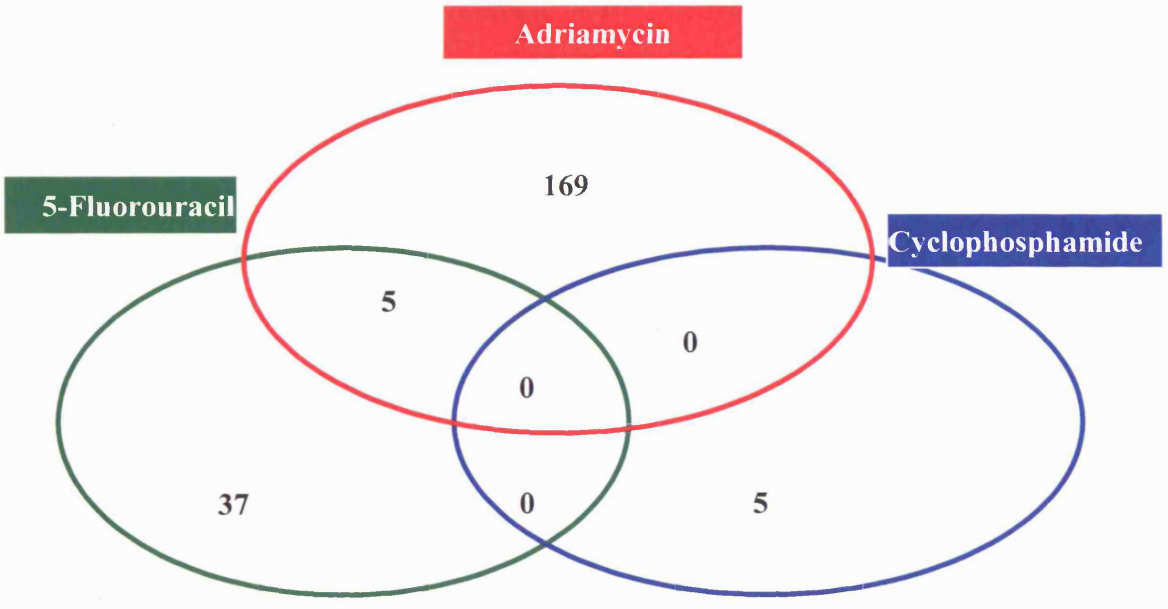


Figure 4.5. The diagram representing the gene expression data obtained after single treatment of CEPH 1206 cells with F, A and C anti-cancer drugs. It shows the number of the genes, which expression has increased by more than 2 fold after the drug treatments. This figure illustrates individual FAC drugs effect on RNA expression of different genes. Only 5-Fu and adriamycin in the experiments conducted induce the same set of genes.

4.2.4. Gene expression studies of the CEPH 1206 lymphoblastoid cells treated with 0.5mM of FAC anti-cancer drug combination.

The experiment to asses the effect of the 0.5 mM FAC drug combination on the gene expression of CEPH 1206 cells was conducted in two parts. The tissue culture experiments were set up as for single treatment experiments (see above). The cells were incubated for 8 hours and 17 hours. The RNA was extracted and the microarray analysis performed. Figure 4.6 shows the results obtained after the 8 hours incubation. Figure 4.7 shows the results obtained from 17 hours incubation. Both experiments were performed twice.

From the data it is clearly visible that there are differences in gene expression between 8 h exposure and 17 h exposure. There are more genes upregulated after 8 h exposure (400) than after 17 h exposure (150). This might indicate that the initial impact of the FAC drugs induced more changes in the gene expression. The longer exposure to drugs might allow cells time to recover and to buffer some of the effects that FAC had. Generated data indicated that combined FAC treatment had more effect on cells, that the single drug treatments. Figure 4.8 shows a diagram of genes that are both upregulated by FAC and single treatments. From the diagram it is clearly visible that only adriamycin upregulates some of the genes that are upregulated by the FAC drug combination. The number of shared genes is only 16. That shows that the combined effect of FAC drugs is much greater that of single treatment and that

the combined FAC dose had different effects that single doses on the gene expression of CEPH 1206 human lymphoblastoid cells.

4.2.5 Synergy interactions

During the analysis of the microarray data it was observed that some genes were induced more that it was previously expected, those were synergistic interactions observed between the FAC drugs. Synergistic interactions occur, where the combined effect of the drugs exceeds the expected outcome. With so many genes to identify and so many possible protein interactions it is important to study the overall response of individual genes to different xenobiotics. One of those responses is synergistic interaction between drugs and its impact on gene expression. For the purpose of this project to assess the synergy an equation was devised:

$$X \times Y \times Z = XYZ$$

where X,Y and Z are gene expression values and XYZ is a predicted value.

In order to assess the synergy index, following equation was devised:

$$\text{Synergy index} = \frac{\text{Actual gene expression value}}{\text{Predicted gene expression value}}$$

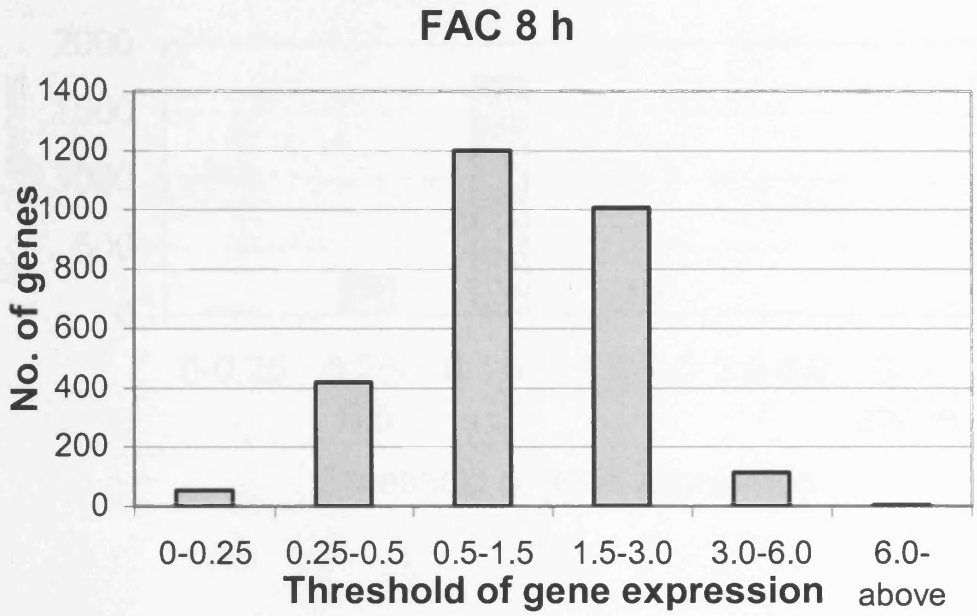


Figure 4.6. Graphs representing gene expression data obtained after the treatment of CEPH 1206 cells with 0.5 mM of FAC drug combination for 8 hours. Graph represents combined results.

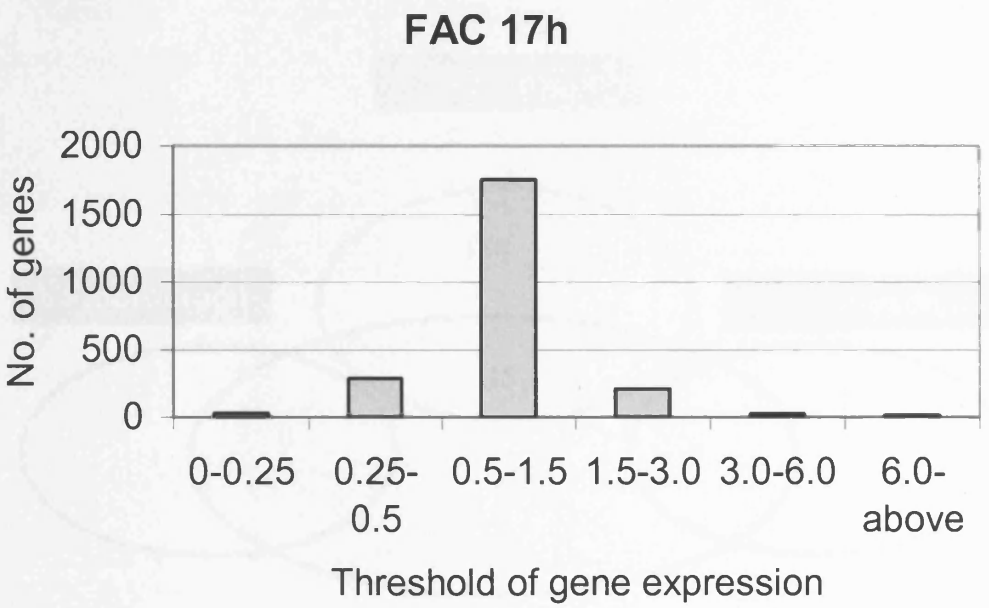


Figure 4.7. Graph representing mean gene expression data obtained after 2 independent treatments of CEPH 1206 cells with 0.5 mM of FAC drug combination for 17 hours.

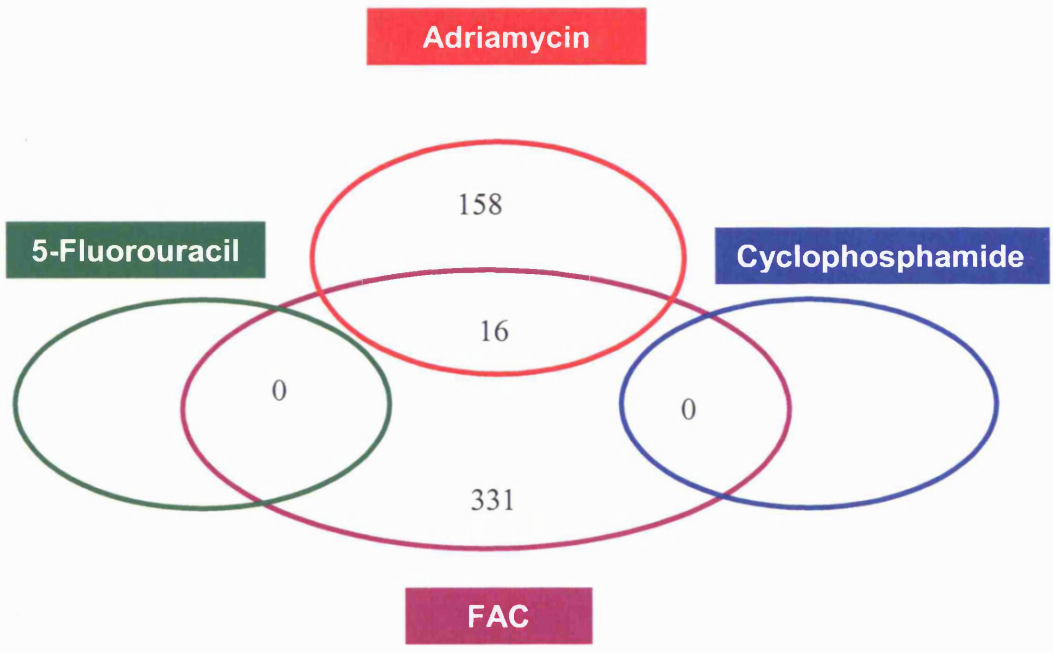
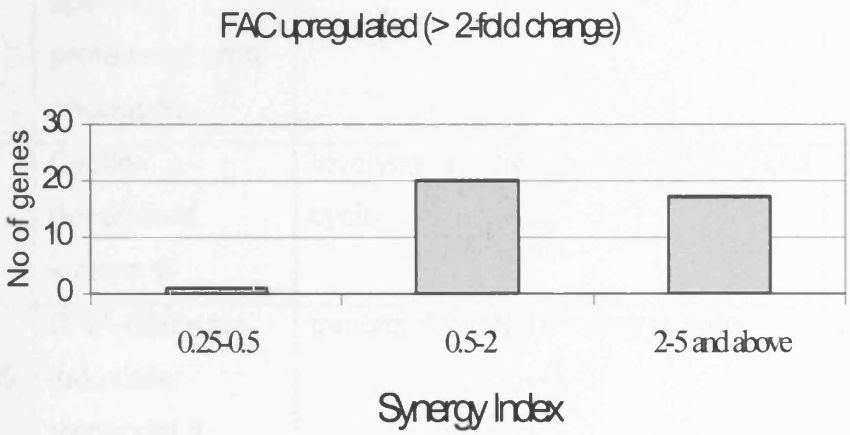


Figure 4.8 Diagram representing the number of genes which expression was upregulated after single drug treatments and combined FAC treatments (17h). It appears that majority of genes upregulated during the treatments are specific to particular treatments. Only a small proportion of genes appears to be both upregulated by combined FAC treatment (17h) and single Adriamycin treatment (17h). The other two drugs Cyclophosphamide (17h) and 5-fluorouracil (17h) do not affect the same genes as in FAC combination treatment.

By using both equations it was possible to determine the number of genes, that had a high synergy index (>1). The effects where the index was higher than 1 were classified as synergistic. Figure 4.9 shows the graph representing the number of genes that displayed the synergistic characteristics. The data used in Figure 4.9 was obtained from FAC treatments (17h incubation time). Exposure to combined drugs caused genes to exhibit synergistic tendencies. Even if only the upregulated genes from FAC 17 h treatment were taken under consideration, the synergistic effects were present. Table 4.4 contains the list of genes, which showed the highest synergy index. The genes represent a selection of different functions; some of them are involved in apoptosis, cellular functions or DNA synthesis. The cluster analysis on the microarray a result was also performed (Figure 4.10).

It revealed that the genes with high synergy index clustered together. That meant that the statistical analysis performed by the Tree View computer programme found that groups of genes behaved statistically in similar fashion. This confirms the synergy data obtained.

A)



B)

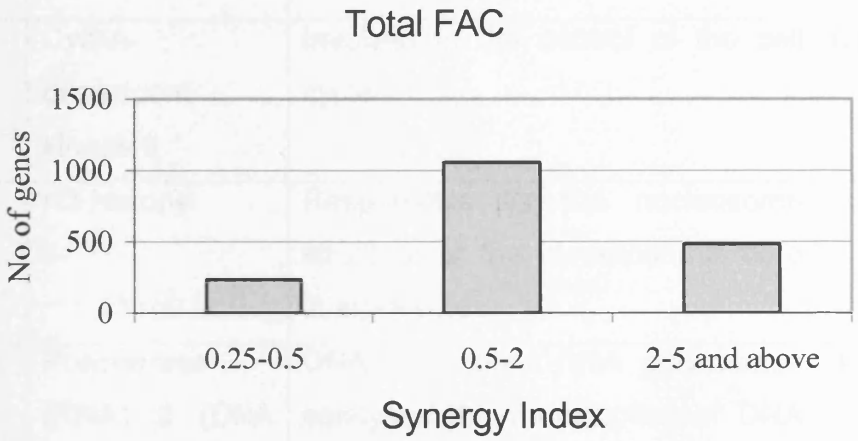


Figure 4.9. Graphs A and B show the data of upregulated genes generated during the gene expression studies of lymphoblastoid cells treated with the 0.5 mM of FAC drug combination as well as single drug treatments (0.5 mM of 5-Fu, 0.05 mM of A and 0.5mM of C). The data has been obtained by calculating the ratios of averages of single drug treatments against average of combined drug treatment. The synergy effect observed within genes upregulated by two-fold or more. Genes in the range of 0.5 – 2.0 fold do not exhibit synergistic tendencies.

Symbol	Full name	Function	Synergy index
Usp4	Ubiquitin specific protease, proto-oncogene	Prevents protein degradation	13.4
CDK4	Cyclin-dependent kinase 4	Involved in the control of the cell cycle	11.8
DDIT3 GADD153	DNA-damage-inducible transcript 3	Inhibits the DNA binding activity	9.2
TNFSF	Tumor necrosis factor (ligand) superfamily, member 12	Capable of inducing apoptosis	7.7
CDK6	Cyclin-dependent kinase 6	Involved in the control of the cell cycle	6.5
H3F3B	H3 histone	Responsible for the nucleosome structure of the chromosomal fibre in eukaryotes	5.8
POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A	DNA dependent RNA polymerase catalyses the transcription of DNA into RNA.	4.1
PURA	Purine-rich element binding protein A	Plays a role in the initiation of DNA replication and in recombination.	4.0
CAV	Caveolin	Can act as a scaffolding protein within caveolar membranes	3.6

Symbol	Full name	Function	Synergy index
CKB	Creatine kinase	Catalyzes the transfer of phosphate between atp and various phosphogens	3.5
DAP3	Death-associated protein 3	Involved in mediating interferon-gamma-induced cell death.	3.2
ADH5	Alcohol dehydrogenase 5	Catalyses the oxidation of long-chain primary alcohols and the oxidation of s-(hydroxymethyl) glutathione.	3.2
COX10	Cytochrome c oxidase subunit X	Converts protoheme ix and farnesyl diphosphate to heme	3.0
PTPN2	Protein tyrosine phosphatase	Involved in the phosphorylation of proteins on tyrosine.	2.4
INPP5D	Inositol polyphosphate-5-phosphatase	Precursor for a number of different messenger molecules.	1.3
TLR2	Toll-like receptor 2	Plays a role in immunity	1.3

Table 4.4 Genes, which showed synergistic tendencies after the treatment with 0.5 mM of FAC, compared with single drug treatment responses.

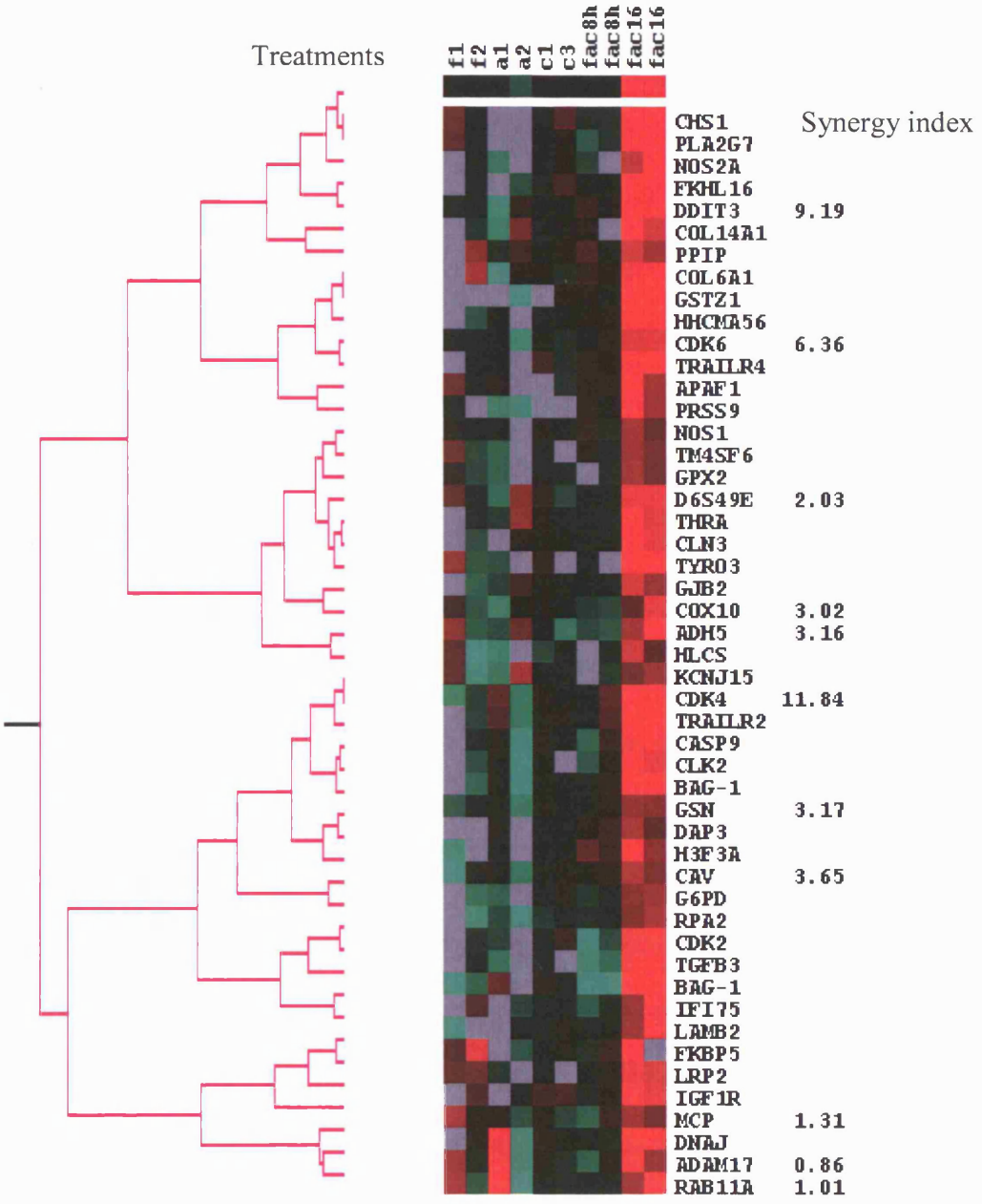


Figure 4.10 Gene cluster showing genes exhibiting synergistic effects clustered together. It also shows that the genes chosen for the further analysis for protein expression also are present in the same cluster. Red colour represents overexpressed genes, green downregulated and black shows no change in expression

4.2.6 Dynamic changes between FAC treatment of 8 hours and 17 hours

The time point experiments performed by treating CEPH 1206 lymphoblastoid cells with the FAC drug combination showed, that the gene expression profiles change with the different time exposure of cells to the anti-cancer drugs. The changes were profound and showed that the gene expressions were susceptible to the amount of time that the cells were exposed to the FAC drugs. When CEPH 1206 cells were exposed to the FAC drug combination for 17 hours, 150 different genes were overexpressed more that 2.0 fold, but when exposed for 8 hours, 337 different genes were overexpressed by 2.0 fold. The differences might be attributed to the defence mechanisms of cells. The longer the cells were exposed to xenobiotics the more time they had to compensate for the impact, hence the less number of genes that were overexpressed at the longer exposure. Figure 4.11 shows the dynamic changes observed after the comparison of gene expression changes after time point experiments. After the 17 hour exposure 7 genes become suppressed and the expression value is less than 0.2 when previously after 8 hour exposure they were overexpressed more than 2.0 times. 165 genes have become suppressed and the expression values of those genes fell between 0.2 and 0.5. 55 genes were showned to return to the normal expression values as in the non-treated cells, where previously they were overexpressed when exposed to FAC drugs for 8 hours. Out of 337 genes upregulated by more than 2.0 fold after 8 hour exposure, 78 were upregulated by 2.0 to 5.0 fold after 17 hour exposure, 32 genes were upregulated by more

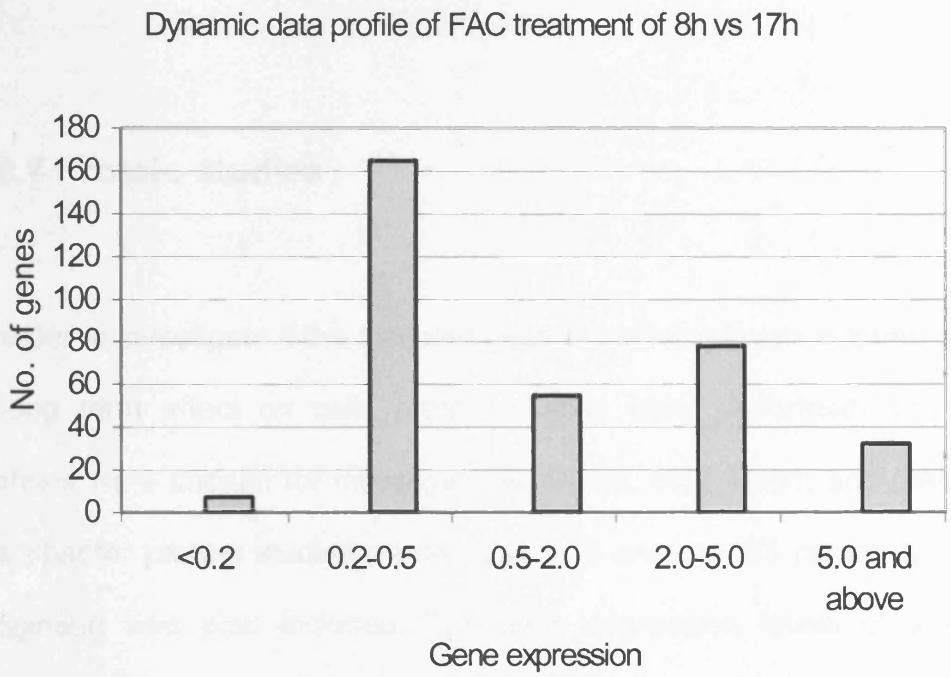


Figure 4.11 Dynamic profile of changes in gene expression after 8 hours and 17 hours exposure of CEPH 1206 CEPH lymphoblastoid cells treated with 0.5 mM of FAC drug combination. The graph shows the gene expression of 337genes that were upregulated more than 2.0 fold at 8 hours that occurred after 17 hours exposure to the FAC drug combination.

than 5.0 fold.

4.2.7 Protein studies

In order to investigate if the elevated gene expression levels in some genes had a long term effect on cells protein studies were performed. The following proteins were chosen for investigations: CDK4, Bcl2, Bag1, and GADD153. In this chapter protein studies on caspase 6, 9 and on p53 carried out by Fiona Higginson was also included. The gene expression levels of the proteins chosen for these investigations were elevated (see previous tables). Also the protein functions were of interest. All of these genes play an important role in cell cycle, DNA damage and apoptosis. Caspase proteins are involved in apoptosis. The disruption of this process has been identified as one of the steps of carcinogenesis. It was of great interest to investigate the impact of FAC anti-cancer drugs on apoptotic activity in lymphoblastoid cells. The CDK4 protein was also of interest, because it regulates cell cycle, which disruption may lead to arrest of cell death. CDK4 is located on chromosome 12. It has been implicated to be involved in control of the cell cycle (Mitchell *et al.,*1995).It belongs to the SER/THR family of protein kinases. Human cell division is regulated primarily at the G1-to-S or the G2-to-M boundaries (Ekholm *et al.*,2000). The sequential activation of cyclin-dependent kinases (CDKs) and their subsequent phosphorylation of critical substrates promote orderly progression through the cell cycle (Sandhu *et al.*, 2000). The complexes formed by CDK4 and the D-type cyclins are involved in the control of cell proliferation

during the G1 phase (Coqueret.2002). CDK4 is inhibited by p16, also known as cyclin-dependent kinase inhibitor-2 (CDKN2A) A large number of human cancers display alterations in the Ink4a/cyclin D/Cdk4 genetic pathway, suggesting that activation of Cdk4 plays an important role in oncogenesis(Zou *et al.*,2002). Genetic aberrations in the regulatory mechanisms that govern the G (1) phase of the cell cycle occur frequently in human cancers. The overexpression of the G (1) phase cyclin, cyclin Dl, is one of the most commonly observed alterations (Diehl, 2002). Activation of the cyclin Dl kinase occurs through increased transcription, protein accumulation and cyclin/CDK4 assembly.. Inhibition of any step, which results in reduced growth factor requirements for cyclin Dl/CDK4 activation, will provide cells with a distinct growth advantage and thus likely represents an early event in neoplasia (Decker *et al.*, 2002). As such this gene is a very interesting candidate to study. Bcl 2 (B-cell CLL/lymphoma 2) is located on chromosome 18 (Aisenberg *et al.*, 1988). Some of the chosen proteins have anti-apoptotic properties such as Bag1 and Bcl2. The upregulation of that protein would mean that the FAC drugs disrupt the lymphoblastoid cells to a great extent. Bcl2 gene suppresses apoptosis in a variety of cell systems including factor-dependent lymphohematopoietic and neural cells (Farlier *et al.*, 1995). It regulates cell death by controlling the mitochondrial membrane permeability (Hockenbery *et al.*, 1990). Appears to function in a feedback loop system with caspases. Inhibits caspase activity either by preventing the release of cytochrome c from the mitochondria and/or bybinding to the apoptosis-activating factor (APAF-1). Overexpression of BCL2 blocks the apoptotic death of a pro-B-lymphocyte cell line (Chen *et al.*, 1996). Thus, BCL2 is unique among protooncogenes, being

localized in mitochondria and interfering with programmed cell death independent of promoting cell division (Jacobson *et al.*, 1993). BAG-1 is a multifunctional protein. It interacts with a wide range of target molecules to regulate apoptosis, proliferation, transcription, metastasis and motility. The pathways regulated by BAG-1 play key roles in the development and progression of cancer (Cutress *et al.*,2001).It binds and induces many proteins from nuclear steroid hormone receptor superfamily (Witcher et al., 2001).

The GADD 153 protein was chosen, because it is expressed in response to growth arrest and DNA damage (Schmitt-Ney et al., 2000).The magnitude of change in the GADD 153 expression is proportional to the extent of the damage (Lin *et al.*,2001). The gene is also involved in mediating apoptosis (Ubeda *et al.*,2000)That was very important, because previous studies carried out in this project revealed that FAC drug combination is DNA damaging. Unfortunately the experiments carried out on GADD 153 did not give any results due to the antibody not working. Figure 4.12 shows the Western blots of the detected proteins. The experiments were conducted on CEPH 1206 lymphoblastoid cells. The cells were treated with 0.5 mM of FAC drug combination for 17 hours. After the incubation the proteins were extracted and concentrations measured using Bradford assay. The proteins were detected using Western Blot analysis, the amount of each protein used in a single experiment was 30 ug. Each experiment was repeated at least twice. The full procedure can be found in Materials and Methods (Chapter 2). In Figure 4.12 it is possible to assess the protein expression and compare them to gene expression results obtained from microarrayer studies. CdK4 gene expression levels were high, than its protein levels were high in one experiment but low in the second experiment. Those

differences in protein expression could be due to protein concentrations varying depending on the cell cycle. It could be that the proteins extracted at different stages of cell cycle are present at varying concentrations. In case of Bag-1 gene expression values were elevated, but the protein levels were the same for treated and untreated protein samples. That illustrates, that in not all cases the higher gene expression means higher protein concentration in cells. The Bcl2 gene expression levels were high in microarray analysis, but the protein studies indicated that there was decrease in protein concentration. The follow up experiment showed higher levels of that protein. This could be also attributed to the differences at different stages of the cell cycle when the proteins were extracted. In the case of p53 protein its gene expression levels were not high, but its protein levels increased after the cells were treated with the FAC drugs. In case of caspases 6 and 9 both of them had high gene expression levels. The protein level for caspase 6 decreased after the cells were treated with FAC drugs. A different story was in the case of caspase 9 where the protein values increase with the FAC treatment of cells.

The experiments studying protein levels that are follow up of the microarrayer data are important as it was demonstrated the elevated gene expression values obtained from microarray studies might not cause protein levels to increase. RT-PCR experiments would also help to determine expression levels of studied genes. This indicates that to obtain a dynamic picture of changes that are happening at the cellular level, it is important to follow microarray experiments with protein experiments.

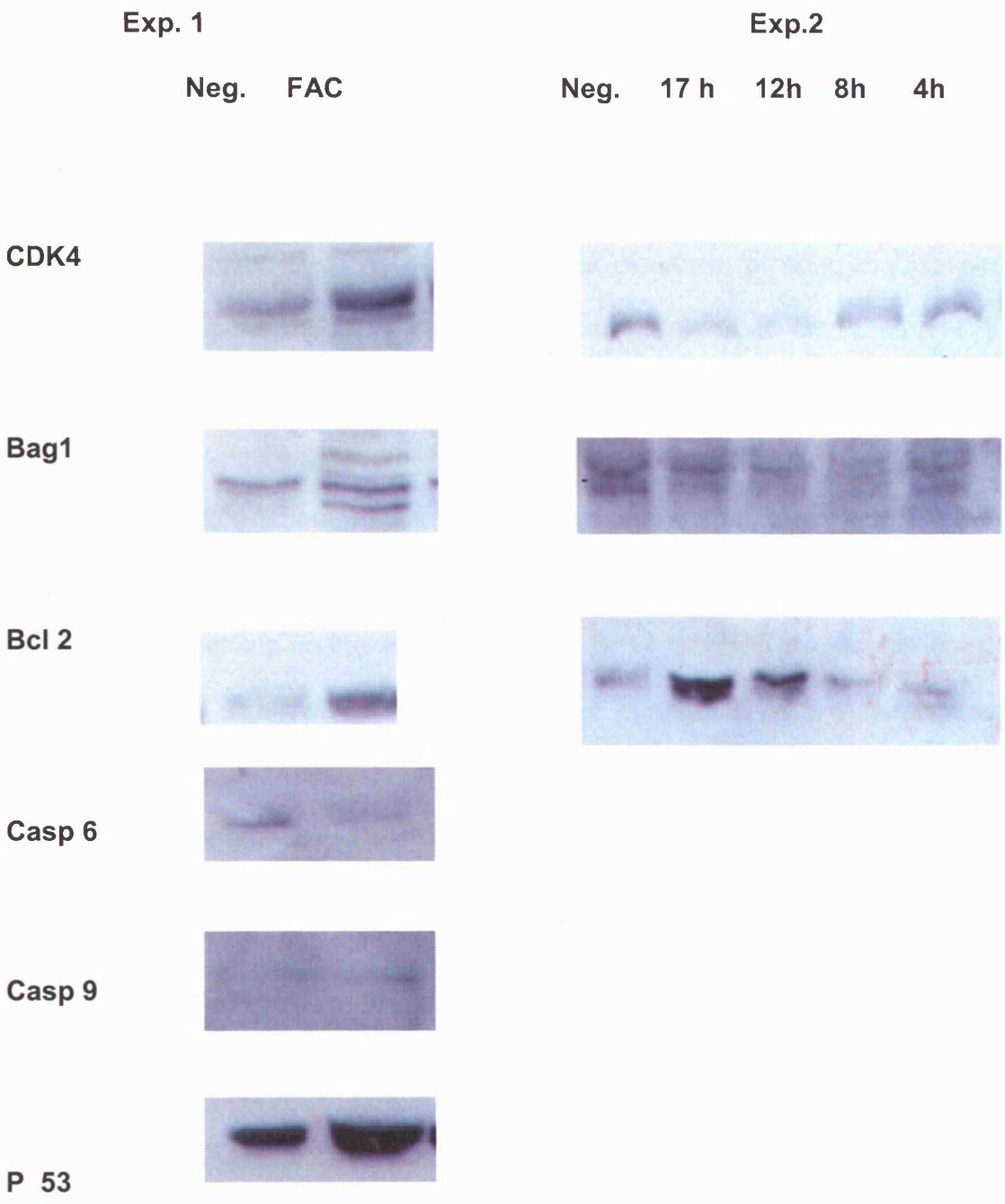


Figure 4.12 Western blots of protein studies conducted on CEPH 1206 lymphoblastoid cells treated with 0.5 mM of FAC for 17 hours. Dilutions of primary antibodies used were as follows: CDK4 - 1/1000, Bag1- 1/500, Bcl2 –- 1/1000, Casp 6 and 9 – 1/1000 and P 53 – 1/1000.

4.3 Discussion

Genomic technologies such as DNA microarrays have been used to study biological processes such as development of diseases, profiling of diseased cells and reaction of cells to xenobiotics (Boeuf *et al.* 2002). Gene expression profiling through the application of cDNA arrays provides comprehensive assessment of gene expression levels in a given tissue or cell population. It also can reveal information about changes of gene expression in altered physiological or pathological situations simultaneously. Microarrays allow the study of interactions in the regulation of a large number of different genes (Curtis 2002). It allows to reveal complex changes in gene expression sometimes thousands of mRNAs change between experiments (Sq *et al.* 2002). The human genome is sequenced, but only a minority of genes have been assigned a function. Whole-genome expression profiling is an important tool for functional genomic studies. Automated technology allows high-throughput gene activity monitoring by analysis of complex expression patterns, resulting in fingerprints of diseased versus normal or developmentally distinct tissues (Ginestier *et al.* 2002). Such studies allow scientists to generate whole profiles of cells and their reaction at the molecular level to xenobiotics to which they were exposed.

In this project the main objective was to find out to what extent FAC drug combination causes DNA damage in human lymphoblastoid cells and also to what extent FAC drugs alter the gene and protein expressions.

It was found out that the FAC anti-cancer drug combination had a great impact on the gene expressions of the treated cells. Some genes became overexpressed and some were suppressed after the exposure to the FAC drugs. It was also discovered that the single dose treatment results varied from the results of combined FAC dose. Single drug treatments generated less upregulated genes, where the combined FAC treatment generated the most upregulated genes. Another discovery was that the gene expression profiles varied depending on the amount of time that the cells were exposed to the FAC drugs. Shorter exposure to FAC drugs generated more overexpressed genes. It shows that the model used in this project is dynamic and capable of detecting changes in cellular gene expressions.

During the microarray analysis it was discovered that combined effect of the FAC drugs had in some cases greater effects than expected and much greater than single doses. Those interactions were called synergistic interactions. 17 genes out of the analysed data were found to have a high synergy index, which means that the combined FAC drug dose had a much greater effect than was predicted. Such synergistic interactions could be very important for future drug discovery and population studies because they are capable of predicting which drugs in which patients might have a greater effect than expected. The Tree View clustering performed on the data obtained from microarray analysis of cells treated with FAC revealed that genes exhibiting high synergistic indexes clustered together. This indicated that those genes "behave " in similar fashion.

Protein studies performed in this project revealed that not always gene expressions correspond to the protein expression. Those studies showed that microarray studies should be followed by protein analysis. Of course it is not

possible at present time to perform thousands of protein studies in the same manner as gene expression microarray analysis. That is why in this project only few proteins were studied. It was found out that the protein expressions were affected by the treatment of lymphoblastoid cells with the FAC drug combination. In only two cases was there no change observed as if the FAC drug did not have an affect on those particular proteins caspase 6 and Bag 1. In both cases the microarrayer analysis revealed that gene expression levels of those genes where very high. Other proteins were affected by the FAC drug combination in different ways. Some of them were induced in levels (CDK4, p53) where the others were suppressed (Bcl2, Casp 9).

Microarray analysis enabled this project to study gene expression patterns of thousands of different genes (the whole data set produced for this project can be found on the CD-ROM included with this thesis). It also showed the synergistic interactions, which occurred when the CEPH lymphoblastoid cells were treated with the FAC drug combination. The information obtained demonstrates the potential to perform drug toxicity and interactions for individual. Such studies have a potential to reduce the number of patients being treated with drugs that give them severe side effects or have no effects at all.

5.0 Minisatellite MS1 (D1S7) as a possible biomarker for detection of DNA damage induced by FAC anti-cancer drugs

Summary

Minisatellites are tandemly repeated loci, characterised by extreme variations in allele lengths and repeat units (Jeffreys *et al.* 1995). Minisatellites are highly variable and spread throughout the human genome, but in most cases they can be found located near the ends of chromosomes. The variations in length of minisatellites arise from spontaneous germline mutations. Because of their unique structures and properties minisatellites are very useful tools for investigating *de novo* mutation events in human germline or somatic tissues (Vergnaud *et al.* 2000). By using minisatellites it is possible to investigate spontaneous and induced tandem-repeat mutations that can occur in the human genome (Jeffreys *et al.* 1995).

In this study the MS1 (D1S7) minisatellite was investigated as a potential biomarker for DNA damage. MS1 is one of the most variable human minisatellites. It has been already studied to some extent (Smith *et al.* 1990, Maleki *et al.* 1997), but it has not yet been assessed as a potential indicator of somatic DNA damage induced by FAC anti-cancer drugs. The anti-cancer drug combination FAC is a powerful DNA damaging mixture. These anti-cancer drugs are widely used in treatment of breast cancer. They can also cause patients to develop secondary leukemia. MS1 was examined to determine if it could pick up DNA damage caused by the FAC drug combination.

A group of DNA samples from the CEPH collection was obtained and the profiles of the MS1 allele were generated for those samples, using a modified MVR-PCR mapping system. As expected the MVR-PCR profiles generated revealed a high diversity between individuals at the MS1 minisatellite. A *Taq* polymerase stop assay performed on CEPH lymphoblastoid cells treated with the combination of FAC showed the presence of DNA damage at the MS1 locus. Those studies demonstrated the possibility of the MS1 for the use as biomarker for DNA damage induced by FAC anti-cancer drug combination.

5.1 Introduction

Minisatellites provide a robust system for studying mutations of tandem repeats in humans. Extensive studies carried out to date revealed, that minisatellite mutation arise both by germline and by somatic mutations (Jeffreys *et al,* 1995). Mutation mosaicism has also been studied and it was concluded that such mutations arise during early embryonic development. Somatic mutations can be found in tumours, but spontaneous somatic mutations of minisatellites have not yet been studied to great depth (Jeffreys *et al*, 1997).

For the investigations of mutational events at minisatellites, very sensitive techniques had to be used. The most powerful technique up to date has been MVR-PCR (Multi Variant Repeat PCR) (Jeffreys *et al,* 1995). This PCR - based technique enables detection of changes in the structure of minisatellites and uses primers of which one is specific for DNA flanking the repeat region and the other primers are specific for variant repeats within the minisatellite. The MVR-PCR system produces patterns in the form of ladders on agarose gel, where each individual band represents a

particular repeat variant of the studied minisatellite. The system is very robust and allows for studying as few or as many repeat variables as desired.

So far the most studied human minisatellite using MVR-PCR has been MS32 (D1S8) (Jeffreys *et al,* 1997). The studies were performed primarily on diploid alleles but also single alleles have been examined. The investigations established variability of variant repeats within the MS32. Furthermore the detailed analysis enabled germline mutation rate of MS32 in the human population to be established. The mean rate of germline mutation was shown to be quite high, being 0.8% per sperm (Jeffreys *et al,* 1995). Knowing the rate of spontaneous mutation makes it possible to investigate mutations that have been induced by xenobiotics such as DNA damaging agents or ionising radiation.

The MVR-PCR technique has been applied in investigations of other human minisatellites as well as in forensic investigations (Andreassen *et al*, 1998), studies of minisatellite distributions in human populations (Armour *et al*, 1996) and in cases of induced mutations (Dubrova *et al,* 1998).

Clinical research is another area that has benefited greatly from studies of minisatellites. It has been found that mutations of simple trinucleotide repeats such as CAG have a significant impact on the development of human diseases. These repeats, although much smaller that minisatellites seemed to behave in similar fashion (Djian 1998). Mutations of the CAG repeats are thought to contribute to such diseases as myotonic dystrophy and fragile X syndrome (Djian 1998). The studies carried out on the mutational processes occurring in microsatellites contributed to the understanding of mutational processes of CAG repeats. Those investigations also contributed to understanding mutational processes of microsatellites.

Microsatellites are similar to minisatellites, but have fewer repeats of shorter length. Microsatellite mutations studies have been very important in colon cancer studies (Eshleman *et al*, 1994). It has been found that in some cases of hereditary non-polypopsis colon cancer there was a higher rate of microsatellite mutations. Cell line studies revealed the existence of a replication error (RER) phenotype, which induces higher mutation rates. The studies indicated that elevated mutations of microsatellites correlated with the mutations of normally expressed genes in colon cancer as well as in some other types of cancer.

The minisatellite investigated as a biomarker in this study was minisatellite MS1 (D1S7), located on chromosome 1(Cederberg *et al*, 1993). MS1 is the second most variable, unstable minisatellite known in the human genome (Smith *et al*, 1990). It consists of tandem repeats 9 bp long and can range from 0.7 to 20 kb in length (Wong *et al* 1987). There are at least 19 variations of minisatellite repeat unit sequences at MS1. Table 5.1 shows some of those variants.

Minisatellites have been described as potentially highly informative markers of mutational processes occurring in both germline and somatic cells (Jeffreys *et al*, 1997). Even the smallest mutational changes occurring may alter the number or sequence of tandem repeats, making it possible to detect mutational events. The MS1 Minisatellite was already used in studies of germline mutations in humans, but somatic mutations had not been yet studied in a great length (Jeffreys *et al,* 1989).

The potential somatic mutations of MS1 have been studied with the aid of the MVR-PCR technique. MVR-PCR was used as a tool by which internal structure of minisatellite MS1 was studied (Andreassen *et al*, 1998). The presence of sequence polymorphism within the tandem repeat array allowed for the development of MVR-PCR (Multiple Variant repeat unit-PCR). In this chapter the mutations were

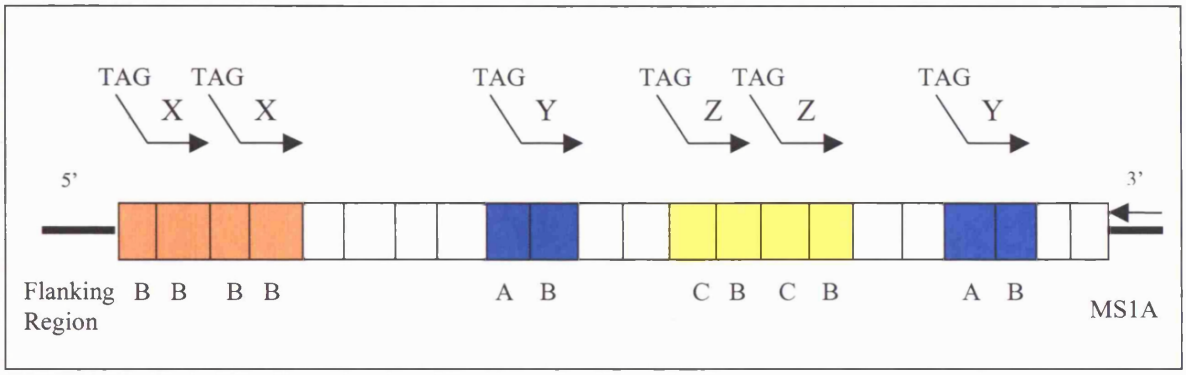
investigated in CEPH lymphoblastoid cell line CEPH1206 treated with the FAC drug combination (5-fluorouracil, cyclophosphamide and adriamycin). The MVR-PCR system was designed to detect any changes in structure of MS1 that could have been caused by exposure of lymphoblastoid CEPH cells to FAC drugs. The system allowed for studying chosen individual repeat types within the MS1. Figure 5.1 demonstrates the principles of the MVR-PCR technique used to detect somatic mutations at the MS1 locus.

The MVR-PCR profiles generated in this study (Figure 5.1) represent both MS1 alleles in each individual. This diploid profile is widely used in such investigations in forensic science, although for allele diversity studies there is a need to generate profiles of single alleles. In this project the repeats of interest were BB, AB and CB dimers. Those repeats are rare at the MS1 allele. The choice of the rare dimers allowed for generating diploid MVR-PCR profiles, where ladder consistent of dimer repeats did not form an unrecognisable smear after Southern blot detection. A number of difficulties were faced, because it was possible that studied individuals may not have had sufficient of these dimer types. There was also a possibility that possible mutational changes did not occur at those particular repeat dimers. The MVR-PCR system developed enabled the detection of any changes that could have occurred within the "B" rich individual if its DNA had been damaged by xenobiotics at the MS1 locus.

Repeat type	Repeat sequence
A	CCCTATCCA
B	CCCTCTCCA
C	CCCTGTCCA
D	CTCTCTCCA
E	CTCTAACCA
F	CCCTACCCA
G	CCCTGCCCA
H	CTGTAACCA
I	CCTTATCCA
J	CCCTATTCA
K	CCCTAACCA
L	CCCTATCTA
M	TCCTATCCA
N	CTCTCCCCA
O	CCCTACCTA
P	CTCTACACA
Q	ACCTAAATG
R	CCCTATCCC
S	CCCTACACA

Table 5.1 Variant repeat unit types found at the minisatellite MS1 locus (D1S7). The table contains all known variant repeat types of the MS1 human minisatellite, which have been defined to date (Gray 1991).

A)



B)

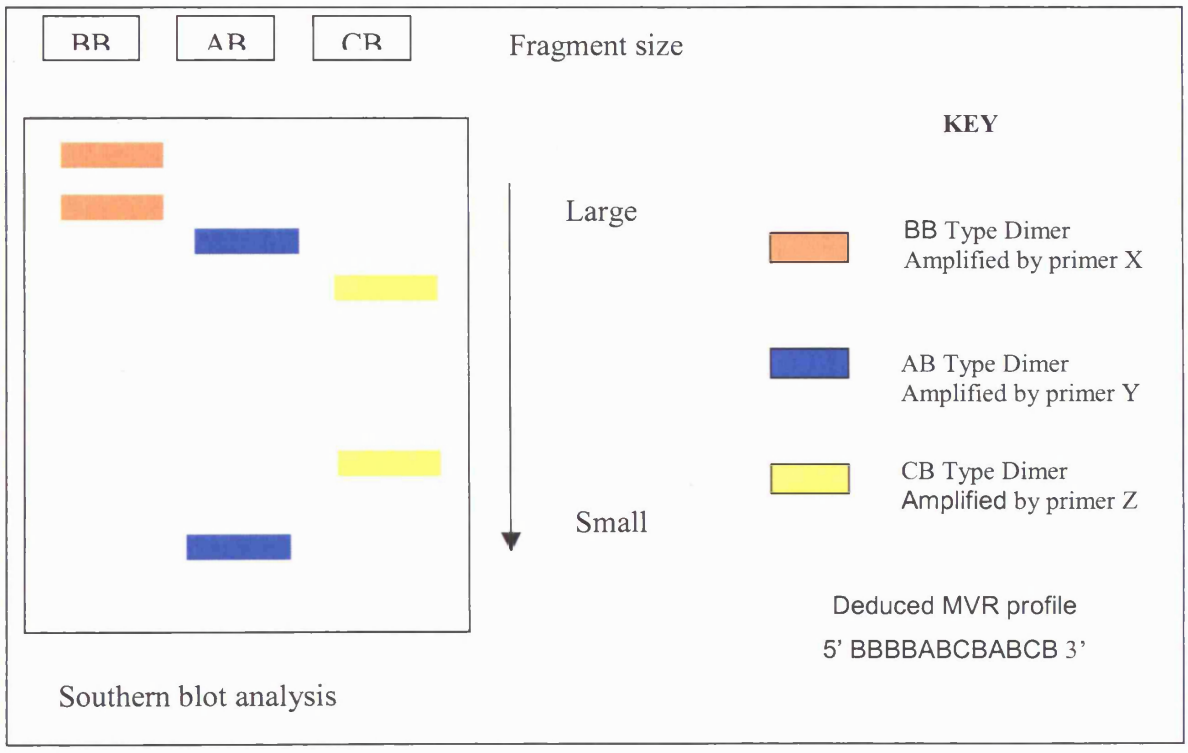


Figure 5.1 MVR-PCR mapping of MS1minisatellite. A) Diagram represents a model fragment of MS1 allele. Orange boxes correspond to BB dimmers, blue boxes represent AB dimers and yellow boxes represent CB dimers at MS1 allele. White boxes represent different types of dimers that are present at MS1 but were not investigated in this project. B) Diagram showing the principles of MVR-PCR profile generation. MVR-PCR profiles of all the DNA samples were to be generated this way.

5.2 Results

5.2.1 Primer design and PCR pilot studies

Generating MVR-PCR profiles of individual human samples from the CEPH collections involved designing a set of specific primers. Designed primers were capable of amplifying only the specific regions of interest of the MS1 allele. Table 5.2 contains sequences of those primers.

Primer Type	Primer Sequence
MS1 A	5' GCTTTTCTGTGATGAGCCTTGATG 3'
MS1 B	5' AAGAAGCATATGCAACCCATGAGG 3'
X (BB)	5' TCATGCGTCCATGGTCCGGAT(AGC)TCCACCCTCTCCACCCTC3'
Y (AB)	5' TCATGCGTCCATGGTCCGGAT(AGC)TCCACCCTATCCACCCTC3'
Z (CB)	5' TCATGCGTCCATGGTCCGGAT(AGC)TCCACCCTGTCCACCCTC3'
TAG	5' TCATGCGTCCATGGTCCGGA 3'

Table 5.2 MS1 Primer Sequences

The primers X, Y and Z were designed to amplify the "B" type repeat dimers of the MS1 locus. Those repeats were called BB, AB and BC. Primer MS1A was designed to prime within the 3' flanking region of MS1 and TAG primer to bind to amplified repeat sequence thus allowing for generation of double-stranded PCR product. TAG prevented shortening of the PCR product by annealing itself to the already amplified

DNA dimer and thus creating a template for further PCR reactions. This "collapsing"of the PCR product was prevented by TAG, which tagged the primers (X, Y and Z) amplifying the repeat regions, therefore preventing amplification of only external dimers. The design of primers allowed for the detection of dimers, which had single base variation in their structure (seeTable 5.2). The degenerate three bases in the middle of each primer ensured that these primers would prime from BB, AB and CB dimers irrespective of the MS1 repeat unit type immediately 5' of that dimer.

Amplified PCR product was separated by gel electrophoresis and detected by Southern blot hybridisation. Because of the need to distinguish between BB, AB and CB repeat unit dimers, careful annealing temperature titrations were carried out over the range of 63^0C – 68^0C. The results obtained revealed that even at the lowest annealing temperatures there was little or no mispriming of AB primers to BB or CB dimers. The same was true for other primer combinations. These pilot studies allowed for determination of PCR conditions and primer concentrations, which were used as standards for all further MVR-PCR experiments at MS1.

Furthermore, the results of the analysis on the individual used in these pilot studies showed that diploid code of first 2 kb of MS1 alleles contained a suitable mixture of BB, AB and CB dimers.

Figure 5.2 shows the autoradiograph of an annealing temperature test of sample CEPH 1206. The temperature range from 68^0C to 63^0C allowed for development of the optimal PCR conditions for generating MVR-PCR profiles of MS1 allele. The conditions were developed in order to avoid non-specific binding of primers. The specificity of the primers was of a great importance because it allowed for precise study of the "B" type dimers at the MS1 minisatellite. The optimal annealing temperature was found to be 67^0C .The photograph shows individual DNA dimers

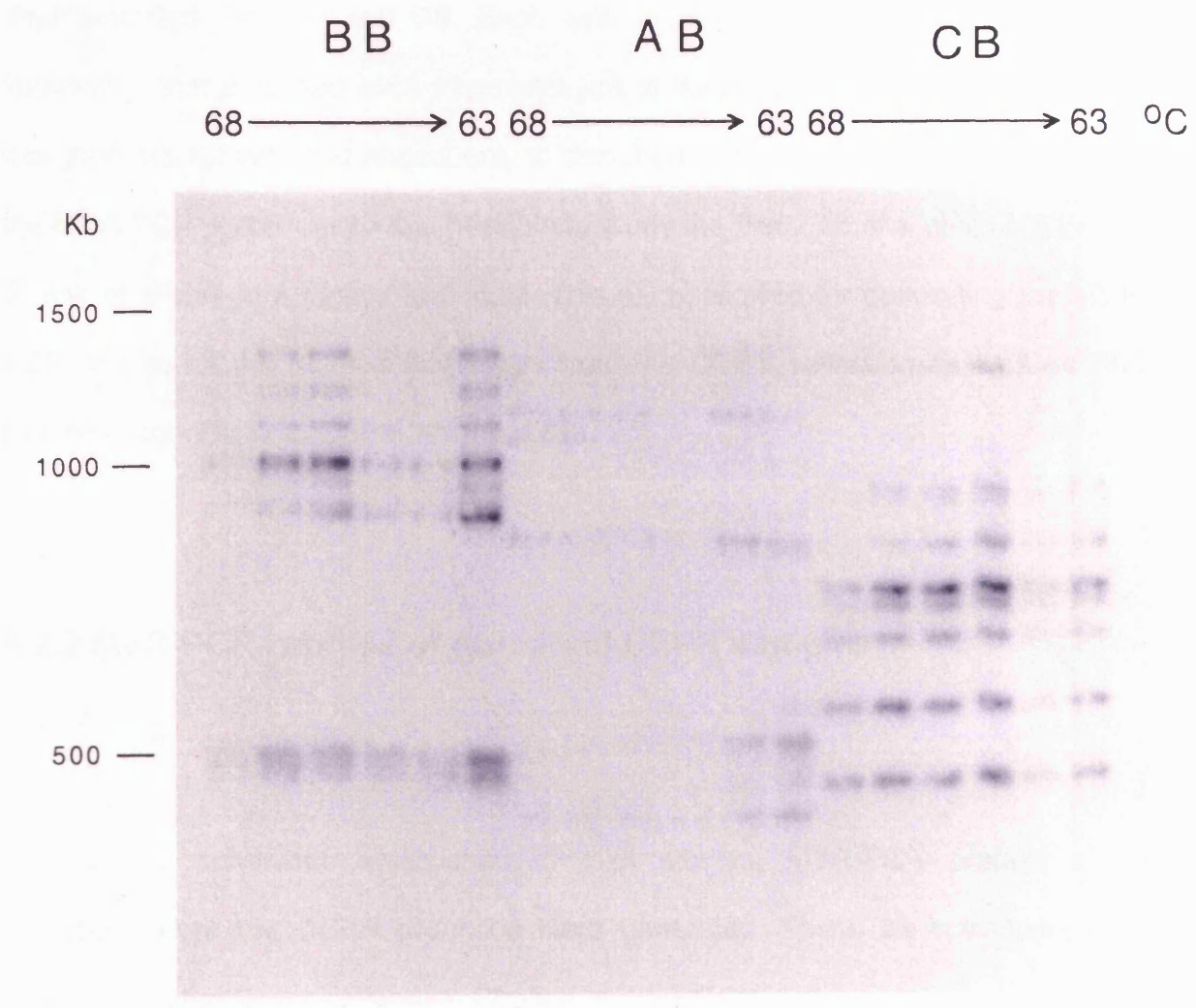


Figure 5.2 Autoradiograph of annealing temperature test of sample CEPH 1206. The annealing temperature range was from 68^0C to 63^0C. The test was performed on "B" type dimers, by amplifying the CEPH sample with primers annealing to BB, AB and CB repeat dimers. This test showed that the best annealing temperature to use for generating MVR-PCR profiles is 67^0C. This temperature was used in all further MVR-PCR experiments at MS1.Cycle number was 19.

that amplified BB, AB and CB. Each type of dimers has an individual pattern, suggesting that amplified MS1 fragments are of different origin i.e. that the primers designed are specific and anneal only to designed sites. It is also apparent, that with the MVR-PCR system used it is possible to study the first 2 kb of a given MS1 allele or pair of alleles in a diploid individual. This study allowed for generating the MVR-PCR profiles of the studied individuals from the CEPH collection as well as FAC treated lymphoblastoid CEPH 1206 cell line.

5.2.2 MVR-PCR profiles of examined CEPH samples

Utilizing the conditions established in pilot studies, MVR-PCR profiles of 24 individuals from the CEPH collection were generated. These 24 individuals were chosen from the larger collection. Chosen individuals had one or in some cases two small MS1 alleles ideally suited for PCR analysis (unpublished data from A.Jeffreys). The main purpose of this experiment was to establish if the developed PCR based methods could be applied to study mutational events in chosen CEPH samples. It was also designed to assess individuals on the basis of the abundance or lack of "B" type dimers in their MVR-PCR profiles of the MS1 allele. The generated profiles were examined for any individual differences that can be detected between studied individuals. It became apparent, that there was a large variation between individual diploid MVR-PCR profiles. In order to have a general picture of those individuals rich in BB, AB and CB dimers versus those with relatively few dimers of these types, diploid MVR-PCR profiles were generated. The generated profiles covered the first 2

kb of the MS1 allele. In order to assess which of the repeat dimers BB, AB and CB were most frequent among studied individuals, the dimers present in the first 2 kb of each MVR-PCR profile were counted. The data was then pooled together and plotted, as a graph to assess which dimer was the most abundant. Figure 5.3 represents the pooled data of the profiles obtained. From Figure 5.4 it is clearly visible, that in an examined population there is a variety of different profiles of "B" type dimers. Most of the MVR-PCR profiles contained large numbers of B type dimers e.g. 140802, 133201; those individuals are suitable candidates for detection of changes in MVR-PCR profiles generated by the technique developed in this project. Only 5 samples 4501,202,142303, 2301 and 1206 contained less than 20 B dimers in total, which suggest that there is a percentage of the population, where this type of investigations would be unsuccessful. The small number of "B" type repeats would lessen the chances of detecting mutational processes that might have occurred. Using the "B" type repeats presented a dilemma. The sparse abundance of those dimers ensured, that the MVR-PCR profiles generated were not in the form of undistinguished smear when detected. At the same time the same situation could cause mutational changes that occurred at the different type of dimer to pass unnoticed. The best approach for this problem could be the detection of all the dimer types present at the MS1 allele one at a time. Thus using the MVR-PCR system developed the profiles of "B" type dimers were obtained for 24 CEPH individuals. Figure 5.5 shows examples of MVR-PCR profiles generated from the CEPH collection. The chosen diploid profiles of first 2 kb of the MS1 allele detected individual to individual differences. The individual 142303 had a following profile of B type repeats CB, AB, BB, CB, AB, CB, BB and AB. The individual was classified as unsuitable for this study due to small number of B type repeats. Individuals with more

than 20 B type dimmers were classified as suitable for investigations. The profiles with the most B type repeats were chosen as the most suitable for further studies of somatic mutation. Large number of repeats created a greater opportunity to detect mutational changes e.g. somatic mutations that may have occurred. The CEPH individuals were not exposed to FAC drugs. In order to investigate the impact of FAC drugs on the human DNA, lymphoblastoid cell lines of some of the CEPH individuals were exposed to the anti-cancer drugs (for details see Chapter 3). The MVR-PCR profiles of the treated CEPH 1206 cells were generated, see Figure 5.6.No changes in B type of profiles were observed with different FAC treatments (17 hours), 0.1 mM,0.5 mM and 1.0 mM. After careful analysis of the obtained profiles no change in B-type MVR-PCR profiles were detected. However by using different levels of FAC drug combination it was possible to detect an effect that could be related to the DNA damage. The data obtained is outlined in the next section.

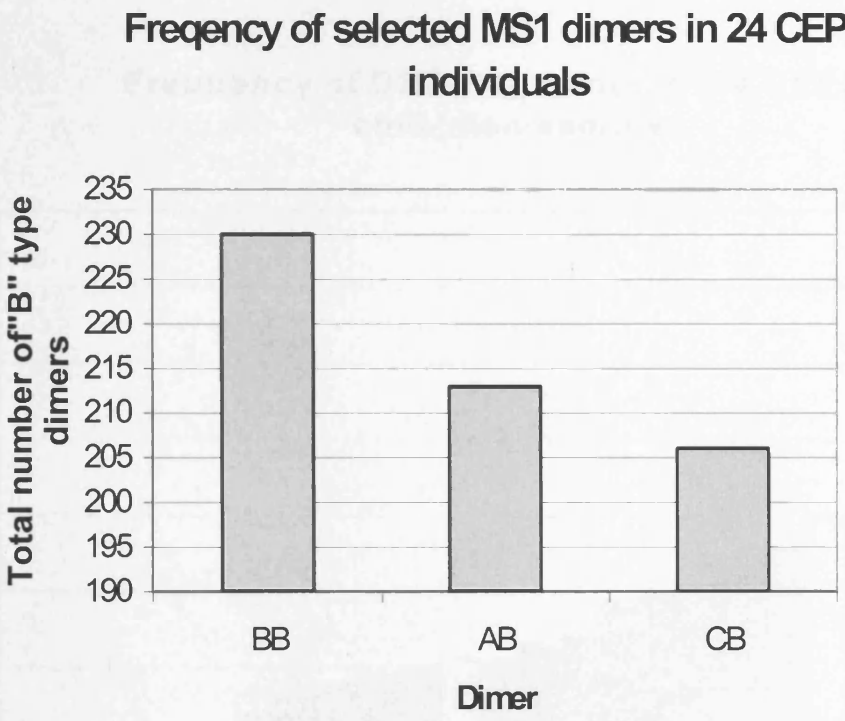


Figure 5.3 Graph showing frequency of selected MS1 dimers in 24 CEPH individuals. The B repeats were detected by Southern blot analysis and then the number of repeats counted for each individual. It shows that BB type of dimer is the most abundant in diploid MVR-PCR profiles of minisatellite MS1 (data from first 2kb only).Standard PCR conditions were used.

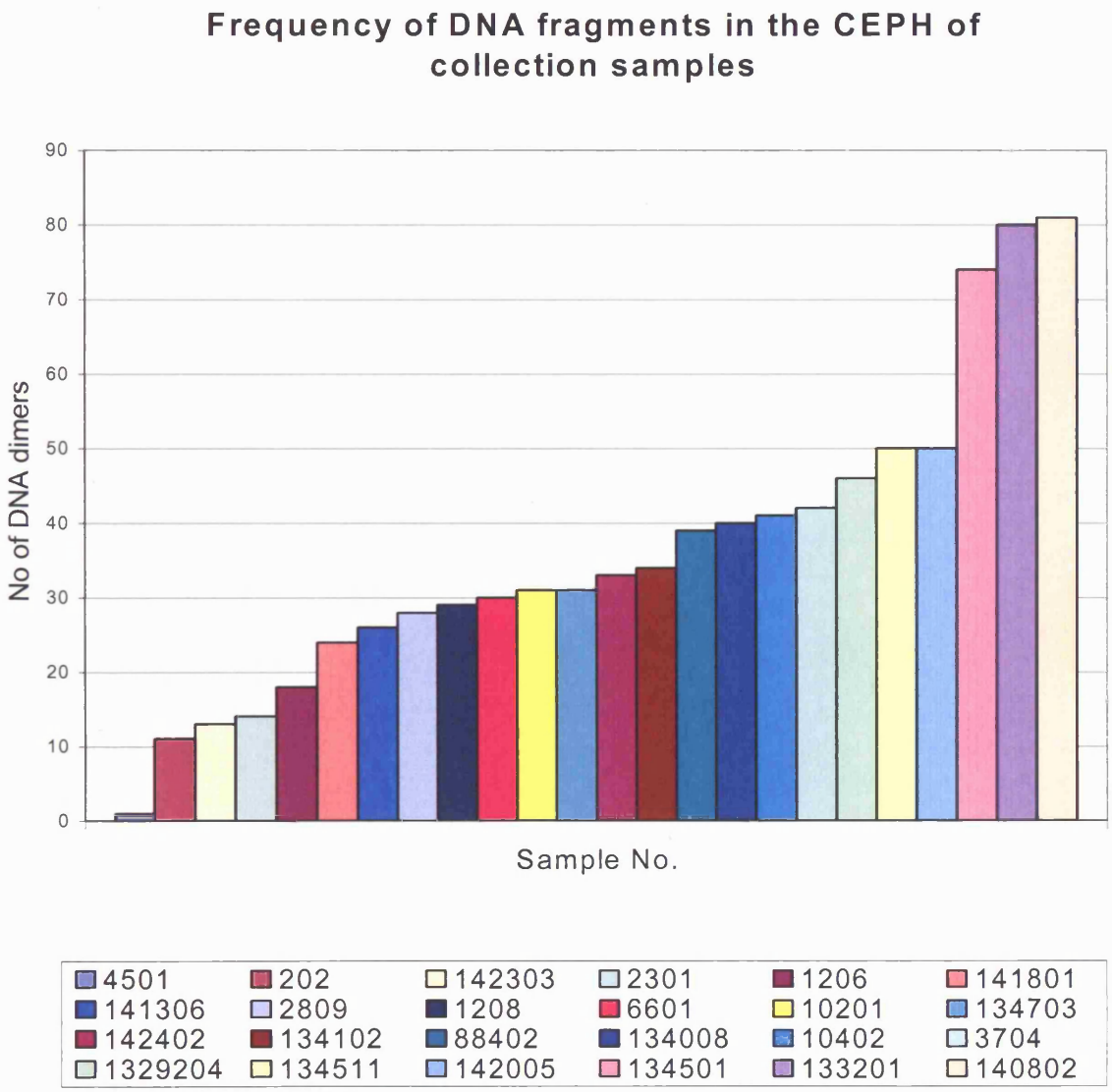


Figure 5.4 The frequency of DNA fragments in CEPH collection samples graph shows the content of B type dimers present in the first 2 kb of MS1MVR-PCR profiles of 24 individuals studied. Those individuals represent a selection of different diploid profiles of "B" dimers that can be found within the human population. The profiles of "B" type dimers generated represent the first 2 kb of the MS1 region.

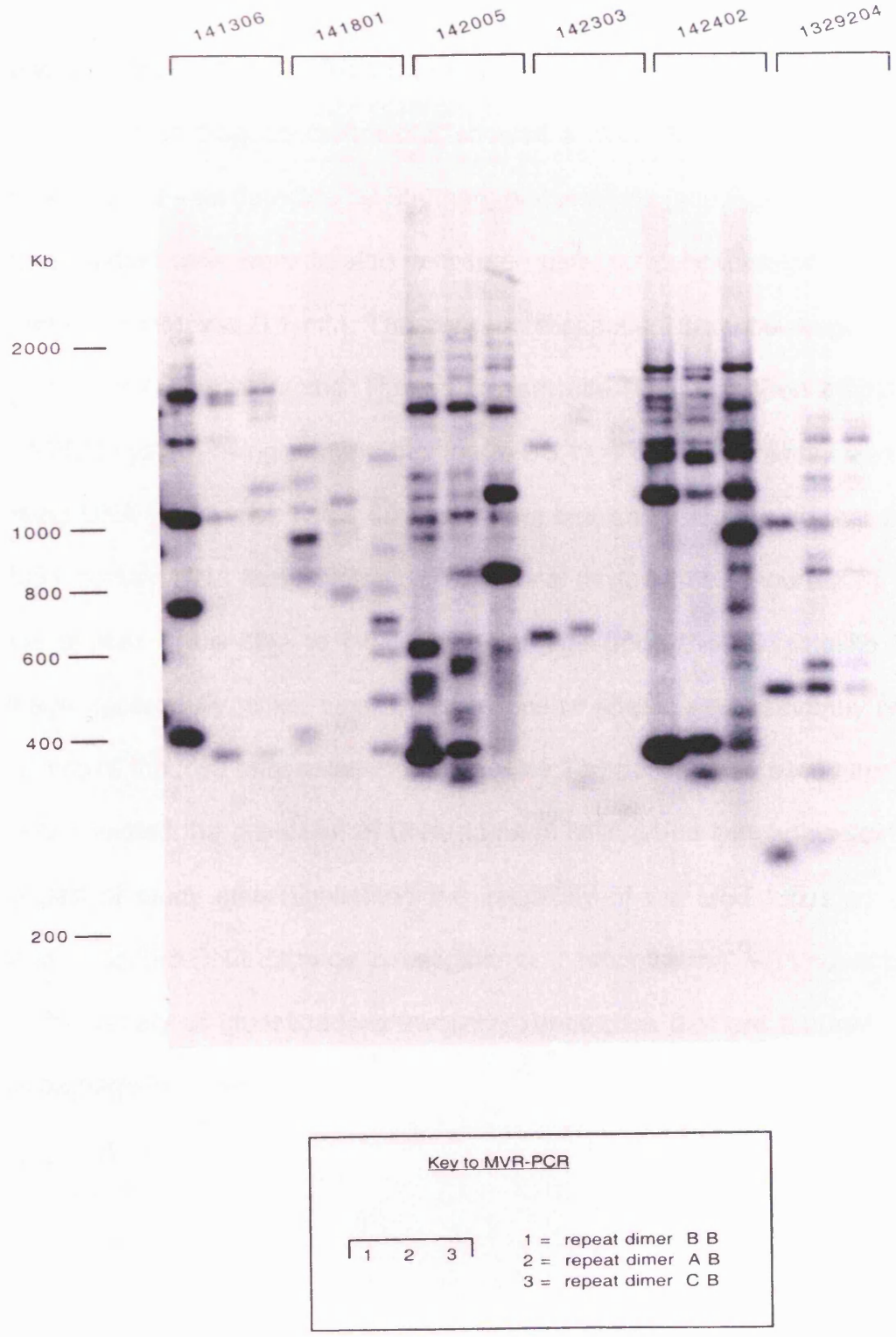


Figure 5.5 The figure presents MVR-PCR profile of several CEPH samples amplified with primers annealing to BB, AB and CB type of dimers in first 2 kb of MS1. Sample differences can be detected between individual profiles. Some of the samples have many B dimers such as samples CEPH 142005 and 142402, but some have very few B type dimers i.e. 142303 and 141306.

5.2.3 *Taq* polymerase stop assay at MS1

Analysis of MVR-PCR profiles of CEPH lymphoblastoid cells treated with differing FAC anti-cancer drug concentrations showed a dose-dependent reduction in DNA fragment signal – as detected by Southern blot analysis (see Figure 5.6). CEPH 1206 lymphoblastoid cells were treated with three different concentrations of FAC drugs, 1.0 mM, 0.5 mM and 0.1 mM. The cells were exposed to those drugs for 17 hours and then DNA was extracted. Human minisatellite MS1 was then amplified by the MVR-PCR system using primers binding to BB type of repeat dimers and within the flanking DNA (MS1, see Table 5.2). Southern blot analysis showed that the amount of MS1 derived DNA was inversely proportional to dose (see Figure 5.7). Thus FAC drugs appear to be able to inflict DNA damage upon the minisatellite MS1. This damage, (potentially nicks, base modifications or abasic sites) severely restricts the efficiency of the *Taq* polymerase reaction. The *Taq* polymerase assay applied in this project revealed the presence of DNA damage inflicted on lymphoblastoid cell lines. This part of study also highlighted the versatility of the MS1 locus as a potential biomarker for the DNA damage investigations. It indicates that MS1 could be possibly used for variety of investigations involving xenobiotics that are thought of inflicting DNA damage in humans.

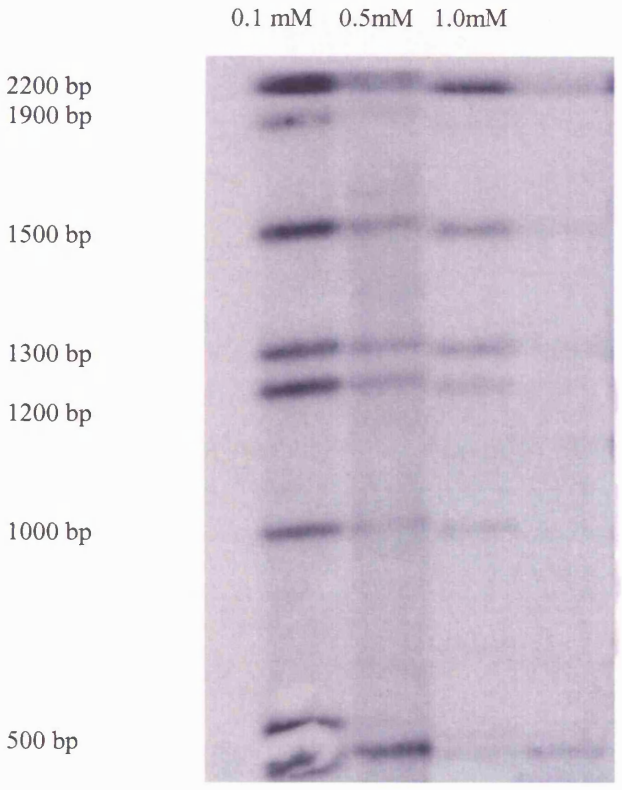


Figure 5.6 BB repeats in CEPH2 1206 sample. Cells were treated for 17h wit 1.0 mM,0.5 mM and 0.1 mM of the FAC drug combination and then collected and DNA extracted. Standard PCR conditions were used to generate the profile. Southern blot analysis was used to detect samples.

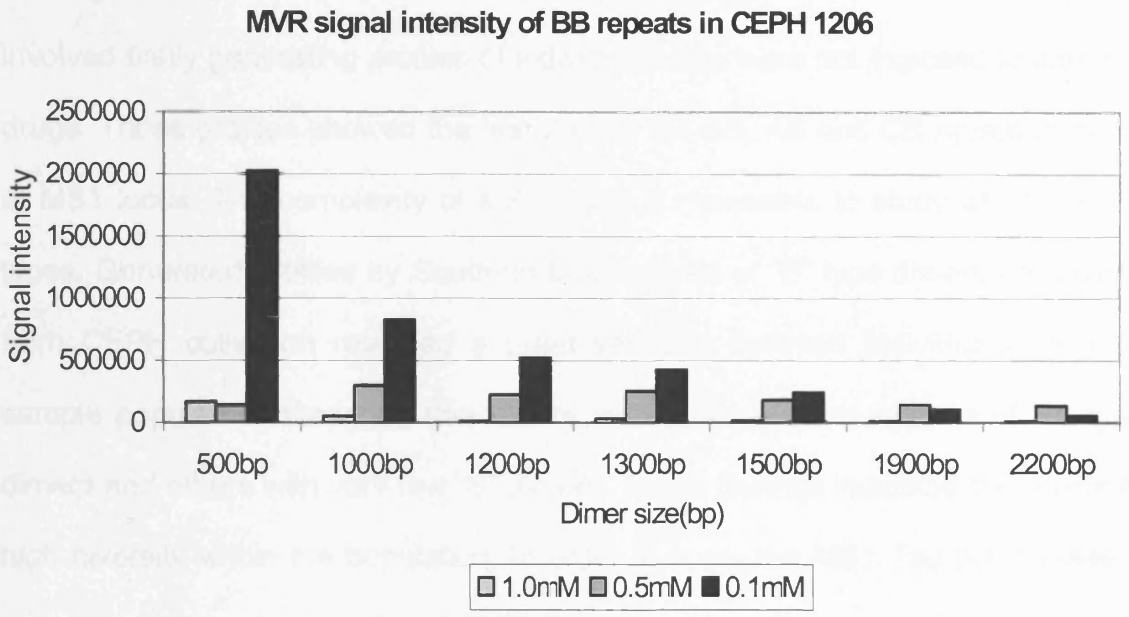


Figure 5.7 MVR signal intensity of BB repeats in CEPH2 1206.Cells were treated for 17h and then collected. The graph shows the intensity of the B type repeats measured by phosphoimaging system in DNA samples treated with different concentrations of FAC drug combination. The signal was measured in each lane. The most intense shades represent the least DNA damage in investigated sample. Standard PCR conditions were used in this experiment.

5.3 Discussion

MS1 has a highly variable internal structure. Because of its variability and unstable nature it was chosen to act as biomarker for the possible FAC induced DNA damage investigations in this project. The strategy employed for its use as a biomarker involved firstly generating profiles of individuals who were not exposed to anti-cancer drugs. Those profiles showed the first 2 kb of the BB, AB and CB repeat units found at MS1 locus. The complexity of MS1 made it impossible to study all of the repeat types. Generated profiles by Southern blot analysis of "B" type dimers from samples from CEPH collection revealed a great variation between individuals. In a small sample population examined there were individuals with abundance of "B" type of dimers and others with very few "B" dimers. These findings indicated the presence of high diversity within the population. In order to apply the MS1 *Taq* polymerase stop assay in further studies it may be necessary to pre-screen individuals because somebody with few "B" types repeats could be difficult or impossible to assess for DNA damage at the MS1 locus. The sparse presence of "B" type dimers will mean that possible mutational changes might not affect those dimers and the profiles might stay unchanged.

The second stage of the strategy was to examine the BB profile at the MS1 region, generated from the DNA treated with anti-cancer FAC drug combination. The FAC drugs due to their biological/toxicological properties have a great impact on the DNA of the individual treated with those drugs. Cyclophosphamide can give rise to mutations by inhibition of DNA synthesis. Doxorubicin causes a decrease in DNA replication and 5-fluorouracil inhibits RNA synthesis. (Chabner 1996) The DNA was

extracted from CEPH 1206 lymphoblastoid cells treated with three concentrations of the FAC drug combination, 1.0mM, 0.5mM and 0.1mM. The internal structure of the three profiles obtained did not differ, but there was significant difference in band intensities of individual dimers in different treatments. Those band intensities were individually measured by phosphoimaging system and it appeared that the stronger the drug concentration applied to the DNA the weaker the band intensity. The band intensity signals with higher drug concentration meant that the DNA damage was greater, because the damaged DNA does not amplify very well. Signal from damaged DNA is much weaker, therefore it is possible to conclude that detectable DNA damage occurred at the MS1 region and it is possible to detect, but the method designed for detecting mutations within MS1 locus needs to be developed further. In the samples treated by the FAC drug combination no mutational changes were observed. Samples were treated for 17 h and harvested. Taking under consideration the nature of the FAC drug combination it was expected to possibly detect some mutations. Minisatellites have been proven to be induced by xenobiotics, so it was expected that such powerful anti-cancer drugs induce changes within minisatellite structure. One of the reasons for not detecting mutations is that the DNA repair took place, therefore removing any mutations. It is possible to examine more repeat unit types at the MS1 locus.

The MS1 minisatellite is a potential powerful biomarker for DNA damage, but further investigations are needed in order to explore its full potential. One method for detecting minisatellite mutation at rates lower than that detectable by MVR-PCR is small pool-PCR (SP-PCR) as has been previously developed by others for the minisatellite MS32 (Jeffrey *et al.* 1997). Whilst this work was being performed I learned that Professor Jeffrey had developed SP-PCR at MS1. His unpublished data

showed high germline rearrangements at MS1, but complete absence of such changes in somatic cells.

Thus it become apparent that a new system needs to be developed in order to assess the impact of FAC anti-cancer drug combination on the DNA rearrangement/mutation. The system chosen for further studies was MLL-AF4 (see Chapter 6).

6.0 Polymerase chain reaction mediated detection of chromosomal translocations t (4; 11)(q21; q23) involving the human genes MLL and AF4

Summary

Despite chemotherapeutic advances in anti-cancer treatments breast cancer is still a disease that claims many human lives. The treatments available give a chance for longer survival, but in some cases may to developing further cancers. One of those treatments is FAC, a combination of 5-fluorouracil, adriamycin and cyclophosphamide. In some patients this drug combination may cause treatment related leukaemia t-AML. However it is not clear why only some patients develop treatment related leukaemia after FAC treatment. Some studies indicate that drug metabolism and differences in genotype within patients make them susceptible to developing cancers. One of the types of treatment related cancer t-AML appears to be a result of chromosomal translocations. In this investigation translocations between MLL and AF4 chromosomes have been explored. The blood samples of patients receiving FAC anti-cancer treatment were examined for the presence of MLL/AF4 translocations. Samples were examined before and after the FAC treatment to determine whether the chromosomal translocations were present in cancer patients even before the treatment. The techniques that were developed to study patient's DNA at the MLL and AF4 regions appeared to be very sensitive, perhaps suggesting detection of a number of translocations even before the

patients received the chemotherapy treatment. However, these finding led to the conclusion that the techniques developed were over sensitive. The presence of so many translocations detected in studied samples could have occurred as PCR artefacts. *In vitro* studies performed on human lymphoblastoid cells gave the same conclusion. Investigations using the *Taq* polymerase stop assay revealed that DNA damage at the MLL region occurs after treatment with FAC drug combination but that the DNA damage could not be classified as chromosomal translocations.

6.1 Introduction

For many decades researchers have been studying different drugs and approaches to combat forms of cancer and new drugs and treatments have become available for cancer patients. Because of many forms of cancer there are many types of anti-cancer treatments available. These treatments can combat many of the tumor cases, but there are still situations where the drugs used to treat cancer unfortunately cause treatment related cancer: this includes as treatment related leukaemia, also known as t-AML (Felix *et al.* 1998).

Approximately 1-20% of the patient population treated for cancer with chemotherapy including alkylating agents and topoisomerase II inhibitors develop t-AML (Felix *et al.* 1998). In the case of breast cancer 1-5% of patients develop treatment related leukaemia as a result of chemotherapy (Diamandidou *et al.* 1996).

Several theories have been put forward as to why only a small portion of the patient population is affected; these include the therapeutic doses administered,

the genotype of patients, the combination of drugs and the age of patients, which all might make them more vulnerable to secondary cancer. In order to find answers to these problems some investigators focussed on analysis of the patients genome. In particular genes involved in drug metabolism pathways required thorough investigation. In some cases it was found, that mutations present at genes involved in drug metabolism made patients more susceptible to side effects (Larson *et al.* 1999).

Treatment related leukaemia (t-AML) could be induced by such factors as translocations between genes that arise in the genome of patients. Those translocations involve chromosomal rearrangements. There are several genes that are known to form prooncogenic translocations, such as MLLT6, AF10 and CEZF (Gregorini *et al.*, 1998). In this project translocations between two such genes MLL and AF4, which cause treatment related leukaemia in cancer patients, were investigated. The aim was to develop methods to help determine whether the translocations of MLL/AF4 are present in cancer patients before or only after they receive FAC chemotherapy and if the numbers of translocations increase as the consequence of the drug treatment. The group of patients studied were breast cancer patients treated with FAC. This drug combination is a mixture of three drugs, cyclophosphamide (C), adriamycin (A, also known as doxorubicin) and 5-fluorouracil (F) (Chamber 1996).

6.1.1 Patient cohort

To study the changes in the genomic DNA of patients, blood samples of patients receiving FAC treatment were collected from patients attending the

Leicester Royal Infirmary Department of Oncology. This work was performed in collaboration with the Professor of Oncology, William Steward. The ethics approval for these studies was granted. The number of samples obtained was 13. Samples were obtained from patients, who did not have any previous anti-cancer treatments. The samples were collected before and after initial FAC treatment. The main objective was to use PCR-based methods to determine the level of chemically induced mutations in these samples. Two particular regions of the human genome have been chosen for study the first being the human minisatellite locus MS1 (see below) and the second is the MLL/AF4 gene translocations (see below). To assist those studies, lymphoblastoid cells from the CEPH panel (The **C**entre d'**E**tude du **P**olymorphisme **H**umain) were also treated with FAC *in vitro* and DNA damage measured.

One of the long-term side effects of FAC, which is observed in 1-5% of the patients, is secondary cancer, most specifically leukaemia. At the moment there is no system available to patients for screening of potential susceptibility for treatment related cancer development after FAC treatment. One of the objectives in this project is to assist in the development of such a system, which would allow an accurate determination of the risk for a given patient developing treatment related cancer after FAC administration.

6.1.2 MLL locus

Treatment-related cancer in patients given anti-cancer drugs are probably caused by many factors such as translocations, point mutations and deletions of part or whole chromosomes. In this project the particular locus to be

investigated was the MLL gene. From previous studies some information on this locus was already known. The MLL gene is situated at 11q23 (Felix *at al.* 1998), and is 100 kb long. Its accession number from GenBank is UO4737 (Gu *et al.* 1994).

In some cases of leukaemia (either spontaneous or induced by anti-cancer drugs) the MLL gene is disrupted by chromosomal translocation. The MLL gene contains a region 8.3 kb long and situated between exon 5 and 13, which seems to be very susceptible to damage by anti-cancer drugs including topoisomerase II inhibitors such as doxorubicin (Gu *et al.* 1994). In this region cleavage of the MLL gene might occur leading to translocation of MLL gene with around 30 possible partner genes. This process is a key step in the development of secondary leukemia (Felix *at al.* 1998). One of the partners involved in translocation event with MLL is the AF4 gene. Such chromosomal mutation occurs in therapy-related leukaemia t-AML. Ability to measure the rates of MLL/AF4 translocation may help to develop PCR-based techniques to distinguish individuals who are more prone to developing treatment related leukaemia.

Whilst the primary mode of investigation of the MLL gene in this project was by examination of DNA samples from hospital patients treated with FAC combination of drugs, the MLL locus in CEPH cell lines was also studied. These cells have been treated *in vitro* with FAC drug combination and the DNA examined for the presence of MLL/AF4 translocations.

6.1.3 AF4 locus

The AF4 gene is located on chromosome 4q21. Marschalek and colleagues identified 3 breakpoint cluster regions present in the AF4 locus, where the DNA helix breaks and forms translocations with the MLL locus (Nilson *et al.* 1997, Marschalek *et al.* 1995). Figure 6.1 shows a diagram of those break point cluster regions and the primers used to study them in this project. There are several Alu elements present at the breakpoint cluster regions of the AF4 locus. Alu elements are repetitive fragments of DNA, which are abundant throughout the human genome and may contribute to PCR artefacts being formed. The second cluster region of AF4 was chosen as first for investigation of translocations with MLL. This region contains the fewest Alu elements (Reichel *et al.*, 1999). The other two cluster regions present in the AF4 locus contain multiple Alu elements therefore were not as good candidates for the investigations. The second candidate for study was the first cluster region of the AF4 locus. The sequence data of AF4 locus was obtained from the GenBank database. The accession number is AJ238093 (Nilson *et al.* 1997).

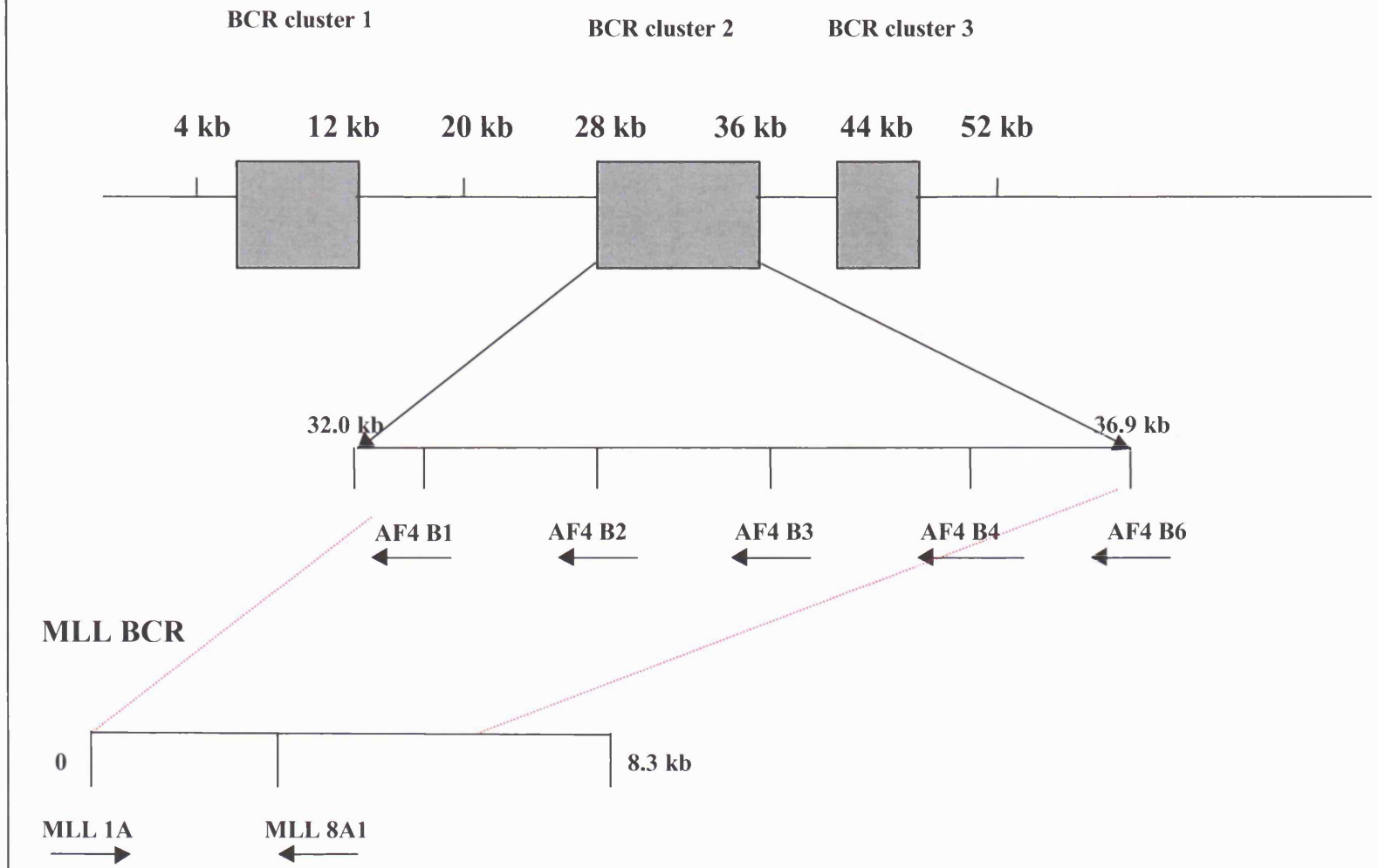
6.1.4 Tissue culture studies

The *in vitro* model designed in this project involved using lymphoblastoid cell lines from the CEPH collection. The cells were exposed to various FAC drugs at the doses that hospital patients might be administered. The chosen doses were as follows; 5-fluorouracil 600 mg/m^2, adriamycin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 . These represented clinical doses administered to patients.After calculating the doses for tissue culture studies the cells were

incubated with the following concentrations of these drugs 1.0 mM, 0.5 mM and 0.1 mM, Table 6.1 shows the doses used in each *in vitro* experiment. In all of the drug concentrations, doxorubicin concentrations used were ten times lower than those of the other drugs. The purpose of these experiments was to establish the extent of MLL/AF4 translocations that occur in lymphoblastoid cells after their exposure to FAC drugs. The viability of treated cells was used to establish the extent to which the FAC combination affected the cells, by counting the ratio of surviving cells compared with dead ones. Several aspects had to be taken into consideration before conducting *in vitro* studies. One of those aspects was that cyclophosphamide, which is a component of FAC, needs to be metabolised to its active form to act as an anti-cancer drug. To overcome this problem human CEPH cells were co-cultured with rat C-450-13 cells. These cells contain the rat cytochrome P450 B1 rat gene needed for bioactivation of cyclophosphamide (Ming *et al.* 1995). Considering all of the above points, the *in vitro* model designed using lymphoblastoid cell lines from the CEPH panel looked a promising system to investigate translocation events that take place in human DNA, after treatment with anti-cancer drugs. In this study a positive control for the detection of chromosomal MLL/AF4 translocations was used. A SEM cell line from a cancer patient with a defined translocation between MLL and AF4 loci was obtained. The translocation linked the 5' end of the MLL BCR (breakpoint at 2770 bp) to AF4 BCR region (breakpoint at 32911 bp). The known position of the translocation made it possible to test if the PCR based techniques developed in this project were sensitive and accurate as a tool to detect rare MLL/AF4 translocations in DNA samples.

Figure 6.1 MLL and AF4 Breakpoint Cluster Regions showing primer positions

Arrows indicate the direction of amplification by particular primers. The grey blocks represent the cluster , where chromosomal breaks and translocations occur.



Dose	Drug	Drug concentration
1.0 mM	5-Fluorouracil Adriamycin Cyclophosphamide	1.0mM 0.1 mM 1.0 mM
0.5 mM	5-Fluorouracil Adriamycin Cyclophosphamide	0.5 mM 0.05 mM 0.5 mM
0.1 mM	5-Fluorouracil Adriamycin Cyclophosphamide	0.1mM 0.01 mM 0.1 mM

Table 6.1 Doses of the FAC drugs administered in tissue culture studies. DNA from treated cells was extracted used in experiments.

6.2 Results

6.2.1 Analysis of defined MLL/AF4 translocation

A range of primers was designed to amplify the MLL/AF4 translocation region present in the SEM cell line. SEM cell line originated from a leukemia patient with a defined MLL/AF4 translocation, a gift from Greil (Greil *et al.* 1994). The MLL break point was at 2770 bp and the AF4 break point at 32911 bp. The PCR primers were designed accordingly. The sequences of the primers can be found in the Materials and Methods section. The results were as expected; the system applied for detecting and amplifying the translocations gave a clear positive result. Figure 6.2 shows a photograph of an agarose gel with the PCR products derived from the MLL/AF4 translocation visible as clear bands. Table 6.2 shows the sizes of PCR products obtained and the primers that were used to amplify them. In this experiment a range of AF4 primers was used to assess to what extent the size and location of translocation can be determined just by the PCR product. The results obtained proved that the system designed in this project is a competent tool for assessing MLL/AF4 translocations. Primers designed and used in this experiment detected the translocation present in SEM cells. The primers amplifying both the MLL and AF4 region were very specific. The AF4 primer AF4 B1, which was positioned out of the area of break point region of AF4 locus, did not yield any product in PCR reaction with the MLL primer (see lane 1 in Figure 5.2). The other AF4 primers used in this experiment were able to detect the translocation between chromosome 4 and chromosome 11. All of these primers were positioned at various distances from the breakpoint cluster

region of the AF4 locus and therefore the PCR products obtained varied in size (see lanes 2,3 and 4 in Figure 6.2). This experiment illustrated, that the use of PCR based techniques might be a valuable tool to detect chromosomal translocations.

6.2.2 Single molecule PCR of SEM DNA

In this project one of the objectives was to determine the presence of MLL/AF4 translocations in patient's blood before or after the FAC treatment. It was also expected that those translocations would be quite uncommon, therefore a system had to be designed in order to identify these occurrences. The most suitable technique appeared to be single molecule PCR. The technique is capable of amplifying single molecules of DNA. The use of Herculase polymerase (see Chapter 2, Materials and Methods) also helped to achieve the goal, due to its high performance. The Herculase polymerase was able to amplify very large fragments of DNA, up to 12 kb in length.

DNA used to achieve single molecule amplification contained known MLL/AF4 translocation. The SEM DNA was diluted and the following amounts of DNA were added to each PCR reactions: 500 pg (around 83 molecules), 120 pg (20 molecules), 60 pg (10 molecules), 30 pg (5 molecules) and 6 pg (one molecule). The samples were then amplified by PCR (35 cycles) and detected by Southern blot analysis. Figure 6.3 shows the results of this experiment. On the autoradiogram it could be seen that it is possible to amplify as little as 30 pg of DNA containing MLL/AF4 translocation and be able to detect it.

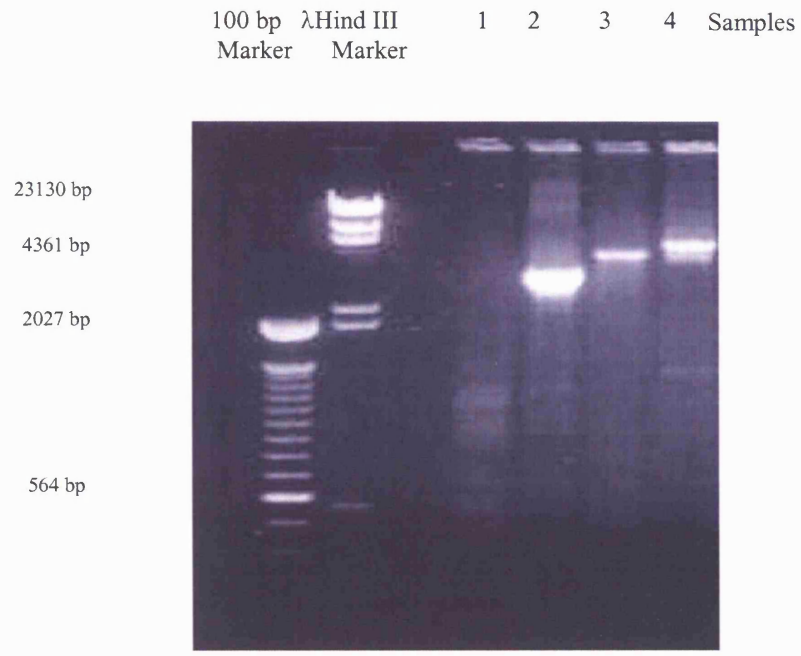


Figure 6.2 Ethidium bromide stained agarose gel with PCR product derived from MLL/AF4 translocation detected in SEM cell DNA.

Well No.	PCR product size (bp)	Primers used
1	No product as expected	MLL 1A & AF4 B1
2	3721, as expected	MLL 1A & AF4 B2
3	4801, as expected	MLL 1A & AF4 B3
4	5867, as expected	MLL 1A & AF4 B4

Table 6.2 PCR product sizes and primers of MLL/AF4 translocations detection in SEM cells (see Materials and Methods section for primer sequences).Standard PCR conditions were applied. Cycle number - 30

In order to amplify the MLL/AF4 translocations from the 6pg of SEM DNA, the genomic DNA was cut with restriction enzyme, for better amplification of the chosen DNA region. The restriction enzyme chosen was Pml I, which do not have restriction sites within the MLL or AF4 regions of interest, so it could not have altered the presence and structure of MLL/AF4 translocations in the studied sample. After the experiment it was discovered that the lowest DNA concentration that can be used to detect MLL/AF4 translocations in genomic DNA samples was 30 pg. This could be due to the uneven dilution of the DNA, where in some cases it is possible to have no DNA molecules in some of the diluted samples.

6.2.3 Detection of MLL/AF4 translocations in clinical samples

Investigations of MLL/AF4 translations involved the use of DNA extracted from the blood samples given by hospital patients (see Chapter 2, Materials and Methods). Samples were given codes P 1 to P 7.

To detect the presence of possible MLL/AF4 translocations in the P samples either before or after the treatment, the DNA was amplified using the MLL 1A primer and AF4 B6 primers. The primers allowed for amplification of any MLL/AF4 translocations joining the MLL bcr with the second bcr of the AF4 locus. PCR products were detected using Southern blot analysis (using a MLL specific radioactive probe). Generated profiles of patients can be found in Figure 6.4. Profiles of the patients obtained differed from patient to patient, in some cases there were more amplified DNA fragments present (that appeared

to be MLL/AF4 translocations) than in the others (e.g. P1U and P2U in Figure 6.4). In some of the samples no translocations could be detected, as where in others many different size translocations could be observed in the range between 2 kb and 20 kb. At this stage results seemed to suggest that some individuals might differ in their susceptibility to have translocations present in their DNA.

Figure 6.5 shows further analysis of P 1 U and P 1 T by Southern blot. The profiles were obtained by PCR with primers MLL 1A and AF4 B6. However this time the samples were amplified using two different DNA concentrations (50 ng, first 5 lanes of P1 untreated and P1 treated and 100 ng, rest of the P1 untreated and treated samples. The DNA concentration did not have effect on the PCR products obtained. The results show that there were PCR products present at around 8 kb, that could represent MLL/AF4 translocations but there were no significant difference in band number or intensity between samples taken before and after the treatment.

There were also lots of PCR artefacts present, see figure 6.5. Artefact could be seen as large bands at the bottom of the picture of the Southern blot. To try to reduce the occurrence of these the extension temperature was reduced from 70^0C to 65^0C. The experiment conducted with extension temperature of 65^0C proved successful, see figure 6.6.In this figure there are no large, intensive bands present at the bottom of the Southern blot picture. Through lowering the annealing temperature the number of PCR artefacts was reduced. A similar procedure was performed earlier, when MS1 profiles of CEPH families were generated.

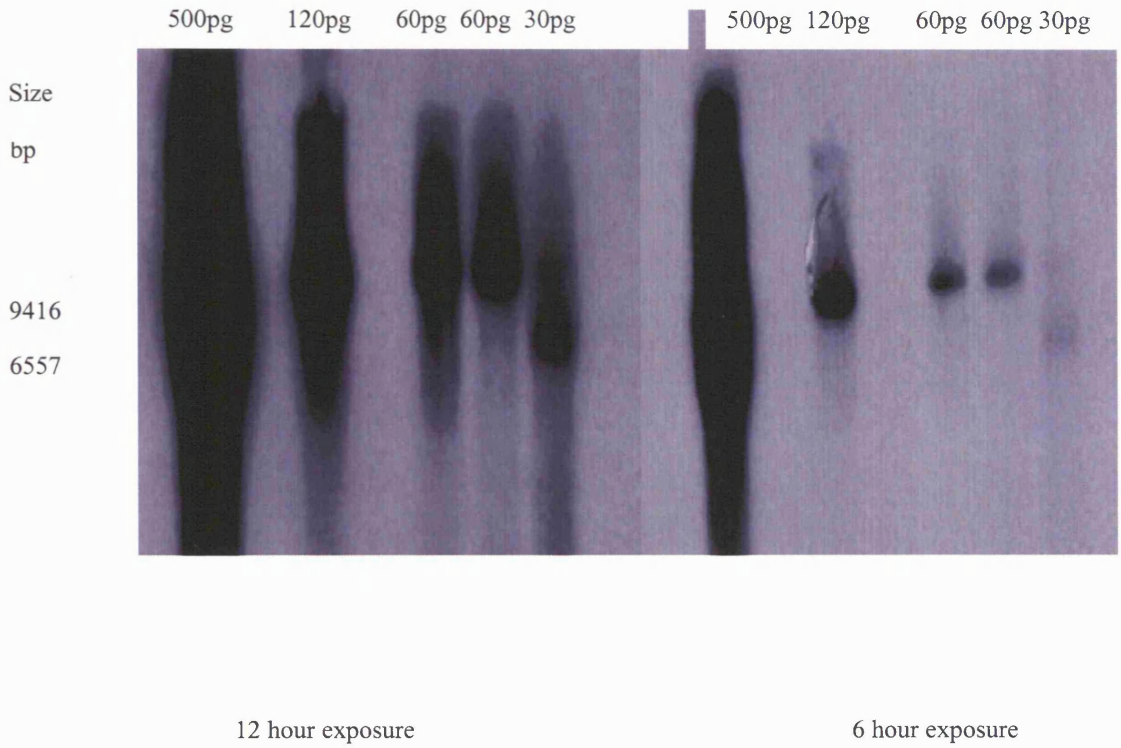


Figure 6.3 Autoradiogram of SEM DNA amplified at single molecular level. The DNA was digested with the restriction enzyme PmI in order to amplify MLL/AF4 translocations from the 6 pg of genomic DNA. It became apparent that the lowest concentration of genomic DNA from which translocations could be amplified was 30 pg. Standard PCR conditions were used for the PCR amplifications. 19 PCR cycles was applied and Southern blot analysis was applied for detection of the samples.

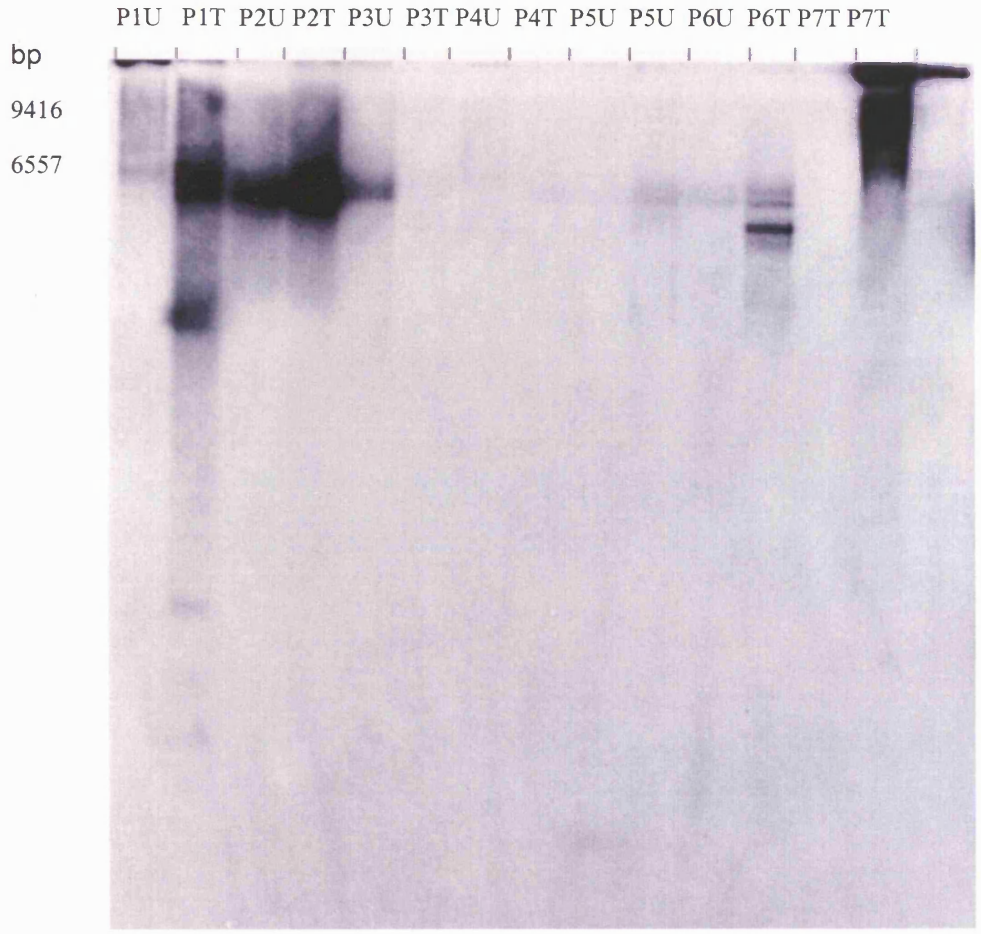


Figure 6.4 Autoradiogram of MLL/AF4 translocations detected in hospital samples. The samples were obtained from patients treated with FAC drug combinations; U samples were taken before the treatment begun, T samples were taken after the FAC treatment. Standard PCR conditions were used to amplify the samples. Samples were detected by Southern blot analysis. There are visible differences between the samples; in some of the MLL/AF4 translocations can be seen (P1U and P2U), but in others (P4U there were no signs of translocations).

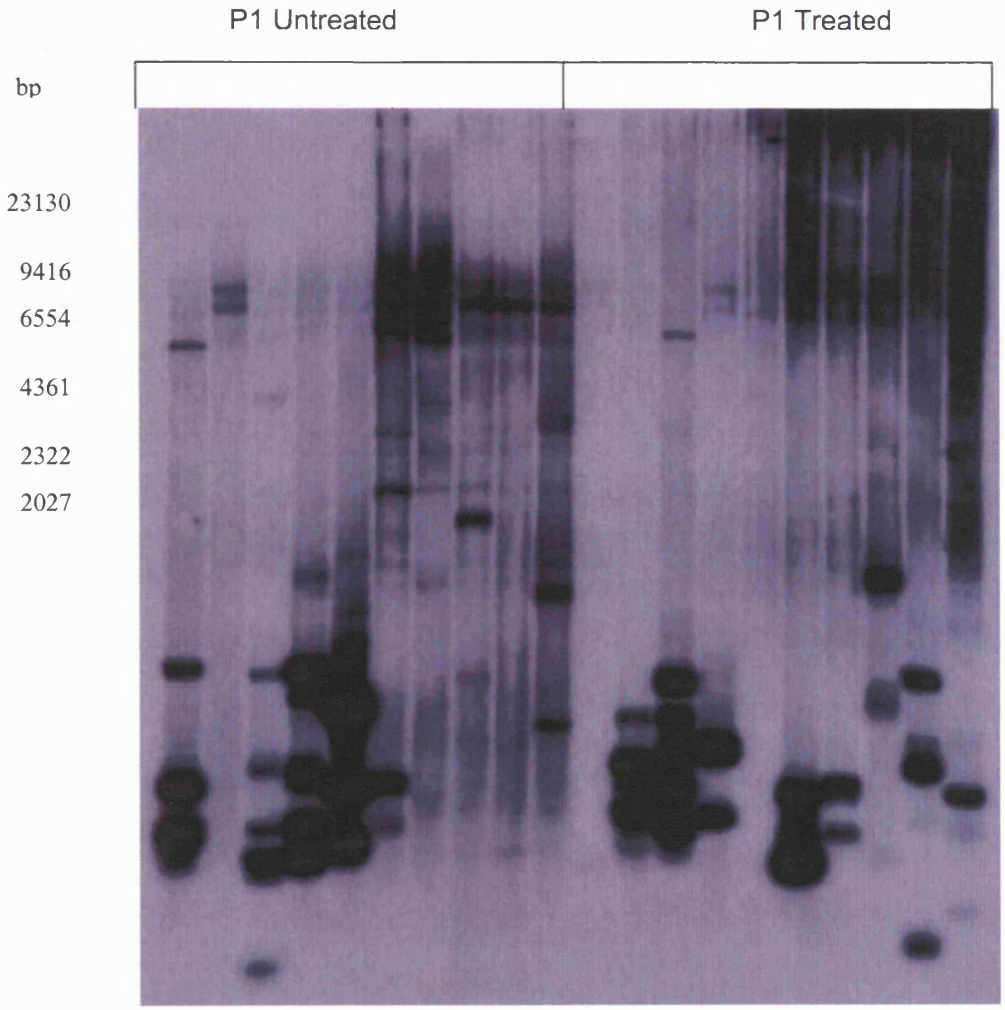


Figure 6.5 The P1U and P1T samples were amplified with MLL 1A and AF4 B6 primers to detect MLL/AF4 translocations. Standard PCR technique was used for amplification. Samples were amplified for 19 cycles and then detected by Southern blot analysis. It has also become apparent that there were PCR artefacts present, which can be seen at the bottom of the blot picture.

6.2.4 Hybrid chromosome 11 and chromosome 4 experiment

During the analysis of patient's DNA for MLL/AF4 translocations, some non-specific bands were present (see above). In order to determine their origin an experiment was designed to determine whether non-specific bands were indeed PCR artefacts resulting perhaps from the non-specific binding of primers to other regions of genomic DNA rather than to MLL and AF4 loci. Hamster DNA extracted from selected monochromosomal somatic hybrid cell lines containing single copies of the human chromosomes 4 and 11 was used. The samples contained MLL and AF4 genes, DNA from the HHW416 cell line contained AF4 gene and DNA from the JICL4 cell line contained MLL gene. Both DNAs were mixed together at a concentration of 50 ng each and amplified with MLL 1A and AF4B6 primers by PCR to detect any possible false translocations. Because the DNA contained one copy of the MLL gene and one copy of the AF4 gene any appearing bands could be classified as false translocations. The samples were also amplified individually using PCR technique with a combination of MLL and AF4 primers to see if any non-specific primer binding occurred. The samples were examined by PCR to confirm if they did contain human MLL and AF4 genes. The results obtained were very encouraging. Detection of PCR product was by Southern blot analysis with MLL probe. No product was detected in single samples and mixed samples amplified with combinations of MLL and AF4 primers. This control experiment indicated that during PCR no false MLL/AF4 translocations were created.

6.2.5 Detection of translocations in CEPH cell lines treated with FAC drugs

The CEPH 1206 cell line was treated with a combination of FAC drugs at different concentrations. The cells were then harvested and DNA extracted. DNA was examined for the presence of MLL/AF4 translocations. The DNA was amplified with MLL 1A primer and AF4 B6 primers. Both primers allowed for amplification of any translocations present at the MLL locus and at second cluster region of the AF4 locus.

The cells used in this experiment were treated with the anti-cancer drugs, which are DNA damaging agents. Thus one might expect to find some DNA damage, possibly MLL/AF4 translocations in surviving cells after FAC treatment. The obtained PCR results were analysed at Southern blot level (MLL radioactive probe was used). Figure 6.6 shows the photograph of the autoradiogram. This result appeared to show that MLL/AF4 translocations were present in FAC treated DNA samples in the region of 8 kb.

6.2.6 Application of the Taq Polymerase stop assay to detect DNA damage at the bcr of the MLL gene.

In order to assess if the FAC drug combination caused DNA damage in CEPH cells treated with those anti-cancer drugs, the polymerase stop assay was performed. The CEPH 1206 cell line was treated with the FAC drug combination and the DNA extracted. The MLL region of that sample was amplified by long range PCR to obtain a PCR product .The region chosen for studies was around

exon 9, which was previously defined as being a DNA damage hot-spot for topoisomerase II inhibitors such as adriamycin (Felix *et al.* 1998). The PCR product was run on agarose gel and detected by using the Southern blot technique. The results showed that DNA extracted from samples treated with higher doses of FAC drugs showed greatest DNA damage. The damage was assessed by measuring band intensities, the more DNA was damaged the less signal was detected. Figure 6.7 shows the autoradiogram of the CEPH 1206 DNA treated with the various FAC drug concentrations. The DNA samples treated with 1.0 mM FAC drug concentration showed the highest DNA damage compared to untreated samples. Figure 6.8 shows a graph on which the DNA damage at MS1 and MLL region are compared, specially for fragments of the similar size from MLL and AF4 locus chosen for comparison. In both cases DNA was extracted from CEPH 1206 treated with 1.0 mM, 0.5 mM and 0.1 mM of FAC drug combination. The DNA damage at both MS1 and MLL loci was clearly detectable and its extent was also similar in both regions (see Figure 6.8). This suggest that despite the MLL locus having a status of a region preferentially damaged by drugs *in vivo*, basic levels of DNA damage *in vitro* were in fact similar to the non-BCR containing locus, MS1. The damaged DNA did not amplify as readily as unexposed DNA and therefore the PCR reaction yielded less PCR product. Thus the bands obtained on the autoradiogram were not as intense as those of undamaged DNA. One of the reasons for bands being weaker could be that fragmentation of the DNA occurred caused by the damaging effect of FAC drugs.

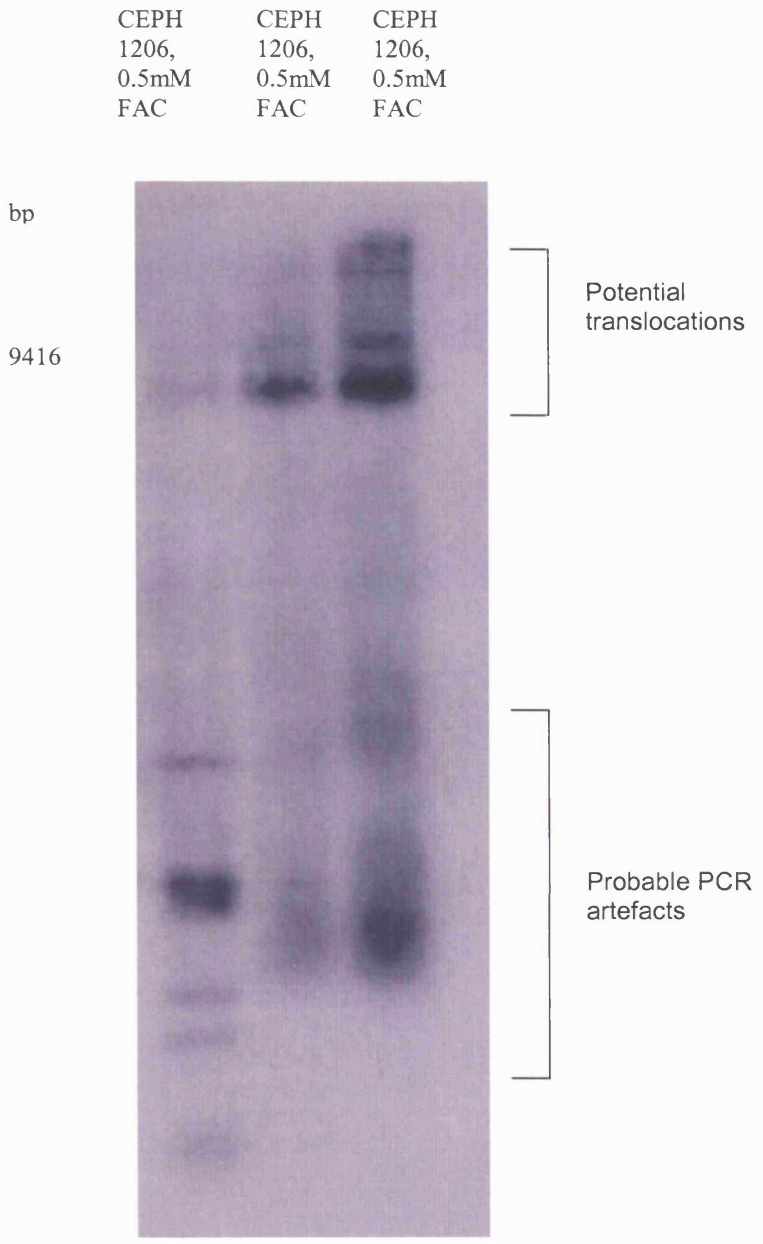


Figure 6.6 An example of CEPH 1206 DNA extracted from cells treated with FAC drug combination for 17 h. The MLL/AF4 translocations have been detected in samples treated with 0.5mM of FAC. The altered annealing temperatures from 70^0C to 65^0C reduced the appearance of PCR artefacts. Cycle number used for amplification was 19. Experiment was repeated 3 times.

The effect of DNA damage might have also limited the ability to detect MLL/AF4 translocations. With this technique it was only possible to asses the DNA damage in the studied area of genome. Non specific DNA damage was not assessed. This type of assessment of DNA damage can be applied in preliminary studies of many xenobiotics that have been suspected of having DNA damaging properties at particular area of genome.

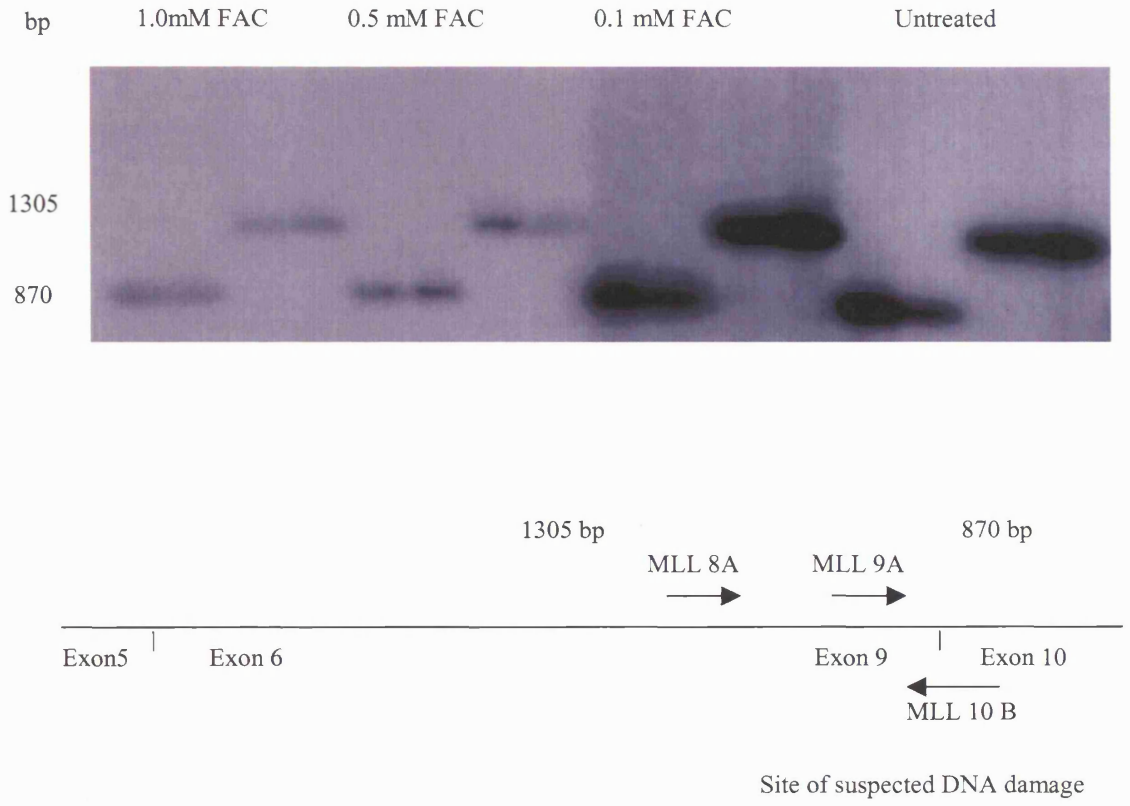


Figure 6.7 Polymerase stop assay performed on DNA extracted from CEPH 1206 lymphoblastoid cells treated with different concentrations of the FAC drug combination for 17 hours. The amplified regions were positioned at MLL exon 9.The primers used were MLL 8 A, MLL 9A and MLL 10 B. Standard PCR conditions were used and cycle number applied was 19.The samples treated with highest doses of the drugs show the highest DNA damage, the bands are very weak. The untreated samples had the strongest bands and samples treated with 0.1 mM FAC showed very little DNA damage.

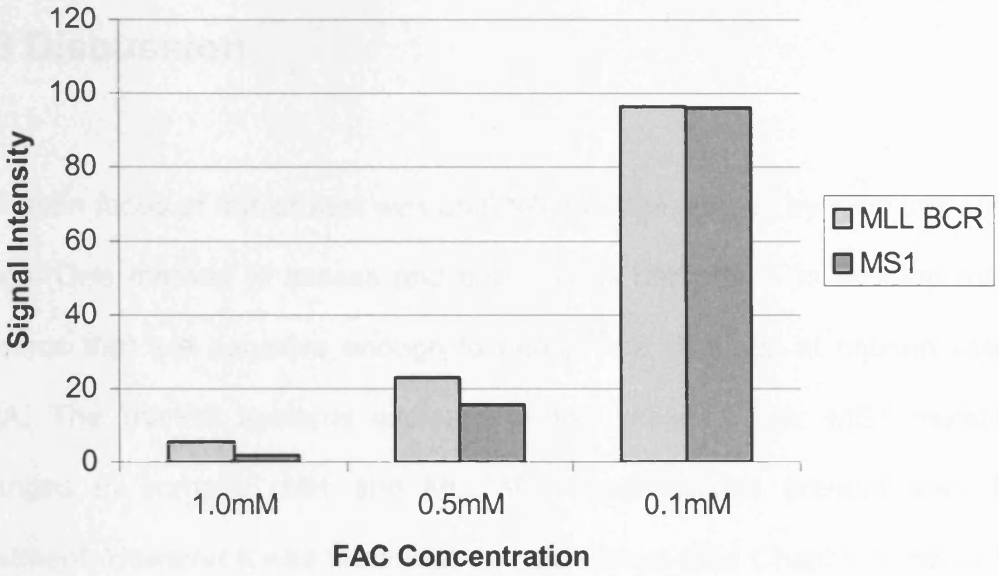


Figure 6.8 *Taq* polymerase PCR stops assay. The data represents the results from the polymerase stop assay performed on DNA extracted from CEPH 1206 cells treated with combination of concentration of FAC drug for. Cells were treated for 17 hours and then DNA was extracted. Standard PCR conditions were used for PCR amplification. Samples were detected by the Southern blot analysis. The dark grey bars represent the density readings taken from the MVR profile of MS1 locus of 1200 bp and 1300 bp fragments. The light bars represent the 1490 bp long PCR product of amplified MLL fragment. As we can see the density readings at both loci are similar, implying that the DNA damage occurred at both sites.

6.3 Discussion

The main focus of this project was on DNA damage caused by FAC anti-cancer drugs. One method to assess and detect DNA damage is to develop marker systems that are sensitive enough to detect rare changes at chosen sites of DNA. The marker systems explored in this project were: MS1 mutational changes in somatic cells and MLL/AF4 translocations present after FAC treatment. However it was found out that MS1 locus (see Chapter 5) possesses limitations that prevented the locus from being a good reporter system of DNA damage. At first it was thought that because minisatellite MS1 behaved like STR's it would have incidents of somatic mutations (Kao *et al.* 1976 & Greil *et al.* 1994). However this was not the case, as MS1 showed no great somatic instability. Therefore the minisatellite MS1 locus was dismissed as a suitable reporter system for DNA damage (personal communication with A. Jeffreys).

An alternative method to detect DNA damage by identification of MLL/AF4 translocations was explored in this chapter. In this system PCR based techniques were employed to identify any MLL/AF4 translocations present both in cell lines and in patients' DNA.

The studies included a use of positive control, the SEM cell line, containing known MLL/AF4 translocation which proved very successful. From the previous studies performed by R.Marshalek the position and the size of the MLL/AF4 translocation was defined (Reichel *et al.*, 1999). In this study the translocation was detected at the level of 30 pg of DNA concentration, equivalent to 5 input molecules. The system of MLL and AF4 primers worked well and the sizes of

PCR products from the translocation present in SEM cells were consistent with its previously reported size/structure (Greil *et al.* 1994).

The studies conducted on both patients DNA and FAC treated CEPH cell lines gave unexpected results. It was expected that patient's samples would have either varied MLL/AF4 translocation profiles or not to have any translocations present. The results obtained during the investigation showed the same MLL/AF4 translocation profile in each investigated sample. This was the case for DNA derived from both hospital samples and treated CEPH cell lines. The obtained profiles were not as individual specific as might be expected. The result therefore can be described to be non conclusive and there are several possibilities as to the cause of such findings: 1) there are no MLL/AF4 translocations present to be detected, 2) there are MLL/AF4 translocations present in investigated samples, 3) despite the satisfactory result obtained with the chromosomes 4 and 11 mixing experiment, the possibility remains that the fragments seen are generated during the process of PCR. Indeed the latter might be increased by the use of the poor quality DNA. Potential translocations appear in the region of 6-8 kb and may be derived from MLL/AF4 translocations, but because they appear in all of the investigated samples, their origin must be confirmed by further study. There are several ways to further investigate these results such as by gel fractionation, prior to the amplification of possible MLL/AF4 translocations. This technique allows for isolation of molecules of interest, in this case MLL/AF4 translocations from wild type molecules. Use of this technique may prove or disapprove the presence of potential MLL/AF4 translocations.

To broaden the knowledge of the events at the MLL and AF4 loci in treated DNA samples, the first cluster region at AF4 locus could be investigated. This would give more information about presence or absence of MLL/AF4 translocations in samples. It would also help to establish if the profiles of translocations already generated contain PCR artefacts only and no MLL/AF4 translocations.

In this project it was shown, that co-culturing two different types of cell cultures, C-450-13, cell line expressing rat cytochrome P450 2B1 and CEPH cell lines is possible and that it allows the full assessment of the effect of FAC drug combination on those cell lines. Cyclophosphamide needed to be metabolised to an active form in order to act as anti-cancer agent. Rat cytochrome P450 2B1 metabolised cyclophosphamide and the active metabolites were released into the tissue culture medium in a similar situation to that found in the blood of FAC treated patients. Without that step *in vitro* studies conducted on cell lines would not be valid if the drug was inactive (Ming *et al.* 1995).

The polymerase stop assay used to detect DNA damage (if any) in treated tissue culture derived DNA samples also proved to be successful. The technique helped to determine, that there is DNA damage present in samples that had been exposed to FAC drug combination. The technique proved that higher concentration of FAC drug causes greater DNA damage. This technique is very robust and it can also be used in other situations, where the DNA damage was suspected to occur.

7.0 General Discussion

Although therapeutic regimens that are currently in use to treat cancer have a high success rate, the cumulative toxicities of those treatments can present barriers to their long-term use. Despite continued gains in survival and other clinical end points in cancer, the goal of offering long-term remission with existing therapies is still far away. However, existing chemotherapy can provide an extension of life span and an improvement in patient quality of life (Aman *et al.* 1989). Because a lot of patients can undergo a series of remissions and recurrences, the additive or cumulative toxicity of cancer therapy must be taken into consideration when designing the treatment plan. Additionally, the potential for patients to experience cumulative toxicity must be carefully weighed against the goals of prolonging the disease-free interval. Particular emphasis should be placed on cumulative side effects. Irreversible toxicity such as gene translocations and mutations leading to developing secondary cancers such as AML (Kelly *et al.* 2002) can limit further treatment options and severely impact the patient's outlook and quality of life. Despite major advances in the understanding of the molecular biology of this disease, the treatment of acute myeloid leukemia (AML) in adults remains challenging. In older adults, AML appears to be a disorder resistant to the available treatments. The outlook for those patients being 4% survival at 5 years (Jackson *et al.* 2002).

In younger patients, regimens available for the treatment have a greater rate of success. Some forms of therapy can produce response rates of 70% with 5-year

relapse-free survival rates of 25% to 40 % (Stone *et al.* 2002). Chromosomal analyses define three prognostic categories with favorable, intermediate, and unfavorable risk. In older adults, AML appears to be an intrinsically resistant disorder of hematopoietic stem cells (Stone *et al.* 2002).

Patients with treatment related leukemia do not respond well to the treatment and their prognosis is very poor. They develop their treatment related cancer 2-5 years after the treatment for the primary cancer (Felix 1998). Not all of the patients develop AML, but it is not clear why this is the case.

In order to carry out investigations of FAC induced DNA damage in this project, an *in vitro* model was designed. It involved co-culturing two different types of cell cultures, the C-450-13, cell line expressing rat cytochrome P450 2B1 and the CEPH cell lines. The designed model allowed for the full assessment of the effect of FAC drug combination on those cell lines. Cyclophosphamide needed to be metabolised to an active form in order to act as anti-cancer agent. Rat cytochrome P450 2B1 metabolised cyclophosphamide and the active metabolites were released into the tissue culture medium.. Without those cells *in vitro* studies conducted on cell lines would not be valid if the drug was inactive (Ming *et al.* 1995). The *in vitro* model provided genetic material for further research.

In order to investigate the changes induced on patients genome by the FAC regime, microarray analysis of gene expression profiles was undertaken. Genomic technologies such as DNA microarray have been used to study biological processes involved in various normal and disease states (Forozan *et al.* 2000). Currently, cDNA microarray analysis enables quantitative measurement of thousands of mRNA expression levels simultaneously. Gene expression profiling

provides comprehensive assessment of gene expression levels in a given tissue or cell population. It also provides information on changes of gene expression in altered physiological or pathological situations (Cooper 2001). Microarrays are particularly suited to study interactions in the regulation of large numbers of different genes, because thousands of genes can be analyzed at the same time. DNA microarrays produce large amounts of data. Complex changes in gene expression are revealed; sometimes hundreds of mRNAs change between experiments. The gene expression profiles obtained during the investigations can be sorted into clusters. Before the microarray analysis the approach to understanding the molecular basis of complex syndromes such as cancer, coronary artery disease, and diabetes was to study the behavior of individual genes. However, it is generally recognized that expression of a number of genes is coordinated both spatially and temporally and that this coordination changes during the development and progression of diseases. The human genome is sequenced, but only a minority of genes have been assigned a function (McPherson *et al.*2001). Whole-genome expression profiling is an important tool for functional genomic studies. Automated technology allows high-throughput gene activity monitoring by analysis of complex expression patterns, resulting in fingerprints of diseased versus normal tissues.

Investigations carried out in this project revealed that the FAC anti-cancer drug combination had a great impact on the gene expressions of the treated cells. Exposure to the FAC drug regime caused some overexpression of some genes and suppression of others. Experiments examining the impact of single doses treatments and combined FAC regime showed variations in gene expression

responses to the xenobiotics. Combined FAC treatment generated the most of overexpressed genes. Gene expression profiles varied depending on the amount of time that the cells were exposed to the FAC drugs. Shorter exposure to FAC drugs generated more overexpressed genes.

During the microarray analysis synergistic interactions between FAC drugs were discovered. Seventeen genes out of the analyzed data was found to have a high synergy index, which means that the combined FAC drug dose had much greater effect than it was predicted from the single drugs. Such synergistic interactions could be very important for future drug discovery and population studies. It would help to tailor drug regime for individuals, thus avoiding administration of drugs which might give severe side effects to particular individuals or not be effective. The data obtained was clustered using Tree View clustering computer software program. It showed that genes exhibiting high synergistic indexes clustered together. Protein studies performed in this project revealed that gene 0expressions do not always correspond to the protein expression. This might be due to factors that might affect transcription. At the present time it is not possible to perform thousands of protein studies as gene expression microarray analysis. That is why in this project only a few proteins were studied. It was found out that the protein expressions were affected by the treatment of lymphoblastoid cells with the FAC drug combination. In only two cases was there no change observed as if the FAC drug did not have an effect on those particular proteins (Caspase 6 and Bag 1). In both cases the microarrayer analysis revealed that gene expression levels of those genes were very high. Other proteins were affected by the FAC drug combination

in different ways. Some of them were induced at level (CDK4) where others were suppressed (Bcl2, Casp 9).

Microarray analysis allowed the detection of the synergistic interactions, which occurred when the CEPH lymphoblastoid cells were treated with the FAC drug combination. Those discoveries are potentially very important for the population studies. They might help to determine drug interactions and prevent individual patients receiving a potentially damaging drug regime.

The impact of the FAC drug combinations on the human genome was assessed on several levels in this project. The possible induction of minisatellite mutations by the FAC regime was investigated. Minisatellites are highly sensitive repeat regions that might be susceptible to changes (Kao *et al.* 1976, Greil *et al.* 1994). MS1 has a particularly highly variable internal structure. Because of its variability and unstable nature it was chosen to act as possible biomarker. The strategy employed to explore its potential as a biomarker involved firstly generating profiles of individuals who were not exposed to anti-cancer drugs. The complexity of MS1 made it impossible to study the whole of the MS1 internal structure. Chosen repeat types were studied – "B" types. Experiments conducted revealed the presence of high diversity within the population with "B" type repeats. The second stage of the strategy was to examine the BB profile at the MS1 region, generated from the DNA treated with anti-cancer FAC drug combination. The FAC drugs due to their biological/toxicological properties have a great impact on the DNA of the individual treated with those drugs. Cyclophosphamide can give rise to mutations by inhibition of DNA synthesis. Adriamycin causes a decrease in DNA replication and 5-fluorouracil inhibits RNA synthesis. (Chabner 1996) The DNA was extracted from

CEPH 1206 lymphoblastoid cells treated with three concentrations of the FAC drug combination, 1.0mM, 0.5mM and 0.1mM. The internal structure of the three profiles obtained did not differ, but there was significant difference in band intensities of individual dimers in different treatments. Those band intensities were individually measured by phosphoimaging system and it appeared that the stronger the drug concentration applied to the DNA the weaker the band intensity. The band intensity signals with higher drug concentration meant that the DNA damage was greater, because the damaged DNA does not amplify very well. This method designed for detecting mutations within MS1 locus needs to be developed further. In the samples treated by the FAC drug combination no mutational changes were observed. Although it might be possible to examine more repeat unit types at the MS1 locus, it was deducted that the evidence made it of low priority to pursue further (personal communication with Professor Sir A.Jeffreys).Longer exposure of cells to the FAC drug combination might have possibly induced greater rate of mutation.

An alternative method to detect DNA damage by identification of MLL/AF4 translocations was explored. In this system PCR based techniques were utilised to detect any MLL/AF4 translocations present both in cell lines and in patients' DNA.

In the investigations the use of a positive control the SEM cell line, containing known MLL/AF4 translocation proved very successful. It allowed for the development of optimal conditions for the study of MLL/AF4 translocations. In this study the translocation was detected at the level of 30 pg of DNA concentration, equivalent to 5 input molecules. The system of MLL and AF4 primers worked well

and the size of PCR products from the translocation present in SEM cells were consistent with its previously reported size/structure (Greil *et al.* 1994).

The studies conducted on both patients DNA and FAC treated CEPH cell lines gave unexpected results. It was expected that patient's samples would have either varied MLL/AF4 translocation profiles or not to have any translocations present. The results obtained during the investigation showed the same MLL/AF4 translocation profile in each investigated sample. This was the case for DNA derived from both hospital samples and treated CEPH cell lines. The result therefore can be described to be inconclusive. Potential translocations appear in the region of 6-8 kb and may be derived from MLL/AF4 translocations, but because they appear in all of the investigated samples, their origin must be confirmed by further study. Examination of blood samples of healthy volunteers might help to determine the extent of translocation occurrence. There are several ways to further investigate these results such as by gel fractionation, prior to the amplification of possible MLL/AF4 translocations. This technique allows for isolation of molecules of interest, in this case MLL/AF4 translocations from wild type molecules. Use of this technique may prove or disapprove the presence of potential MLL/AF4 translocations.

It would also help to establish if the profiles of translocations already generated contain PCR artefacts only and no MLL/AF4 translocations.

Advances in molecular biology have led to a modern pharmacogenetics. Pharmacogenetics as a science is focused on investigating drug metabolizing enzymes and their genetic variability (Guzey *et al.* 2002).

With more information available on the structure of drug targets and the genes coding for them, increasing attention has been directed towards variability in therapeutic response and adverse drug reactions to particular drugs (Guzey *et al.* 2002). Traditionally, genetic drug safety research has focused on variations in single genes with known functions and confirmed association to given adverse drug reactions. A few such examples, malignant hyperthermia, the long QT syndrome and venous thromboembolic disease have been assessed in such way (Berul *et al.*, 2002, Kullman *et a*l., 2002, Lee 2002). The advent of Human Genome Project together and DNA microarray technology, high-output screening systems and advanced bioinformatics have opened new possibilities in the field of drug impact on patients genome. It can be expected that pharmacogenetic research with help of microarray gene profiling will identify situations where a drug should be avoided in certain individuals in order to reduce the risk for adverse drug reactions. With help of microarray analysis it could be possible to generate profiles of selected drugs that might not be safe to use by the individual patient.

Recent advances in technology made it possible for the development of a new approach to protein analysis. Proteomics is a new, fast growing protein analysis, which allows the characterization of the proteome of cells or tissues (Debouck *et al.* 2000). Traditional investigations have yielded abundant information about individual proteins, but those methods did not provide the scientists with an integrated understanding of biological systems. Proteomics allows for studying many proteins simultaneously and gives clearer answers to how proteins interact with each other, as well as with non-protein molecules in order to control complex processes in cells, tissues and even whole organisms (MacBeath 2002). The

complete human genome sequence is now available and the goal for researchers is to assign molecular and cellular functions to thousands of predicted gene products. The new field of proteomics research has a capacity to determine protein expression and function of novel genes on a large scale (Adam *et al.* 2002).

In a field of cancer therapy scientists recently begun to focus on identification of single nucleotide polymorphisms (SNPs) in DNA sequences. It was discovered that those polymorphisms may cause clinically significant alterations in drug metabolizing enzyme activities. Because of SNPs scientists have begun to understand the nature of interindividual differences in drug-induced adverse reactions, toxicity, and therapeutic responses. Those variations in DNA sequences explain some of the variability in drug metabolizing enzyme activities which contribute to alterations in drug clearance and impact patients' response to drug therapy.

The frequency of variant alleles for drug-metabolizing enzymes often differs among ethnic groups (Ma *et al.*, 2002). Continued research in a field of pharmacogenetics will further our understanding in individual differences in drug reactions of patients. The application of this knowledge will ultimately help tailor drug dosing and drug therapy selection, predict toxicity or therapeutic failure, and improve clinical outcomes for individual patients (Ma *et al.* 2002).

The application of DNA microarrays for gene expression profiling opens new horizons not only for pharmacogenetics but also for other biological and medical sciences. This new technique already is used by molecular biologists, pharmacologists and physicians. The practical applications of this technique can be used in drug target discovery studies, toxicological risk assessment of many

xenobiotics, it can possibly help to determine a course of therapy for individual patients.

In this project investigations of the DNA damage induced by the FAC drug combination were undertaken. Great care was taken to study the DNA damage using techniques to investigate gene expression and chromosomal aberrations. Results obtained showed that the anti-cancer drug combination FAC inflicts complex changes in gene expressions and associated chromosomal anomalies. The complexity of those changes illustrated that subtle interaction between gene transcription and chromosomal changes play a very important role in assessing the impact of xenobiotics on the genome and made this project very interesting to undertake.

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