FLUORESCENCE IN SITU HYBRIDIZATION AS A DIAGNOSTIC TOOL FOR THE DETECTION OF THE FANCA delE12-31 AND delE11-17 MUTATIONS.

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Dissertation submitted in accordance with the requirements for the degree

Magister Scientiae in Medical Science
(M. Med. Sc.)

in the

FACULTY OF HEALTH SCIENCES
DIVISION OF HUMAN GENETICS
UNIVERSITY OF THE FREE STATE
BLOEMFONTEIN

NOVEMBER 2005

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DECLARATION

I declare that the dissertation hereby submitted by me for the M. Med. Sc. degree at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. Where help was sought, it was acknowledged. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

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S.J. Nogabe

Dedicated to my parents and in loving memory of my dearest grandmother

ACKNOWLEDGEMENTS

After all those years, I've got quite a list of people who contributed to the completion of this research study, for which I would like to express my gratitude for they have shared in my progress, frustrations, and joyous moments.

It is difficult to overstate my gratitude to my co-supervisor, **Dr M Theron,** for an enormous amount of faith in me and lots of encouraging words, enthusiasm, great efforts, good teaching, lots of good ideas, and inspiration, which was always there when I needed it. I would have been lost without her commitment to helping see this project through to its final completion.

Dr T Pearson for all the supervision he provided.

Prof Gert van Zyl and Prof GHJ Pretorius for helping me overcome difficulties during my studies.

Prof S Jansen, who devoted a great deal of his time to reviewing the manuscript. His valuable advice, proof-reading and comments on the work were really great.

Dr B Visser and Dr J Albertyn for allowing me to use their services.

The **Research Committee** of the Faculty of Health Sciences, University of the Free State, and the **Medical Research Council** for the financial support to conduct this study.

T Muller, whose knowledge of computers and assistance made the writing of this dissertation easier.

IZ Spies, for all the assistance she provided with regard to the cytogenetic section.

All my laboratory personnel for helping out with all the things a student in our lab needs helping out with.

TJA Mongake for helping me get through the difficult times, and for all the emotional support and care he provided.

My family for their unconditional support both financially and emotionally in the last 26 years. Without their faith in me, I could not have survived. In particular, I am in great debt to **my beloved mother** as I will never be able to compensate the number of fine lines that have appeared on her face over this period of time.

Lastly, **My Heavenly Father**, who mercifully gave me the strength and determination to complete this research study.

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ABBREVIATIONS

AFP	α-fetoprotein
AML	acute myelogenous leukemia
amp	ampicillin
AT	ataxia telangiectasia
ATP	adenosine tri-phosphate
bp	base pair
BRCA	breast cancer
$CaCl_2$	calcium chloride
cDNA	complementary DNA
ºC	degrees Celsius
CGH	comparative genomic hybridization
cis-DPP	cis-diaminedichloro-platinum II
DAPI	4′-6′-diamidino-2-phenylindole
dATP	deoxyadenosine-5′-triphosphate
dCTP	deoxycytidine-5′-triphosphate
ddUTP	dideoxyuridine-5′-triphosphate
DEB	diepoxybutane
dGTP	deoxyguanosine-5′-triphosphate
DIG	digoxigenin
DMSO	dimethysulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside 5′-triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
dTTP	deoxythymidine-5′-triphosphate
dUTP	deoxyuridine-5′-triphosphate

EDTA	ethylenediaminetetra-acetic acid
E. coli	*Escherichia coli*
FA	Fanconi anaemia
FAA (C,D1,D2,E,F,G,)	FA group A (C,D1,D2,E,F,G,) genes
FANC A/B/C/D1/D2 E/F/G/H/I/J/L	FA complementation group A,B,C,D1,D2,E,F,G,I,J,L
FICTION	fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocynate
FP	FlexiPrep
g	gram
G2	second gap period of the cell cycle
GFP	green fluorescent protein
hr(s)	hours
HCl	hydrochloric acid
H_2O	water
HSC	haematopoietic stem cells
HLA	human leukocyte antigen
HNPCC	hereditary non-polyposis colorectral cancer
ICL	interstrand cross-links
IPTG	isopropyl-β-D-thiogalactosidase
KAc	potassium acetate
kb	kilobase
KCl	potassium chloride
l	litre
LB	Luria bertani
LOH	loss of heterogeneity
μ	micro

m	milli
M	molar/mitosis
MDS	myelodysplastic syndrome
$MgCl_2$	magnesium chloride
min	minutes
MOPS	3-[N-morpholino] propanesulfonic acid
MMC	mitomycin C
n	nano
N	normal
NaAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
NBS	Nijmegen's breakage syndrome
NP40	nonidet P40
pMol	pico mole
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
pH	potential of hydrogen
PHA	phytohaemagglutinin
PRINS	primed *in situ* labeling
RE	restriction enzyme
RNA	ribonucleic acid
RNase	ribonuclease
RxFISH	cross species colour banding
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
sec	seconds
SET	sodium EDTA tris
SO_4	sulphate

SSC	sodium saline citrate
SSCP	single strand conformation polymorphism
ssDNA	single stranded DNA
Taq	*Thermus aquaticus*
TAR	thrombocytopaenia-absent radii
TBE	tris borate EDTA
TE	tris EDTA
Tm	melting temperature
UTR	untranslated region
UVA	ultraviolet A-rays
UV	ultraviolet
V	volts
VATER/VACTERL	vertebral defects, tracheo-oesophageal atresia, and renal and radial ray defects
vol	volume
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4 chloro-3-indonyl-β-D-galactosidase
XP	xeroderma pigmentosum

CHAPTER 1

INTRODUCTION

1.1 Historical background

Fanconi anaemia (FA) was first described in three brothers with a syndrome of congenital physical anomalies, anaemia, and a fatty aplastic bone marrow (Auerbach, Rogato and Schroeder-Kurth, 1989). According to Fanconi's original description, the three boys, all between five and seven years of age, also had microcephaly, intense brown pigmentation of the skin, skin hemorrhages, hypogonadism, genital hypoplasia, internal strabismus and hyperreflexia. The erythrocytes were hyperchromic and there was no evidence of haemolysis. Subsequently, other FA patients were identified who suffered from leukopaenia and thrombocytopaenia, in addition to anaemia, as a consequence of an aplastic marrow (Alter, 1992; Liu et al., 1994; Alter, 2000; Gluckman, Socié and Guardiola, 2000).

In 1931, Naegeli proposed the name Fanconi's anaemia to distinguish this familial anaemia from the exogenous anaemias caused by inadequate nutrition (Liu et al., 1994). Approximately 20 years later, Estren and Dameshek reported two families in which several siblings had aplastic anaemia, but whose appearances were normal. It took another 30 years before these two syndromes, FA and Estren and Dameshek Type II constitutional aplastic anaemia were demonstrated to be part of the same spectrum of disease, one of the original Estren and Dameshek families was found later to have a relative with classical FA and diepoxybutane (DEB) induced chromosome breakage (Alter, 1993a). FA is usually grouped with inherited cancer-prone syndromes such as Bloom's syndrome, ataxia telangiectasia (AT), and xerodema pigmentosum (XP). For some of these disorders the genetic

defect has been identified and correlated with some aspects of deoxyribonucleic acid (DNA) repair (Liu et al., 1994).

1.2 Differences between FA and Fanconi syndrome

FA has to be considered as a syndrome and not a specific disease. A syndrome is a collection of findings which characterize a condition, for which the specific pathophysiology may not yet be identified and FA certainly belongs in that category (Alter, 1993a). Fanconi syndrome, of which there are six types described, with different modes of inheritance, is a rare and serious disorder of kidney dysfunction, occurring mainly in childhood. In this syndrome, several important nutrients and chemicals are lost in the urine. This leads to failure to thrive, stunted growth, and bone disorders, such as rickets (Frohnmayer and Frohnmayer, 2000).

Fanconi syndrome is already used to describe a specific constellation of renal tubular dysfunction including proteinuria and glycosuria. FA patients may be born with abnormal kidneys and may experience growth problems, but the treatment of FA is very different from that for Fanconi syndrome. The two disorders should not be confused with each other. The primary defects are not haematopoietic, dermatologic, or orthopaedic, but presumably related in some as yet undefined manner to DNA repair (Alter, 1993a; Frohnmayer and Frohnmayer, 2000).

1.3. Haematological aspects

The most important clinical feature of FA is haematological and this is responsible for the greatest morbidity and mortality in homozygotes. At birth, the blood count is usually normal and macrocytosis is often the first detected abnormality. Thrombocytopaenia and anaemia follow this, and pancytopaenia typically presents between the ages of five and ten years, with the median age of onset being seven years. Clinically, the affected FA patient may present with bleeding, pallor, and/or recurring infections. FA patients with abnormal radii have a 5.5 times increased risk of developing bone marrow failure compared with the number of heart, kidney, head, hearing, and developmental abnormalities present (Tischkowitz and Hodgson, 2003). In children without congenital abnormalities the development of haematological abnormalities can be the first presenting feature of FA and can occasionally be the presenting feature in adulthood (Glanz and Frazer, 1982). The true proportion of FA patients, who present in adulthood, may be underestimated even when they exhibit haematologic pathology, because testing for chromosomal fragility is not routine in this age group (Kwee et al., 1997; Liu, Auerbach and Young, 1991). The anaemia is caused by a progressive loss of haematopoietic stem cells (HSC) and thus affects all blood lineages (Grompe and D'Andrea, 2001).

The considerable clinical variability of the disease is shown by the occurrence of severe congenital abnormalities and death from anaemia or acute myelogenous leukaemia (AML) in the first decade of life at one extreme of the clinical spectrum, and at the other end by the presentation of mild anaemia and death from oral cancer during the fifth decade of life (Butturini et al., 1994b; Joenje and Patel, 2001). The classical presentation is progressive bone marrow failure, which first manifest as low platelet counts and which eventually leads to transfusion dependent anaemia in the first two decades of life (Gluckman et al., 1989; Tischkowitz and Dokal, 2004). Actuarial risks of bone marrow failure and leukaemia

(MDS and AML) by 40 years of age are 98 % and 52 %, respectively. The cause of bone marrow failure in FA is unknown. In addition to pancytopaenia, FA patients show other haematologic and immunologic abnormalities, including an elevated level of foetal haemoglobia and a low level of natural killer cell function (Rosselli, Briot and Pichierri, 2003).

Although the most common and well characterized malignancies in FA are haematologic, FA patients have been found to develop a wide array of different neoplasms. FA patients are highly predisposed to non-haematologic (solid) tumours, particularly to AML and squamous cell carcinoma (SCC) of the upper aerodigestive and anogenital tract (Alter, 1996; Kruyt et al., 1996; Faivre et al., 2000; Rosselli, Briot and Pichierri, 2003). This increased cancer susceptibility is most likely due to the high degree of genomic instability and is not well characterized. Other syndromes with a high degree of genomic instability and strong cancer predisposition include AT, Nijmegen's breakage syndrome (NBS), Bloom syndrome, hereditary non-polyposis colorectral cancer (HNPCC), and hereditary breast/ovarian cancer syndromes (Kruyt et al., 1996). The common feature of these disorders is an impaired capacity to maintain genomic integrity, which result in the accelerated accumulation of key genetic changes that promote cellular transformation and neoplasia. Cancer predisposition in these diseases is an indirect result of the primary genetic defect (Joenje and Patel, 2001). Since tumour cells are characterized by chromosomal instability, FA group A gene (*FAA)* was postulated to be a candidate for the gene targeted by loss of heterogeneity (LOH) at 16q24.3 (Levran et al., 1997, Joenje and Patel, 2001; D'Andrea, 2003; Kutler et al., 2003). The term pre-leukaemia has been used for FA patients with either myelodysplastic marrow morphology or clonal marrow cytogenetics. Leukaemia in FA is primarily myeloid, which is also the leukaemia that develops in non-FA patients with myelodysplastic syndrome (MDS) (Alter et al., 1993b).

1.4 Phenotypical features

FA patients display a wide range of clinical features: patients may be severely affected, with multiple congenital malformations, or may have a mild phenotype, with no major malformations (Verlander et al., 1995). The non-haematological phenotype in FA is highly heterogeneous and individuals can have a wide variety of clinical abnormalities. Generalised skin hyper-pigmentation, *café au lait* spots and areas of hypo-pigmentation are often present and may sometimes be the only features present (Tischkowitz and Dokal, 2004). Furthermore, in the age group over 16 years, the most common anomalies of short stature and skin hyper-pigmentation may go unrecognized (Liu, et al., 1991). Skeletal abnormalities commonly include radial ray defects such as hypoplasia of the thumbs and radial ray hypoplasia; other skeletal defects that may occur include congenital hip dislocation, scoliosis and vertebral anomalies. Around one-third of FA patients have renal anomalies including unilateral renal aplasia, renal hypoplasia, horseshoe kidneys; or double uterus. FA is associated with altered growth both *in utero* and postnatally; low birth weight is common and the median height of FA individuals lies around the fifth percentile. This can sometimes be related to growth hormone deficiency or hypothyroidism. Microphthalmia, microcephaly, microstomia, conductive deafness and developmental delay are all often present (Auerbach, Adler and Chaganti, 1981; Strathdee and Buchwald, 1992a; Faivre et al., 2000; Tischkowitz and Dokal, 2004).

Males have a high incidence of genital abnormalities such as hypogenitalia, undescended testes and hypospadias with infertility being the norm, although there have been reports of males with FA fathering children. Females may also have underdeveloped genitalia and uterine anomalies (Tischkowitz and Dokal, 2004). Sexually mature females may have sparse, irregular menses, secondary amenorrhoea, anovulatory periods, premature menopause, and increased risk of gynaecological malignancies (Alter, et al., 1991b). Females can become pregnant if not on androgen therapy. Other abnormalities which are less commonly seen in FA include gastrointestinal defects such as atresia (oesophageal, duodenal, jejunal),

imperforate anus, tracheo-oesophageal fistulae; as well as genital, hearing loss, mental retardation; cardiac defects such as patent ductus arteriosus, ventricular septal defect, pulmonary stenosis, aortic stenosis, aortic coarctation; and central nervous system defects including hydrocephalus, absent septum pellucidum and neural tube defects (Giampietro et al., 1993; Faivre et al., 2000; Tischkowitz and Dokal, 2004).

Anomalies such as vertebral defects, tracheo-oesophageal atresia, and renal and radial ray defects found in the sporadic VATER/VACTERL association, overlap with those found in FA. Thrombocytopaenia-absent radii (TAR) syndrome, which is autosomal recessive, presents with thrombocytopaenia at birth or around the neonatal period and radial ray defects but, unlike FA, thumbs are invariably present bilaterally. Unlike FA, there is no documented increase in haematological or solid tumour malignancies in TAR. Diamond-Blackfan anaemia is characterized by defective erythroid progenitor maturation and usually presents in the first year of life with normochromic or macrocytic anaemia. Over one third have congenital malformations, often involving the head, upper limbs, and genitourinary system. It is slightly more common than FA and most cases are sporadic with evidence of autosomal dominant or less frequently, recessive inheritance (Auerbach, 1994; Tischkowitz and Hodgson, 2003)

1.5 Cytogenetic characteristics

The cellular feature of chromosome instability and sensitivity to DNA bifunctional cross-linking agents were the first to be systematically described in FA (Auerbach, Rogatko and Schroeder-Kurth, 1989; Grompe and D'Andrea, 2001). Many cellular phenotypes have been reported in FA cells, but the most consistent and accepted of these is their hypersensitivity to agents that induce interstrand DNA cross-links (ICLs), such as mitomycin C (MMC), DEB, photoactivated psoralens, furocoumarins in combination with ultraviolet A-rays (UVA), cis-diaminedichloro-platinum II (cis-DPP), nitrogen mustard and cyclophosphamide (Ishida and Buchwald, 1982; Cervenka and Hirsch, 1983; German et al., 1987; Strathdee and Buchwald, 1992a; Buchwald and Carreau, 2000; Gluckman, Socié and Guardiola, 2000; Grompe and D'Andrea, 2001; Rosselli, Briot and Pichierri, 2003).

After ICL treatment FA cells display several phenotypes. These include increased chromosome breakage, radial formation, and other cytogenetic abnormalities seen in metaphase chromosome spreads. The hypersensitivity can manifest itself as apoptosis or growth arrest depending on cell type (Kruyt et al., 1996). FA cells also have a more modest hypersensitivity to other DNA damaging agents such as ionizing radiation and oxygen, or free radicals anomalies in the S-phase checkpoint activation. A second phenotype, which has been established by many studies, is an increase of the proportion of cells with 4N DNA content. This has generally been interpreted as a G2/M delay. The increase of cells with 4N DNA content can occur spontaneously in some FA cells, but become more pronounced after ICL treatment (Dutrillaux et al., 1982; Grompe and D'Andrea, 2001).

Since the chromosomal hypersensitivity to ICL agents is the most consistent characteristic of the FA cells, it is used as a criterion for both pre- and postnatal diagnosis. Chromosome breakage studies can be carried out on amniotic cells, chorionic villus cells, or foetal blood. Importantly, not only the frequency of induced chromosomal aberrations, but also the type of aberrations is indicative of FA. Tri- and quadriradial figures are typically observed in FA cells exposed to ICL

agents. The relationship between these different characteristics and response to DNA damage is not clear (Rosselli, Briot and Pichierri, 2003).

1.6 Diagnosis

The basic criteria for a positive diagnosis of FA are the presence of phenotypic and haematological abnormalities, as well as an increase in chromosome breakage. At least two of these criteria must be met. Early and accurate diagnosis of FA is important, because it profoundly affects patient monitoring and treatment decisions and permits early genetic counselling of family members. Correction between the molecular defect, cellular defects and clinical manifestation is an important task which will lead to better diagnosis and management of affected subjects with the ultimate goal of developing effective gene therapy. Improved haematological management is leading to improved survival of FA patients, but this has resulted in larger numbers of homozygotes reaching the age where they are likely to develop solid tumours and further research is needed to determine optimum treatment for these malignancies (Alter, 2000). Patients with acute leukaemia have been diagnosed with FA after developing toxicity from their bone marrow transplant conditioning regimen (Alter, 2000; Shimamura et al., 2002)

Thirty to forty percent of FA patients lack developmental malformations or a positive family history. Although MMC/DEB testing is highly specific for FA, interpretation is complicated in cases of somatic mosaicism. Another diagnostic technique with comparable accuracy to chromosome breakage studies is based on flow cytometric analysis of cells exposed to DNA cross-linking agents to measure the prolonged progression through, and arrest within, the G2 phase, which is characteristic of FA cells. Such an approach has the advantage that it is less time-consuming and does not require cytogenetic expertise. However, it is not reliable in cases with concurrent myelodysplasia or leukaemia (Shimamura et al., 2002; Tischkowitz and Dokal, 2004; Magdalena et al., 2005).

Serum α-fetoprotein (AFP) levels are consistently elevated in FA patients irrespective of whether liver abnormalities are present and this could be used as a fast and cheap screening test in the sizeable group of individuals with early onset leukaemia or cancer, or other FA-like features. However, diagnostic precision varies with the type of AFP assay technique used; thus, new AFP assays must first be carefully validated prior to complementation. Recently, a new diagnostic test was developed which assays primary lymphocytes for Fanconi anaemia complementation group D2 (FANCD2) protein monoubiquitination by immunoblot. This assay could be used in conjunction with retroviral techniques or direct gene sequencing to provide a rapid diagnostic and subtyping assay (Tischkowitz and Dokal, 2004).

Until recently, there has been no method to determine the complementation group apart from time consuming cell fusion assays, but it has now been shown that retroviruses expressing Fanconi anaemia complementation groups A, C, or G (FANCA, FANCC, or FANCG) complementary DNA (cDNA) can be used to correct the phenotype of T cells from FA patients and thereby determine the complementation group in a rapid, accurate manner. Occasionally, an FA case can be due to biallelic breast cancer type 2 (BRCA2) mutations, which seems to be associated with an increased risk of medulloblastoma or Wilm's tumour that may precede the development of aplastic anaemia or an earlier onset of leukaemia. Subsequent management in all cases of FA depends on the age of presentation and the absence or presence of haematological abnormalities. All patients should have a full haematological assessment that should include examination of the bone marrow, and human leukocyte antigen (HLA) typing in anticipation of possible bone marrow transplantation, which should also be considered (Tischkowitz and Hodgson, 2003).

1.7 Treatment

Optimal treatment regimens for aplastic anaemia depend on the etiology of the bone marrow failure. Given the striking sensitivity of patients with FA to DNA damaging agents, timely diagnosis is critical prior to the use of chemotherapy or radiation therapy in the bone marrow transplant setting (Gluckman, Socié and Guardiola, 2000). The only long-term treatment for FA has been transplantation of bone marrow or umbilical cord haematopoietic cells. The success rate for bone marrow transplantation is only fairly high with HLA-matched siblings but is, unfortunately, low with siblings who do not match as well as HLA-identical unrelated donors (Alter, 1992). Complications often occur after transplantation, such as graft-versus-host disease and tumour formation. In addition, conditioning regimens for transplantation are highly toxic to FA patients, reduced doses of cyclophosphamide and irradiation are now used, but they still contribute to the complications. An alternative curative treatment might be gene transfer into haematopoietic stem cells (Carreau and Buchwald, 1998; Wang and D'Andrea, 2004). Once marrow failure ensues, many patients have a protracted period of pre-aplasia, during which observation and periodic blood counts are needed. The proportion that will ultimately develop full-blown aplastic anaemia is unknown, but it is certainly very high (Magdalena et al., 2005).

The usual treatment for those who do have a donor but wish to delay transplant is androgen. Although oral androgens provide more risk of liver disease, they are easier to manage than injectable medication (Alter, 2000). Therapy for FA is directed at the haematologic manifestations, typically the most life-threatening complications. Although bone marrow transplantation is potentially curative of the haematologic pathology, patients may go on to develop secondary malignancies, often solid tumours of the head and neck. Umbilical cord blood transplantation has also been successfully applied for FA patients. For FA patients lacking a suitable stem cell donor, other approaches to treatment are needed (Fu et al., 1997).

1.8 Genetics of FA

1.8.1 Genetic classification by complementation analysis

The importance of using complementation analysis for the genetic classification of FA patients is several fold. First, complementation analysis is a powerful means to discover new FA genes and would permit an estimate of FA subtype prevalence in the human population even before the corresponding gene has been isolated. In addition, classification of FA families by complementation analysis would allow positional cloning of FA genes through linkage analysis. Furthermore, complementation analysis can be a critical tool in ascertaining the pathogenic status of sequence alterations found in FANCC by mutation screening methods. Finally, in view of upcoming clinical trials designed to correct the bone marrow failure in FA patients by gene therapy, complementation analysis is an important means to select patients who are eligible for such treatment (Joenje et al., 1995). Complementation analysis may be used to assign a patient to a specific group, and to determine population frequencies and founder effects (Alter, 2000).

Complementation analysis by somatic cell fusion (Duckworth-Rysiecki and Taylor, 1985) and correction of cross-linker hypersensitivity has delineated at least twelve complementation groups, FANCA, B, C, D1, D2, E, F, G, I, J, L and M (Kennedy and D'Andrea, 2005; Rodriguez et al., 2005; Taniguchi and D'Andrea, 2006) and eleven FA genes (*FAA, FAC, FAD1, FAD2, FAE, FAF, FAG, and FAL*) have been cloned (Table 1.1) (Strathdee et al., 1992b; Lo Ten Foe et al., 1996; de Winter et al., 1998; de Winter et al., 2000a; de Winter et al., 2000b; Timmers et al., 2001; Howlett et al., 2002; Meetei et al., 2003; Meetei et al., 2004; Levitus et al., 2005; Meetei et al., 2005). FA cells derived from all complementation groups appear to have the same heightened sensitivity to bifunctional cross-linking agents. The assumption that each group corresponds to a distinct FA disease gene has been supported by the identification of genes with pathogenic mutations in most of

these groups (Joenje and Patel, 2001). There is little if any correlation between the clinical and cellular phenotypes of the patients and the complementation groups. Unlike other FA subtypes, FANCD1 cells also have spontaneous chromosome breakage and quadriradial chromosome formation. Clinically, FA patients from each complementation group are similar. A further complementation group, FANCH, has subsequently been shown to belong to the FANCA complementation group (Joenje et al., 2000). The genetic basis of FA is as complex as its highly varied clinical presentation.

Complementation group	Gene	FA patients estimated %	Chromosomal location	Protein AA	Protein products kDa	Exons
FANCA	*FAA*	60	16q24.3	1455	163	43
FANCB	*FANCB*	0.3	Xp22.31	853	95	9
FANCC	*FAC*	15	9q22.3	558	63	14
FANCD1	*FAD1/BRCA2*	4	13q12-13	3418	380	28
FANCD2	*FAD2*	3	3p25.3	1451	155,162	44
FANCE	*FAE*	1	6p21.3	536	60	10
FANCF	*FAF*	2	11p15	374	42	1
FANCG	*FAG/XRCC9*	9	9p13	622	68	14
FANCI	-	rare	-	-	-	-
FANCJ	*BRIP1*	1.6	17q23.2	1249	130	20
FANCL	*FAL*	0.1	2p16.1	375	43 (E3 Ubiquitin ligase)	14
FANCM	*FAM*	rare	14q21.21	2048	250	16

Table 1.1 Genetic data on FA genes and proteins (Kennedy and D'Andrea, 2005; Taniguchi and D'Andrea, 2006)

1.8.2 Cloning of FA genes

The extreme rarity of the disease, together with its genetic heterogeneity has long been obstacles to the cloning of FA genes. Only after a substantial number of families had been assigned to group A by complementation analysis, was the gene defective in this subtype mapped to chromosome 16q24.3 by linkage analysis. A genome wide search using microsatellite markers led to the initial linkage to marker D16S520 (Pronk et al., 1995; Gschwend et al., 1996). Complementation cloning has proved to be the most successful approach to identify FA genes. This method relies on the capacity of a plasmid that expresses a normal copy of the defective FA gene to correct the MMC-sensitive phenotype of an FA lymphoblast cell line (Joenje and Patel, 2001). The *FAB* gene is located on the X chromosome, and all of the reported patients with FANCB are males (Meetei et al., 2004).

The first gene belonging to complementation group C was cloned in 1992 by functional complementation of the cellular phenotype using a cDNA expression library. Cloning of the second gene from complementation group A was achieved independently by functional complementation, like *FAC,* and by positional cloning (Joenje and Patel, 2001). The *FAC* gene, mapped to chromosome 9q22.3 by *in situ* hybridization, is composed of 14 exons, two non-coding 5' regions and three alternate untranslated regions (UTRs) (Strathdee et al., 1992b). The gene for FANCD1 subtype, FAD1, is identical to a breast/ovarian cancer susceptibility gene, *BRCA2*. A third method, which led to the cloning of *FAD2*, combined features of both the complementation and positional cloning approaches (Timmers et al., 2001). This method is relatively laborious and time consuming, but it is also robust. To clone the *FAD2* gene the chromosome that carries the disease gene was first identified by fusing a panel of microcells which contain single human chromosomes with an immortalized FA-D fibroblast cell line. The critical region of the *FAD2* gene was mapped to the short arm of chromosome 3p25.3 and eventually narrowed to a

200kb interval, where *FAD2* was identified as the disease gene (Whitney et al., 1995). These data suggest that for FA the "one group = one gene" concept does seem to hold up (Buchwald, 1995). The *FAE* (de Winter et al., 2000a) and *FAF* (de Winter et al., 2000b) genes were both cloned by functional complementation (Tischkowitz and Hodgson, 2003). The *FAF* gene has been mapped to chromosome 11p15 (Gschwend et al., 1996). The identification of *XRCC9,* a gene proposed to be involved in cell cycle regulation or post-replication repair (Garcia-Higuera et al., 2000), as the equivalent of *FAG* places the latter locus at 9p13 (de Winter et al., 1998).

1.8.3 Incidence

FANCA and FANCC mutations are the most prevalent, accounting 65% and 5-15% of FA patients. The world-wide prevalence of distinct FA gene mutations varies strongly depending on the geographic region or ethnic background of each population studied (Savoia et al., 1996; Tischkowitz and Hodgson, 2003; Magdalena et al., 2005; Rodriguez et al., 2005). Founder mutations have been described in Ashkenazi Jews (FANCC) who has an appropriate carrier frequency of 1 in 89 (Yamashita et al., 1996), and an even higher prevalence was reported in Gypsy families (FANCA) from Spain with an estimated carrier frequency of 1 in 64 to 1 in 70 (Callén et al, 2005). Morgan and co-workers described the FANCG deletion as an ancient founder mutation in Bantu-speaking populations of sub-Saharan Africa (Morgan et al., 2005), while the white Afrikaans speaking population of South Africa, the so-called Afrikaner (FANCA), presented with a carrier frequency of ~1 in 83 (Tipping et al., 2001; Tischkowitz and Hodgson, 2003).

1.8.4 The founder effect in the Afrikaner population

Tipping et al (2001) genotyped FA families of the Afrikaner population and detected the FANCA haplotype (Tipping et al., 2001). Mutation screening of the *FAA* gene revealed association of these haplotypes with four different mutations. The most common was an intragenic deletion of exons 12-31 (delE12-31), accounting for approximately 60% of all clinical FA cases, followed by deletion of exons 11-17 (delE11-17), which accounts for 13% of the FA phenotype, and a single nucleotide deletion in exon 34 (3398delA) which accounts for 7% of clinical FA cases. Screening for these mutations in the European populations ancestral to the Afrikaners detected one patient from the Western Ruhr region of Germany who was a carrier for the major deletion. The mutation was associated with the same unique FANCA haplotype as in the Afrikaner patients. Genealogical investigation of twelve Afrikaner families with FA revealed that all were descended from a French Huguenot couple who arrived at the Cape on 5 June 1688. Mutation analysis showed that the carriers of the major mutations were descendants of this same couple. The molecular and genealogical evidence is consistent with transmission of the major mutation to western Germany and the Cape near the end of the 17th century, confirming the existence of a founder effect for FA in South Africa (Tipping et al., 2001).

1.8.5 Mutational profile in *FAA*

A large number of gene mutations have been identified in each complementation group, but no clear association between the mutation and the clinical or cellular phenotypes has been established (Wang and D'Andrea, 2004). Over 100 different mutations have been reported in *FAA* (Lo Ten Foe et al., 1996; Levran et al., 1997; Morgan et al., 1999), with 30% point mutations, 30% with one to five base pair microdeletions or microinsertions, and 40% with large deletions, removing up to 31 exons from the gene. Small duplications have also been reported. The large deletions often occur at specific breakpoints and have been shown to arise as a result of *Alu* mediated recombination. The tremendous heterogeneity of the mutation spectrum and the frequency of intragenic deletions present a considerable challenge for the molecular diagnosis of FA. Mutation screening of the *FAA* gene is a difficult task, since the coding sequence consists of 43 exons, and the mutational spectrum is very heterogeneous (Ianzano et al., 1997; Morgan et al., 1999; Adachi et al., 2002; Tischkowitz and Hodgson, 2003). Joenje et al. (1995) screened the *FAA* gene for mutations and found the cell line to be a compound heterozygote for two mutations: a missense mutation in exon 29 and a mutation that removes exons 17-31 from the open reading frame. Table 1.2 lists reported mutations in *FAA*. The identification of patients with specific mutations in the FA genes may lead to a better clinical description of this condition, also providing data for genotype-phenotype correlations, to a better understanding of the interaction of this specific mutation with other mutations in compound heterozygote patients, and ultimately to the right choices of treatment of each patient with improvement of the prognosis (Magdalena et al., 2005).

1.8.6 Molecular interactions of FA proteins

Sensitivity to DNA-damaging agents is often associated with a defect in a DNA repair pathway, as is the case with AT, Bloom's syndrome, HNPCC, Werner syndrome and the nucleotide excision repair syndromes, XP, Cockayne syndrome and trichothiodystrophy (Levran et al., 1997; Carreau and Buchwald, 1998). In FA, sensitivity to DNA cross-linking agents suggests a role for the FA proteins in a DNA repair pathway specific for cross-link repair (Carreau and Buchwald, 1998). The breast tumour suppressor genes BRCA1 and BRCA2 play a role in DNA repair (Cleton-Jansen et al., 1999). Biochemical studies have indicated that the FA proteins, FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM form a multisubunit nuclear core complex (Yamashita et al., 1998; Garcia-Hinguera, 2000; Adachi et al., 2002; Shimamura et al., 2002; Zdzienicka and Arwert, 2002; Wang and D'Andrea, 2004). The monoubiquitination of FANCD2 by FANCL (an E3 ubiquitin ligase) is impaired in cells lacking any member of the upstream FA core complex, thus explaining their common hypersensitivity to DNA cross-linking agents (Medhurst et al., 2001; Shimamura et al., 2002). Direct interactions between FANCA and BRCA1, FANCG and FANCD1/BRCA2 and between FANCD2 and FANCD1/BRCA2 have been described (Kruyt and Youssoufian, 1998; Grompe and D'Andrea, 2001; Folias et al., 2002; Ventikaraman, 2002).

Exons	Mutation	Published name	Amino acid change	Mutation type	Proper Nomenclature
1	1A>G	1A>G	M1V	AA substitution	p.M1? (c.1A>G)
1	c.2T>C	2T>C	M1T	AA substitution	p.M? (c.2T>C)
1	24C>G	24C>G	N8K	AA substitution	p.Asn8Lys (c.24C>G)
1	44_69del26 c.44_69del26			Deletion	c.44_del26
1	65G>A	65G>A	W22X	Stop codon	p.Try22X (c. 65G>A)
1	66G>A	66G>A	W22X	Stop codon	p.Try22X (c. 66G>A)
1	1_2981del2981	1-2981del		Deletion	DelExon1-30 (c. -32-? 2981del)
1	DelExon1	c.-32-?_79+?del		Exon skip	DelExon1 (c.-32-?_79+?del)
2	100A>T	100A>T	K34X	Stop codon	p.Lys34X (c. 100A>T)
2	154C>T	154C>T	R52X	Stop codon	p.Arg52X (c.154C>T)
2	163C>T	163C>T	Q55X	Stop codon	p. Gln55X (c.163C>T)
2	IVS2-1G>T	IVS2-1G>T		RNA splicing	c.190-1G>T
2	IVS3+3A>C IVS3+3A>C			RNA splicing	c.283+3A>C
4	401dupC	401insC		Frameshift	c.401dupC
4	416_417delTG	c.416_417delTG		Frameshift	c.416_417delTG
5	*427-522*del *427-522*del			Deletion	DelExon5 (c.427-?_522+?del)
5	513G>A	513G>A	W171X	Stop codon	p.Try171X (c.513G>A)
6	523_1359del836	523-1359del		Deletion	DelExon6-14 (c. 523_1359del836)
6	523_3066del2544			Deletion	
6	542C>T	542C>T	A181V	AA substitution	p. Ala181Val (c.542C>T)
6	IVS6-2A>G	IVS6-2A>G		Deletion	c.597-2A>G
7	597_1826del1229	597-1826del		Deletion	DelExon7-20 (c.597_1826del)
7	597_3066del2470	597-3066del		Deletion	DelExon7-31 (c.577_3066del)
7	IVS7+1G>A IVS7+1G>A			RNA splicing	c.709+1G>A
7	IVS7+5G>A 709+5G->A			RNA splicing	709+5G>A
7	c.709+5G>T IVS7+5G>T			RNA splicing	IVS7+5G>T
8	732G>C	732G>C	L244F	AA substitution	p.Leu244Phe (c. 732G>C)
8	790C>T	790C>T	Q264X	Stop codon	p.Gln264X (c.790C>T)
9	795_808del14	795-808del		Frameshift	
9	811C>T	c.811C>T	Q271X	Stop codon	p.Gln271X (c. 811C>T)
9	IVS9+3delA IVS9+3delA	793del34		Exon skip	c.826+3delA
9	IVS9-1G>T	IVS9-1G>T		RNA splicing	c.827-1G>T
10	Ex10_12del* Deletion Ex –10-12			Deletion	DelExon10-12 (c.827-?1083+?del)
10	Ex10_17del* Deletion Ex 10-17			Deletion	DelExon10-17 (c.827-?_1626+?del)
10	827_1225del399	827-1225del		Deletion	delexon10-13 (c.827_1225del)
10	856C>T	856C>T	Q286X	Stop codon	p.Gln286X (c.856C>T)
10	862G>T	862G>T	E288X	Stop codon	p.Glu288X (c. 862G>T)
10	890_893delGCTG	890-893del		Frameshift	c.890_893delGCTG
10	IVS10+1G>T IVS10+1G>T			RNA splicing	
10	IVS10-1G>A IVS10-1G>A			RNA splicing	c.894-1G>A
10	IVS10-2A>G c.894-2A>G			RNA splicing	c.894-2A>G
10	IVS10-2A>G IVS10-2A>G			RNA splicing	
11	894_1006del113	938-1050del		Frameshift	
11	894_1359del466	894-1359del		Frameshift	delExon11-14 (c. 894_1359del)
11	987_990delTCAC	987-990del		Frameshift	c. 987_990delTCAC
11	IVS11-1delG IVS11-1delG			RNA splicing	c.1007-1delG
12	1034_1035delAG	c.1034_1035delAG		Frameshift	c.1034_1035delAG
13	1115_1118delTTGG	1159-1162delTTGG		Frameshift	c.1115_1118delTTGG

Exons	Mutation	Published name	Amino acid change	Mutation type	Proper Nomenclature
13	1164_1165delAG	1164-1165del		Frameshift	c.1164_1165delAG
13	1191_1194delTGTG	1191delTGTG		Frameshift	c.1191_1194delTGTG
13	IVS13(-6)_(-2)del	c.1226(-6)_(-2)del		Unknown	c.1226(-6)_(-2)del
14	1303C>T	1303C>T	R435C	AA substitution	p. Arg435Cys (c.1303C>T)
14	IVS14+1G>C IVS14+1G>C			RNA splicing	c.1359+1G>C
15	1360_1626del270	1360-1626del		Deletion	DelExon15-17 (c.1360_1626del
15	1360_1826del467	1391del467		Frameshift	DelExon15-20 (c.1360_1826del)
15	1459dupC	1459-1460insC		Frameshift	c.1459dupC
15	IVS15-1G>T IVS15-1G>T			RNA splicing	c.1471-1G>T
16	1471_1626del156	1515-1670del		Deletion	
16	1471_1826del355	1471-1826del		Deletion	DelExon16-20 (c.1471_1826del)
16	1475A>G	1475A>G	H492R	AA Substitution	p. His492Arg (c.1475A>G)
16	IVS16+3A>C IVS16+3A>C			Exon skip	c.1566+ 3A>G
16	IVS16-20A>G	IVS16-20A>G		RNA splicing	c.1567-20A>G
16	IVS16-2A>G IVS16-2A>G			RNA splicing	c.1567-2A>G
17	1606delT	1606delT		Frameshift	c.1606delT
17	1615delG	1615delG		Frameshift	c. 1615delG
17	1693delT	1693delT		Frameshift	
18	1627_1900del274	1671-1944del		Frameshift	delExon18-21 (c.1627-?_1900+?del)
19	1751_1754delTCCC	1751-1754del		Deletion	c.1751_1754delTCCC
19	1771C>T	1771C>T	R591X	Stop codon	p. Arg591X (c.1771C>T)
19	IVS19-7del10 IVS19-7del10			Unknown	c.1777_7del10
20	1792G>A	1792G>A	D598N	AA substitution	p.Asp-598Asn (c.1792G>A)
21	1827_2778del951	1827-2778del		Deletion	del Exon21-28 (c.1827_2778del)
22	2005C>T	2005C>T	Q669X	Stop codon	p. Gln669X (c.2005C>T)
22	1944delG	1944delG		Frameshift	1944delG
22	IVS22-1G>T IVS22-1G>T			RNA splicing g	p.Leu684Pro(c.2015-1G>T)
23	1901_2778del878	1932del879		deletion	delExon22-28 (c.1901_2778del)
23	2026C>T	c.2026C>T	Q676X	Stop codon	p.Gln676X (c.2026C>T)
23	2051T>C	c.2051T>C	L684P	AA substitution	c.2051T>C
23	2066delG	2066delG		Frameshift	c.2066delG
23	2107C>T	2107C>T	Q703X	Stop codon	p. Gln703X (c.2107C>T)
24	2167_2169delCTG	2167-2169delCTG	723delL	Deletion	p. Leu723del (c.2167_2169delCTG)
24	2172dupG	2172-2173insG		Frameshift	c.2172dupG
24	IVS24+166A>G	IVS24+166A>G		Insertion	c.2222+166A>G
25	2290C>T	c. 2290C>T R764W		AA substitution	p.Arg764Try (c.2290C>T)
25	2303T>C	c.2303T>C	L768P	AA substitution	p. Leu768Pro (c.2303T>C)
25	2314C>T	2314C>T	Q772X	Stop codon	p. Gln772X (c.2314C>T)
26	2450T>C	2450T>C	L817P	AA substitution	p. Leu817Pro (c.2450T>C)
26	2495_2497delTCT	2495-2497del 832delF		Deletion	p. Phe832del (c. 2495_2497delTCT)
16	IVS26+2T>C IVS26+2T>C			RNA splicing	
26	IVS26+134A>G	IVS26+134A>G		Frameshift	c.2504+134A>G
27	2524delT	2524delT		Frameshift	c. 2524delT
27	2533_2536delCTCT	c.2533_2536delCTCT		Frameshift	c.2533_2536delCTCT
27	2534T>C	2534T>C	L845P	AA substitution	p. Leu845Pro (c. 2534T>C)
27	2535_2536delCT	2535-2536del		Frameshift	c. 2535_2536delCT
27	2546delC	2546delC		Frameshift	c.2546delC
27	2574C>G	2574C>G	S858R	AA substitution	p.Ser858Arg (c.2574C>G)
27	IVS27-1G>A	IVS27-1G>A		Deletion	c.2602-1G>A
27	IVS27-2A>T	IVS27-2A>T		Deletion	c.2606-2A>T
28	2604_2609delTCAGTT	c.2604_2609delTCAGTT		Frameshift	c.2604_2609delTCAG

Exons	Mutation	Published name	Amino acid change	Mutation type	Proper Nomenclature
28	2606A>C	c.2606A>C	Q869P	AA substitution	p.Gln869Pro (c.2606A>C)
28	2678G>A	c.2678G>A	W893X	Stop codon	Try893X (c.2678G>A)
28	2708G>A	c.2708G>A	W903X	Stop codon	p.Try903X (c.2708G>A)
28	2730_2731delCT	c.2730_2731delCT		Frameshift	c.2730_2731delCT
28	2738A>C	c.2738A>C	H913P	AA substitution	p.His913Pro (c.2738A>C)
28	IVS28+83C>G	IVS28+83C>G	928ins28aa+Stop	RNA splicing	c.2778+83C>G
29	2779_3066del287	2779-3066del		Deletion	DelExon29-31 (c. 2779-3066del)
29	2779_3348del570	2779-3348del		Deletion	DelExon29-33 (c. 2779_3348del)
29	2806G>A	c.2806G>A	E936K	AA substitution	p.Glu936Lys (c.2806G>A)
29	2807A>G	c. 2807A>G E936G		AA substitution	p.Glu936Gly (c.2807A>G)
29	2812_2830dup19	2831dup2812-2830		Frameshift	
29	2815_2816ins19	2815_2816ins19		Insertion	c.2815_2816ins19
29	2840C>G	2840C>G	S947X	Stop codon	p.Ser941X (c. 2840C>G)
29	2851C>T	c.2851C>T	R951W	AA substitution	p.Arg951Trp (c.2851C>T)
29	IVS29-2A>C c.2853-2A>C			RNA splicing	c.2853-2A>C
29	IVS29(-19)_1del19	IVS29-19del19		RNA splicing	c.2853-19del19
31	2982_3066del85	2982-3066del		Deletion	delExon31 (c.2982_3066del)
31	*2982_4365del1383	*2982-4365del		Deletion	delExon31-43 (c.2982-?_4365del)
31	3061_3154del94	3061-3154del		Frameshift	delExon31-32 (c.3061_3154del)
31	IVS31+1G>A c.3066+1G>A			RNA splicing	c.3066+1G>A
32	3091C>T	3091C>T	Q1031X	Stop codon	p.Gln1031X (c.3091C>T)
32	3130C>T	3130C>T	Q1044X	Stop codon	p.Gln1044X (c.3130C>T)
32	3163C>T	3163C>T	R1055W	AA substitution	p.Arg1055Try (c.3163C>T)
32	3164G>T	3164G>T	R1055L	AA substitution	p.Arg1055Leu (c.3164G>T)
32	3188G>A	3188G>A	W1063X	Stop codon	p.Try1063X (c. 3188G>A)
32	3239G>T	c.3239G>T	R1080L	AA substitution	p.Arg1080Leu (c.3239G>T)
32	IVS32-1G>A IVS32-1G>A			RNA splicing	c.3240-1G>A
33	3288G>C	c. 3288G>C Q1096H		AA substitution	p.Gln1096His (c.3288G>C)
33	3329A>C	3329A>C	H1110P	AA substitution	p.His1110Pro (c. 3329A>C)
34	3349A>G	3349A>G	R1117G	AA substitution	p.Arg1117Gly (c.3349A>G)
34	3382C>G	3382C>G	Q1128E	AA substitution	p.Gln1128Glu (c. 3382C>G)
34	3391A>G	3391A>G	T1131A	AA substitution	p.Thr1131Ala (c.3391A>G)
34	3396_3399delCCAC	3396-3399del		Frameshift	c.3396_3399delCCAC
34	3398delA	3398delA		Frameshift	c.3398delA
34	3403_3405delTTC	3403-3405delTTC	1135delF	Deletion	p.Phe1135del (c.3403_3405delTTC)
36	3520_3522delTGG	3520-3522del	W1174del	Deletion	p.Try1174del (c.3520_3522delTGG)
36	3558dupG	3558dupG		Frameshift	
36	3559insG	3559insG		Frameshift	c.3558dupG
36	3592C>T	3592C>T	Q1198X	Stop codon	p.Gln1198X (c.3592C>T)
37	3629dupT	3629-3630insT		Frameshift	c.3629dupT
37	3639delT	3639delT		Frameshift	c.3639delT
37	3703C>G	c.3703C>G	Q1235E	AA substitution	p.Gln1235Glu (c.3703C>G)
37	3715_3729del15	3715-3729del	1239del5	Deletion	p.Glu1239_Arg1243del (c.3715_3729del15)
37	3760G>T	3760G>T	E1254X	Stop codon	p.Glu1254X (c. 3760G>T)
37	3760_3761del GA	3760-3761del		Frameshift	c. 3760_3761del GA
37	3762_3763insAG	3762_3763insAG		Frameshift	c.3762_3763insAG
38	3786C>G	3786C>G	F1262L	AA substitution	p.Phe1262Leu (c. 3786C>G)
38	3788_3790delTCT	3788-3790del	F12 63del	Deletion	p.Phe1263del (c. 3788_3790delTCT)
38	Exon 38del	Exon 38del		Exon skip	delExon 38 (c.3766_3828del)
38	3813dupA	c.3813dupA		Frameshift	c.3813dupA

Exons	Mutation	Published name	Amino acid change	Mutation type	Proper Nomenclature
38	IVS38-1G>C IVS38-1G>C			RNA splicing	c.3829-1G>C
39	3846_3856del11	3846_3856del11		Deletion	c.3846_3856del11
39	3884T>A	3884T>A	L1295X	Stop codon	p.Leu1295X (c.3884T>A)
39	3920delA	3920delA		Frameshift	c.3920delA
39	3904T>C	3904T>C	W1032R	AA substitution	p.Trp1032Arg (c.3904T>C)
40	3971C>T	3971C>T	P1324L	AA Substitution	p.Pro1324Leu (c.3971C>T)
40	4010delG+18 4010delG+18		Exon skip	c.4010delG+18	
40	IVS40+(1-18)del	IVS40+1-18del		Exon skip	c.4010+(1_18)del
41	4015delC	4015delC		Frameshift	c.4015delC
41	4017_4021delCTCCT	c.4017_4021delCTCCT		Frameshift	c.4017_4021delCTCCT
41	4069_4082del14	4069-4082del		Frameshift	c.4069_4082del14
41	4075G>T	4075G>T	D1359Y	AA Substitution	p.Asp1359Try (c.4075G>T)
41	4080G>C	4080G>C	M1360I	AA Substitution	p.Met1360Ile (c. 4080G>C)
41	IVS41-2A>G IVS41-2A>G		Deletion	c.4168-2A>G	
42	4195G>C	4195G>C	A1399P	AA substitution	p.Ala1399Pro (c.4195G>C)
42	4198C>T	4198C>T	R1400C	AA substitution	p.Arg1400Cys (c.4198C>T)
42	4249C>G	4249C>G	H1417D	AA substitution	p.His1417Asp (c.4249C>G)
42	4267_4404del138	4267del138		Deletion	delExon43 (c.4261_4404del)
42	IVS42(-19)_(-12del)	c.4261(-19)_(-12del)		Unknown	c.4261(-19)_(-12del)
43	4275delT	4275delT		Frameshift	c.4275delT
	Del Exon 1-6 c.-32-?_596+?del			Exon skip	Del Exon 1-6 (c.-32-?_596+?del)
	Del Exon 1-43	Del Ex 1-43		Deletion	DelExon 1-43 (c.-32-?_5481del)
	Del Exon 6-31	Del Ex 6-31		Deletion	DelExon6-31 (c.523_3066del)
	Del Exon 8-42	Del Ex 8-42		Deletion	delExon8-42 (c.710-?4260+?del)
	Del Exon 11-17	Del Ex 11-17		Deletion	DelExon11-17 (c.894_1626del)
	Del Exon13	c.1084-?1225+?del		Deletion	delExon13 (c. 1084-?1225+?del)
	Del Exon16	c.1471-?_1566+?del		Deletion	DelExon16 (c. 1471-?_1566+?del)
	Del Exon16-17	DelEx16-17		Deletion	DelExon16-17 (c .1471_1626del)
	Del Exon16-22	c.1471-?2014+?del		Deletion	DelExon16-22 (c.1471-?2014_+?del)
	Del Exon 16-23	c.1471-?_2151+?del		Deletion	delExon16-23 (c.1471-?_2151+?del)
	Del Exon 16-26	c.1471-?_2504+?del		Deletion	Delexon16-26 (c.1471-?_2504+?del)
	c. -32-?_1900del			Exon skip	5'UTR-21 (c.-32-?_1900del)
	c.427-?_1006+?del			Deletion	c.427-?_1006+?del
	c.427-?_3066+?del			Deletion	DelExon5-31 (c.427-?_3066+?del)
11-31	1007_3066del1060	1007-3066del		Deletion	DelExon12-31 (c.1007_3066del)
c.1471-?_4010+?del	c.1471-?_4010+?del			Deletion	DelExon16-40 (c.1471-?_4010+?del)
c.1567-?_3066+?del				Deletion	delExon20-40 (c.1777-?_4010+?del)
c.1827-?_1900+?del				Deletion	delExon21 (c.1827-?_1900+?del)
c.1901-?_2014+?del	c.1901-?_2014+?del			Deletion	DelExon22 (c.1901-?_2014+?del)
c.1901-?_2981+?del	c.1901-?_2981+?del			Deletion	DelExon22-30 (c.1901-?_2981+?del)
c.3240-?_3828+?del	c.3240-?_3828+?del			Deletion	DelExon33-38 (c.3240-?_3828+?del)
c.3240-?_4010+?del	c.3240-?_4010+?del			Deletion	DelExon33-40 (c.3240-?_4010+?del)
c.3766-?_4010+?del	c.3766-?_4010+?del			Deletion	DelExon38-40 (c.3766-?_4010+?del)
c.3935-?_4260+?del	c.3935-?_4260+?del			Exon skip	delExon40-42 (c.3935-?_4260+?del)
	Ex 24-28del Exon 24-28del			Deletion	delExon24-28 (c.2152_2778del)
	*5'UTR_522*del	*5'UTR-522*del		Deletion	-32-?_522+?del
	*5'UTR-1900*del	*5'UTR-1900*del		Deletion	
	*5'UTR-3066*del	*5'UTR-306*del		Deletion	c.-32-?_3066del

Table 1.2 Mutations detected in the FAA gene (http://www.rockefeller.edu/fanconi/mutate)

An asterisk (*) denotes that the deletion endpoint is undefined.

1.8.7 Mutation detection methods

Several methods have been described to identify exonic deletions and duplications, including Southern blotting (Sellner and Taylor, 2004), long range PCR, real-time PCR (Shimamura et al., 2006), reverse-transcriptase PCR (Bouchlaka et al., 2003; Savino et al., 2003), multiplex ligation-dependent probe amplification (MLPA) (Sellner and Taylor, 2004; Hearle et al., 2006), multiplex amplification and probe hybridization (MAPH) (Sellner and Taylor, 2004), quantitative fluorescent PCR (Morgan et al., 1999; Tipping et al., 2001; Callén et al., 2004) and fluorescent *in-situ* hybridization (FISH) (Ligon et al., 1997). Limitations imposed by some of these screening strategies involved the failure to detect intragenic deletions in heterozygous state. This problem was overcome by the use of gene dosage analysis combined with a mutation detection method.

1.8.7.1 Multiplex ligation-dependent probe amplification

MLPA is a high resolution method used to detect copy number variation in genomic sequences. This technique has rapidly gained acceptance in genetic diagnostic laboratories due to its simplicity compared to other methods, relatively low cost, capacity for reasonably high throughput and perceived robustness (Hearle et al., 2006). MLPA analysis are used to detect exonic deletions up to deletion of the entire *FAA* gene. This technique relies on the ligation and subsequent PCR amplification of two adjacently hybridizing probes using fluororescently labeled universal oligonucleotides complementary to synthetic sequence tags present on every probe. Each probe is design to ensure that a spectrum of uniquely sized PCR products is generated that can be individually quantified by electrophoretic analysis (Sellner and Taylor, 2004; Hearle et al., 2006).

Deletions of probe recognition sequences will be apparent by a 35-50% reduced relative peak area of the amplification product of the probe. However, molecular lesions or polymorphisms close to the probe ligation site may also result in a reduced relative peak area. Another possible drawback will be the requirement of an additional method to confirm the presence of an apparent deletion of a single exon (Shimamura et al., 2006).

1.8.7.2 Multiplex amplification and probe hybridization

In contrast to MLPA, where genomic DNA is hybridized in solution to probe sets, this method relies on the fixation of genomic DNA on a membrane followed by hybridization with a set of probes corresponding to the target sequence. Probes are generated by cloning the target sequences into a plasmid vector followed by PCR amplification with primers directed to the vector. This resulted in amplification products with the same flanking regions. Multiplexed probes have to be of different size to resolve during electrophoresis, which may be a restriction to this method. After hybridization the unbound probes are removed and the specifically bounded probe, proportional to its target copy number, is stripped from the membrane and simultaneously PCR amplified with the universal primer pair. Products are subsequently separated by electrophoresis and a relative comparison is made between band intensities or peak heights, depending on the detection method. Band intensities or peaks are compared to the internal control probe. A reduction in peak or band intensity will result in a decrease in gene copy number (deletion), while an increase in band intensity or peak results in a increase in copy number (duplication) (Sellner and Taylor, 2004).

1.8.7.3 Quantitative PCR

A quantitative fluorescent PCR gene dosage assay has been successfully employed in detecting large intragenic deletions in FA (Morgan et al., 1999; Tipping et al., 2001; Callén et al., 2004). Morgan and co-workers developed a two-step fluorescent based multiplex PCR to detect both small mutations and heterozygous deletions. In the first step the entire coding region was amplified by RT-PCR and sized on agarose gels. Aberrantly sized fragments were further characterized by automated sequencing. Secondly, a quantitative fluorescent multiplex PCR was employed to simultaneously amplify 11 exons of the *FAA* gene. Dosage analysis was established to collectively screen for heterozygous deletions (Morgan et al., 1999). Taking advantage of this novel method Tipping and co-workers described the most common founder mutation in the Afrikaner, delE12-31 (Tipping et al., 2001). With some minor modifications and optimization to this novel method, Callén and co-workers applied this strategy and described a founder intragenic deletion in Spanish FA patients (Callén et al., 2004).

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1.9 Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) involves the evaluation of DNA in metaphase or interphase cells using labelled DNA probes and microscopic signal counting. The use of different photoreactive dyes allows various chromosomal sequences of interest to be colour-coded and multiple sequences to be probed simultaneously. It offers many advantages, one of the most important being the localization of molecular abnormalities within the cellular context of the tissue. This allows one to distinguish changes occurring in *in situ* carcinoma versus invasive carcinoma and to compare these signals with those of admixed normal stromal or epithelial cells. Limitations of this technology include the requirement to develop robust protocols for different types of tissue specimens, and the need to design and combine probes for specific applications. Differences in fixation times, such as are commonly encountered in routine surgical pathology practice, may also limit the efficient application of FISH to all specimens (Dillon, 2002).

The applications of FISH include microdeletion analysis, identification of marker chromosomes, characterization of structural rearrangements and gene rearrangement associated with neoplasia, ploidy analysis for both prenatal and tumour diagnosis, preimplantation analysis, and gene amplification studies. It allows the detection of nucleic acids with exquisite sensitivity and specificity while the integrity of the cells and the morphology of tissues remain preserved. A main advantage is the ability to detect heterozygous deletions, which allows the identification of disease carriers (Blancato, 1999). FISH has gained infinite and most valuable application in the routine diagnosis of several microdeletion syndromes. Various commercially available FISH probes are successfully implemented as a diagnostic tool in Williams syndrome, Prader-Willi/Angelman syndromes, Smith-Magenis syndrome, DiGeorge/velocardiofacial syndrome and many more (Ligon et al., 1997).

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1.9.1 Labeling methods

A variety of isotopic and nonisotopic labels can be incorporated into probes. The label must be stable when exposed to the chemicals, solvents and high temperatures used and must be easily incorporated into the DNA/RNA (ribonucleic acid) using a reproducible labeling system. Finally the labeled nucleotide must be designed in such a way that it does not obstruct the labeling reaction and avoids steric hindrance in the subsequent detection system by having a spacer arm of appropriate length. There are two main types of labeling strategy: the direct and indirect labeling (Southern and Herrington, 1998).

1.9.1.1 Direct labeling

The detectable molecule is bound directly to the nucleic acid probe so that the probe target hybrid can be visualized under a microscope immediately after the hybridization reaction (Bauman et al, 1980, Bauman et al, 1984 and Renz & Kurz 1984). It includes direct incorporation of fluorescent tags, or cross-linking enzyme molecules directly into nucleic acid. The label is integral to the probe; adding a fluorescent dye at the end of a sequencing reaction. Direct labels include derivatives of rhodamine, fluorescein isothiocynate (FITC), and Texas Red fluorescent dyes (Sinclair, 1999). For such methods it is essential that the probe-reporter bond survive the rather harsh hybridization and washing conditions. Perhaps more important, however, is that the reporter molecule does not interfere with the hybridization reaction (Gilliam and Tener, 1986; Reisfeld et al., 1987; Viscidi et al., 1986).

1.9.1.2 Indirect labeling

Most systems are based on indirect detection, in which hapten-modified nucleotides are detected with a secondary reagent. In these systems, hapten-modified nucleotides are incorporated into a probe molecule either by internal incorporation, end labeling or chemical modification. After hybridization, the hapten is detected using a labeled antibody or other specific binding protein (Sinclair, 1999). The presence of the label should not interfere with the hybridization reaction or the stability of the resulting hybrid (Landegent et al, 1984).

1.9.2 Types of non-radioactive labels

1.9.2.1 Digoxigenin

The digoxigenin (DIG) system is an effective system for the labeling and detection of DNA, RNA, and oligonucleotides. The digoxigenin-labeled nucleotides may be incorporated, at a defined density, into nucleic acid probes by DNA polymerases (such as *E. coli* DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, reverse transcriptase and *Taq* DNA polymerase) as well as RNA polymerase (SP6, T3, or T7 RNA polymerase), and terminal transferase. The DIG label may be added by random primed labeling, nick translation, PCR, 3' end labeling or tailing, or in vitro transcription. Hybridized DIG-labeled probes may be detected with high affinity anti-DIG antibodies that are conjugated to alkaline phosphatase, peroxidases, fluorescein, rhodamine, amino-methylcoumarin, or collical gold. Alternatively, unconjugated antidigoxigenin antibodies may be used. Detection sensitivity depends upon the method used to visualize the anti-DIG antibody conjugate (Mühlegger et al., 1990).

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1.9.2.2 Biotin

In the biotin system biotin is incorporated in the probe by using biotinylated dNTPs during probe synthesis. The incorporated biotin is detected directly by avidin or streptavidin or an anti-biotin antibody conjugated to a fluorochrome or an enzyme such as alkaline phosphatase or horseradish peroxidase. Avidin is a 68kD glycoprotein derived from egg white and streptavidin is a 60kD protein from *Streptomyces avidinii* (http://www.kpl.com/docs/techdocs/TECHGUID.PDF and Langer et al., 1981). In both systems the probe is detected with chromogenic (colorimetric) substrates, fluorescence or chemiluminescence.

1.9.2.3 Fluorescein

Fluorescein nucleotide analogues can be used for direct as well as indirect *in situ* hybridization experiments. Fluorescein dUTP/UTP/ddUTP can be incorporated enzymatically into nucleic acids according to standard techniques. A fluorescein-labeled nucleotide can be detected with an anti-fluorescein antibody or a fluorescein labeled secondary antibody during indirect labeling (Dirks et al., 1991; Wiegant et al., 1991).

By using combinations of digoxigenin, biotin and fluorochrome-labeled probes, multiple simultaneous hybridizations can be performed to localize different chromosomal regions or different RNA sequences in one preparation (Dirks et al., 1991; Wiegant et al., 1991).

1.9.3 Enzymatic labeling procedures

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1.9.3.1 Nick translation

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In this method, labeled nucleotides are introduced into double stranded DNA by two enzymes: DNaseI and DNA polymerase I. DNA template can be supercoiled or linear. DNaseI introduces random nicks along the DNA and the endonuclease activity of the polymerase pulls the labeled nucleotides into place at the 3' hydroxyl terminus of the nicks. The ratio of DNA polymerase I to DNaseI is important in order to achieve an efficient labeling and to get a suitable probe size distribution, ideally 200-500 bases. The amount of non-radioactively labeled DNA is about 200ng in the standard assay when using 100ng to 3μg of DNA (Brunning et al., 1993 and Langer et al., 1981).

1.9.3.2 Random prime labeling

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Random primed labeling is performed on linearized denatured DNA to which random oligonucleotide or hexanucleotide primers are annealed. Similarly, random primer extension involves hybridizing short random sequence oligonucleotides to the target dsDNA and extending from their 3' ends using the Klenow fragment of DNA polymerase I or a cloned fragment of this or a similar enzyme, using the random oligonucleotides as primers (Brunning et al., 1993 and Feinberg and Vogelstein, 1984). The random primed labeling method allows efficient labeling of small (10ng) and large (up to 3μg) amounts of DNA per standard assay. The labeling method also works with both short DNA (200bp fragment) and long DNA (cosmids or λDNA) fragments (www.bio.vu.nl/mnb/downloads/nonradlb.pdf). Probes prepared by random primed labeling are often preferred for blot applications because of the high incorporation rate of nucleotides and the high yield of labeled

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probe. The method produces from 30-70ng (for 10ng template) to 2.10-2.65μg of non-radioactively labeled DNA (Feinberg and Vogelstein, 1983).

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1.9.3.3 PCR labeling

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The generation of hybridization probes by PCR is a well-established and convenient technique. Large amounts of a specific probe can be obtained within hours from minimal amounts of plasmid or even genomic DNA template. This approach requires significantly less bacterial culture and plasmid purification than random primed labeling does (Komminoth and Long, 1995). PCR labeling can be performed on cloned DNA with specific primers. The principle is similar to normal PCR amplification, except that one of the four nucleotides (dTTP) is partly replaced by dUTP (www.bio.vu.nl/mnb/downloads/nonradlb.pdf). Two oligonucleotide primers hybridize to opposite DNA strands and flank a specific target sequence. Incorporation of a labeled nucleotide during PCR can produce large amounts of labeled probe from minimal amounts (10-100pg) of linearized plasmid or even from ng amounts of genomic DNA. PCR allows easy production of optimally sized hybridization probes. Changing the sequence of the PCR primers controls fragment length. The reaction is performed with a selected ratio of dTTP: dUTP. In general a good reaction may be seen with ratios of between 1:1 and 20:1. The actual ratio selected is dependent on the reaction product required (Komminoth and Long, 1995).

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1.9.4 Detection of fluorescent labels

Certain molecules have the ability to emit light of a specific wavelength following absorption of light of a shorter, higher energy wavelength. This process of light absorption and re-emission is termed 'fluorescence' and the molecules, which exhibit this behaviour, are termed 'fluorochromes'. All fluorochromes have characteristic light absorption and emission spectra. Upon absorption of photons of the excitation wavelength, fluorochromes become excited into a higher, unstable energy state. This instability is then relieved by the subsequent production of photons of a lower energy emission wavelength. If a fluorescent dye can be made to interact with specific cellular components then it can be used as a probe for microscopy. A specimen stained with this probe may be illuminated with pure, filtered light corresponding to its excitation wavelength and then viewed through an emission filter, which is opaque to all other light except for its emission wavelength. The structures tagged with the fluorescent probe will appear to light up against a black background in a high contrast image (Rogers, 2003).

CHAPTER 2

OBJECTIVES OF THE STUDY

The gold standard for the diagnosis of FA has been chromosome breakage analysis by DEB and MMC, which usually is a reliable technique to identify FA homozygotes but could not be depended on to identify FA carriers (Giampietro et al., 1993). Once the diagnosis of FA has been established cytogenetically, molecular testing should demonstrate pathogenic mutations in an FA gene. The heterogeneity of the mutational spectrum and the frequency of intragenic deletions present a considerable challenge for the molecular diagnosis of FA. Molecular analysis seems to be a more appropriate method for the detection of FA carriers and often provides a faster and simpler method for mutation screening. To distinguish between homozygotes and carriers further analysis by dosage analysis and/or sequencing of the specific region are to be performed, which are not only labour intensive but also expensive and time consuming. FISH provides a route to bypass these problems encountered in molecular screening and leads the way to a more direct diagnosis.

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The objectives of this study were:

- ☆ To develop and establish a method for the detection of the two Afrikaner founder mutations, delE12-31 and delE11-17, by means of FISH.
- ☆ To evaluate FISH as a diagnostic tool in FA patients and carriers.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Ethical approval

This project was approved by the Ethics Committee of the Faculty of Health Sciences, University of the Free State (reference number ETOVS 229/01).

3.2. DNA isolation from whole blood

Blood samples were obtained with informed consent, from unaffected individuals without a family history of FA. Genomic DNA was extracted using a standard protocol. Ten ml of whole blood was drawn in a vacutest blood sample tube containing EDTA as anti-coagulant, transferred to 50ml Nunc tubes and frozen (-20°C) overnight to enhance lysis of red blood cells. Samples were defrosted on an orbital shaker and once thawed, lysis buffer was added to each sample to a final volume of 45ml to complete lysis of red blood cells. The tubes were centrifuged for 15 min at 2800 x g (4°C), to collect white blood cells. After the white cells were washed with lysis buffer, the pellet was resuspended in 4.5ml 1 x SET, 250µl 20% SDS and 100µl 10 mg/ml Proteinase K to induce lysis. The tubes were incubated overnight at 37°C, followed by a phenol-chloroform:isoamylalcohol (24:1) extraction. The contents of the tubes were mixed on an orbital shaker for 60min, and subsequently centrifuged for 15 min at 2800 x g (4°C). The supernatant was transferred to a sterile Nunc tube and a chloroform:isoamyl alcohol (24:1) extraction was performed.

Precipitation of the DNA was obtained by means of absolute ethanol (-20°C) (2,5 volumes) and 3M NaAc (pH 5.4) (0,1 volume) for 2 hrs to 16 hrs at -20°C. DNA was scooped out with a Pasteur pipette and transferred to an eppendorf tube. Five hundred µl of 70% ethanol (v/v) was added and the tubes were gently shaken to remove excessive salts, thereafter the supernatant was discarded. If the amount of DNA was too little to be scooped out, the tubes were subsequently centrifuged for 30 min at 2800 x g (4°C). The supernatant was discarded and the pellet was washed with 500µl 70% ethanol (v/v). Again the tubes were centrifuged at 2800 x g for 20 min and the supernatant discarded. The DNA pellets were left to air dry and subsequently dissolved in sterilized 200µl-400µl 1 X TE at 37°C. Before usage, the DNA quality and concentration were determined by agarose gel electrophoresis and spectrophotometry. The samples were stored at 4°C.

3.3 Amplification of genomic DNA by PCR

3.3.1 Primer construction

The delE12-31 and delE11-17 mutations overlap, therefore, to detect delE12-31 the FISH probe must be a DNA fragment that complements an area between introns 17 and 31 of the *FAA* gene. To detect delE11-17, an area between introns 10 and 17 must be covered. The PCR primer pairs (Table 3.1) were designed manually according to the literature (Abd-Elsalam, 2003). Amplification with the FISHIBF forward primer in intron 10 and the reverse primer FISHIBR in intron 11 of the *FAA* gene produced a 2.3kb DNA fragment. Both the FISHIIBF (forward primer) and the FISHIIBR (reverse primer) are located in intron 20 of the *FAA* gene, giving rise to a 1.8kb amplified product (Table 3.2).

Mutation type	Oligo-nucleotide	Sequence (5'-3')	Tm	GC%	No of bases	Product size (kb)
delE12-31	FISHIBF	AGTGCAGTGGTGCGATCTC	58.8	53.35	19	2.3
	FISHIBR	TGGGTACAGTCTGTCATACAT	55.9	42.86	21	
delE11-17	FISHIIBF	GAGCTCAGAAGATGATCTAC	55.3	45	20	1.8
	FISHIIBR	CTCGTGACCTTGTGATTCAC	57.3	50	20	

Table 3.1 Primers used for PCR amplification

3.3.2 PCR regime

PCR was performed on a thermal cycler (MyCycler, Biorad). Amplified products served as FISH probes in further analysis. The PCR for the delE12-31 mutation consisted of 10pMol each of the forward and reverse primers, 10 X *ExTaq* ™ buffer including 20mM $MgCl_2$ (TaKaRa Bio Inc.), 2 units TaKaRa *ExTaq* ™ (5 units/µl), 2,5mM dNTP mixtures (TaKaRa Bio Inc.), 10% DMSO (Fluka), and 10ng human genomic DNA from normal healthy individuals. The total reaction volume for all reactions was 50µl. The PCR for the delE11-17 mutation consisted of 50pMol of each of the forward and the reverse primers, 10 X *ExTaq* ™ buffer including 20mM $MgCl_2$ (TaKaRa Bio Inc.), 1,5 units TaKaRa *ExTaq* ™ (5 units/µl), 2,5mM dNTP mixtures (TaKaRa Bio Inc.), 10% DMSO (Fluka), and 10ng genomic DNA. The total reaction volume for all reactions was 50µl.

Intron10

102224acatagctgggactataggtgtgtgccaccatgcctggctaatttttttattttctttttttgagacaagaatttcgctctgtcgc
ccatgct102319gg**agtgcagtggtgcgatctctg**ctcactgcaagctccgctccctgggttcacaccattctcctgcctcagtc
tcccgagtagctg102405gaactacaggtgcccaccaccatgcccggctaatttttctatttttttagtagagatggggtttcgttgg
gttggccgggatggtctcga102496gctcctgaccttgtcatctgccccccttggcctcccaaagtgctgggattacaggtgtgag
ccaatgcgcctggcctaatattttggta102585tttttagtgtagttcggagtgttcagtggacacacgcttggcagtgtaatttccac
agatcctctgaagaggttcttcagtcataccctgaIntron11

103893cacagccctgtgctgaaaggtaggcccagtgtggaggctgctcagttgctacccatttggggattgctgttgccttgttgc
cttggtat103983tgtttgtttgtttgagtcagagtctcactcacttgcccatgctggagtgcagtggcgtgatctcagctcactgcaa
cctctgcctcccag104073gttcaagcaattctcgtgtctcagcctctcaagtagctgggactacaggcgccaaccaccacacc
cggttaatttttgtatttttagtat104164gagactgggttcaccatgttggccaggctggcctcgaactcctgaccttcggtgctcca
cctgccttggcttcccagagtgtcgaagt104252gttgggattacaggtgtgagccaccgcacccagcgtaaaatagaaatatc
tttatttttatttatttatttatttatttatttatttattg104350gagatggagtcttgctctgttgccaggttggagtacagtggcgtg
atttgggctcactgcaacctccacctcccgggttcaaaccatt104438ctcctgcctcagcctccacagtagctgggattacggg
catgtgccaccatgcccggctaatttttgtatttttagtagagacgggctttc104528accatgttggcctggctacccttgccttgctg
tgactgtgtcatggttctcagctagactcggtggcgtgtttgatggcccaggcatcgt104618ctgcccagtgggtgtcctgttaccc
atgtatgacagactgtacccaaga

Intron20
121015gagtgcaattgcacaatcttggctcactgcaagctctgcctcctgggttggagcaattgtcctgcctcagcctcctgagta
gctggga121103ttataggtatgcaccaccacgccctgctaatttttgtatttttagtacagacagggttttgccatattggccaggc
ggttctcaaactcct121194***gagctcagaagatgatctac***ccacctcatcctcacagagtgctgggattacagatgtgagcca
ccgctcctggccttttttttttttttt121284ttttgagatgaagtctcactctgttgcccaggctggagtgcagtggtgcgatcttggctca
ctgcaccctccacctcctgggttcaagg121373aattctcctgcctcagcctcctgagtagctgggattacaggcgcgcaccacc
acgcccggctaatttttgtatttttagtagagacgg121461ggtttcaccatgttagtcaggctggtcttgaactcctgggctcaagc
gatctgcccacctcggcctcccaaagtgctgggattatagg

Intron20

122666cgtgagctaccgcgcaatctcggctcactgcaagctccaccttctgggttcatgccattctcccacctcagcctaccaagt
agctga122756gactgcaggcgcccgccaccacgcccggctaattttgtttttgtatttttagtagacacagggtttcactgtgttag
ccaggatggtctc122852aatctcctgacctcctgatctgcctgcctcggcctcccaaagtgctgggattacaggcgggatccac
cgcgcctggcccaattttgc122942atatttttaatgacacgaactaaacgtttttcttagtattaatcattaaaattaacaaagattca
tgttttgctgggcgtggtggctcacgc123035ctgtaatcccagcactttgggaggctgaggcag***gtgaatcacaaggtcacg***
agttcgaggccagcctggtgaacatggtgaa123120actccgtctctactaaaaatacaaaaaattagctgggcctggtggca
ggcgcctgtaatcccacctactcgggagggtgaggca123206ggagaattgcttgaacctgggaggcggaggttgcagtgag
ccgagatcgcaccactgcactccagtgtgggtgatagtgggag123290actctgtctcaaaaaaaaaaaaaaaaaaaaaa
aaaagaaaaacaaagattcgtgttttgaatttggaaattaccaaaaattgtatgagagaaatagaaataatttataatttcact

Table 3.2 Primer positions as indicated on the DNA sequence

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3.3.3 PCR conditions

The following PCR program was used for DNA amplification as indicated in Table 3.3: the PCR regime for the synthesis of the probe FISHI (FISHIBF and FISHIBR primers) was initiated with an initial 8 min denaturation step at 95°C followed by 30 cycles of 95°C for 1 min, 66°C for 1 min and 72°C for 2 min. The PCR concluded with a 10 min extension at 72°C. The samples were cooled down to 4°C.

The PCR for the synthesis of the probe FISHII (FISHIIBF and FISHIIBR primers) was initiated with an 8 min denaturation step at 95°C, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 2 min. The PCR concluded with a 10 min extension at 72°C. The samples were cooled down to 4°C.

PCR Step	Number of cycles	Temperature	Duration
Denaturation	1	95°C	8 min
Denaturation Annealing Extension	30	95°C 60°C / 66°C 72°C	1 min 1 min 2 min
Extension	1	72°C	10 min

Table 3.3 PCR conditions

3.3.4 Agarose gel electrophoresis

Amplified PCR products were analyzed by horizontal agarose gel (1%) electrophoresis and stained with ethidium bromide in 1 X Tris/Borate/EDTA (TBE) running buffer. Products were visualized under ultraviolet light (UV).

3.4 Purification of probe

Amplified DNA was loaded on a 1% agarose gel with standard markers and run overnight at 45V. The desired band was excised from the agarose gel under UV light using surgical blades, removing as much of the agarose, surrounding the band of interest, as possible. The gel fragments were placed into a 50ml Nunc tube, and washed twice with sterile water. The gel was smashed into small pieces in a sterile Petri dish and transferred back into the Nunc tubes, and mixed with adequate (3-5ml) 1 X TE (pH 8.0). It was then incubated at 50°C in a water bath, with occasional shaking (every 10 min) for 1 hr, and centrifuged at 12, 000 x g for 20 min. The supernatant (DNA) was transferred to a sterile Falcon tube and precipitated using ethanol (2,5 volumes) and 3M NaAc (0,1 volume) (pH 5.4) at -20°C overnight. The tubes were centrifuged at 12, 000 x g for 30 min. The supernatant was discarded and the pellet washed with 70% ethanol (v/v) and centrifuged for 30 min at 12, 000 x g. The ethanol was discarded and the pellet was air-dried. The pellet was resuspended in 50µl 1 X TE (pH 7.6). A small sample was loaded with Lambda DNA/*Hind III* DNA marker (Roche Molecular Diagnostics) on a 1% agarose gel to determine quality and the concentration was determined spectrophotometrically.

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3.5 Multiplication of probe

Two different strains of *E. coli,* HB101 and JM109, served as competent cells. The standard ampicillin resistance is the sole criterium for selecting recombinants. The HB101 strain is compatible with electroporation, and these cells are useful for cloning into a vector that does not require α-complementation for blue/white screening as selective criteria. While the JM109 strain is compatible with heat shock, the JM109 contains the lacIqZΔM15 genotype and is compatible with α-complementation, providing blue/white screening and standard ampicillin resistance as selective criteria for recombinants. Both transformation assays were performed in the plasmid pGEM®-T. Applying different transformation assays enhanced the probability of obtaining the recombinant DNA.

3.5.1 Ligation into vector pGEM®-T

The purified PCR fragments (probes FISHIB and FISHIIB) were ligated to the plasmid pGEM®-T using the Easy Vector system I Kit (Promega, WI, USA). For the ligation reaction 1µl plasmid (50ng/µl) was mixed with 3µl of PCR product, one µl T4 DNA ligase, and 5µl 2 X Rapid ligation buffer. This was subsequently incubated at 4°C for 72 hrs. The ligated plasmids were divided in two for further use in both transformation assays.

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3.5.2 Preparation of *E. coli* competent cells (HB 101 probe FISHIB)

A single colony of *E. coli* (strain HB101) cells was inoculated into a 5ml LB medium and grown overnight at 37°C with moderate shaking. One ml of the overnight culture was transferred into 100ml LB medium in a sterile 2 litre flask and grown at 37°C with moderate shaking, also overnight. The culture was transferred to 50ml pre-chilled, sterile Falcon tubes and left on ice for 5–10 min. Thereafter, the cells were centrifuged at 12,000 x g for 7 min at 4°C and the supernatant was discarded. The pellet was resuspended in a total volume of 8ml ice cold 100mM $CaCl_2$ containing 15% glycerol (v/v). The cells were stored in 200µl aliquots at -70°C.

3.5.3 Transformation in *E. coli* competent cells (HB101 probe FISHIB)

Approximately 5µl of the ligated plasmid was mixed with 100µl competent cells (HB101 strain). The mixture was run through the electroporation machine overnight at 37°C, and then added to LB medium without agar, mixed well, and transferred into a new Falcon tube and incubated overnight in a waterbath at 37°C. About 350µl of the electroporation product was evenly spread with a "hockey stick" over the LB ampicillin plate containing 100µg/ml ampicillin, and incubated at 37°C for 16 hrs. Ampicillin resistant colonies were picked up, streaked on LB ampicillin master plates and again incubated at 37°C for 16 hrs. Single colonies were inoculated into 1ml LB broth containing 100µg/ml ampicillin and incubated with moderate shaking at 37°C for 16 hrs. Minipreps were performed, followed by restriction enzyme (RE) analysis and PCR amplification to confirm the presence of the appropriate DNA insert (probe).

3.5.4 Transformation in *E. coli* competent cells (JM109 probe FISHIIB)

Sterile Falcon culture tubes were chilled on ice, one per transformation. Frozen competent cells were removed from a -70°C freezer and placed on ice for 5 min, or until just thawed. Once the cells have thawed, they were pipetted quickly to prevent them from warming above 4°C. The thawed competent cells were gently mixed by flicking, and 100µl was transferred to each of the chilled culture tubes. Fifty ng of ligated plasmid was added per 100µl of competent cells. The pipette tip was moved through the cells while dispensing. The tube was quickly flicked several times and was immediately returned to ice for 10 min. The cells were heat shocked for 45-50 sec in a water bath at exactly 42°C. The tubes were immediately placed on ice for 2 min. Nine hundred µl of cold SOC medium was added to each transformation reaction and incubated for 60 min at 37°C, during which time moderate shaking was applied.

For each transformation reaction, the cells were diluted 1:10 and 1:100. One hundred µl of the undiluted, 1:10 and 1:100 dilutions were plated on LB plates containing 100µg/ml ampicillin, 0,5mM IPTG (isopropyl-β-D-thiogalactosidase) and 40µg/ml X-gal (5-bromo-4 chloro-3-indonyl-β-D-galactosidase) to enable blue/white screening for recombinants. Plates were incubated overnight at 37°C. White colonies representing recombinant DNA were picked and inoculated on a master plate (LB-amp/X-gal/IPTG) and again incubated overnight at 37°C. Single colonies were inoculated into 1ml LB broth containing 100µg/ml ampicillin. Cultures were incubated with moderate shaking at 37°C for 16 hrs. Minipreps were performed followed by RE analysis and PCR amplification to confirm the presence of the insert (probe).

3.5.5 Restriction enzyme (RE) analysis

Recombinant DNA (15µl) was digested with 50-100U *EcoRI* by adding 5-10µl *EcoRI* (Roche Molecular Diagnostics), 5-10µl SuRE/Cut Buffer H for restriction enzymes (Roche Molecular Diagnostics) and distilled water to a final volume of 50-100µl and incubated at 37°C for 3 hrs. The restriction fragments were separated on a 1% agarose gel together with a DNA marker, insert DNA and uncut recombinant DNA. RE fragments, which contained the DNA insert, were selected and the appropriate cell lines were again inoculated into larger volumes of medium.

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3.5.6 Isolation and purification of plasmid DNA from *E. coli* cultures (Maxiprep procedure)

3.5.6.1 Probe FISHIB

Five hundred ml of an overnight culture of *E. coli* (strain HB101) was transferred into a bottle and centrifuged at 7700 x g for 10 min. The supernatant was carefully decanted without disturbing the cell pellet and leaving it as dry as possible. The pellet was resuspended in 100ml of ice-cold SET solution by pipetting, centrifuged and drained. This wash step was repeated in 30ml of suspension solution by pipetting and centrifuged at 7700 x g for 10 min. In order to digest the *E. coli* cell membranes one ml of lysozyme was added and mixed, and placed on ice for 5 min. To induce lysis of the *E. coli* cells 30ml of cell lysis solution was added, mixed by inverting the tube several times and incubated at room temperature for 5 min. Thirty ml of the neutralization solution was added and mixed by inverting the tube several times. The solution was placed on ice for 10 min and centrifuged at 12,000 x g for 15 min at room temperature. The supernatant was carefully

decanted into a clean centrifuge tube. Sixty three ml (0.7 volumes) of ambient-temperature isopropanol was added to the supernatant and mixed well; incubated for 10 min at room temperature and centrifuged at 12,000 x g for 20 min to pellet the plasmid DNA.

The supernatant was decanted and the tube was inverted to drain. Thirty ml of the Sephaglas ™ FP suspension was added to the DNA pellet and vortexed gently for 1 min to dissolve the pellet. Plasmid adhesion to the Sephaglas was obtained by leaving the samples at room temperature for 10 min gently vortexed every 2 min to keep the Sephaglas in suspension, and then centrifuged at 1000 x g for 2 min. The supernatant was carefully removed without disturbing the Sephaglas pellet. Thirty ml of wash buffer was added to the Sephaglas pellet, vortexed gently to resuspend it, and centrifuged at 1000 x g for 2 min. The supernatant was carefully removed without disturbing the pellet. Thirty ml of 70% ethanol was added to the Sephaglas pellet, vortexed gently, and centrifuged at 1000 x g for 2 min. The supernatant was carefully removed without disturbing the pellet. Following the ethanol rinse, the pellet was centrifuged without a cap at 1000 x g for 1 min and any residual ethanol was carefully removed with a pipette. The pellet was allowed to air dry for 20-30 min at room temperature. To elute the bound plasmid from Sephaglas, 5ml of 1 X TE buffer was added to resuspend the pellet and incubated for 10min at room temperature, vortexing occasionally to keep the Sephaglas in suspension. The suspension was centrifuged at 1000 x g for 5 min and the supernatant was transferred to a clean eppendorf tube.

3.5.6.2 Probe FISHIIB

A single colony (*E. coli* strain JM109) from a freshly streaked selective plate (LB-amp/X-gal/IPTG) was picked and a starter culture of 2-5ml LB medium containing ampicillin (100μg/μl) was inoculated and incubated for approximately 8h at 37°C, with vigorous shaking. The 1/500 starter culture was diluted to 1/1000 into selective LB medium. One hundred ml of the medium was inoculated and grown at 37°C for 12-16 hrs with vigorous shaking. The bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C. The bacterial pellet was resuspended in 10ml buffer P1 (Appendix B). Ten ml buffer P2 (Appendix B) was added and mixed gently but thoroughly by inverting the tube and incubating at room temperature for 5 min. Ten ml of chilled buffer P3 (Appendix B) was added and immediately mixed by gently inverting the tube and incubating it on ice for 20 min. The sample was centrifuged at 20,000 x g for 30 min at 4°C, and the supernatant containing the plasmid DNA was removed. The plasmid DNA was separated from *E. coli* DNA by means of column chromatography. A QIAGEN-tip 500 was equilibrated by running 10ml buffer QBT (Appendix B) through the column by gravity flow. The supernatant was allowed to flow through the QIAGEN-tip by gravity flow. The plasmid DNA stayed behind in the column and was washed by adding 2 X 30ml buffer QC (Appendix B) to the column. The plasmid DNA was eluted from the column with 15ml buffer QF (Appendix B). Precipitation of the DNA was affected by adding 10.5ml isopropanol to the eluted DNA at room temperature. The sample was mixed and immediately precipitated by centrifugation at 15,000 x g and 4°C for 30 min. The supernatant was carefully decanted. The DNA pellet was washed with 5ml 70% ethanol at room temperature, and centrifuged at 15,000 x g for 10 min. The supernatant was carefully decanted without disturbing the pellet. The pellet was air dried for 5-10 min, and the DNA was redissolved in 50μl of

1 X TE buffer, pH 8.0. The DNA concentration and quality were determined respectively by a spectrophotometer and by 1% agarose gel electrophoresis.

3.6 Labeling of probes

3.6.1 Direct labeling

3.6.1.1 Nick translation assay

The labeling protocol for Digoxigenin-11-dUTP was followed with the exception of using fluorochrome labeled dUTP for direct labeling (Roche Molecular Diagnostics). Probe FISHIB was labeled with SpectrumGreen and probe FISHIIB with SpectrumOrange (Vysis Inc., Downers Grove, IL, USA). The following were added into a microcentrifuge tube and kept on ice: 1-2μg of probe (FISHIB or FISHIIB or CEP16 – a control probe), 10μl dNTP mixture, 2μl 10 X buffer, 4-5μl sterile double distilled water, and 2μl of the enzyme mixture (DNA polymerase I repairs nicks, DNaseI introduce nicks in 50% glycerol). The assay was mixed and centrifuged briefly at 1000 x g and incubated overnight at 15ºC. The reaction was stopped by adding 1μl 0.5M EDTA (pH8.0) or incubation at 65ºC for 10 min.

3.6.1.2 Thermo cycled amplification labeling

A PCR was performed according to the optimized parameters or conditions described on page 38, with the exception that the ratio of dTTP: dUTP was adapted to 20:1, 10:1, 5:1 and 3:1 (Vysis Inc., Downers Grove, IL, USA). SpectrumGreen was used for the FISHIB probe and SpectrumOrange was used for the FISHIIB probe.

3.6.2 Subjects

Blood samples were obtained from three FA patients of Afrikaner lineage, with a clinical and molecular diagnosis of FA, as well as sensitivity to DEB. Molecular analysis revealed one patient to be a carrier of the Afrikaner founder deletion delE11-17, one a carrier of the delE12-31 lesion and the third to be a compound heterozygote with genotype delE11-17/delE12-31. A normal healthy individual served as negative control. Blood samples were obtained with informed consent.

3.6.2.1 Culturing of peripheral lymphocytes

Two lymphocyte cultures per person/patient were initiated in sterile 15ml plastic screw cap centrifuge tubes by adding 0.3ml heparinized blood (lithium heparin as anticoagulant) to 4ml of a stock solution consisting of 400ml RPMI 1640 medium, 50ml foetal calf serum, 20ml phytohaemagglutinin (PHA) and 2ml 1% gentamycin. The contents were mixed gently and the cultures incubated at 37°C for 69 hrs, with the tubes tilted at an angle of approximately 30 degrees.

3.6.2.2 Harvesting of lymphocytes

After an incubating period of 69 hrs, 0.2ml colcemid (10μg/ml) was added to each culture to arrest dividing lymphoblasts in the metaphase stage of mitosis. The culture contents were mixed gently and incubated at 37°C for another 90 min. Thereafter the cultures were terminated. This was initiated by centrifugation at 700 x g for 10 min, removing the supernatant and resuspension of the cells in a hypotonic solution of 5ml 0.075M KCl, and incubated at 37°C for 17 min. The tubes were centrifuged at 700 x g for 10

min and the supernatant removed. Cells were fixed by adding 5ml freshly made fixative (3 parts methanol and 1 part glacial acetic acid), precooled to -20°C. The cells were resuspended and stored overnight at -20°C. This was followed by centrifuging the tubes at 700 x g for 10 min, removing the supernatant, the addition of 5ml 3:1 fixative, and resuspending the cells by means of a vortex. The fixation step was repeated four times, using 6:1 (6 methanol: 1 acetic acid) fixative during the last two steps. The tubes were stored at –20°C.

3.6.2.3 Preparation of slides

Pre-cleaned slides were soaked in absolute alcohol for at least 24 hrs at room temperature; they were then dried and polished with facial tissue paper. The fixed cell suspension was centrifuged at 700 x g for 10 min, the supernatant removed, leaving approximately 1ml, depending on size of the cell pellet. The cells were resuspended, using a narrow calibre glass pipette. Five to seven drops of the cell suspension were dropped onto each marked slide from a height of approximately 50cm. Slides were left to dry at room temperature overnight for maturation. The preparations were now ready for application of the FISH procedure.

3.6.2.4 Probe preparation

The labeled DNA was mixed with 1μg/ml of 50 X Cot Human DNA (Roche Molecular Diagnostics) and precipitated with 0.1 volume of 3M NaAc (pH 5.4) and two and half volume pre-chilled absolute ethanol. The sample was mixed and incubated at –70ºC for 30 min subsequently centrifuged at 12,000 x g for 30 min at 4ºC to pellet the DNA, after which the supernatant was discarded. The pellet was washed by adding 400μl of 70% ethanol (v/v) and again centrifuged for 15 min at 12,000 x g, the supernatant was

discarded, and the pellet air-dried. The pellet was resuspended in 50μl 1 X TE (pH 8.0) and the concentration was determined. The probe was further diluted to a concentration of 100ng/μl in 1 X TE (pH 8.0) and stored in the dark at 4ºC. The undiluted probe was stored at -20ºC in the dark (Roche Molecular Diagnostics).

A commercially available fluorescent labelled probe (Vysis Inc., Downers Grove, IL, USA Inc), located on the centromere of chromosome 16 (CEP 16), was used as internal control.

3.6.2.5 Pre-hybridization washes

The matured preparations were incubated in a coplin jar containing 2 X saline sodium citrate (SSC) (pH 7.4) for 1 hrs at 37ºC. Proteins were digested using a pepsin treatment, for 30 min at 37ºC. The preparations were rinsed for 5 min in a coplin jar containing 1 X phosphate buffered saline (PBS) at room temperature, followed by post fixation in formaldehyde for 15 min at room temperature. The PBS treatment was repeated and preparations were left to air-dry. The preparations were dehydrated through an ethanol series (70%, 85%, 100%; 1 min each) and air-dried.

3.6.2.6 Denaturation and hybridization

The preparations were denatured for 5 min at 73±1ºC in 70% formamide/2 X SSC solution. Simultaneously 10μl of labeled probe (Vysis Inc., Downers Grove, IL, USA) was denatured for 5 min at 73±1ºC. The preparations were immediately dehydrated for 1 min each in 70%, 85%, and 100% ethanol. Excess ethanol was drained on a paper towel. The preparations were placed on the side of a waterbath (73ºC) and 10μl of the denatured probe was applied on each marked area and the probe solution was covered with a coverslip and sealed with clear adhesive (Bostik). The

preparations were incubated in a moist chamber overnight at 37ºC for hybridization (Roche Molecular Diagnostics).

3.6.2.7 Post-hybridization washes

After hybridization, the coverslips were removed and preparations were washed at 46ºC for 5 min in each of three changes of formamide solution divided into three coplin jars, followed by 5 min in 2 X SSC (pH 7.0) and finally in 2 X SSC/0.1% Nonidet P40 (NP40) for 10 min. The preparations were air-dried and counterstained with 10µl 4'-6'-Diamidino-2-phenylindole (DAPI II, Vysis Inc., Downers Grove, IL, USA), coverslipped and sealed. Preparations were stored at –20°C in the dark for at least 30 min before analysis by fluorescence microscopy.

3.6.3 Indirect labeling

3.6.3.1 Thermo cycled amplification labeling

A PCR was performed according to the optimized parameters or conditions described on page 38, with the same exception as for direct labeling by PCR. The ratio of dTTP: dUTP was adapted to 20:1, 10:1, 5:1, and 3:1. Ten µM Dig-dUTPs (Roche Molecular Diagnostics) was prepared by reconstituting 50nmol of lyophilized dUTPs in 50µl deionized water (dH_2O).

3.6.3.2 Probe preparation

Labeled probe DNA was precipitated overnight at –20°C with 2.5 volume absolute ethanol and 0.1 volume 3M NaAc. The probe was centrifuged at 12,000 x g for 30 min at 4ºC. The supernatant was discarded

and the pellet was washed with 100μl ice cold 70% ethanol (v/v). The tube was centrifuged at 12,000 x g for 20 min at 4ºC. The supernatant was discarded and the pellet air-dried. The pellet was resuspended in 20μl 1 X TE (pH 8.0). The concentration was determined and diluted to a concentration of 20ng/μl in 1 X TE (pH 8.0) (Roche Molecular Diagnostics).

3.6.3.3 Pre-hybridization washes

The same pre-hybridization wash conditions and buffers were applied as for direct labeling.

3.6.3.4 Denaturation and hybridization

The same denaturation and hybridization conditions and buffers were applied as for direct labeling.

3.6.3.5 Post-hybridization washes

The same post-hybridization wash conditions and buffers were applied as for direct labeling.

3.6.3.6 Detection

One hundred μl TNB (Roche Molecular Diagnostics) was pipetted onto the preparations, covered with 24 X 50mm coverslips and sealed with Bostik. The preparations were incubated for 30 min at 37ºC in a moist chamber and immersed in TNT (Roche Molecular Diagnostics) for 5 min to loosen the coverslips. Fresh working solutions of two antibodies (2μg/ml anti-digoxigenin-rhodamine-Fab fragments and anti-digoxigenin-fluorescein-Fab

fragments, Roche Molecular Diagnostics) were prepared. One hundred μl of the antibody working solution was pipetted onto each preparation, coverslips were put on and sealed, and the preparations were incubated for 30 min at 37ºC in a moist chamber. The preparations were washed twice for 5 min in the dark at room temperature in TNT and once in 1 X PBS for 5 min, dehydrated through an ethanol series (1 min in 70%, 1 min in 85%, and 1 min in 100% ethanol solution), and air-dried. The preparations were counter-stained with 10μl DAPI II, cover-slipped, sealed and stored at -20ºC. Preparations were examined with a Nikon fluorescence microscope using DAPI/Green/Orange triple-bandpass filter as well as orange and green single band filters (Vysis Inc., Downers Grove, IL, USA).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Synthesis of probe

The first stage of this investigation was to amplify by PCR those DNA regions which are deleted if the two specific mutations are present. Initially the salt method of DNA isolation was attempted (Morgan et al, 1999). With this approach, amplification of the isolated DNA resulted in production of smears on agarose gel electrophoresis. This was obtained even after further washes with 70% ethanol. Amplification was inconsistent and not reproducible. This may be ascribed to poor DNA quality and high salt content of DNA. The purity and integrity of the DNA template is very critical for molecular analysis. The phenol method was used to overcome these problems. This approach produced a high quality of DNA, but still unsatisfactory results were obtained after amplification of the delE12-31 fragment. Even though the PCR products amplified from the delE11-17 DNA fragment revealed an exact band at 1.8kb (Figure 4.1 B), the delE12-31 amplified product resulted in a multiple of bands migrating from 500bp to 3,0kb in size with an exact band at 2.3kb (Figure 4.1 A). Even after intense standardization ($MgCl_2$ concentrations, annealing temperature, DNA concentrations) the banding pattern persist unchanged. This may be ascribed to poor primer design, as this set of primers was also complementary to other DNA sequences in the genome. (www.ncbi.nlm.nih.gov/BLAST/).

The gel purification method was used to separate the desired PCR product from non-specific products, primer dimers and dNTPs. A small cube

of agarose surrounding the band of interest was excised and the DNA was purified from the gel. The purity and specificity of the amplified DNA, which will be used as FISH probes in further analysis, will determine the specificity of the probe to the target molecules. The purified amplified products were again subjected to PCR to confirm specificity and the concentrations were determined spectrophotometrically.

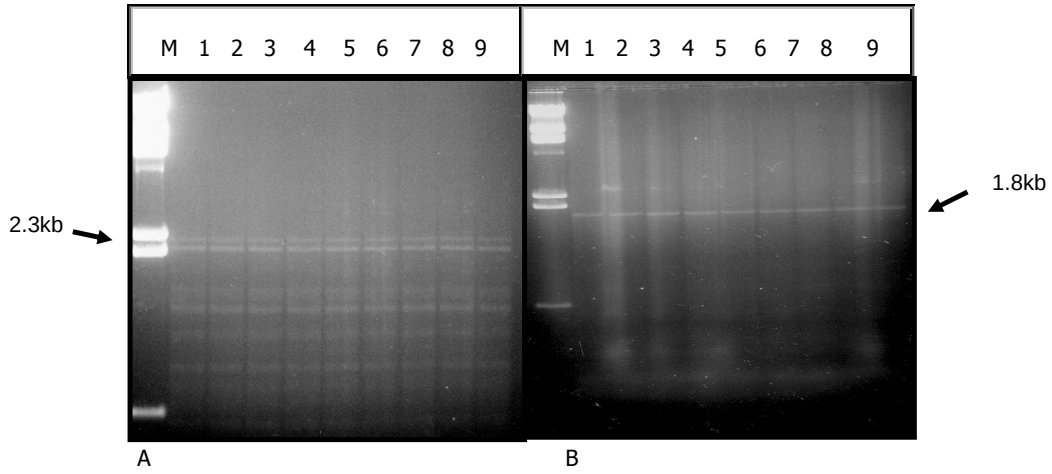
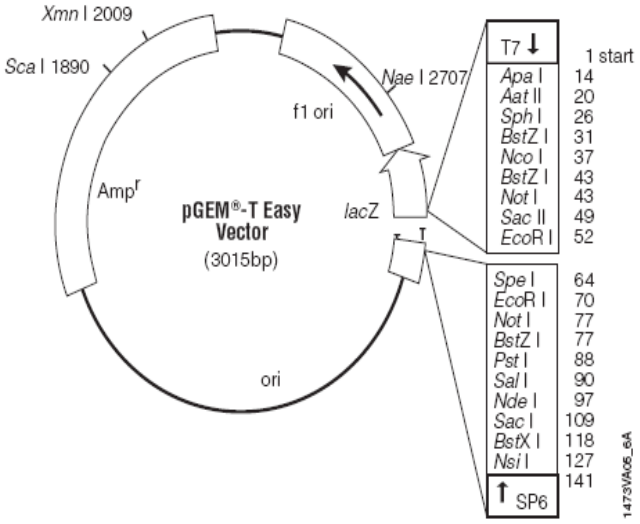


Figure 4.1 Analysis of (A) delE12-31 and (B) delE11-17 PCR products by agarose gel electrophoresis (1%). Lane M, molecular marker (Lambda DNA/Hind III, Roche Molecular Diagnostics), lanes 1-9 bands indicating amplified PCR products.

4.2 Multiplication of probe

The second stage of the investigation was to clone the purified PCR products (probes FISHIB and FISHIIB) into the pGEM®-T vector (Promega, Figure 4.2). The product was ligated directly into the pGEM®-T plasmid using the adenine overhangs added to the 3' ends of the PCR product by *Taq* polymerase. At pGEM®-T pre-cut insertion sites, thymidine overhangs have been added to the 3' end, which allows direct ligation of PCR product.

By applying two different transformation strategies the possibility of finding a recombinant clone was enhanced. *E. coli* strain HB101 competent cells are compatible to transformation with electroporation, but only allows selection of positive clones (plasmid plus DNA insert) by ampicillin resistance. Competent cells from strain JM109 (Promega), which are compatible to heat shock, on the other hand, allow both ampicillin resistance as well as α-complementation for blue/white screening as selection criteria.



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Figure 4.2 pGEM®-T easy vector circle map and sequence reference points (Promega Technical manual Cat.# A1360).

Fifteen clones (from each probe), derived from the HB101 competent cell transformation, were selected and streaked out on master plates (LB-amp). The percentage of positive clones (white colonies) derived from the transformation with the JM109 strain were much higher, and fifty colonies from each DNA insert were selected and streaked out on master plates containing ampicillin, X-gal and IPTG. Mini-preps were performed, followed by both RE analysis with *EcoR1* (Figure 4.3 I) and PCR amplification (Figure 4.3 II). Only recombinants which contain the appropriate DNA insert, either a 1.8kb fragment for delE11-17 or a 2.3kb fragment for delE12-31, (after RE digestion) and amplified well in an exact band of the right size, were further analysed. Various aberrant restriction (Figure 1A, lanes 1 and 3) and amplification (Figure IIA, lanes 1 and 3) patterns were observed, which may be attributed to either a PCR artefact or recombination of the plasmid vector. PCR amplified products were originally excised from a gel before re-amplification and subsequently, RE digestion. Hence contamination with another piece of amplified DNA from the gel might occur, causing amplification of both the desired and non-wanted piece of DNA. Secondly, the plasmid vector itself could undergo recombination, causing either the creation or abolishment of *EcoR1* restriction sites resulting in an unfamiliar RE banding pattern.

Based on these criteria, one clone for each of the probes was selected and inoculated into large quantities of LB-amp broth for maxipreps. Recombinant DNA was again subjected to both RE analysis and amplification by PCR to ensure the presence of the appropriate DNA fragment. The quality and concentration of the DNA fragments were determined by agarose gel electrophoresis and by spectrophotometer. The recombinant DNA (plasmid and DNA insert) served as template in the following labeling reactions.

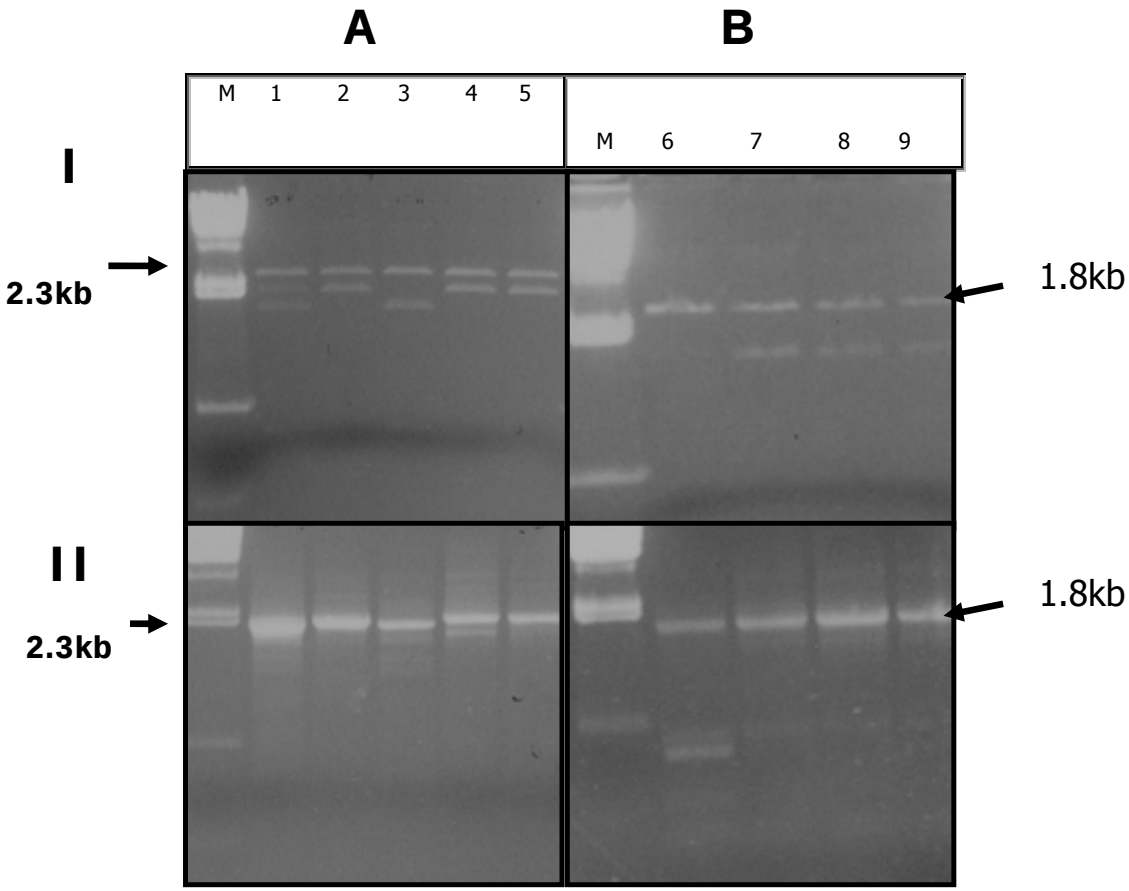


Figure 4.3 **(I)** Restriction analysis and **(II)** PCR amplification of the **(A)** delE12-31 and **(B)** delE11-17 recombinant DNA. M= Lambda DNA/Hind III marker, **(A)** lanes 2, and 5, and **(B)** 7, 8 and 9 **(I)** show the appropriate insert, 2.3kb and 1.8kb respectively; and **(II)** PCR amplified products.

4.3 FISH

Various methods for labeling of *in situ* probes have been described, each with its own advantages and specific applications. There are mainly two types of non-radioactive labeling strategies, direct and indirect labeling. In the direct method the detectable molecule or reporter is bound directly to the nucleic acid probe so that the probe-target can be visualized microscopically immediately after hybridization (Bauman et al, 1980 and Bauman et al, 1984).

The indirect procedure requires the probe to contain a reporter molecule, introduced chemically or enzymatically and can be detected only after affinity cytochemistry (Langer et al., 1981).

4.3.1 Direct labeling

4.3.1.1 Nick translation

Direct labeling was the first choice as it is simpler, faster and more cost effective in contrast to indirect labeling that requires secondary antibodies. To generate labeled probes nick translation and thermal cycling by PCR were performed for the direct detection. Nick translation labeling method produces a heterogeneous population of DNA strands, many of which have overlapping complementary regions. This may lead to signal amplification during hybridization. The 50:50 ratio dUTP:dTTP was used to allow high sensitive detection and to decrease unacceptable elongation times of the slower reaction kinetics of labeled dUTP (Langer et al., 1981). Various probe (recombinant plasmid) concentrations (1μg to 2μg) were used in the labeling reactions and both labeled well.

Labeling was successful as the DNA probe was sufficiently nicked in the reaction and the fragments produced range was approximately between 300 to 500bp in size. The fragments were not larger than 500bp as this may hinder penetration into the cells efficiently resulting in an increased background. Probes, which are too small, tend to bind non-specifically and rehybridize so that a smaller amount of probe can hybridize to the target DNA, leading to poor hybridization efficiency and sensitivity (Feinberg and Vogelstein, 1983). The size of the fragment was determined after the labeling reaction by gel electrophoresis using a 1% agarose gel. The light smear ranging from approximately 300bp to 500bp represents the fragment distribution. The overall success of this method was due to the correct concentration of the probe DNA in the labeling reaction. CEP16, a commercially available labeled probe (Vysis Inc., Downers Grove, IL, USA), was used as a control. This was in the first instance to ensure that the pretreatment and hybridization reactions were optimal, and secondly to serve as internal control. Unincorporated nucleotides were removed by ethanol precipitation.

4.3.1.2 PCR amplification

Since the PCR method was optimized, the preferred method for labeling was amplification by PCR, using dUTP labeled with fluorochromes. Commercially obtainable dUTP-SpectrumGreen (for delE12-31) and dUTP-SpectrumOrange (for delE11-17) (Vysis Inc., Downers Grove, IL, USA) were used in the amplification reactions. Direct labeling enabled the application of both probes, labeled with different fluorochromes, in the same hybridization reaction. This introduced a second advantage as one probe could serve as control for the other.

PCR labeling is useful for producing highly labeled probes from limited amounts of template DNA. A range of probe dilutions was applied to

determine the optimal amount of template DNA necessary in the labeling PCR reactions (Figure 4.4). To reduce background and to prevent interference with the signal the labeled amplified product (probe) has to be as specific as possible. Optimal results were concentration of 0.022ng and 0.32ng of template DNA, for the delE12-31 and delE11-17 deletions, respectively. Secondly, the recommended concentrations of dUTP-Spectrum Green/Orange (Vysis Inc., Downers Grove, IL, USA), 10μM (20 dTTP: 1dUTP), 20μM (10 dTTP: 1 dUTP), 40μM (5 dTTP: 1 dUTP) and 60μM (3 dTTP: 1 dUTP) substituting the dTTP in the non-labeling PCR reaction were optimized (Figure 4.4). The ratio of 20:1 (dTTP: dUTP) proved to be the optimum and was used in the labeling reaction.

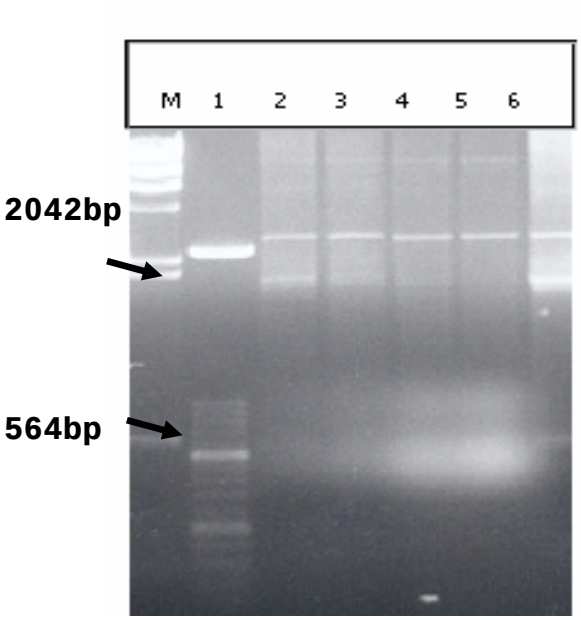
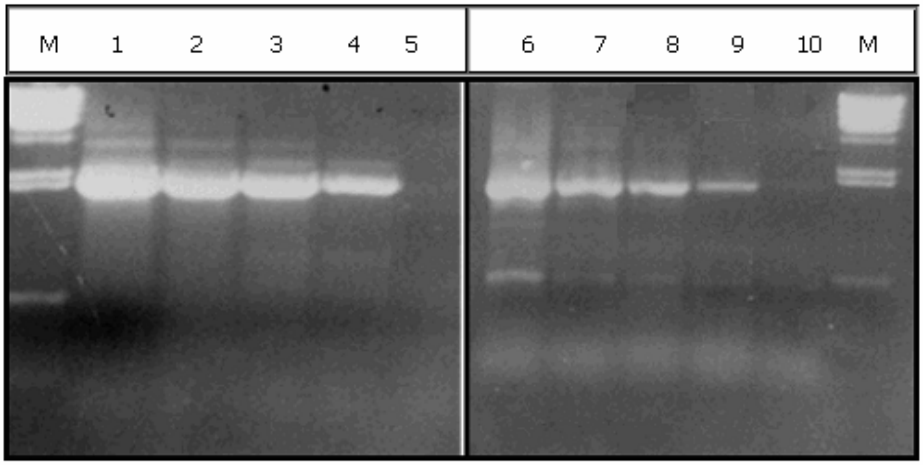


Figure 4.4 Optimization of dUTP-SpectrumOrange (delE11-17) concentration. M = Lambda DNA/Hind III marker, lanes 2 (10μM dUTP), 3 (20μM dUTP), 4 (40μM dUTP), 5 (60μM dUTP) and 6 (without dUTP) acted as control.



A B

Figure 4.5 Range of dilutions of the recombinant DNA probe. M = Lambda DNA/Hind III marker, lanes 1-5 delE12-31 mutation, lanes 6-10 delE11-17 mutations. Dilutions were as follows: lanes 1 (22ng), 2 (2.2ng), 3 (0.22ng), 4 (0.022ng), 6 (32ng), 7 (3.2ng), 8 (0.32ng), 9 (0.032ng), with lanes 5 and 10 acting as zero controls.

Although both these labeling methods provided highly labeled probes it was impossible to visualize the hybrids by fluorescent microscopy. This may most probably be due to probe size (1.8kb for delE11-17 and 2.3kb for delE12-31). Even though direct labelling is the most cost-effective and rapid method of detection the mayor limitation is imposed by probe length. Fuller and Perry (2002) suggested much longer probes for application in direct fluorescent detection.

4.3.2 Indirect labeling

Indirect labeling, utilizing secondary antibodies provides an extra level of sensitivity by enhancing the signal and may provide a way around the problems encountered by direct labeling. In contrast with direct labeling where the fluorochrome is incorporated into the probe and become part of the inherent character of the probe, indirect labeling involves introduction of the fluorochrome (antibody) to the hybrid molecule (target and probe) only after hybridization. Beneficial to direct labeling is the use of many probes labeled with different fluorochromes, in the same reaction, whereas indirect labeling only allows one fluorochrome per detection. PCR conditions for direct labeling were adopted with the exception of digoxigenin-dUTP (Roche Molecular Diagnostics). Various labeled probe concentrations, 20ng, 50ng, 100ng, 150ng and 200ng, were utilized in the hybridization reactions. Afterwards, detection with anti-digoxigenin fluorescent labels rhodamine or fluorescein was applied. Hybridization was performed on normal cell lines.

No signals, for both probes, were visible after hybridization with the 20ng probe concentration, whereas very faint signals were observed with the 50ng probes. Clear signals with no background were observed with the 100ng delE12-31 probe (2.3kb) but tended to be non-specific to the target (2–4 signals per interphase cell), while the delE11-17 probe (1.8kb) of the same concentration (100ng) produced a faint signal with less specificity to the target (2–6 signals per interphase cell). Clear signals were visible after hybridization with the 150ng concentration probes. Although no background was visible with the delE12-31 probe (Figure 4.6), background may interfere with the signal in the case of the delE11-17 probe (1.8kb). Target specificity for the delE12-31 probe (2, 3 or 4 signals per interphase cell) was better than that obtained for the delE11-17 probe (1–6 signals per interphase cell). More than 70% of all cells analysed for both probes, however, revealed two bright

signals. The 200ng delE12-31 probe produced even brighter signals, with no background, but forfeited target specificity (>6 signals per interphase cell). The background tended to be a problem with the smaller delE11-17 probe (200ng concentration), since signal intensity was reduced and target specificity was almost lost. The cut-off value for probe concentration ranges between 100ng and 200ng. The 150ng concentration probes resulted in clearly visible signals generated almost no background and tended to be more target specific. To improve target specificity the stringency of the hybridization buffer (the formamide content was elevated from 50% to 70%) was intensified and hybridization temperature was increased to 39°C, but this rendered no hybridization at all.

	*Probe delE12-31 (2.3kb)	Probe concentration (ng)	*Probe delE11-17 (1.8kb)
Signal intensity	None	20ng	None
Target specificity			
Background			
Signal intensity	++	50ng	+
Target specificity	?		?
Background	-		-
Signal intensity	++++	100ng	+++
Target specificity	2-4		2-6
Background	-		-
Signal intensity	+++++	150ng	++++
Target specificity	2-4		1-6
Background	-		+
Signal intensity	++++++	200ng	+++
Target specificity	>6		Almost lost
Background	-		++

Table 4.1. Standardization of probe concentration in interphase cells of normal controls.

? signals were not clear enough to determine quantity.

* stringency of hybridization conditions: hybridization temperature at 37ºC and hybridization buffer contains 50% formamide.

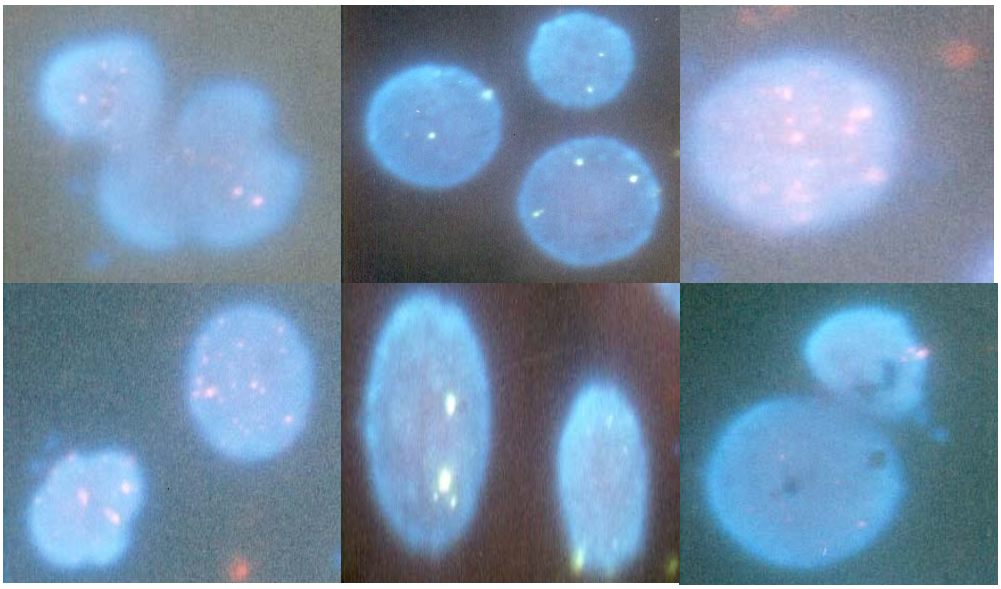


Figure 4.6 FISH analysis of normal control cells (A) delE12-31 and (B) delE11-17, [(I) 100ng, (II) 150ng, (III) 200ng].

Exploiting the more stringent hybridization buffer (70% formamide content), hybridization temperature (37°C) and optimal probe concentration (150ng), indirect labeling was repeated on three molecularly characterized Afrikaner patients. The father was characterized as heterozygous for the Afrikaner founder mutation delE12-31, the mother as heterozygous for delE11-17, while the affected child was a delE12-31/delE11-17 composite heterozygote. Easily visible and clear signals with no background were obtained in all three patients. Hybridization with the delE12-31 probe yielded one signal in the majority (>70%) of the father's interphase nuclei, whereas two signals were observed (>70% cells) with the delE11-17 probe. In the

mother, the majority of cells (>70%) revealed two signals after hybridization with the delE12-31 probe, and one signal (>70% cells) with the delE11-17 probe. The FA daughter's (delE12-31/delE11-17) FISH results with both probes revealed predominantly (>70%) only one signal per interphase cell. Even though the number of signals in the majority of cells (>70%) were in agreement with the ploidy of the specific DNA sequence, the remaining cells revealed a mixture of either one, two, or three signals. Although, after hybridization, both probes resulted in easily visible fluorescent signals, the smaller delE11-17 probe (1.8kb) tended to be more prone to background interference with the signal and to be less target-specific. Probe hybridization efficiency and background are influenced by the size of the labeled probe. Efficiency of hybridization detection decreases with decreasing probe size. For *in situ* hybridization the length of the probe molecule is critical for probe diffusion and hybridization to the specific target sequence.

Probe		Father delE12-31	Mother delE11-17	Child delE12-31/delE11-17
*delE12-31	Signal intensity	+++	+++	+++
	Target specificity	1 (>70%)	2 (>70%)	1 (>70%)
	Background	-	-	-
*delE11-17	Signal intensity	+++	+++	+++
	Target specificity	2 (>70%)	1 (>70%)	1 (>70%)
	Background	-	-	-

Table 4.2 FISH results in interphase cells from molecularly characterized FA patients. *hybridization conditions: hybridization temperature 37°C, hybridization buffer contains 70% formamide, probe concentration 150ng.

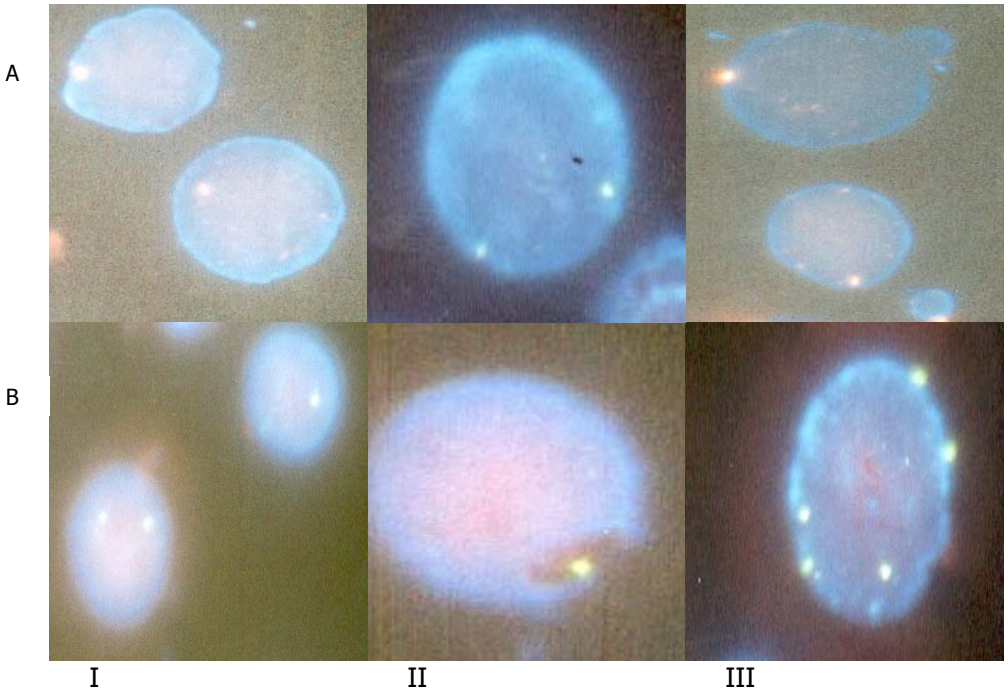


Figure 4.7 FISH results in interphase cells from molecularly characterized FA patients (A) delE12-31 probe and (B) delE11-17 probe, [(I) father, (II) mother, (III) child].

CHAPTER 5

CONCLUSIONS

Investigation of the clinical phenotype associated with specific mutations in FA is difficult due to the wide heterogeneity of mutations causing the disease (Magdalena et al, 2005). A single large intragenic deletion in exons 12-31 and exons 11-17 of the *FAA* gene was previously found to be present in 60% and 13% of the Afrikaner patients. FISH, a new avenue in routine diagnosis setting, opens doors to a cost effective and unequivocal diagnosis in genetic diseases caused by deletions and insertions. Fluorescent *in situ* hybridization techniques allow specific nucleic acid sequences to be detected in morphologically preserved chromosomes, cells or tissue sections. Using the FISH route, we attempted to implement a population-directed diagnosis focusing on the two major gene defects underlying the FA phenotype in Caucasians of Afrikaner lineage (delE12-31 mutation and delE11-17 mutation) and to evaluate whether FISH can be used as a diagnostic tool in the screening of the Afrikaner delE12-31 and delE11-17 mutations in FA patients and carriers. The phenotypic heterogeneity of FA can delay or impair clinical diagnosis. The current laboratory test consists of cytogenetic analysis looking for chromosomal breakage in response to DEB or MMC. Mutation screening mostly involves nucleotide substitutions, small insertions and deletions, while FISH is par excellence applied in the detection of hemizygous microdeletions. To date, the detection of hemizygous deletions is restricted to only two methods, other than FISH (Morgan et al., 1999; Tipping et al., 2001; Callèn et al., 2004). Both these screening strategies depend on dosage analysis and therefore require sophisticated and expensive apparatus such as a DNA sequencer and appropriate software. Since our laboratory offers FISH as routine diagnostic service, the most

expensive tool needed in the analysis, a fluorescent microscope is available and therefore contributes to the choice of FISH as diagnostic tool in FA.

Various non-radioactive techniques for probe labeling with fluorochromes exist and are widely applied. Labeling by thermal cycling was the first method of choice since PCR is commonly used and offers a familiar avenue to embark on. Probe length provides some consideration and cloning into a plasmid vector not only provides a means of multiplication of the probe, but, in addition, enlarges probe size for application in the second labeling method, the nick translation assay. Using this method a heterogeneous population of DNA strands are formed which enhance the kinetics and may lead to signal amplification in the hybridization experiment. The labeling density allows optimal enzymatic incorporation of the modified nucleotide and produces the most sensitive targets for direct (immunological) detection.

Direct labeling offers an efficient and rapid way to FISH. Like every method the advantages, disadvantages and applicability of the specific technique have to be considered and in this study the probe size hinders the direct approach. Even though direct labeling by both thermal cycling and nick translation was optimal, visualization by fluorescent microscopy was unsuccessful. Hybridized DIG-labeled probes may be detected with high affinity anti-digoxigenin antibodies that are conjugated to a fluorescent dye. However, indirect labeling with secondary antibodies are more timeous, but it proved to be a more sensitive method for detection. We again embarked upon labeling by thermal cycling using DIG-dUTP and immunochemistry. Bright, clearly defined signals were obtained after hybridization. Predominantly (>70% of interphase nuclei) the number of signals were in agreement with the ploidy of the specific DNA sequence, but target specificity tends to be a problem especially with the smaller probe. Probes that are too small tend to bind non-specifically and re-hybridize so that a smaller volume of probe is available for hybridization to the target.

Probe size should be improved in order to provide an extremely sensitive method for the detection of the delE12-31 and delE11-17 gene deletions. A longer probe will improve target-specificity and reduce the possibility of hybridization to other complementary regions in the genome. The identification of patients with specific mutations in the FA gene will lead to a better clinical description of this condition, also providing data for genotype-phenotype correlation studies and lead to a better understanding of the interaction of the specific mutation with other mutations in compound heterozygotes.

Using this population-directed approach rather than offering all-out testing for all patients seems to be the ethical and cost-effective option. This study has emphasised the importance of a population-directed molecular screening strategy for the diagnosis of FA. Although the ethical and psychological consequences of genetic screening have to be considered, potential harmful effects of a molecular diagnosis of FA would be largely outweighed by the benefits of preventive treatment.

Future perspectives

The importance of genetic testing in assessing prognosis and identifying high-risk family members is increasingly recognized and it can be expected that medical advances linked to the mapping of the human genome will radically change the healthcare process.

A recently experimental apparatus, the PCRJet, is the fastest thermocycler for high-speed amplification of DNA available. This apparatus can be applied in future to alleviate the burden of instrument sharing by reducing cycling time. A further more exciting benefit is the amplification and construction of synthetic genes or regions of DNA which can serve as FISH probes. Applying this technology, longer FISH probes for the diagnosis of FA

can be constructed fairly easy and more cost-effective than with present techniques (Moore, 2005).

SUMMARY

Fanconi anaemia (FA) is a rare autosomal recessive and X-linked disorder characterized by a very high frequency of bone marrow failure and many other manifestations. These include, but are not restricted to, severe birth defects and marked predisposition to malignancies, especially acute myeloid leukaemia and, to a lesser extent, solid tumours (Rodriguez et al, 2005). Cells from FA patients are hypersensitive to agents that produce DNA cross-links, and after in vitro treatment with these agents, display marked chromosome breakage and other cytogenetic abnormalities.

FA shows genetic heterogeneity with mutations in any of twelve genes resulting in a similar phenotype. Current diagnostic criteria for FA relies mainly on cytogenetic quantification of chromosomal breakage in response to DEB and/or MMC. The diagnostic value of induced chromosome instability does not appear to be feasible for differentiating between FA carriers and non-carriers, since overlapping in quantitative values between the two groups is common place.

In this investigation a population based screening strategy was followed. The method based on fluorescent *in situ* hybridization (FISH) was applied to allow a rapid and unequivocal identification of two founder Afrikaner *FAA* gene deletions, in both homozygous and carrier states.

Direct labeling by both nick translation and thermal cycling amplification, using dUTP-labeled fluorochromes, resulted in no visible signals after hybridization, even though labeling proved to be successful. This restriction may be ascribed to the relatively small size (1.8kb and 2.3kb, respectively) of the DNA probes. Efficiency of hybridization detection decreases with decreasing probe size and a more sensitive detection method may solve this problem.

Indirect labeling by polymerase chain reaction (PCR) amplification using digoxigenin-dUTP (DIG-dUTP) and antibodies (anti-DIG fluorochromes), provides an extremely sensitive method of detection, albeit more time consuming and costly. Bright, clearly defined signals were visualized after hybridization, using fluorescent microscopy. Stringent hybridization conditions, such as formamide contents of the hybridization buffer (70%) and optimal probe concentration (150ng), enhanced target-specificity and reduced background interference to almost none.

Predominantly (>70% of interphase nuclei) the number of signals were in agreement with the ploidy of the specific DNA sequence, but the remaining cells revealed a mixture of either one, two or three signals. Target specificity tends to be a problem, especially with the smaller probe. Probes that are too small tend to bind non-specifically and re-hybridize, leaving smaller amounts of probe available for hybridization to the specific target. Even though, after hybridization, both probes resulted in easily visible fluorescent signals, the smaller delE11-17 probe (1.8kb) tended to be more prone to background interference with the signal, and, in addition, less target-specific. Probe hybridization efficiency and background are both influenced by the size of the labeled probe. The length of the probe molecule is critical for probe diffusion and hybridization to the specific target sequence.

Probe size should be improved in order to provide a reliable and unequivocal diagnostic tool in the diagnosis of both FA patients and carriers. Longer probes will improve target-specificity and reduce the possibility of hybridization to other complementary regions in the genome.

In conclusion, making use of this unique application of FISH offers an effective population directed screening for FA carriers and affected.

KEYWORDS: Fanconi anaemia, *FAA*, FISH, founder mutations, delE12-31, delE11-17, PCR, Afrikaner population.

OPSOMMING

Fanconi anemie (FA) is 'n seldsame, outosomaal resessief oorgeërfde toestand, gekenmerk deur 'n hoë voorkoms van beenmurgversaking asook verskeie ander defekte. Laasgenoemde sluit erge geboortegebreke en 'n verhoogde neiging to maligniteite soos akute miëloiëde leukemie in, maar is nie noodwendig daartoe beperk nie (Rodriques et al., 2005). Die selle van FA pasiënte is hipersensitief teenoor agense wat DNA-kruisverbindings vorm. In vitro behandeling hiermee veroorsaak 'n verhoogde insidensie van chromosoombreuke en ander sitogenetiese veranderinge.

FA toon 'n genetiese heterogeniteit, met mutasies in enige van elf gene wat 'n ooreenstemmende fenotipe tot gevolg het. Die teenswoordige diagnostiese kriteria vir FA berus hoofsaaklik op die sitogenetiese kwantifisering van chromosoombreuke uitgelok deur DEB en/of MMC. Diagnosties is geïnduseerde chromosoomonstabiliteit egter nie 'n betroubare maatstaf vir die onderskeid tussen FA draers en nie-draers nie, angesien oorvleueling in kwantitatiewe waardes tussen die twee groepe te groot is.

'n Strategie wat sifting van 'n bevolkingsgroep ten doel het, is met hierdie ondersoek gevolg. 'n Tegniek gebaseer op Fluoressensie In Situ Hibridisasie (FISH) is aangewend vir vinnige diagnose van en ondubbelsinnige onderskeid tussen draers en aangetastes van twee Afrikaner *FAA* geendelesies.

Direkte merking deur beide kerfvertaling en hittesiklus-amplifisering deur gebruik van dUTP-gemerkte fluorochrome, het na hibridisasie geen waarneembare seine opgelewer nie. Merking was oënskynlik suksesvol. Die relatiewe klein grootte van die DNA peilers (1.8kb en 2.3kb, onderskeidelik), is waarskynlik hiervoor verantwoordelik. Die effektiwiteit van hibridisasie daal met afname in grootte van die peiler. Hierdie probleem kan aangespreek

word deur gebruik te maak van 'n meer sensitiewe tegniek vir waarneming van die peiler.

Indirekte merking deur middel van van polimerase kettingreaksie (PCR)-amplifisering en die gebruik van digoksigenien-dUTP (DIG-dUTP) en antiliggame (anti-DIG fluorochrome), is 'n hoogs sensitiewe opsporingstegniek, alhoewel tydrowend en duur. Na hibridisasie het die seine helder en duidelik afgebaken onder die fluoressensiemikroskoop vertoon. Die toepas van streng hibridisasiekriteria soos 70% formamiedinhoud van die hibridisasiebuffer en 150ng as optimale peilerkonsentrasie, verhoog teikenspesifisiteit en verminder agtergrondsteurnis tot feitlik niks.

In meeste gevalle (>70% van die interfase kerne) was die aantal seine in ooreenstemming met die ploiëdie van die spesifieke DNA volgorde, terwyl die oorblywende selle 'n mengbeeld van een, twee of drie seine vertoon het. Teikenspesifisitiet was veral problematies by die kleiner peiler. Peilers wat te klein is neig om nie-spesifiek te bind of te herhibridiseer, wat kleiner hoeveelhede vir hibridisasie laat om aan die spesifieke teiken te bind. Alhoewel dit maklik was om seine van beide peilers na hibridisasie waar te neem, was die kleiner delE11–17 peiler (1.8kb) meer onderhewig aan agtergrondsteuring, asook minder teikenspesifiek. Die effektiwiteit van peilerhibridisasie sowel as agtergrondsteurnis word beide deur die grootte van die gemerkte peiler beïnvloed. Die lengte van die peilermolekuul is van kritiese belang vir peilerverspreiding en hibridisasie aan die spesifieke teikenvolgorde.

Die grootte van die peiler is van deurslaggewende belang in die daarstelling van 'n betroubare en ondubbelsinnige diagnostiese tegniek vir die diagnose van beide FA pasiënte en draers. Deur van langer peilers gebruik te maak, kan teikenspesifisiteit vehoog en die kanse vir hibridisasie aan ander komplementêre streke verminder word.

Die slotsom is dat FISH op ‘n unieke wyse aangewend kan word om ‘n bevolkingsgerigte sifting vir FA draers en aangetasdes op koste-effektiewe manier uit te voer.

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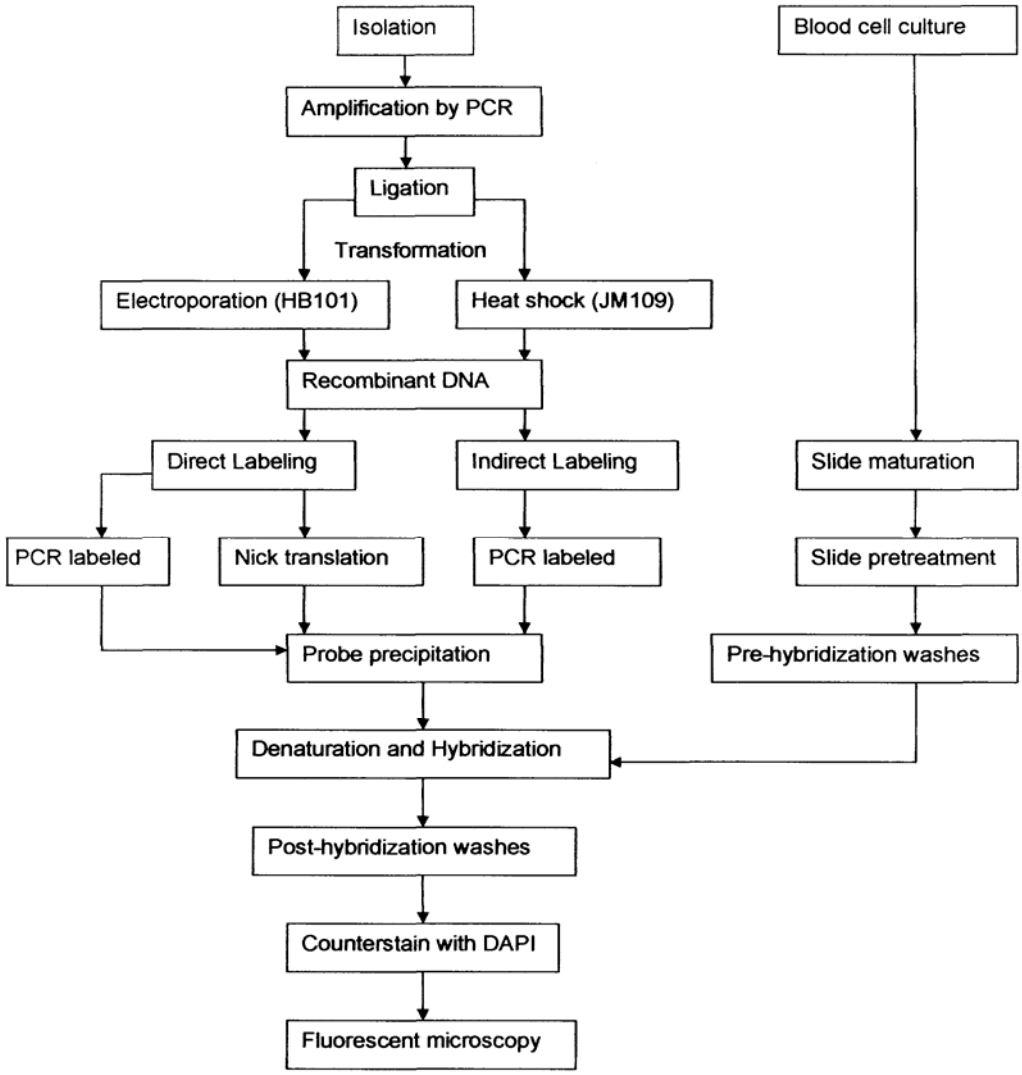
APPENDIX A

FLOW CHART OUTLINING THE RESEARCH FOR THIS DISSERTATION



APPENDIX B

SOLUTIONS COMPONENTS

1 X SET	100mM NaCl, 1mM EDTA, 10mM Tris-HCl pH 8.0
1 X TE	10mM Tris pH 7.6, 1mM EDTA pH 7.8
2 X Rapid ligation buffer	60mM Tris-HCl pH 7.8, 20mM $MgCl_2$, 20mM DTT, 2mM ATP, 10% PEG
2 X SSC	7ml 20 X SSC, pH 5.4, 14ml distilled water, mixed well; pH 7.0-8.0
70% formamide	49ml deionized formamide (500ml formamide and 50g of Amberlite MB-1A, mixed bed exchanger; Sigma, thoroughly mixed, overnight stirring at room temperature, filtered twice through Whatman No1 filter paper, aliquoted and stored at 4ºC)
Buffer P1	50mM Tris·HCl, pH 8.0; 10mM EDTA; 100µg/ml RNase A
Buffer P2	200mM NaOH, 1% SDS (w/v)
Buffer P3	3.0M potassium acetate, pH 5.5
Buffer QBT	750mM NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)
	LB medium 10g Bacto®-Tryptone, 5g Bacto®-Yeast extract, 5g NaCl
Buffer QC	1.0M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v)
Buffer QF	1.25M NaCl; 50mM Tris·HCl, pH 8.5; 15% isopropanol (v/v)

dNTP mixture	1.5 vol 0.4mM SpectrumGreen/Orange dUTP, 1.5 vol 0.4mM dTTP, 3 vol 0.4mM dATP, 3 vol 0.4mM dGTP and 3 vol 0.4mM dCTP
Formaldehyde	39ml 1 X PBS, 1ml 37% formaldehyde, 0.18g $MgCl_2$
Formamide solution	50% formamide/2 X SSC (105ml deionized formamide, 21ml 20 X SSC, 84ml dH_2O; pH 7.0-8.0)
Labeled probe	1µl labeled probe, 2µl dH_2O and 7µl LSi hybridization buffer
Lysis buffer	109.5g sucrose, 10ml 1M Tris-HCl pH 7.5, 5ml 1M $MgCl_2$, 10ml Triton X-100 made up to 1 litre with distilled water
Pepsin treatment	40µl of 10% pepsin solution in 40ml 0.01N HCl
SOC medium	2.0g Bacto®-Tryptone, 0.5g Bacto®-Yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1 ml Mg^{2+} stock (1M $MgCl_2 \bullet 6H_2O$, 1M $MgSO_4 \bullet 7H_2O$), 1ml 2M glucose, filter-sterilized
T4 DNA ligase	10mM Tris-HCl pH 7.4, 50mM KCl, 1mM DTT, 0,1mM EDTA, 50% glycerol
TNB	100mM Tris-HCl pH 7.5, 150mM NaCl, 0.5% blocking reagent
TNT	100mM Tris-HCl pH 7.5, 150mM NaCl, 0.05% Tween 20