TRACKING DOWN GENE INTEGRITY WITHIN FRAGILE SITES: DO THEY PLAY A ROLE IN OESOPHAGEAL CANCER?

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

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ABSTRACT

Oesophageal cancer (OC) is the third most common malignancy in South Africa (SA), affecting 1 in 20 and 1 in 76 black males and females respectively. Squamous cell carcinoma (SSC) is an aggressive disease showing a poor prognosis due to late diagnosis. Identification of genetic changes associated with these tumours may shed light on its pathophysiology and aetiology in SA. The chromosomal status of five OC cell lines, established in SA, was assessed to identify possible common chromosomal alterations by M-FISH (multicolour fluorescence *in situ* hybridisation) and specifically the fragile site loci, *FRA3B* and *FRA16D* by FISH (Fluorescence *in situ* hybridisation). The genes at these loci, *FHIT* (Fragile Histidine Triad) and *WWOX* (WW domain containing oxidoreductase) respectively, were analysed by RT-PCR (Reverse transcriptase polymerase chain reaction). *FHIT* was aberrantly expressed in four of the five cell lines while *WWOX* expression was normal. The *EGFR* (epidermal growth factor receptor) locus is frequently amplified and this gene is also over-expressed in OC. Increased *EGFR* expression was previously found in three of the cell lines, for this reason, particular attention was paid to markers involving the *EGFR* locus on 7p. An interesting marker chromosome seven was identified in one of the cell lines and further analysis, using a specific *EGFR* probe, revealed an amplification unit involving *EGFR* in this cell line. Common translocations involving chromosomes 3 and 1 as well as 3 and 22 were identified in two cell lines; these may involve a locus involved in OC and warrants further investigation.

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CHAPTER ONE – INTRODUCTION

1.1 Oesophageal Carcinoma: Epidemiology, molecular biology and treatment

Oesophageal cancer (OC) is the 5th most common cause of cancer death in the world (Vos *et al*, 2003) and is the third most common malignancy in South Africa, the risk of developing the disease is estimated at 1 in 20 for black South African males and 1 in 76 for black South African females (Pacella-Norman *et al*, 2002). Major cancers of the oesophagus in South Africa are squamous cell carcinoma (SCC) and the less frequent adenocarcinoma (AD) (Cancer Association of South Africa available at http://www.cansa.co.za/registryoesophageal.asp).

Oesophageal carcinoma has been linked to geographically located endemic regions such as the Transkei, Johannesburg and the Western Cape (Van Rensburg, 1987). In the Transkei it was found that with more than 35 years residence, there is a greater risk for women than men to develop OC (Pacella-Norman *et al*, 2002). Urbanisation is thought to be linked to OC incidence through the life-style changes, which have taken place for black South Africans such as increased alcohol consumption and cigarette smoking (Pacella-Norman *et al*, 2002). Smoking and alcohol consumption are known major risk factors for the development of OC and seem to have independent as well as a combined effect (Wang *et al*, 2004; Pacella- Norman *et al*, 2002). The tobacco in Transkei is home grown along with marijuana, which is more mutagenic than commercial cigarettes (Van Rensburg, 1987). It has also been observed that OC incidence is higher in those individuals that smoke black tobacco compared to those that smoke blonde tobacco. The reason is likely that black tobacco contains a higher content of tobacco

specific, carcinogenic, N-Nitroso compounds (Vos *et al*, 2003). Tobacco is considered to be the leading risk factor for OC and seems to contribute to early and late carcinogenesis while alcohol seems to contribute to late carcinogenesis (Pacella – Norman *et al*, 2002; Vos *et al*, 2003). The link between urbanised populations and the rural endemic regions such as the Transkei is that urbanised populations are subject to economic deprivation and like the poorer rural populations demonstrate restricted diets (Pacella-Norman *et al*, 2002). High risk for OC seems always to be associated with a diet based on maize or wheat (Van Rensburg, 1987). The effect of tobacco or alcohol consumption on OC risk appears to some extent to be associated with the nutritional level as well (Van Rensburg, 1987). Maize contaminated with *Fusarium moniliform* seems to be strongly associated with the development of OC (Van Rensburg, 1987; Pacella- Norman *et al*, 2002) and it seems that a set of micronutrients must be deficient for the risk of developing OC to increase. These nutrients include Vitamins A and C, riboflavin, nicotinic acid, zinc, magnesium, calcium and folate (Van Rensburg, 1987; Jaskiewicz *et al*, 1987). It has also been suggested that the use of liquid paraffin seems to be a significant risk factor for OC in women (Pacella-Norman *et al*, 2002).

The precise molecular mechanism for the development and progression of OC is still unclear (Kuroki *et al*, 2002). However, certain genetic abnormalities have been identified, which appear to play a role in this disease. International research groups have shed light on these genetic factors but whether these apply to the South African scenario remains to be investigated thoroughly. *p53* mutations seem to be a common occurrence in SCC and often are dinucleotide transversions, which supports the effect of exogenous carcinogens on the development of this disease (Metzger *et al*, 2004).

p53 mutations usually span exons 5 to 8 of the gene and are usually point mutations (Metzger *et al*, 2004). It has been observed that 57% of SCC had *p53* abnormalities in the surrounding non-malignant tissue supporting the idea that *p53* mutation is an early event in the development of SCC of the oesophagus (Metzger *et al*, 2004). Vos *et al* (2003) has found genetic alterations in exons 5 to 8 of *p53* in OC patients from the Transkei. Only 13% of SCC showed mutations, which could potentially lead to structural and functional changes to *p53*. These findings are in contrast to what American and Chinese studies found (Vos *et al*, 2003). Vos *et al* (2003) also looked at the flanking regions of these exons to look for mutations in the promoter region and found many polymorphisms, in particular, either a proline or an arginine at codon 72 in exon 4. Arginine at this position is thought to present a risk factor for the development of cancer and is associated with Human Papilloma virus (HPV) (Vos *et al*, 2003). The E6 oncoprotein produced by Human Papilloma viruses binds to *p53* amino acid sequence and results in its degradation and *p53Arg* alleles are more susceptible to E6 binding and degradation (Storey *et al*, 1998). Vos *et al* (2003) found HPV prevalent in 71% of their OC samples, however, they did not look at any relationship between the HPV and the disease or the prevalent subtype and it was suggested that these factors should undergo further investigation. It is thus hypothesised that polymorphisms in *p53* may either contribute to susceptibility or resistance to the development of OC (Vos *et al*, 2003).

Another role for polymorphisms in the development of OC is demonstrated in the Type I and Type II metabolic enzymes. Type I metabolic enzymes such as *CYP1A2*, are responsible for activating aromatic hydrocarbons (procarcinogens in tobacco) to their

reactive electrophilic forms, which can initiate carcinogenesis (Wang *et al*, 2004). Polymorphisms within the *CYP1A2* gene have been found to increase its activity, specifically the *CYP1A2/Val/Val* polymorphism in exon 7, which was found with incidences of 2-5% in whites, 19, 8% in Japanese and 22, 3% in Chinese with OC (Wang *et al*, 2004). At the same time the Type II metabolic enzymes are responsible for inactivating these reactive electrophilic compounds. Deletion of the gene coding for one of these enzymes, *GSTM1*, is associated with an increased risk for OC (Wang *et al*, 2004; Metzger *et al*, 2004). These findings demonstrate the synergistic relationship between genetic susceptibility and environmental factors (Wang *et al*, 2004). It would be interesting to determine the incidence of polymorphisms and deletion of these two genes in the South African black population.

SCC of the oesophagus has been shown to demonstrate increased expression of cellular growth factors and growth factor receptors such as Epidermal Growth Factor Receptor (EGFR). EGFR induces the expression of cyclooxygenase –2 (*COX-2*). *COX-2* has been found to be overexpressed in SCC and premalignant lesions (Altorki, 2004). It is responsible for inhibiting apoptosis and the tobacco constituent benzo(a)pyrene induces *COX-2* as well. Cyclooxygenases also play a rate-limiting role in the production of prostaglandins, which also have a role in uncontrolled growth (Altorki, 2004). *COX-2* derived prostaglandins have been shown to promote tumour invasion and metastasis (Altorki, 2004). *EGFR* over expression is correlated with a poor prognosis in many epithelial cancers (Khalil *et al*, 2003; Bulgaru *et al*, 2003). New cancer therapies are being designed to target EGFR, such as monoclonal antibodies and tyrosine kinase inhibitors. Cetuximab (Erbitux) is a drug currently being tested in phase III clinical

trials in combination with radiation or chemotherapy (Khalil *et al*, 2003). Another tyrosine kinase inhibitor, erlotinib (Tarceva), proved in phase II clinical trials to be a promising drug targeting *EGFR* over expression (Bulgaru *et al*, 2003). Determining whether an OC patient is over expressing *EGFR* may be worthwhile in terms of selecting therapy in the future.

Transforming growth factor β (TGF-β) strongly inhibits epithelial cell proliferation and many tumour cells lose their sensitivity to this factor (Fukuchi *et al*, 2004). The loss of sensitivity to TGF- β results in an accumulation of this protein, TGF-β then stimulates tumour invasion by promoting angiogenesis and extracellular matrix production, inhibiting the immune response (Fukuchi *et al*, 2004). Lower expression of TGF-β specific receptors and Smads (signal transducer proteins in the TGF-β growth inhibition pathway) or higher local TGF-β expression was found to be associated with oesophageal cancer progression. Blood taken from the azygos vein, the vein responsible for venous return from the oesophagus, was analysed for TGF-β levels. Increased levels were shown to correlate with metastasis to distant lymph nodes in patients with oesophageal cancer (Fukuchi *et al*, 2004). TGF-β levels in the azygos vein may prove to be a useful prognostic marker but needs further research. Thirty five percent of OC tumours show over expression of *TGF-α* (Metzger *et al*, 2004) as well.

The *p16* gene product is responsible for the inhibition of cell proliferation through its interaction with CDK6. Loss of *p16* expression has been shown in 50% of OC and is associated with a poor prognosis (Metzger *et al*, 2004). The oncogene, *Cyclin D1* is overexpressed in SCC and is associated with lymph node metastasis, high proliferation,

poor response to chemotherapy, and poor survival (Metzger *et al*, 2004). Table 1 summarises some of the genes thought to contribute to the development of OC.

Oesophageal carcinoma is an aggressive disease showing poor prognosis and so early diagnosis and treatment is in high demand (Mori *et al*, 2000). Surgery and radiotherapy or combined radiotherapy and chemotherapy are the current methods for treatment. The late diagnosis results in difficulty to perform surgery and the over all outcome is usually poor (Metzger *et al*, 2004). Further developments in the molecular carcinogenesis of this disease could improve the chances for early detection and improved therapies.

Table 1. Genes considered to be involved in OC development and progression

GENE	CHROMOSOME	CHROMOSOME ALTERATION
PK13CA, hTR	3q	Gain
FHIT	3p	Loss
CDH6, CDH12, CDH14	5p15.1-15.2	Gain
APC, MCC	5q21	Loss
EGFR, cyclin D1	7p12.13	Gain
C-MYC	8q24	Gain
p16	9p21-22	Loss
c-ras-Ha-1	11q13-15	Loss
TFDPI	13q34	Gain
RB1	13q14	Loss
p53	17p13	Loss
DCC, DPC4, SMAD2, SMAD4	18q21	Loss

Table adapted from Metzger *et al* (2004). APC= Adenomatosis polyposis coli, CDH= cadherin, C-MYC= c-myc myelocytomatosis oncogene, DPC= dystrophin associated protein complex, EGFR= epidermal growth factor receptor, DCC= deleted in colorectal cancer, hTR= human telomerase RNA, MCC= mutated in colorectal cancer, PK13CA= gene for catalytic subunit of phosphaticlylinositol-3-kinase, RB1= retinoblastoma locus 1, SMAD= signal transducer in the TGF-β pathway, TFDPI= transcription factor.

1.2 Fragile sites and cancer

Fragile sites are chromosomal regions expressed as gaps or breaks appearing under specific culture conditions such as folate deficiency or aphidicolin treatment (Yunis & Soreng, 1984). Folate deficiency and aphidicolin treatment results in the inhibition of DNA synthesis. Rare or heritable fragile sites are found in less than 5% of individuals, while common or constitutive fragile sites are found in all individuals (Richards, 2001). Fragile sites were originally studied under artificial culture conditions, which raised the

concern as to whether they ever occurred *in vivo* or if they were only artifactual. The discovery that the *FRA11B* fragile site is predisposed to 11q breakage, resulting in Jacobsen Syndrome cases, gave the evidence that fragile sites not only occurred *in vitro* but also *in vivo* (Richards, 2001). The most commonly expressed fragile sites are *FRA3B, FRA16D, FRA7G, FRA7H and FRAXB* (Richards, 2001). The hypothesis that fragile sites may be linked to cancer first arose due to the observation that these sites seemed to coincide with the chromosome regions frequently altered in cancer (Heubner & Croce, 2001). The first real link between fragile sites and cancer was made when it was discovered that a translocation involving 3p14 at *FRA3B* is associated with a familial renal cancer (Heubner & Croce, 2001). Since these discoveries twenty years ago, much more evidence linking fragile sites to cancer has been uncovered.

Expanded repeats in the DNA sequence of rare fragile sites form part of their molecular basis for fragility, however the molecular basis for the fragility of common fragile sites remains obscure (Richards, 2001). Studies looking into the sequence of *FRA3B* have given some insight into the reasons for their fragility. Aphidicolin (reversible inhibitor of eukaryotic DNA replication) stimulated breaks were found to be within so called high flexibility regions, which are thought to contribute to the fragility of *FRA3B* (Mimori *et al*, 1999). These regions are however unremarkable in sequence except for being AT-rich at the high flexibility regions (Heubner & Croce, 2001). These AT islands are said to act as scaffold attachment regions involved in genome organisation on the nuclear matrix and are preferentially targeted by DNA-alkylating anti-tumour drugs (Woynarowski, 2004). These AT sites have been shown to behave differently in cancer cells compared to normal cells and are abnormally expanded. This may sensitise

cancer cells to AT island targeting drugs, potentially a new cancer therapy (Woynarowski, 2004). Since it has been discovered that fragile sites replicate late in S-phase and gaps and breaks are incompletely replicated regions, this late replication could be contributing to fragile site instability (Brown, 2003). Fragile sites are proposed to be initiators of amplification of certain regions of DNA by acting as end points for the breakage-fusion-bridge mechanism of DNA amplification (Richards, 2001).

Fragile site induction has been shown to increase the frequency of deletion, translocation and foreign DNA integration (Inoue *et al*, 1997). Since these fragile sites coincide with chromosomal aberrations in cancer cells, those sites susceptible to carcinogen-induced alterations may play a major role in cancer cell rearrangements (Inoue *et al*, 1997). Fragile sites have been seen to interact with each other in metaphase, which may facilitate translocation (Richards, 2001). *FRA3B* has not only been found to be the site of a translocation in familial renal carcinoma (Gemmill *et al*, 1998) but also in an adenocarcinoma of the oesophagus (Ohta *et al*, 1996). Deletions involving the *FRA3B* locus are frequent in tissues exposed to the environment, indicating *FRA3B* as a possible "hot spot" for translocations, deletions and its possible sensitivity to environmental carcinogens (Richards, 2001). The most common fragile sites have been found to encompass genes proposed to be tumour suppressors; *FRA3B* and *FHIT*; *FRA16D* and *WWOX* as well as *FRA7G* and *Caveolin 1* and *2*. Since fragile sites have been associated with these genes that may contribute to the neoplastic process, it is thought that mutation at these fragile site loci may have a causative role in cancer (Richards, 2001).

Fragile sites are features of most mammalian genomes and specific regions of fragile sites seemed to be conserved across species (Heubner & Croce, 2001), indicating that they may have a specific function. It is thought that fragile sites are difficult to replicate and even small doses of replication inhibitors lead to the stalling of the replication fork, which could cause the expression of these fragile sites (Cimprich, 2003).

It has been discovered that the stability of fragile sites is controlled by a checkpoint kinase, ATR (Ataxia Telangiectasia and Rad 3 related protein kinase). ATR deficiency results in fragile site expression with or without replication inhibitors (Casper *et al*, 2002). ATR deficiency in rats results in death and upon looking at the blastocysts, massive chromosome fragmentation was observed (Casper, 2002). ATR is responsible for the repair of DNA at fragile sites (Brown, 2003). The gene responds to stalled DNA replication due to DNA damage during the cell cycle and is responsible for activating repair proteins and is thus thought to have a key role in genome maintenance (Casper *et al*, 2002).

It is proposed by Casper *et al* (2002) that fragile sites are single-stranded breaks resulting from collapsed replication forks following inhibition of DNA replication. Gaps represent single stranded breaks. Double stranded breaks are likely to be secondary events leading to chromosome rearrangements. In a study analysing the patterns of fragile site expression in smokers and non-smokers, it was noted that one normal individual displayed twice the level of expression as compared with the smokers (Stein *et al*, 2002). This lead to the hypothesis that some individuals in the population may have polymorphisms resulting in susceptibility to fragile site

expression in stressed DNA replication conditions. Another study looking at genetic susceptibility to oral cancer, found that two patients without any history of smoking, had enhanced expression of fragile sites, reinforcing this hypothesis (Subhadra *et al*, 2003). These individuals may even be ATR deficient and as a result be more susceptible to developing cancer, an interesting avenue for further research.

1.3 Nutritional and environmental effects on fragile sites, their genes and cancer

Caffeine, an inhibitor of DNA repair, induces a ten-fold increase in fragile site expression (Yunis, Soreng, 1984) and active tobacco exposure results in increased expression of *FRA3B*, which is reversed upon reduction of tobacco exposure (Sozzi *et al*, 1997; Stein *et al*, 2002). This indicates that the *in vivo* breakage of fragile sites appears to be affected by environmental factors producing DNA damage (Sozzi *et al*, 1997; Stein *et al*, 2002). The DNA damage could be associated with tumourgenesis through impaired DNA replication and repair. For example, nicotine has adverse effects on DNA replication through its induction of single stranded nicks (Stein *et al*, 2002). Since fragile sites are often associated with oncogenes, individuals with high carcinogen exposure and increased fragile site expression are proposed to be pre-disposed to malignancy (Yunis & Soreng, 1987).

Fragile site expression is induced by folic acid deficiency *in vitro*; perhaps folic acid deficiency could play a role in *FHIT* disruption through *FRA3B* expression *in vivo,* since folate plays a role in DNA synthesis, repair and methylation (Heimburger, 1992). Folate is involved in the re-methylation of homocysteine to methionine (Hiraoka *et al*,

2004). The methylenetetrahydrofolate reductase (MTHFR) is responsible for directing folate into this process. A polymorphism in this gene, the C677T polymorphism, results in lowered activity thereby causing genomic hypomethylation and reduced availability of pyrimidines and purines for DNA repair and synthesis, specifically in low folate status (Ueland *et al*, 2001; Beilby *et al*, 2004). This is another gene that has not been studied in South African populations with oesophageal cancer. Carcinogens inactivate folate co-enzymes, thereby reducing folate activity possibly linking its deficiency to cancer development (Heimburger, 1992).

High alcohol intake interferes with folate absorption and results in increased excretion through the kidney (Pelucchi *et al*, 2003). Alcohol also interacts with the *MTHFR* gene (Giovannucci *et al*, 2003). Some tissues may require higher levels of folate, specifically those cells undergoing rapid turnover such as the gastrointestinal tract (Heimburger, 1992). Nutrient deficiencies are said to place individuals at risk for the development of oesophageal carcinoma, especially in conjunction with carcinogen exposure (Van Rensburg, 1987, Jaskiewicz *et al*, 1987). Alcohol intake is known to aggravate mineral deficiencies, so does tobacco (Van Rensburg, 1987). Rural endemic regions show nutrient deficiencies as well as high exposure to the more carcinogenic, homegrown tobacco and marijuana as well as alcohol (Van Rensburg, 1987). Smokers were seen to have the highest frequency of fragile site expression, specifically in active smokers (Stein *et al*, 2002).

There is also the influence of fungal infections or toxin exposure due to the primary diet of endemic regions for oesophageal carcinoma being maize, which is commonly

infected with the Fungus, *Fusarium moniliforme.* The fungus releases toxins, such as Aflatoxin and Fumonisin, which are often detected at high levels in maize foods. Maize is often eaten several times a day and so these individuals are exposed to the toxins on a daily and long-term basis (Dutton *et al*, 2001). Exposure to these toxins has been correlated with the incidence of oesophageal cancer (Van Rensburg, 1987). Fumonisin B1 was found in tissues and blood of patients with oesophageal cancer and was found in high amounts within the cancerous tissue itself (Dutton *et al*, 2001). The exact effects are not known but could link it to the aetiology of the disease (Dutton *et al*, 2001).

Human Papilloma virus is an important risk factor for the progression of cervical cancer. The oncogenic E6 and E7 open reading frames are almost always retained and this is important in maintaining malignancy (Thorland *et al*, 2000). In a study of 50 oesophageal cancer patients in South Africa, 23 samples had detectable HPV DNA. The predominant HPV subtype detected in these OC samples was HPV-11, a lower risk subtype (Matsha *et al*, 2002). The dominant types detected in other international studies of OC are usually subtypes 16 and 18, higher risk subtypes (Matsha *et al*, 2002). It was demonstrated that common fragile sites are frequent targets for integration by these viruses in cervical cancer specimens and so genes within these regions may be disrupted and participate in the initiation or progression of cervical cancer (Thorland *et al*, 2000). The high prevalence of low risk HPV subtypes in South African oesophageal cancer supports the hypothesis that HPV integration within fragile sites may be disrupting these genes and participates in initiation or progression of cancer.

1.4 The *FHIT* gene and cancer

The cytogenetic analysis of many solid tumours and tumour derived cell lines revealed that the short arm of chromosome 3 is often involved in deletions in cancer (Croce *et al*, 1999). The finding of loss of heterozygosity and the fact that they coincided with the location of the t(3;8)(14.2; q24) in a familial renal cancer, lead to the discovery and cloning of the *FHIT* gene encompassing the *FRA3B* fragile site (Croce *et al*, 1999). Alterations in this gene, resulting in the inactivation of both alleles, are frequent in a variety of cancers (Mimori *et al*, 1999; Fang *et al*, 2001; Stein *et al*, 2002; Sozzi *et al*, 1997). Loss of heterozygosity (LOH) or loss of *FHIT* expression has been detected in oesophageal, head and neck squamous cell carcinoma, non-small cell lung carcinoma, oral, hepatocellular, tongue, cervical, ovarian and breast carcinomas (Fang *et al*, 2001; Ohta *et al*, 1996; Mori *et al*, 2000; Menin *et al*, 2000; Virgilio *et al*, 1996, Tseng *et al*, 1999; Tanimoto *et al*, 2000; Yuan *et al*, 2000; Lee *et al*, 2001; Connolly *et al*, 2000; Ozaki *et al*, 2001; Ingvarsson *et al*, 1999). It was indicated through these studies that loss of Fhit protein not only correlated with tumour aggressiveness but also was detected in pre-cancerous lesions, pointing out the possible importance of the *FHIT* gene in the initiation of cancer (Mori *et al*, 2000).

The *FHIT* gene is the second largest spanning locus in the human genome consisting of more than 1 Mb of DNA (Ishii *et al*, 2001b). The gene consists of 10 exons transcribed into a messenger RNA of 1,1kb. The Fhit protein is 16,8kD and is the human diadenosine triphosphate hydrolase (Barnes *et al*, 1996). This enzyme is homologous to the amino acid sequence of the Histidine Triad (HIT) proteins, with little known about its function (Barnes *et al*, 1996). The Histidine triad sequence is the catalytic

component of the protein, critical for the activity of Fhit in the hydrolysis of dinucleotide triphosphate (AP_3A). The HIT sequence also binds zinc, which seems to inhibit this function (Barnes *et al*, 1996). It was thought that increasing levels of AP_3A in the cell could possibly lead to carcinogenesis through enhancement of signal transduction or inhibition of apoptotic pathways (Barnes *et al*, 1996). It has since been shown that although decreased levels of AP_3A are linked to apoptosis (Sard *et al*, 1999), the tumour suppressor activity of the Fhit protein does not necessarily require the catalytic unit as introduction of the *FHIT* gene lacking the HIT coding sequence into cancer cells still resulted in tumour suppression and apoptosis (Sard *et al*, 1999, Siprashvili *et al*, 1997).

The evidence for *FHIT* as a tumour suppressor has been growing with numerous experiments involving introduction of *FHIT* into *FHIT* negative tumour cells. These experiments resulted in the induction of apoptosis and upon transplantation into nude mice, considerable tumour suppression and prevention of tumour growth was observed (Siprashvili *et al*, 1997; Roz *et al*, 2002; Dumon *et al*, 2001; Ishii *et al*, 2001a; Vecchione *et al*, 2001). It was also observed that *FHIT* over expression, that is the introduction of *FHIT* into *FHIT* expressing cells, did not have this result (Siprashvili *et al*, 1997). The exact mechanism of this tumour suppression is still unknown but increased expression levels of pro-apoptotic molecules such as Bak, caspase 8, caspase 3, caspase 9 was observed as well as Bid, caspase 8 and 9 cleavage was noted (Ishii *et al*, 2001b; Dumon *et al*, 2001; Sard *et al*, 1999; Roz *et al*, 2002). This suggests that these molecules are downstream of *FHIT* in a signalling pathway and induction of apoptosis is through the extrinsic or cytoplasmic pathway rather than through the

mitochondrial pathway (Roz *et al*, 2002). The mechanism of induction also appears to be independent of *p53*, since *p53* levels were unaffected in these experiments (Sard *et al*, 1999). Recently, Fhit was found to be a target for tyrosine phosphorylation by Src protein kinase. Src phosphorylates Y114 of Fhit in vitro and in vivo (Pekarsky *et al*, 2004). This phosphorylation may cause gain of function to Fhit and thereby stimulate apoptosis by interaction with target proteins, which have yet to be identified (Pekarsky *et al*, 2004). At the same time the exact role of Src in apoptosis is unknown as it is associated with apoptosis in thymocytes and 293 cells but inhibits apoptosis in fibroblasts and gall bladder epithelial cells. Phosphorylation of Fhit by Src may result in loss of tumour suppressor function (Pekarsky *et al*, 2004). Fhit is further linked to apoptosis by the finding that *FHIT* has an E2F-1 recognition site in the 5' region (Ishii *et al*, 2004a). E2F is key in cell proliferation and induction of apoptosis. When E2F-1 was over expressed in oesophageal cancer cell lines, apoptosis was induced by increased expression of *FHIT* (Ishii *et al*, 2004a). In *FHIT* deleted cells, E2F-1 had a lesser effect on the induction of apoptosis. Fhit protein was also seen to act on the cell cycle, arresting it at the G2 phase, as increased levels of $p21^{waf}$ were detected (Sard *et al*, 1999; Roz *et al*, 2002). Fhit was also found to be associated with microtubules and human ubiquitin-conjugating enzyme 9, which is involved in the degradation of S and M-phase cyclins (Ishii *et al*, 2001b).

The *FHIT* gene is expressed at low levels in most tissues of the body but interestingly the highest expression was detected in epithelial cells and tissues (Croce *et al*, 1999). *FHIT* is inactivated in epithelial tumours and specifically those exposed to environmental carcinogens (Croce *et al*, 1999). This loss of expression seems to occur

early in the development of these cancers while, in other cancers, it may be a later event corresponding to progression and aggressiveness. Deletion of *FHIT* usually results in the loss of exons and thus loss of full length *FHIT* transcript and protein (Siprashvili *et al*, 1997). Although some deletions occur within the non-coding introns, reduced *FHIT* expression or loss of expression is still observed in these tumours indicating that the introns still affect expression, possibly through the instability of the mRNA (Mimori *et al*, 1999). These deletions, leading to loss of expression, are cancer specific, a hallmark of a tumour suppressor (Siprashvili *et al*, 1997). No point mutations have been detected in the gene, indicating that the *FRA3B* fragile site is probably mechanistically involved in the deletion of the *FHIT* gene (Siprashvili *et al*, 1997). It is suspected that the frequent expansion of these clones with deletions in the *FHIT* gene, provide cells with a selective growth advantage (Siprashvili *et al*, 1997; Kholodnyuk *et al*, 2000).

The t(3;8) translocation involving *FHIT* found in familial renal cell carcinoma disrupts intron 2 while the *FRA3B* site is located over intron 4 and 5 and includes a Human Papillomavirus-16 (HPV16) integration site. These sites flank the first coding exon 5, which is the most commonly deleted exon, resulting in the loss of functional protein due to loss of the first methionine (Ishii *et al*, 2001b; Kholodnyuk *et al*, 2000; Druck *et al*, 1997). The mechanism of these deletions in exon 5, which forms greater than 60% of the gaps, could be attributed to the homologous recombination between LINE 1 elements, which have been found to be located near exon 5 (Inoue *et al*, 1997; Croce *et al*, 1999). Insertions are also detected between exons 4 and 5 resulting in loss of expression further indicating the importance of intron sequences for the synthesis of Fhit protein (Campiglio *et al*, 1999). Most breaks have been found to occur in the

proximal region of *FRA3B* between exon 4 and intron 5 as well as telomeric to exon 5 (Corbin *et al*, 2002).

The *FHIT* gene and the *FRA3B* site thus appear to have an important role to play in the development of cancer of tissues exposed to environmental carcinogens.

The FHIT gene and oesophageal cancer

Loss of heterozygosity studies of gastric cancers detected a high frequency of allelic deletion close to exon 5 in *FHIT* and barely detectable *FHIT* transcripts (Ohta *et al*, 1996). These events were detected in 50% of oesophageal, stomach and colon cancers (Ohta *et al*, 1996). Loss of heterozygosity (LOH) was 80% in SSC of the oesophagus and 44% in adenocarcinoma of the oesophagus with the highest incidence occurring in heavy smokers, indicating *FHIT* as a target for carcinogens (Menin *et al*, 2000). To elucidate the level at which these LOH events occur, Mori *et al*. (2000) looked for LOH in primary tumours, mild dysplasia and normal epithelium of the oesophagus in individuals with high exposure to carcinogens. Seventy six percent of primary oesophageal tumours displayed LOH and the cases of mild dysplasia lacked Fhit protein, as did the surrounding normal epithelium. This indicates that loss of *FHIT* is more than likely an early event in oesophageal carcinogenesis (Mori *et al*, 2000). These results were similar to those obtained for studies in lung cancer, indicating cigarettes and alcohol as major risk factors (Mori *et al*, 2000). Fang *et al* (2001) reported the finding of two translocations in an early passage oesophageal adenocarcinoma cell line. These translocations resulted in the formation of one non-functional chimeric transcript involving 16p13.3 sequences and *FHIT*. The other translocation resulted in the

juxtaposition of the 5' portion of *FHIT* on to a region on 4p. These findings provided evidence that fragile sites participate in translocations and suggested that translocations involving *FHIT* may not be infrequent events in oesophageal carcinoma (Fang *et al*, 2001).

Oesophageal cancer risk has also been related to folic acid, riboflavin and zinc deficiencies (Jaskiewicz *et al*, 1987). Fragile site expression is induced by folic acid deficiency *in vitro*. Perhaps folic acid deficiency could play a role in *FHIT* disruption through *FRA3B* expression *in vivo,* since folate plays a role in DNA synthesis, repair and methylation (Heimburger, 1992). As stated before, nutrient deficiencies are said to place individuals at risk for the development of oesophageal carcinoma, especially in conjunction with carcinogen exposure (Van Rensburg, 1987; Jaskiewicz *et al*, 1987). Carcinogens inactivate folate co-enzymes, thereby reducing folate activity possibly linking its deficiency to cancer development (Heimburger, 1992).

A Human Papilloma virus-16 (HPV 16) integration site has been mapped within *FHIT* and HPV sequences have been found integrated within *FHIT* coding sequences (Terry *et al*, 2002). LOH of *FHIT* was found in cervical carcinomas where there was HPV infection (Vecchione *et al*, 2001) and the high risk for HPV infection in South Africa could be associated with *FHIT* LOH in the oesophagus. However, loss of *FHIT* expression has not yet been associated with HPV integration at the *FRA3B* locus. SSC of the oesophagus has been found to contain a broad spectrum of HPV types, suggesting its etiological contribution to oesophageal carcinoma incidence (Lavergne *et al*, 1999). Human embryonic oesophageal epithelial cells infected with HPV 18 and

transplanted into nude mice, resulted in the development of oesophageal tumours (Shen *et al*, 2000). This provided further evidence for HPV and oesophageal carcinoma etiology and pathogenicity.

The finding, that loss of *FHIT* occurs in pre-cancerous lesions of the lung, indicates an early event and that it seems to be more common than *p53* mutation in lung cancer. These findings suggested *FHIT* expression as an indicator of carcinogen induced damage and initiation of the multi-step process leading to lung carcinogenesis (Croce *et al*, 1999; Varella-Garcia *et al*, 1998; Tseng *et al*, 1999). Many of the findings for oesophageal cancer are similar to those of lung cancer (Mori *et al*, 2000) including smoking and alcohol as risk factors for *FHIT* disruption. This suggests *FHIT* integrity as a marker for cancer risk in individuals with nutrient deficiencies and high carcinogen exposure.

Aberrant methylation of normally unmethylated CpG islands at the 5' promoter region of genes is associated with transcriptional inactivation (Sarkar *et al*, 1993). Methylation of the CpG island at the 5' promoter region of *FHIT* was found in breast, non-squamous cell lung carcinoma cell lines, as well as primary tumours. The gene itself had no other structural alterations (Sarkar *et al*, 1993). Methylation of *FHIT* was also found in 5 primary tumours of the oesophagus indicating that although structural changes may not be detected, methylation of the 5' promoter region may exist, thereby inactivating transcription of *FHIT* and subsequent loss of *Fhit* protein (Tanaka *et al*, 1998). Smoking can be a cause of methylation (Sarkar *et al*, 1993) and smoking is a risk factor for the development of oesophageal carcinoma. Methylation also seems to

occur early in disease progression (Zöchbauer-Müller *et al*, 2001; Tanaka *et al*, 1998) so could possibly act as a marker for early detection or susceptibility where structural changes in *FHIT* are not found.

1.5 The *WWOX* gene and oesophageal cancer

FRA16D has frequently demonstrated loss of heterozygosity (LOH) in hormonally active tissues including cancer of the testis and breast (Bednarek *et al*, 2000; Paige *et al*, 2000). The putative tumour suppressor gene, *WWOX/FOR,* was mapped within the LOH region of this fragile site (Bednarek *et al*, 2000). Src phosphorylates Wwox, like Fhit, at the first WW domain and this enhances Wwox binding of the *p53* homologue, *p73* (Aqeilan *et al*, 2004). Wwox physically interacts with *p73* at its first WW domain and triggers *p73* accumulation in the cytoplasm; loss of *WWOX* is thought to result in reduced apoptotic activity of *p73* (Aqeilan *et al*, 2004). *WWOX* expression was also found to increase when E2F-1 was introduced into oesophageal cancer cell lines and induction of apoptosis ensued (Ishii *et al*, 2004[a]). JNK1 is involved in cell survival through its transient activation but when JNK1 is activated persistently, apoptosis is induced. JNK1 phosphorylates Wwox at its first WW domain, inhibiting Wwox in apoptosis (Chang *et al*, 2003). However, at a certain stage of apoptosis, JNK1 may act synergistically with Wwox in facilitating cell death (Chang *et al*, 2003). A high LOH frequency was detected in SCC of the oesophagus and the most common region of deletion was found to be within intron 8, an area prone to breakage in *FRA16D* as well as the site of a translocation breakpoint found in a myeloma (Kuroki *et al*, 2002). *FRA16D* alterations have also been associated with Aflatoxin B1 (Kuroki *et al*, 2002), the *Fusarium moniliforme* fungus toxin found on maize plants, which has also been

associated with oesophageal cancer incidence through primary diet (Van Rensburg, 1987). Fhit and Wwox losses in invasive breast tumours are found to be strongly correlated, probably because they both encompass common fragile sites (Guler *et al*, 2004). This may be true for oesophageal cancer too.

1.6 The five oesophageal carcinoma cell lines

To analyse the possible roles for *FHIT* and *WWOX* in oesophageal cancer in South Africa, five oesophageal cancer derived cell lines were used. Professor Rob Veale developed the WHCO series of cell lines (Wits Human Carcinoma of the Oesophagus). The WHCO series were developed from moderately differentiated tumours from four South African black males (summarised in Table 2 in materials and methods). SNO is a cell line, which was established from a well-differentiated tumour taken from a 62-year-old black South African male in 1976. Cytogenetically SNO was found to be deficient in group D chromosomes, have excess group E and F chromosomes and a subtelocentric marker (Bey *et al*, 1976).

WHCO1, WHCO3 and SNO have been analysed for over expression of epidermal growth factor receptors (EGFR) by binding assays (Veale *et al*, 1989). The average number of receptors for normal epithelial cells is in the region of 2X 10^5 receptors per cell. Two kinds of receptors are usually expressed by oesophageal cancer cells, high affinity, associated with stimulation from low concentrations of epidermal growth factor (EGF) and low affinity receptors, associated with stimulation from high concentrations of EGF (Veale *et al*, 1989). These three cell lines express only low affinity receptors and between 4 and 20 times more receptors per cell than normal

keratinocytes (Veale *et al*, 1989). WHCO5 and WHCO6 also have over expression of EGFR found by binding assays. However this is unpublished data from R Veale.

1.7 Rationale for the project

1.7.1 Hypotheses

Numerous events are likely to contribute to the development of oesophageal carcinoma. The compound effect of alcohol consumption, smoking, folate and nutrient deficiencies and Fumonisin exposure are hypothesised to be linked to an increase in fragile site expression. All of these factors have been associated with the increased incidence of oesophageal carcinoma. Fragile sites are thought to play a major role in the initiation of cancer development through induction of deletions, translocations and viral integration leading to inactivation of tumour suppressor genes. It is thus hypothesised that the *FHIT* and *WWOX* genes, located at two fragile sites, may be disrupted or down-regulated early in the development of oesophageal carcinoma and detection thereof could act as markers for early diagnosis. It is further hypothesised that shared chromosomal genetic breakpoints resulting from a common pathway of genetic instability may be present in SA oesophageal carcinoma.

1.7.2 Aim and Specific objectives

This project used a model for oesophageal cancer in the form of the five oesophageal cancer derived cell lines. The aim was to assess the chromosomal status in these five oesophageal cancer cell lines in order to identify the presence of common chromosomal alterations. These common alterations could have resulted from common carcinogenic

exposures. The specific aim was to assess the fragile sites *FRA3B* and *FRA16D* and evaluate the expression of *FHIT* and *WWOX* transcripts.

Specific Objectives

1. To assess the integrity of the *FHIT* loci using a sequence specific, break apart rearrangement probes with Fluorescence *in situ* Hybridisation (FISH) on metaphase preparations from the 5 cell lines. (This probe set was used to assess the *FHIT* locus on interphase cells in a previous unpublished honours project by Brown and Stafne).
2. To assess the expression of the genes at fragile sites, *FHIT and WWOX* in the 5 cell lines using reverse transcriptase polymerase chain reaction.
3. To look for common chromosomal breakpoints and rearrangements in these cell lines using Multicolour Fluorescence *in situ* Hybridisation (M-FISH).
4. To assess *EGFR* DNA copy number since *EGFR* is over expressed in three of the cell lines. EGFR is considered as a therapeutic target in cancer.

The FISH, RT-PCR and M-FISH methodology and findings are discussed in the following chapters.

CHAPTER TWO – MATERIALS AND METHODS

2.1 SAMPLING

2.1.1 Cell lines

Five oesophageal cancer derived cell lines were used for this study. They are listed in table 2. The ovarian carcinoma cell line, UWOV2, was used in fluorescence *in situ* hybridisation experiments as a positive control for the break-a-part probe used for analysis of the *FHIT* locus. This cell line was previously found to have a translocation, t(3;11), spanning the *FHIT* locus (Rekhviashvili, 2001).

Table 2. Oesophageal cancer cell lines

CELL LINE	S	R	AGE	GRADE	SOURCE	YEAR
WHCO1	M	Black	47	Moderately differentiated SSC	Professor R Veale (WITS University)	1986
WHCO3	M	Black	71	Moderately differentiated SSC	Professor R Veale	1987
WHCO5	M	Black	47	Moderately differentiated SSC	Professor R Veale	1988
WHCO6	M	Black	39	Moderately differentiated SSC	Professor R Veale	1989
SNO	M	Black	62	Well differentiated SSC	Bey *et al.* (Virus Cancer Research Unit, Johannesburg)	1976

S= Sex, R= Race

RNA Isolation

The cell lines were cultured using standard cell culture protocol developed for the WITS cell lines, WHCO1, WHCO3, WHCO5, WHCO6 (R Veale, 1989) and SNO (Bey, 1976). The cells were grown in petri dishes and were harvested for RNA isolation upon full confluence. RNA was stabilised in RLT buffer from the Qiagen® RNeasy® midi kit. The cells were isolated using radiation sterilised scrapers (Nunc®) and lysed in 2ml of RLT buffer containing 20µl of β-mercaptoethanol. The cells were immediately stored at minus seventy degrees Celsius until further processing. The RNA was then extracted using the RNeasy® midi kit protocol. RNA was eluted into 100µl of RNase-free water; the elution was then repeated using 50µl of RNase-free water.

Metaphase Preparation

The cells were grown until the petri dishes were just over half confluent. The day before harvesting, the medium was changed. The cells were then incubated with colcemid (Karyomax® Colcemid®, Invitrogen Corporation) at a final concentration of 0,4µg/ml for 4 hours to induce chromosome condensation and halt the cells in metaphase by arresting mitotic spindle formation. The cells were then harvested using radiation sterilised scrapers (Nunc®) and poured into tubes for centrifugation at 900rpm for 10 minutes. The supernatant was removed and 0,5ml of 0,075M KCl (Appendix A) was added to re-suspend the cells, and then 7,5ml of 0,075M KCl and 1ml of Foetal Calf Serum (Sigma®) were added. The cells were incubated for 30 minutes at 37°C. 5 drops of fixative (Appendix A) were added before centrifugation at 900rpm for 10 minutes. The supernatant was then removed and the cells re-suspended in 5ml of fixative. The cells were then placed at minus twenty degrees Celsius for 1

hour. Two more fixative changes were then performed. The pellets were transferred to 1,5ml Eppendorf tubes and subsequent washes were performed by centrifugation at 6000rpm for 2 minutes. The cell pellets were then stored at –20 °C until slides were made.

2.1.2 Blood

Peripheral blood samples were isolated from two normal individuals to be used as controls for the study. The blood was collected in EDTA BD Vacutainers™ for RNA isolation as well as Sodium Heparin BD Vacutainers™ for cell culture.

RNA isolation

RNA was extracted from blood using the QIAamp® RNA Blood mini kit (Qiagen®). The blood was centrifuged at 1000rpm for 10 minutes and the buffy coat was removed for RNA extraction. The kit's protocol was followed but the cells were homogenised using a twenty-gauge needle and syringe instead of the Qiashredder™. RNA was eluted into 50µl of RNase-free water and stored at -70°C.

Metaphase preparation

500µl of whole blood was planted in 5ml of complete medium (Appendix A). The cultures were incubated for 96 hours at 37°C. At 72 hours, 50µl of methotrexate working solution (Appendix A) was added (final concentration of 10^{-7}M) to synchronise the cells and the cultures placed back into the incubator for 16 hours. Then 100µl of thymidine working solution (Appendix A) was added and the cultures incubated for 4 and a 1/2 hours. 100µl of Karyomax® Colcemid® (Invitrogen

Corporation, 10μg/ml) was then added (final concentration of 0,4μg/ml). Incubation was 30 minutes at 37°C. The cultures were then centrifuged at 1000rpm for 10 minutes and the supernatant removed. 5ml of 0,075M KCl was added and incubated for 20 minutes at 37°C, 5 drops of fixative was added before centrifugation. The supernatants were removed and 5ml of fixative added. The cells were incubated for 30 minutes at -20°C. The pellets were then transferred to 1,5ml eppendorf tubes and subsequent washes were performed at 6000rpm for 2 minutes. Ten fixative changes were done and the cell pellets were stored at -20°C until slides were made.

2.2 RNA EVALUATION

RNA was evaluated for concentration, purity and integrity by spectrophotometry and gel electrophoresis. All glassware used for the preparation of RNA buffers and solutions was baked at 200°C for 2 hours prior to use.

2.2.1 Spectrophotometry

A one in ten dilution was made of each RNA sample to be read by the GeneQuant Pro (Amersham Biosciences). Readings were taken at absorbancies of 280ηm and 260ηm. These readings were done to check the purity and concentration of the samples respectively.

2.2.2 Gel electrophoresis

Running a 1% denaturing agarose gel (Appendix B) checked the integrity of the RNA. 1μl of RNA was loaded with a drop of 5X RNA loading dye (Appendix B).

2.3 FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

2.3.1 Probe design and production

Bacterial artificial chromosomes (BACs)

The bacterial artificial chromosome is based on the Fertility plasmid of bacteria. This cloning vector is an artificial, self -replicating chromosome, which can accept an insert of about 300kb (Klug and Cummings, 2000). The vector carries an antibiotic resistance marker allowing for the selection of those clones carrying the insert. *E. Coli* serves as the host cells for the generation of BAC clones. The BAC clones chosen for this project are demonstrated in figure 1 (Obtained from BACPAC resource centre, Children's Hospital Oakland Research Institute, CA, USA). BAC 240 C07 lies centromeric to the *FHIT* locus and BAC 201J24 covers the region centromeric of exon 6 to within intron 8 (Fig.1). These two probes together are quite distant from each other on the chromosome allowing for the detection of breaks, which may occur in a large region including the regions of highest fragility.

Culturing of BACs

200ml of BAC growth medium, LB top agar (Appendix C), was aliquoted into autoclaved flasks. 20µg/ml of chloramphenicol (Sigma®) was added to the growth medium. 5ml of the 200ml volume was aliquoted into Nunc® tubes for inoculation with BACs from frozen glycerol stock cultures (Appendix C). The inoculated medium was incubated at 37˚C, orbiting at 200rpm for 2-6 hours. When the medium was turbid, the cultures were transferred to the flasks containing 195ml of medium. If they were not turbid they were left overnight. The 200ml cultures were incubated at 37˚C, orbiting at 180rpm overnight.

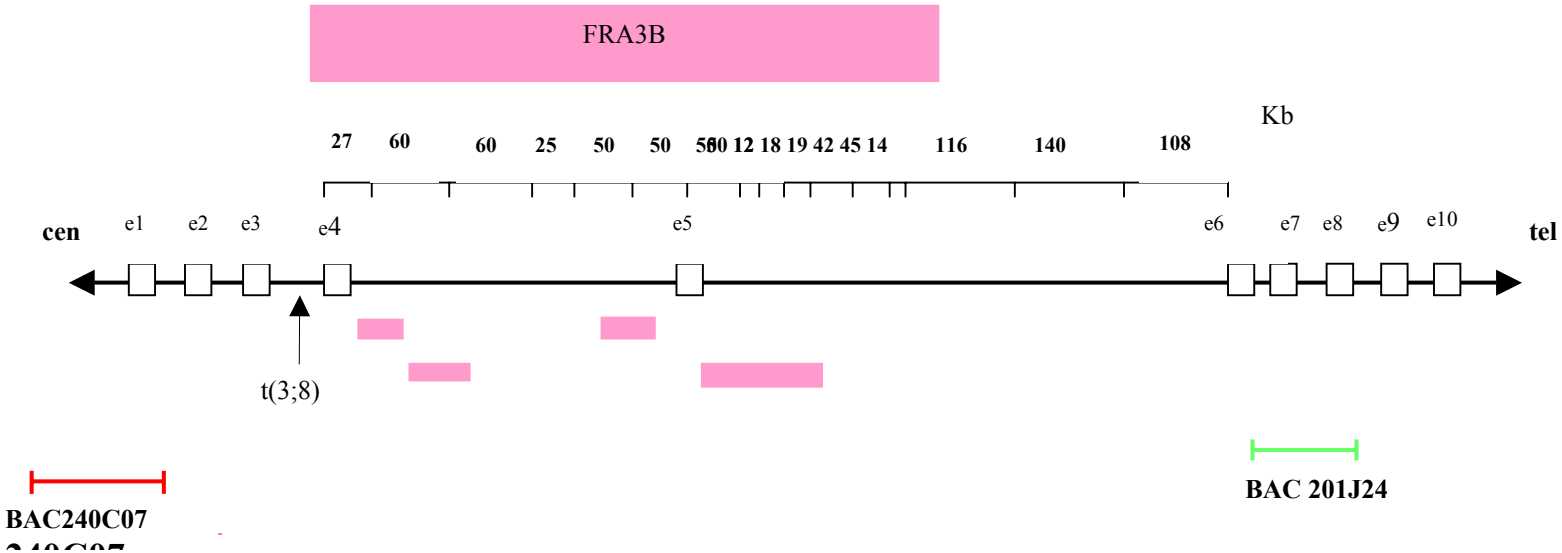

Figure 1. Schematic representation of the *FHIT* gene, indicating the position of the *FRA3B* fragile site and BACs used. The positions of the exons are indicated as well as the regions of highest instability (pink bars). Figure adapted from Corbin *et al* (2002).

BAC DNA extraction

The cultures were harvested by aliquoting them into 50ml Nunc® tubes and centrifuging at 5000 rpm for 15 minutes. DNA extraction was done using the Qiagen® Plasmid Purification Kit. Utilisation of the QIAfilter™ Plasmid Midi procedure resulted in very pure, protein and RNA free insert DNA with no chromosomal DNA. The protocol for the kit was strictly followed. The lysate is cleared through the Qiagen®-tip 100. Plasmid DNA is bound to the anion- exchange resin, while RNA, proteins and other impurities are removed by low salt washing. The high salt buffers then elute the DNA. The DNA is precipitated to remove the salt by adding isopropanol and centrifuging at 12000xg for 30 minutes at 4˚C. Washing with 70% ethanol followed and then centrifugation at 12000xg for 10 minutes at 4˚C. The pellets were air dried and re-suspended in an appropriate volume of 1X TE buffer (Appendix C) for the size of the DNA pellet.

Estimation of DNA yields

The concentration and purity of DNA extracted needs to be clarified for the efficient labelling of DNA in probe production. This was achieved by agarose gel electrophoresis. The spectrophotometer was found to be inaccurate for BAC DNA estimation, the readings given, were 2 fold that given by estimation by gel electrophoresis and so the latter was used instead.

To estimate concentration of DNA, the sample DNA was compared to a control DNA with known concentration. Lambda DNA (Sigma) was diluted to a 1/10 dilution, which was approximately 25ng/µl of DNA. 1µl of BAC DNA was loaded into a 2% agarose

gel (Appendix C) along with 25ng, 50ng and 100ng of Lambda DNA. The gel was run for 10 minutes at 100V. The electrophoresis tank contained 1X TAE buffer (Appendix C) in which the agarose had also been dissolved. DNA integrity could be determined by the presence of a band of DNA or a smear, in which case the DNA would be degraded.

Labelling of BAC DNA

The BAC DNA was directly labelled using the nick translation method. This method involves the nicking of the two DNA strands by DNase I and the 5' to 3' exonuclease activity of DNA polymerase removes nucleotides from the point of the nick and incorporates labelled nucleotides by its 5' to 3' endonuclease activity (Horton *et al*, 1996). BAC240C07 was labelled with SpectrumOrange-dUTP (Vysis Inc.) and BAC201J24 was labelled with SpectrumGreen-dUTP (Vysis Inc).

The reaction was made up to a final volume of 100µl, which consisted of final concentrations of 1x nick translation buffer (Appendix C), 0.01M β-mercaptoethanol (BDH), 0.8X nucleotide stock, which contained the fluorescent nucleotides. The fluorescent nucleotides were kept in a 50:50 ratio to thymine nucleotides to minimise steric hindrance (Appendix C). 210u/ml of DNA Polymerase (Promega), 1µl of a 0.48µl/1000µl dilution of DNase I solution (Appendix C) for BAC240C07 and 1µl of a 0.40µl/1000µl dilution for BAC201J24, 2µg/100µl of DNA. The reaction was incubated at 15˚C for 2 hours in the Eppendorf Mastercycler. The optimum probe size is 200-500bp for efficient penetration of the nucleus and hybridisation. The probe size and fluorescence incorporation was evaluated by 2% gel electrophoresis.

The probe was purified to remove unincorporated nucleotides by co-precipitation with 20µg of Cot1-Human DNA (Roche). 1/10 volumes of 3M-sodium acetate and 2, 5 volumes of ice-cold 100% ethanol were used to precipitate the labelled DNA by incubating at -70°C for 30 minutes and centrifuging at 13000xg for 30 minutes. The precipitated DNA was then washed in 70% ethanol to remove salts by centrifuging at 13000xg for 10 minutes. The probes were air dried and re-suspended in hybridisation buffer (Appendix C), usually 100ng of DNA per 10µl of hybridisation buffer.

2.3.2 Sample preparation

Cell lines as well as blood cultures were prepared for FISH in the same manner. Cell pellets were washed by changing fixative at least six times by centrifugation at 6000rpm for 2 minutes and removal of the supernatant. The cells were re-suspended in fresh fixative each time. These washing steps ensure the sufficient removal of impurities and loosen the cytoplasm for more efficient spreading of the chromosomes. Glass slides were then washed in methanol, rinsed in distilled water and wiped with paper towel. The slides were dipped in distilled water before dropping the cells onto the slide using a fine glass pipette and steaming for the appropriate time to obtain sufficient spreading of metaphase chromosomes. Passing them through 70, 90 and 100% ethanol respectively for 5 minutes each dehydrated the slides. The slides were aged overnight before using them for FISH. For long-term use they were stored at -70°C with silica gel crystals. However it was preferred to use freshly made slides aged overnight.

The slides for the UWOV2 cell line were stored slides from the previous study done on this particular cell line. Slides had been stored at -70°C. These slides were dehydrated in 70, 90 and 100% ethanol for 5 minutes each before hybridisation.

2.3.3 Hybridisation

FHIT break apart rearrangement

100ng of each of the probes BAC201J24 and BAC240C07 were denatured at 96˚C for 7 minutes and then pre-annealed by incubating at 37˚C for 30 minutes. Pre-annealing allows the COT1 human DNA to bind repetitive sequences so that the probe will bind specifically. The Cep 3 Alpha SpectrumOrange probe (Vysis Inc.) was denatured for 5 minutes at 76˚C. The recommended amount of probe was used according to the manufacturer's instructions. The probe for *FHIT* was then added to Cep 3 Alpha and mixed before adding to the slide.

EGFR gene amplification

The LSI EGFR SO/CEP 7 SG probe from Vysis Incorporated was used according to the manufacturer's instruction.

Samples and washing

The slides were denatured at 76˚C for 5 minutes and then dehydrated in ice-cold 70, 90 and 100% ethanol for 5 minutes each. The slides were air-dried and the probes were applied to the slide. Hybridisation was overnight or at least 15 hours at 37˚C. Washing was done at 42˚C, three washes in 50% formamide (Appendix C) for 10 minutes each, one wash in 2X SSC (Appendix C) for 10 minutes and one wash in 2X SSC with

Tween® 20 (Appendix C) for 5 minutes. The slides were then stained in DAPI (Appendix C) for 15 minutes and washed in DAPI wash solution (Appendix C) for 2 minutes. The slides were mounted with Vectashield (Vecta Laboratories) and a coverslip. The slides were analysed by fluorescent microscope and all images were captured using the Genus™ CytoVision 3.0 program from Applied Imaging.

2.4 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR).

The OneStep RT-PCR kit (Qiagen®) was used for all the PCR reactions done. In a twenty microlitre final volume, the PCR reaction consisted of the following final concentrations, 1X reaction buffer, 0.4mM dNTPs, 0.8µl enzyme mixture and 0.5ρmol forward and reverse primers (Table 4), 500ng-1µg of RNA. Cycling was done on the Eppendorf Mastercycler. Blanks were always included to exclude the presence of artefacts. PCR products were resolved on 2% agarose gels containing ethidium bromide (Appendix C). Primers are summarised in table 3.

2.4.1 *GAPDH*

The reaction was performed with the following cycles, 50°C for 30 minutes, 95°C for 15 minutes, then 35 cycles each of 95°C for 1minute, 56°C for 1 minute, 72°C for 1 minute and a final extension at 72°C for 7 minutes.

2.4.2 *FHIT* full transcript

The reaction was performed with the following cycles, 50°C for 30 minutes, 95°C for 15 minutes, then 35 cycles each of 95°C for 1minute, 62, 5°C for 1 minute and 30 seconds, 68°C for 3 minutes and a final extension at 68°C for 7 minutes. This longer extension time at a lower temperature allowed the extension of the large product while the high annealing temperature prevented the formation of spurious bands.

2.4.3 *Exon 5*

The reaction was performed with the following cycles, 50°C for 30 minutes, 95°C for 15 minutes, then 35 cycles each of 95°C for 1minute, 60°C for 1 minute, 72°C for 1 minute and a final extension at 72°C for 7 minutes.

2.4.4 *WWOX* full transcript

The reaction was performed with the following cycles, 50°C for 30 minutes, 95°C for 15 minutes, then 35 cycles each of 95°C for 1minute, 66°C for 1 minute, 72°C for 1 minute and a final extension at 72°C for 7 minutes.

Table 3. Primer sequences

GENE	PRIMERS	BINDING	REFERENCE	MANUFACTURER
GAPDH (589bp)	**F 5'** CCC TTC ATT GAC CTC AAC TAC ATG **3'** **R 5'** CAT GCC AGT GAG CTT CCC GTT CAG **3'**		Unknown	Genosys
FHIT (987bp)	**F 5'** 5' CTT TTT GCC CTC TGT TCC CG **3**' **R 5'** TGC CTG TCT GAG CCG TTT AGG TC **3'**	Exon 1 Exon 10	Fang *et al* 2001	MWG Biotech (F) Genosys (R)
EXON 5 (100bp)	**F 5'** ATG TCG TTC AGA TTT GGC C **3'** **R 5'** CTG GTA CCA CAG GTT TCC TA **3'**	Exon 5 Exon5	Fang *et al* 2001	MWG Biotech
WWOX (983bp)	**F 5'** GAG TTC CTG AGC GAG TGG A **3'** **R5'** GCT CGT TGG AGA AGA GGA T **3'**	Exon 1 Exon 9	Designed in this study	Inqaba Biotec

2.5 MULTICOLOUR FLUORESCENCE IN *SITU* HYBRIDISATION (M-FISH)

M-FISH was performed using the SpectraVysion™ Assay (Vysis®). The protocol was changed very slightly from the manufacturer's instructions. The pre-treatment buffers and wash buffers were made fresh each time instead of making larger volumes and storing them as they were found to go off very quickly. 20X SSC (Appendix C), 10X PBS and 1M $MgCl_2$ was prepared and stored for up to 6 months.

2.5.1 Sample preparation

Metaphase slides were prepared fresh, the day before hybridisation to achieve the best results. Slides were cleaned with methanol and then with distilled water and dried. A few drops of the cell pellets, which had been washed 10 times in fresh fixative, were dropped onto slides previously dipped in distilled water and then steamed for an appropriate amount of time to adequately spread the metaphase chromosomes. The slides were dehydrated in 70, 90 and 100% ethanol for 5 minutes each. The slides were aged overnight. Metaphase quality is very important for this technique to work. Slides were selected by chromosome colour, spreading and that no cytoplasm should be present. Metaphase chromosomes must not overlap and should be grey in colour.

2.5.2 Enzymatic pre-treatment

20µl of 500µg/ml DNase-free RNase (Roche) was diluted in 80µl of 2X SSC (Appendix C). The 100µl of RNase solution was added to the slide and incubated in a moist chamber at 37°C for 30 minutes. The slides were washed in 2X SSC twice for 5 minutes at room temperature. The slides were treated with the pepsin according to manufacturer's instructions for strictly 5 minutes and then washed twice in 1X PBS

(Appendix C) for 5 minutes. Fixation was done in formaldehyde according to the manufacturer's instructions; this was done for a strict 2 minutes before washing twice in 1X PBS for 5 minutes. The slides were then dehydrated in 70, 90 and 100% ethanol for 3 minutes each and air-dried.

2.5.3 Hybridisation

The probe was pre-warmed at 37°C for 5 minutes and then vigorously vortexed for about 1 minute to ensure that all SpectrumFRed probe was re suspended. This step was very important for even hybridisation. It was then spun down briefly before aliquoting 10µl per slide. The probe was denatured at 72°C for 5 minutes. The slides had already been denatured at 72°C for 2 minutes and dehydrated in ice-cold 70, 90 and 100% ethanol for 5 minutes each. The probe was applied to the appropriate area and incubated overnight for at least 18 hours at 37°C in a moist chamber.

2.5.4 Post hybridisation washing

The slides were washed in 0,4XSSC with 0,3% Tween® 20 (Appendix C) at 72°C by agitating the slides for 30 seconds and allowing to stand for 2 minutes. The second wash was in 2XSSC with 0, 1% Tween® 20 (Appendix C) at room temperature. The slides were agitated for a few seconds and then washed for 30 seconds. The slides were air-dried before applying Vectashield (Vecta Laboratories) containing DAPI for M-FISH (Appendix C). A cover slip was applied. The slides were analysed using the Applied Imaging program, Genus™ CytoVision 3.0. Six single band pass filters were used to visualise each of the fluorophores, which are listed in Table 4. The software allows the capture of each of the six images and a composite image comprising of the

computerised pseudo-colours for each of the chromosomes. 10 metaphases were analysed per cell line.

Table 4. List of the M-FISH fluorophores and their spectra.

PROBE	EXCITATION PEAK	EMISSION PEAK
SpectrumFRed probes	655nm	675nm
SpectrumRed probes	592nm	612nm
SpectrumGold probes	530nm	555nm
SpectrumGreen probes	497nm	524nm
SpectrumAqua probes	433nm	480nm
DAPI	367nm	452nm

CHAPTER THREE – RESULTS FOR ANALYSIS OF *FHIT* LOCUS INTEGRITY AND *FHIT* AND *WWOX* GENE EXPRESSION

3.1 Fluorescence *in situ* hybridisation (FISH) for *FHIT* locus integrity

3.1.1 Introduction

FISH is a powerful tool in detecting genetic or chromosomal alterations or rearrangements in cancer and has had the highest impact in molecular cytogenetics (Popescu, 2000). FISH is most powerful with its ability to couple cytological and molecular information directly. With the production of specific oligonucleotide probes with incorporated labelled nucleotides, the sensitivity of FISH has been brought to the intragenic level. Almost any chromosomal rearrangement, regardless of its complexity could be resolved (Popescu, 2000). FISH can overcome the limitation of PCR in LOH studies since abnormalities may be analysed on a cell-to-cell basis.

FISH was performed on metaphase cells from the 5 cell lines in order to corroborate results obtained on interphase nuclei (Brown & Stafne, 2002, unpublished honours work) to that of the metaphases for the integrity of the *FHIT* locus. The BAC201J24 and BAC240C07 were used in combination as a break apart probe. Breaks occurring between exons one to eight would be detected. The Cep3 alpha probe (Vysis Inc.) was added to this experiment to determine chromosome three copy number.

3.1.2 FISH Results regarding *FHIT* locus integrity

In normal interphase cells, no break apart signals were detected (Figure 2A and B), while break apart of *FHIT* was found in 20% and 18% of the interphase cells in WHCO6 and SNO respectively (during the previous honours project). Figure 3A shows the break apart of the probe system in WHCO6 and figure 3B shows a possible break or deletion in SNO where one derivative is lost. Table 5 shows the FISH results on interphase cells (study previously done: Brown & Stafne, Honours thesis).

In this study, FISH was performed on metaphases of the 5 cell lines. 100 metaphases were analysed per cell line. Results are summarised in Table 6. The *FHIT* locus was analysed in relation to the number of cep3 signals seen in each metaphase. In the normal control, two copies of cep3 and two copies of *FHIT* were seen in 91% of cells (Figure 4A) thus the ratio of *FHIT* to Cep3 is 1. The positive control had two cep3 signals and two to four break apart signals for *FHIT* in 100% of the cells, showing that the probe design was detecting the break apart of the *FHIT* locus resulting from a translocation within this locus in the UWOV2 cell line (Figure 4B). WHCO1 had consistent results for metaphase and interphase analysis. No rearrangement of the *FHIT* locus was detected, there was a double amount of cep3 signals to *FHIT* signals, the *FHIT*/cep3 ratio is thus 0.5, most likely due to unbalanced rearrangements of chromosome three (Figure 5A). In 5% of cells, deletion of *FHIT* was evidenced with two or more cep3 signals and one *FHIT* signal. FISH on the metaphases of WHCO3 showed that the *FHIT* locus was intact and in normal copy number (*FHIT*/cep3 ratio is 1). However one copy of *FHIT* was found to be translocated to another chromosome in 98% of these cells shown in Figure 5B; this translocation does not seem to involve the

FHIT locus as defined by the present probe system. Within this 98% of cells, 5% had a partial duplication of the other *FHIT* locus (Figure 6B). WHCO5 metaphase analysis showed four to six cep3 signals and two to four *FHIT* signals; the *Fhit*/Cep3 ratio being 0,5 showing that *FHIT* signals relative to the cep3 copy number were once again in reduced amount (Figure 7A).

WHCO6 showed no rearrangement of *FHIT* but three cep3 signals were seen and two *FHIT* signals giving an average *FHIT*/Cep3 ratio of 0.6. This could be a deletion of *FHIT* relative to chromosome three copy number (Figure 7B). 6% deletion of *FHIT* was detected, where there was normal cep3 copy number and one *FHIT* signal only. A full deletion of one allele of *FHIT* was defined when the chromosome three centromeric probe was present in normal amount (2 signals) and only one *FHIT* signal was present. This was the case for SNO, which has a ratio of 0.4 (Figure 8). Relative loss of *FHIT* signals was however observed in WHCO1, WHCO5 and WHCO6 wherever the *FHIT*/Cep3 ratio is less than one.

Table 5. Table of Fluorescence *in situ* hybridisation results on interphase cells.

Slide	Normal	Split	Extra red	Extra green	Full Deletion	Other*	Average copies of *FHIT*
Negative control	91%	0%	0%	0%	2%	7%	2
WHCO1	87%	2%	0%	0%	2%	9%	2
WHCO3	96%	2%	0%	0%	0%	2%	2
WHCO5	91%	1%	0%	3%	0%	5%	3-5
WHCO6	71%	**9%**	**11%**	**3**%	4%	2%	2-4
SNO	67%	**5%**	**13%**	**2**%	5%	8%	2
UWOV2 Positive Control	0%	**100%**	3%	0%	**6%**	9%	2-4 copies

*Other refers to missing red or green signals, which were not significantly different from the controls

Table 6. Fluorescence *in situ* hybridisation results on metaphase cells

Slide	Centromeric 3 copy number	*FHIT* copy number	*FHIT* signal not with Cep 3	Split	*FHIT* Deletion	Other	*FHIT* to Cep3 ratio
Negative control	2 signals	2 signals= 92%	0%	0%	2%	0%	1
UWOV2 Positive control	2 signals	2-4 signals	N/A	100%	0%	0%	1
WHCO1	4- 8 signals	2-4 signals = 86%	0%	1%	**5**%	0%	0,5
WHCO3	2 signals	2 signals = 98%	**98%**	1%	0%	**5%**	**1**
WHCO5	4-6 signals	2-4 signals= 100%	0%	0%	0%	0%	0,5
WHCO6	3 signals	2 signals = 80%	0%	0%	**6%**	0%	0,6
SNO	3-5 signals	2-3signals = 50%	0%	0%	**50%**	0%	0,4

Other refers to duplication in WHCO3.

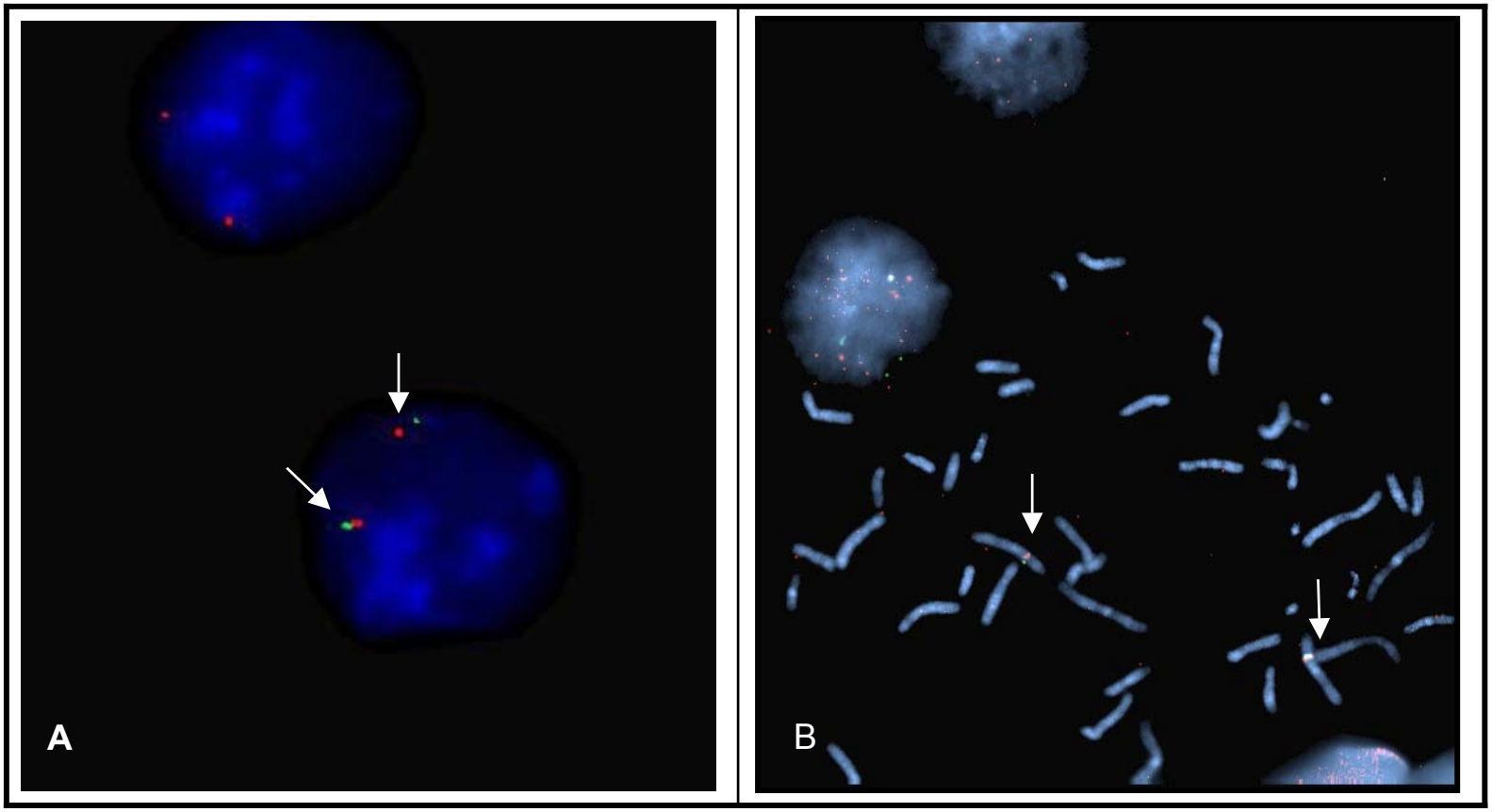

Figure 2. Fluorescence *in situ* hybridisation results from negative control. **A** Interphase cell showing the slight separation of BAC 240C07 (red) and BAC 201J24 (green) due to the chromatin being less compacted. **B** Metaphase showing the close position of the probes, BAC 240C07 (red) and BAC 201J24 (green).

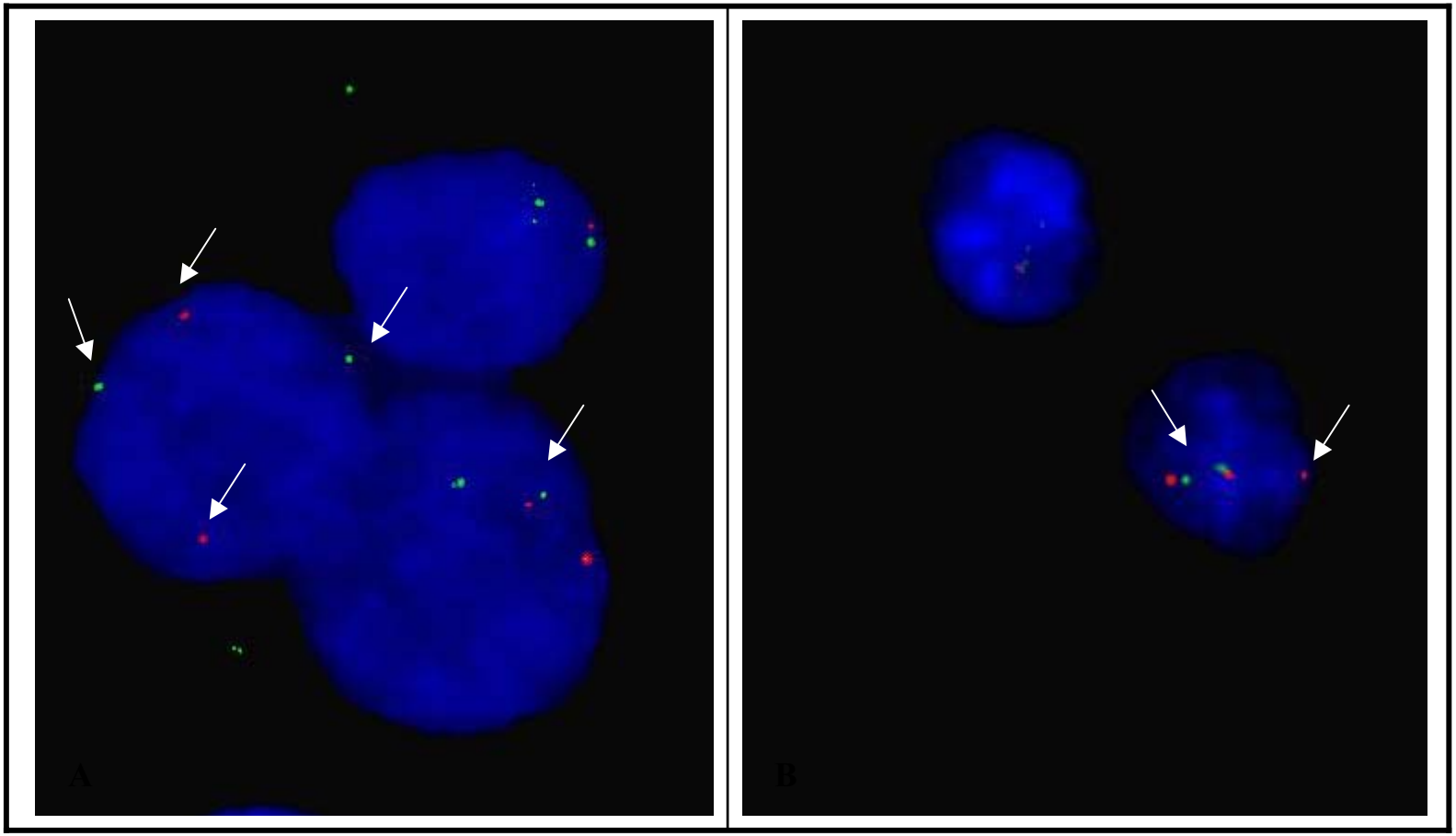

Figure 3. **A** WHCO6 interphase showing the break apart of BAC201J24 (green) and BAC240C07 (red) i.e. the split of both copies of the FHIT gene in one cell and one copy in another cell. **B** SNO showing two normal copies of FHIT with an extra red (BAC240C07), the derivative containing BAC201J24 (green) is likely to have been lost. These images are from the honours study (J Brown, A Stafne, 2002).

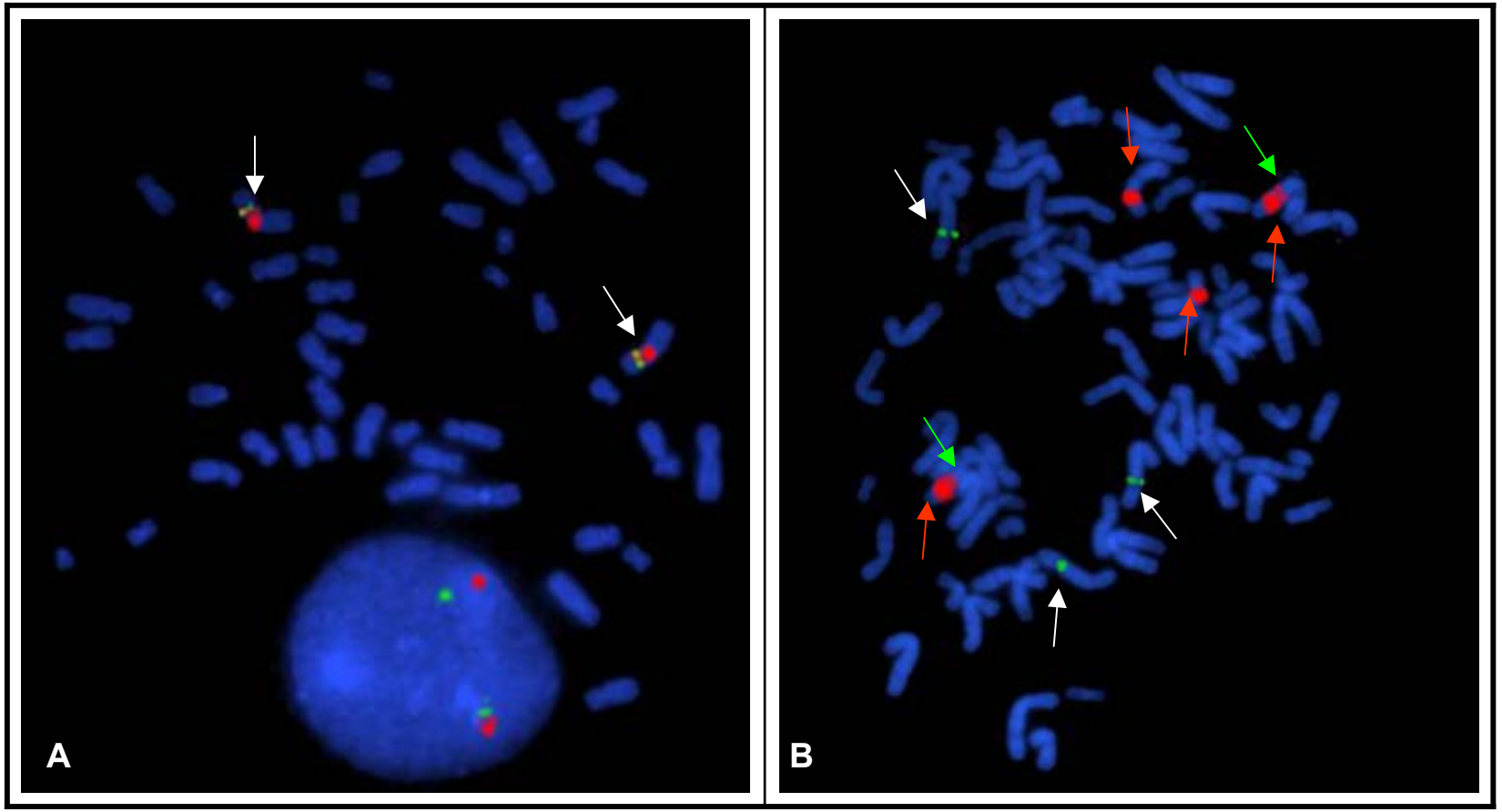

Figure 4. **A** Normal control showing cep3 probe fluorescing red with normal *FHIT* fluorescing yellow (white arrows). **B** UWOV2 cell line showing translocation of BAC201J24 fluorescing green (white arrows), cep3 (red arrows) with BAC240J24 fluorescing red (green arrows) and two extra cep3 signals (red arrows).

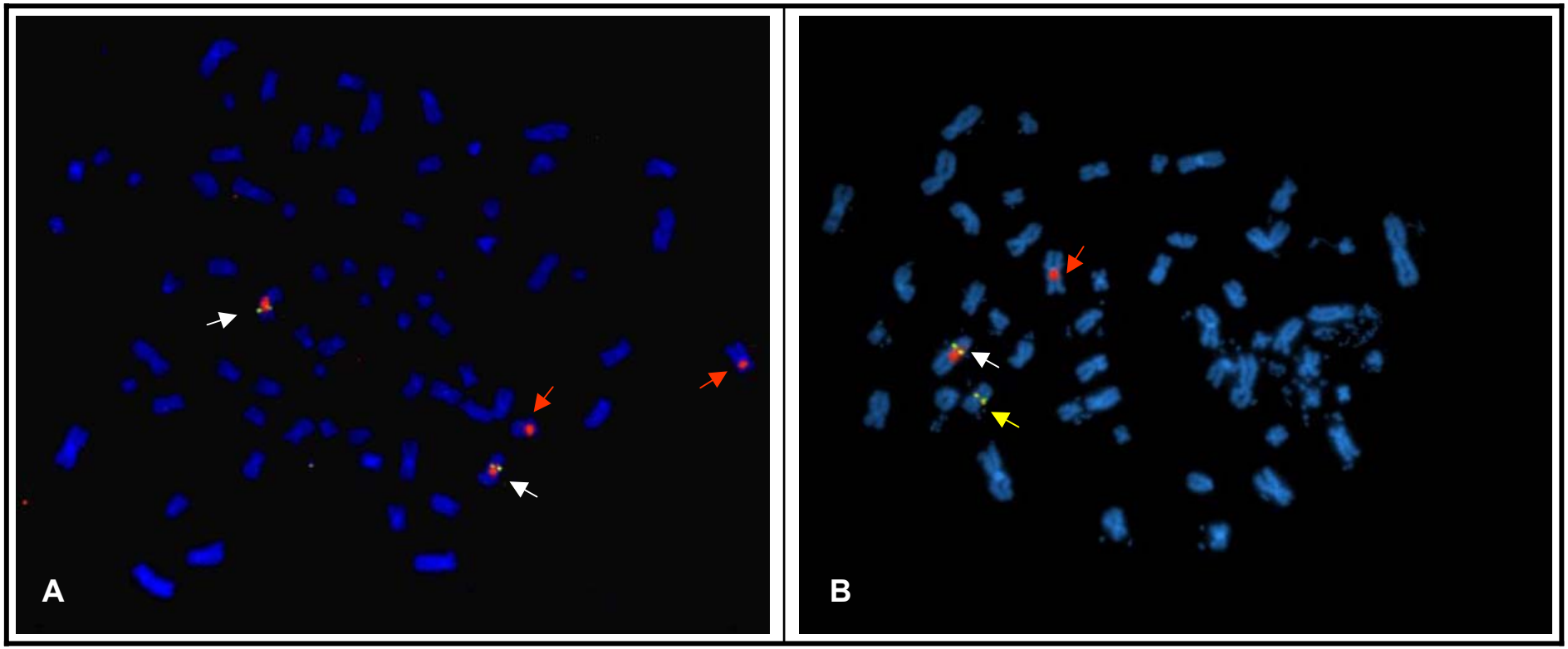

Figure 5. **A** WHCO1 metaphase with two cep3 signals (red fluorescence) with *FHIT* fluorescing yellow (white arrows) and two extra cep3 signals fluorescing red (red arrows). **B** WHCO3 metaphase with one chromosome three with *FHIT* (white arrow), translocated *FHIT* gene fluorescing yellow (yellow arrow) and the other chromosome three cep3 (red arrow).

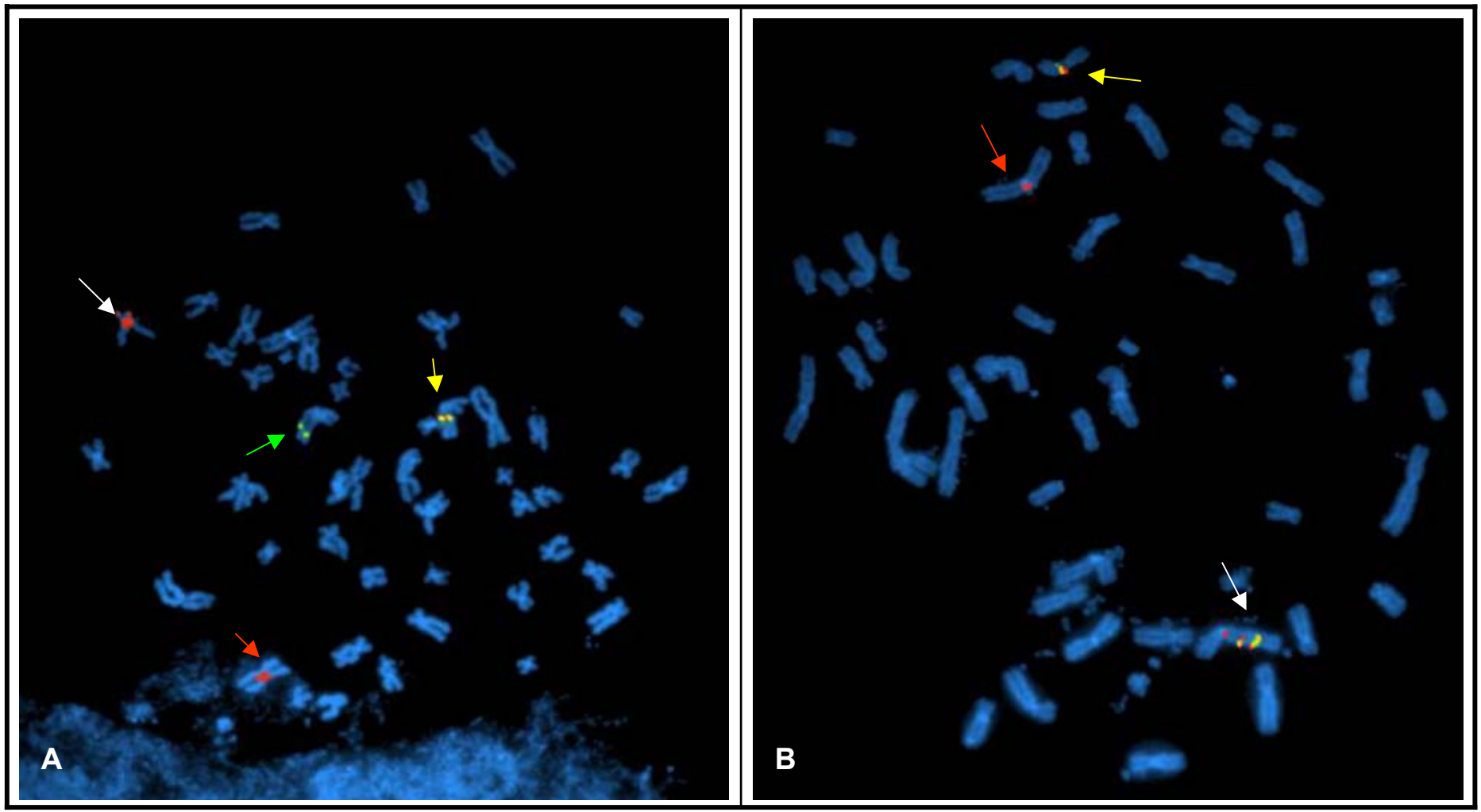

Figure 6. **A** WHCO3 metaphase with one copy of *FHIT* fluorescing yellow on another chromosome (yellow arrow), BAC201J24 (green fluorescence) translocated (green arrow) and BAC240C07 (red fluorescence with cep3 (white arrow) and chromosome three (red arrow). **B** WHCO3 with *FHIT* (yellow arrow) on another chromosome, partial duplication of *FHIT* (white arrow) on chromosome three and the other chromosome three (red arrow).

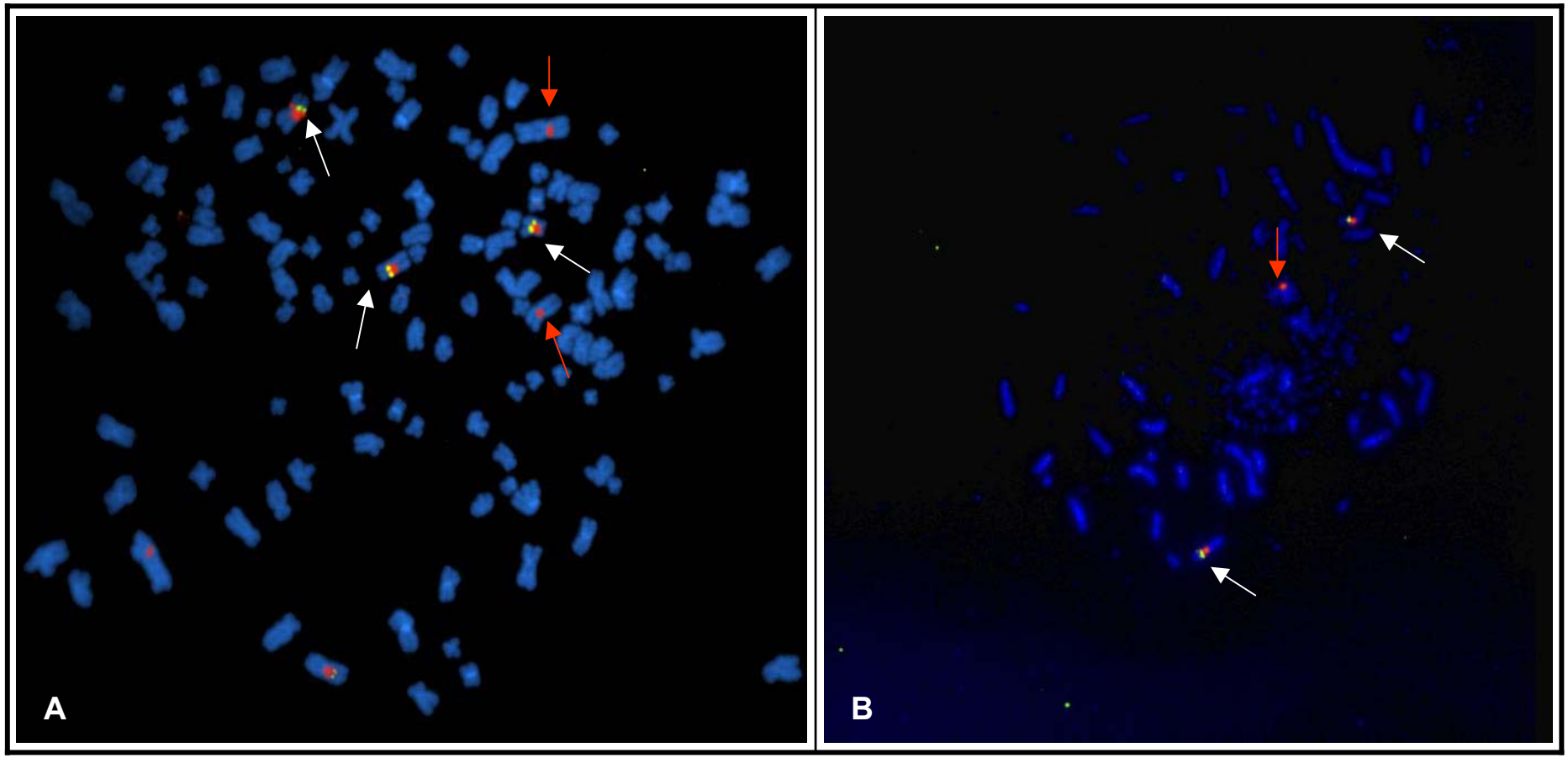

Figure 7. **A** WHCO5 metaphase with four cep3 fluorescing red with *FHIT* fluorescing yellow (white arrows) and three extra cep3 signals (red arrows). **B** WHCO6 metaphase, with three cep3 signals (red arrow) and two *FHIT* fluorescing yellow (white arrows).

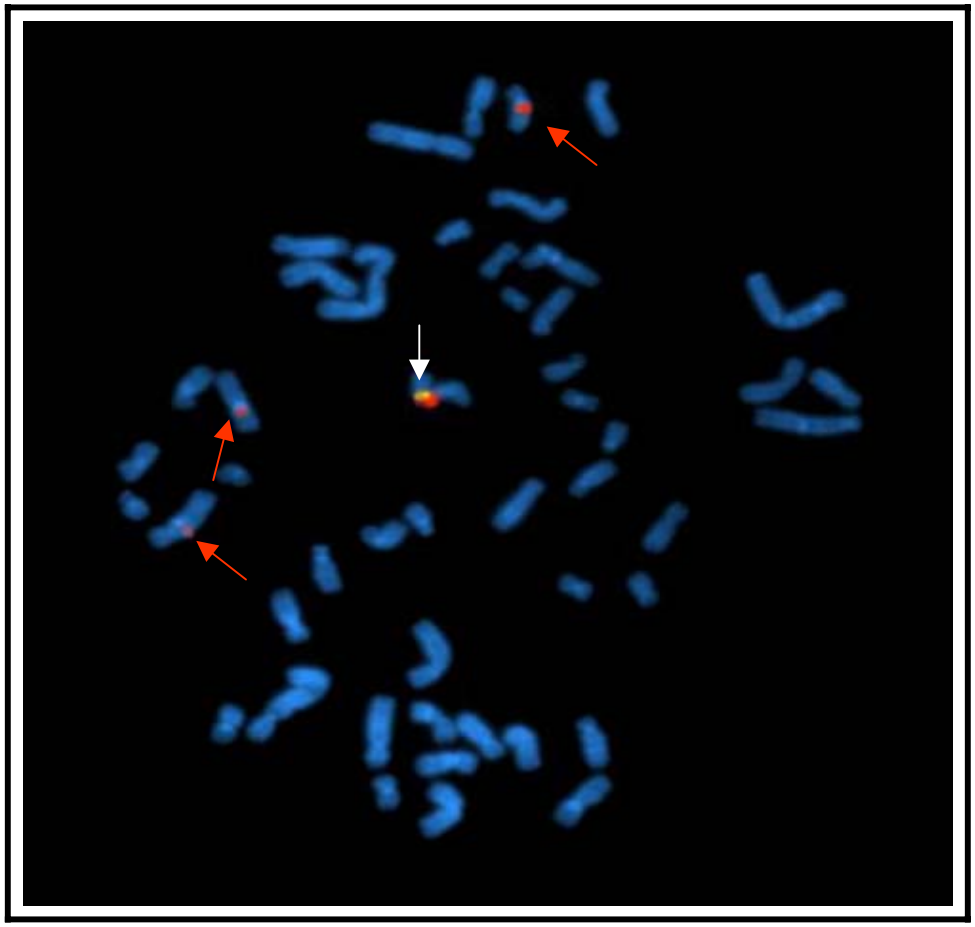

Figure 8. SNO metaphase with four cep3 signals (red arrows) and one with *FHIT* fluorescing yellow (white arrow).

3.2 RT-PCR results for *FHIT* and *WWOX*

3.2.1 Introduction

RT-PCR was performed on the cell lines and two controls. This allowed analysis of expression of the *FHIT* and *WWOX* genes. Aberrant, non-functional transcripts or alternatively sliced products would also be detected. A housekeeping gene, *GAPDH* (glyceraldehydes-3-phosphate dehydrogenase) was amplified as a control to verify mRNA integrity. RT-PCR was compared for sensitivity in detection of *FHIT* gene aberrations with FISH. Primers for the full *FHIT* transcript were those described by Fang *et al* (2001). RT-PCR was also performed for the detection of expression of exon 5 of the *FHIT* gene using primers designed by Fang *et al* (2001). Primers for the *WWOX* full transcript were designed using the Primer3 program (http://flypush.imgen.bem.tmc.edu/primer/primer3_www.cyi).

3.2.2 Results

RNA isolation

The RNA extraction from the cell lines was successful. The RNA was found to be intact (Figure 9) and in good concentration after gel electrophoresis and spectrophotometer readings. The spectrophotometer readings can be seen in table 7.

Table 7. Table of RNA concentration and purity

SAMPLE	CONCENTRATION	PURITY (260/280 RATIO)
C1	198,8ng/µl	1,972
C2	171,2ng/µl	2,11
WHCO1	273,2ng/µl	1,876
WHCO3	476,8ng/µl	1,98
WHCO5	509,6ng/µl	2,042
WHCO6	609,2ng/µl	1,94
SNO	608,8ng/µl	2,008

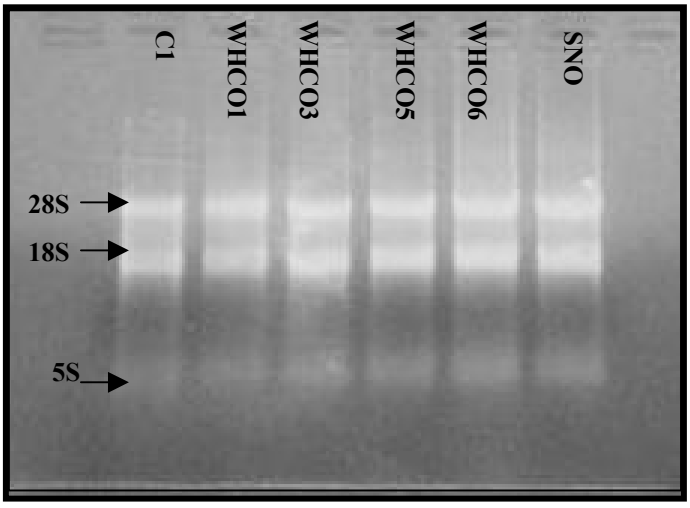

Figure 9. Denaturing gel showing the integrity of the RNA for the control sample (C1) and all five cell lines. The 28S, 18S and 5S RNAs can be seen.

GAPDH RT-PCR

The five cell lines and two controls had *GAPDH* amplification confirming that the mRNA was intact. (Figure 10).

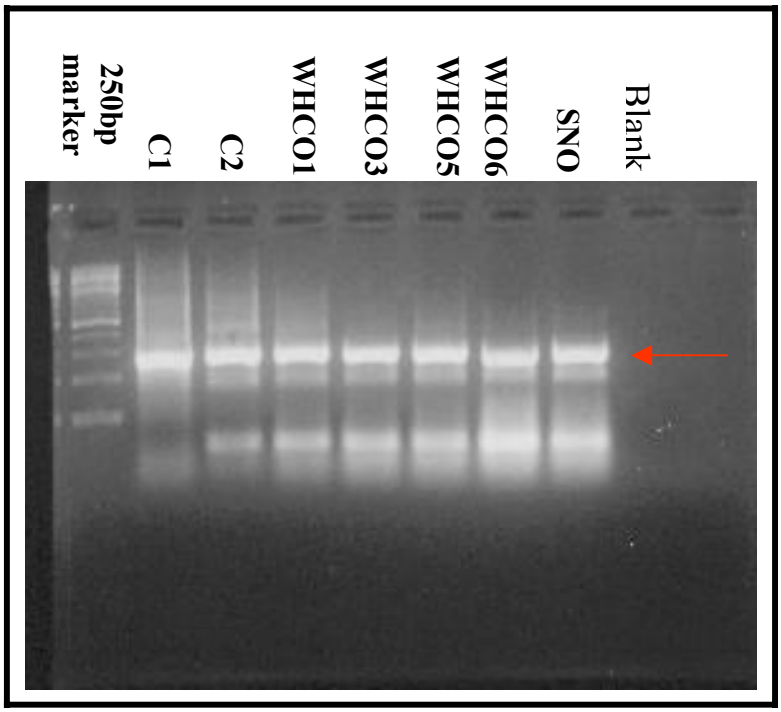

Figure 10. 2% agarose gel showing results for *GAPDH* RT-PCR, red arrow indicates the GAPDH product at 589bp.

FHIT RT-PCR

Normal *FHIT* transcripts were detected in WHCO1 and WHCO3, however all cell lines appear to have down-regulation of the gene in comparison to a normal control, this would have to be confirmed by semi-quantitative PCR. Aberrant transcripts were detected repeatedly in WHCO1, WHCO5, WHCO6 and SNO (See Fig 11).

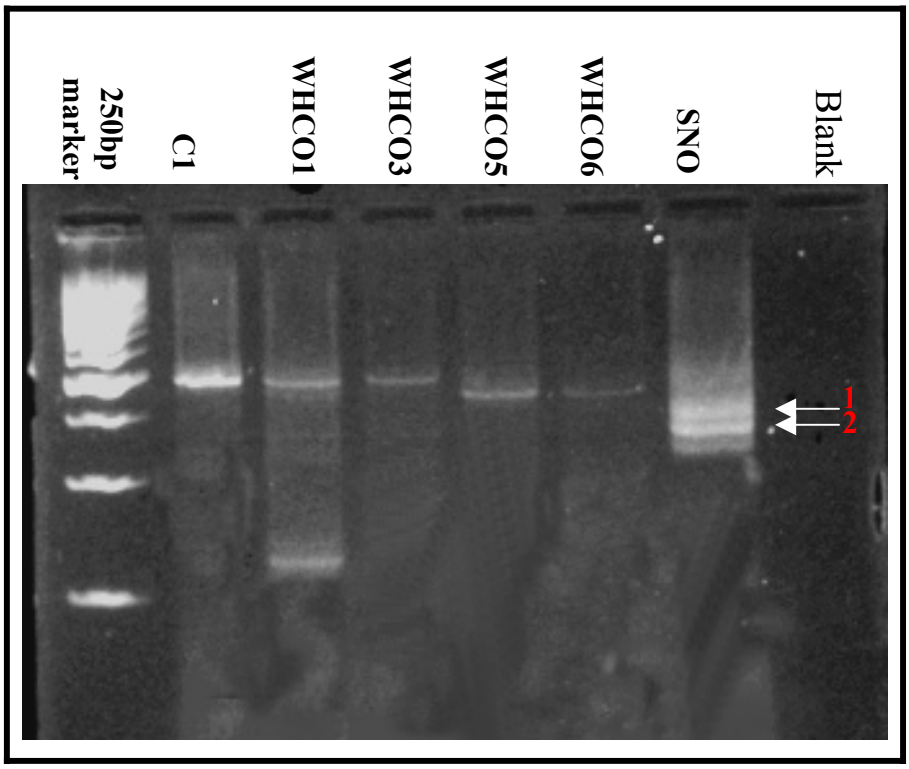

Figure 11. 2% Agarose gel showing expression of *FHIT* in one control and the five cell lines. An aberrant transcript is seen in WHCO1, WHCO5 and WHCO6 show abnormal transcripts only and SNO has two major abnormal transcripts indicated by the 1 and 2, these two bands and bands for WHCO1, WHCO5 and WHCO6 were extracted for sequencing.

Exon 5 RT-PCR

WHC01, WHC03, WHC05 and WHC06 all have exon 5 expression although expression appears to be significantly down regulated, while SNO appears to have lost exon 5 (Fig 12).

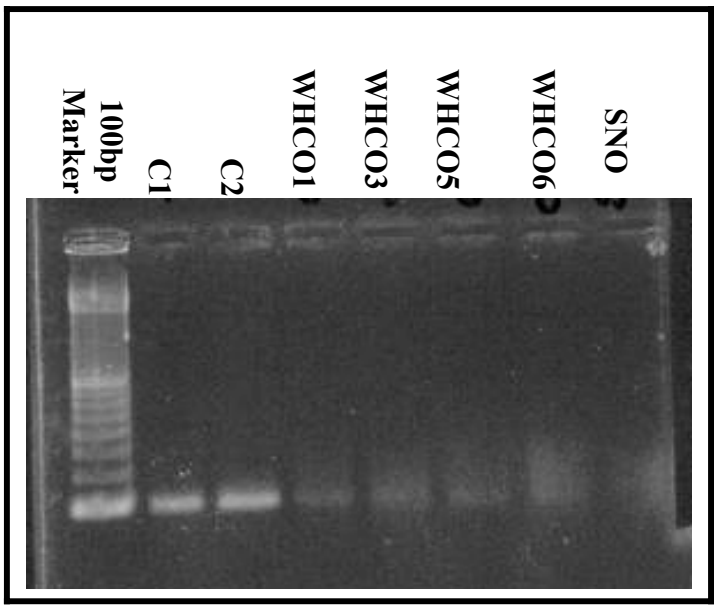

Figure 12. 2% agarose gel showing exon 5 expression by the controls and what appears to be lowered expression in WHCO1, WHCO3, WHCO5 and WHCO6. SNO has almost no exon 5 expression.

WWOX RT-PCR

The expression of *WWOX* was found to be normal in all the cell lines (Fig 13). It even appears that *WWOX* is being expressed at an increased level in comparison with the controls.

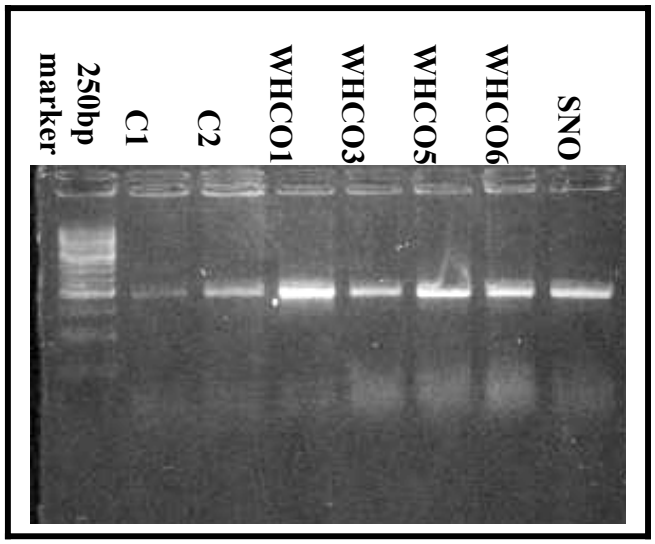

Figure 13. 2% agarose gel showing expression of *WWOX*. The controls expression seems to be lower as compared with the cell lines.

3.3 Discussion

3.3.1 *FHIT* locus integrity

It can be concluded from this probe system used for analysis of *FHIT* locus integrity, that in the five cell lines, the *FHIT* locus was not broken apart by rearrangement except in 1% of cells for WHCO1 and WHCO3. This 1% may reiterate the instability of the *FRA3B* region and the susceptibility of *FHIT* to rearrangement because FISH has a high specificity and sensitivity in detecting translocations, especially with break apart probes (Gozzetti and Le Beau, 2000). However, the current probe system does not span the entire locus and perhaps using a different combination of probes; specifically a BAC beyond exon 10 instead of BAC 201J24, more rearrangements, such as internal deletions, may be detected.

SNO displayed a consistent deletion of one copy of the *FHIT* gene as opposed to the break apart seen in this line in the previous interphase analysis. This discrepancy may be due to the different cell passages used in these two separate studies, the deletion may have evolved through passage.

The translocation that was seen in WHCO3 did not affect the *FHIT* gene but clearly the locus was very involved and this shows the instability of the *FRA3B* region and this may have an effect on the down-regulation of expression of the gene in these cells as seen in the RT-PCR results.

There was a definite hypo representation of the *FHIT* loci in relation to cep3 and overall ploidy of the cells. This could be representing a deletion of the gene and may

have a bearing on growth selection in these cells. The M-FISH results later in the study revealed that chromosome three was involved in a number of translocations. The particular arms of chromosome three which are involved in these events should be elucidated and this may answer the question of *FHIT* representation as compared with the chromosome three copy number.

3.3.2 *FHIT* and *WWOX* expression

FHIT expression appeared to be down regulated in all the oesophageal cancer cell lines in comparison to the controls, however this can only be confirmed by semi-quantitative RT-PCR (relative quantification). In brief, semi- quantitative RT-PCR refers to the use of real time PCR (continuous monitoring of a fluorescent reporter for example, SYBR green). It relates the PCR signal of the gene in question in a test sample to that of the signal in a control sample, which is compared in turn to that of a housekeeping gene in the test and control samples (Kenneth & Schmittgen, 2001).

The expression of *FHIT* is found to be higher in epithelial tissues (Croce *et al*, 1999) and white blood cells were used as controls, so for true comparison of expression a semi-quantitative RT-PCR using normal epithelial cells as controls would be more accurate in determining the level of expression. WHCO5 and WHCO6 had transcripts, which were smaller than the expected size indicating that they may have non-functional fhit protein or may be alternatively spliced. SNO had two major aberrant transcripts and did not express exon 5 indicating that SNO more than likely does not have functional fhit protein as Exon 5 is most commonly deleted and is the first coding exon (Druck *et al*, 1997). Deletion of this exon results in non-functional fhit protein. Band number 2

(Figure11) was cloned and sequenced and the first 120bp showed homology to the *FHIT* wild type mRNA sequence indicating that this aberrant transcript is an *FHIT* transcript (data not shown as sequencing of all the transcripts is still incomplete). WHCO1 had one aberrant transcript, which could be an alternatively spliced mRNA or mRNA giving rise to non-functional fhit protein. Exon 5 expression seemed to be diminished in the other cell lines as well. (This deletion of exon 5 has been confirmed in a parallel study using MLPA in the process of publication, Willem *et al*, 2004).

WWOX was not lost or decreased in these cell lines. They do appear to express *WWOX* at higher levels than the controls, but the implications of this are not known. One explanation could be, that possibly like *FHIT, WWOX* may be expressed at higher levels in epithelial tissues than white blood cells and this is being seen in these RT-PCR results. This would be verified by semi-quantitative RT-PCR.

CHAPTER FOUR – M-FISH ANALYSIS AND ANALYSIS OF *EGFR* DNA COPY NUMBER

4.1. M-FISH

4.1.1 Introduction

M-FISH is a technique used to identify all structural abnormalities of chromosomes (Gunawan, 2001; Heng, 2004). It is powerful in the detection of translocations and insertions where conventional cytogenetics can only identify 70% of this type of abnormality specifically in complex karyotypes typically associated with cancer cell lines. For example translocations involving more than two chromosomes. This technique will help identify primary and secondary abnormalities and identification of loci, which may be involved in tumour development and progression (Gunawan, 2001).

The SpectraVysion Assay (Vysis Inc.) is a 52-probe mixture labelled with five fluorophores. The probes are labelled in a combinatorial format where one probe is labelled with a combination of two or three fluorophores giving each chromosome a distinct colour when analysed using an imaging system. The five fluorophores used are SpectrumRed, SpectrumGreen, SpectrumGold, SpectrumFarRed and SpectrumAqua (Vysis Inc.) and then counterstained with DAPI. These fluorophores are visualised using six single-band pass filters. Cross talk between filters must be kept to a minimum to prevent inaccurate results. The Genus™ program from Applied Imaging puts the five images captured into a composite image of pseudocolours. The metaphases are karyotyped automatically by chromosome size and colour. Abnormalities are analysed by using the fluoromap. Although this is a commercially available kit, the technique is difficult technically in terms of sample preparation as well as image acquisition and

analysis. Metaphases have to be of exceptional quality, chromosome length, colour and spreading are very important for the technique to work. The capture of metaphase images is very time consuming and the analysis of these images for abnormalities is also exceptionally time consuming.

This technique was used to analyse genome integrity and identify possible marker chromosomes, which are common between the cell lines. These marker chromosomes could be involved in carcinogenesis of oesophageal cancer and may help identify genomic regions, which should be further analysed in oesophageal cancer.

4.1.2 RESULTS

The M-FISH results for each of the cell lines gave complex karyotypes, highlighting a number of structural abnormalities. Composite karyotypes representing the common abnormalities of ten cells for each of the cell lines are summarised in table 8. A common translocation between chromosomes one and three was found in WHCO5 and SNO (highlighted in red) (Figure16). A translocation involving chromosomes 3 and 22 are common to WHCO3 and WHCO5 (Figure 15 and 16) as well. A trisomy 21 was common to WHC01, WHOC3 and WHCO6 (highlighted in blue). Translocations involving chromosomes 11, 13 and 22 seem to be common as well (highlighted in purple). WHCO5 is highly polyploid. It is also noted that chromosome 3 is highly involved specifically in SNO and so are chromosomes 9 and 12.

Table 8. Table of M-FISH results. Composite karyotypes of recurring abnormalities in 10 cells analysed per cell line.

Oesophageal cancer cell line	M-FISH analysis
WHCO1	Trisomy 1, 2 and 3, t(5;19), t(6;12), t(6;13), t(7;18), 50% trisomy 12, t(1;14), t(7;14), t(15;19), t(5;19), aneuploidy 20, t(19;21), t(8;22), XXY
WHCO3	t(1 ;11;15), trisomy 2, t(5;8), t(8;9;20), t(7;9;16;18), trisomy 12, t(6;12), t(13;14;20), t(9 ;13;20), trisomy 14, t(15;22), t(11;15 ;22), trisomy 17, t(13;21), t(3;16;22), X
WHCO5	t(1;3), t(1;19), t(2;9), t(3;22), t(5;19), t(9;19), t(9;14), t(2;11), t(3; 11 ;13;22), t(8;14;18), t(7;15), t(15;Y), t(2;19), t(3; 11;22), t(4;10;22;X), XY
WHCO6	t(1;18), t(5;10), aneuploidy 7, t(9;15), t(10;14), trisomy 12, t(17;19), monosomy 21, t(6;22) , YY
SNO	t(1;3), t(1;16), t(2;X), t(1;2), t(3;20), t(3;12), t(3;9), t(1;5), del 6, t(2;8;7;11 ;20), t(3;7), t(2;8), t(9;16), t(3;10), t(10;22), t(4;11), t(11;13;22), t(12;21), t(2;11;13), t(14;22), t(14;19), t(12;15), t(6;17), t(8;18), monosomy18, t(5;19), trisomy 20, t(20;21), t(14;22), t(15;y) del X, XXY

Note: This table represents the consistent aberrations from 10 cells analysed for each cell line. Thus it is not exhaustive in describing translocations occurring in these cell lines.

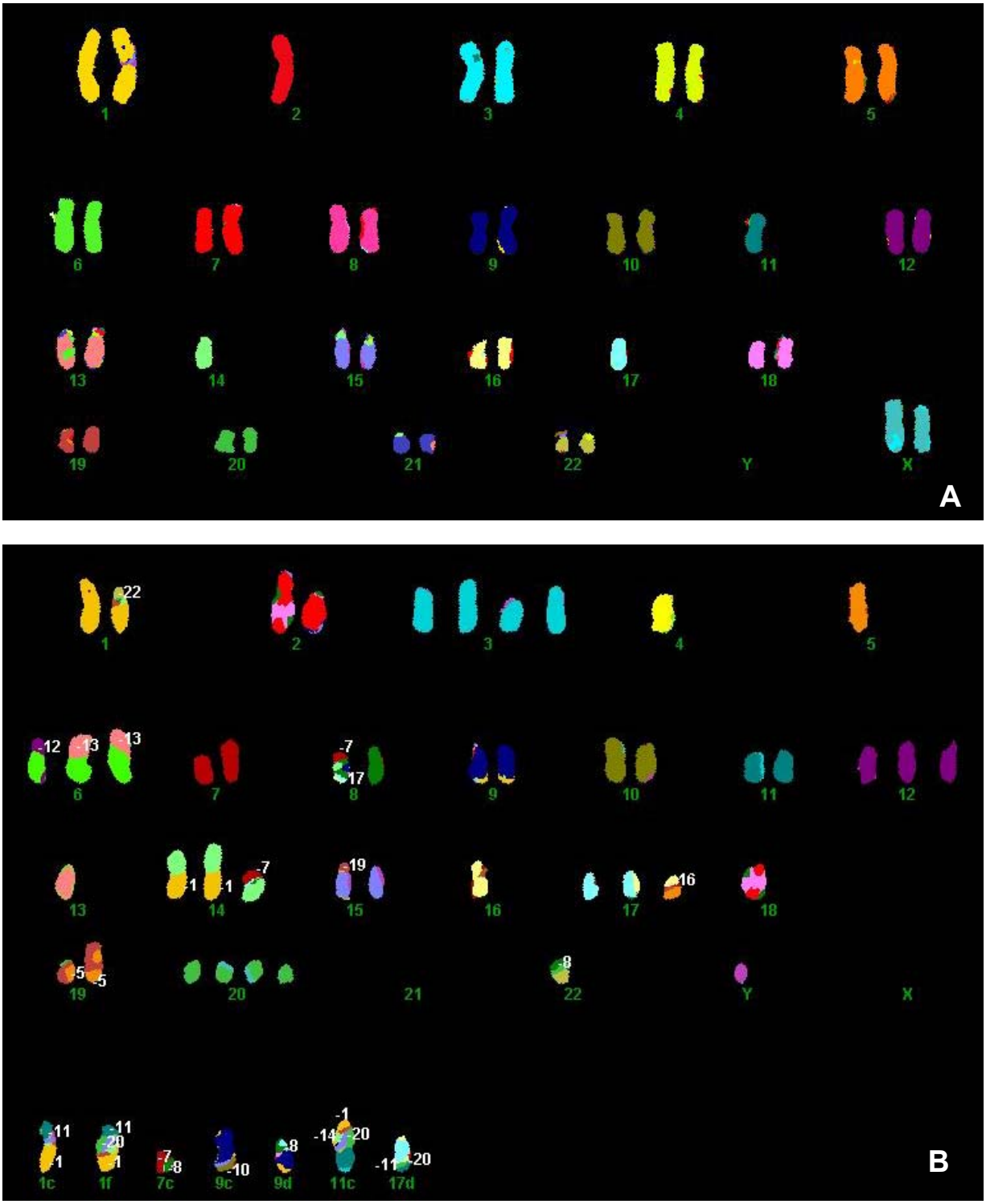

Figure 14. **A** M-FISH on a normal female control. **B** M-FISH on WHCO1 showing the remarkable t(6;13), t(6;12), tri 12, t(1;14), t(5;19). Note that this image is the most representative karyotype out of 10 cells.

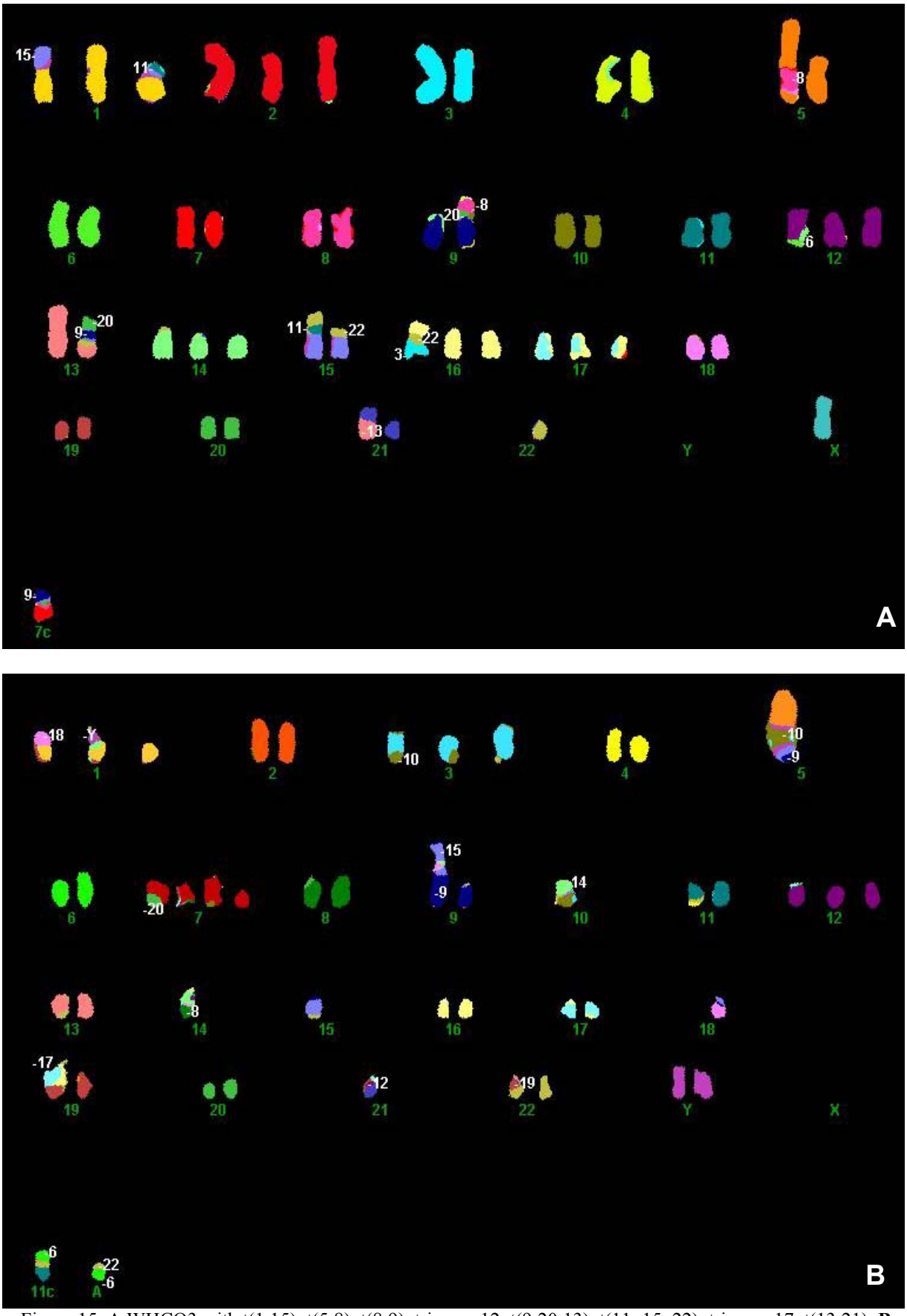

Figure 15. **A** WHCO3 with t(1;15), t(5;8), t(8;9), trisomy 12, t(9;20;13), t(11 ;15 ;22), trisomy 17, t(13;21). **B** WHCO6 with t(1;18), t(5;10), aneuploidy 7, t(9;15), t(10;14), trisomy 12, t(17;19), monosomy 21, t(6;22), YY. Note that this image is the most representative karyotype out of 10 cells.

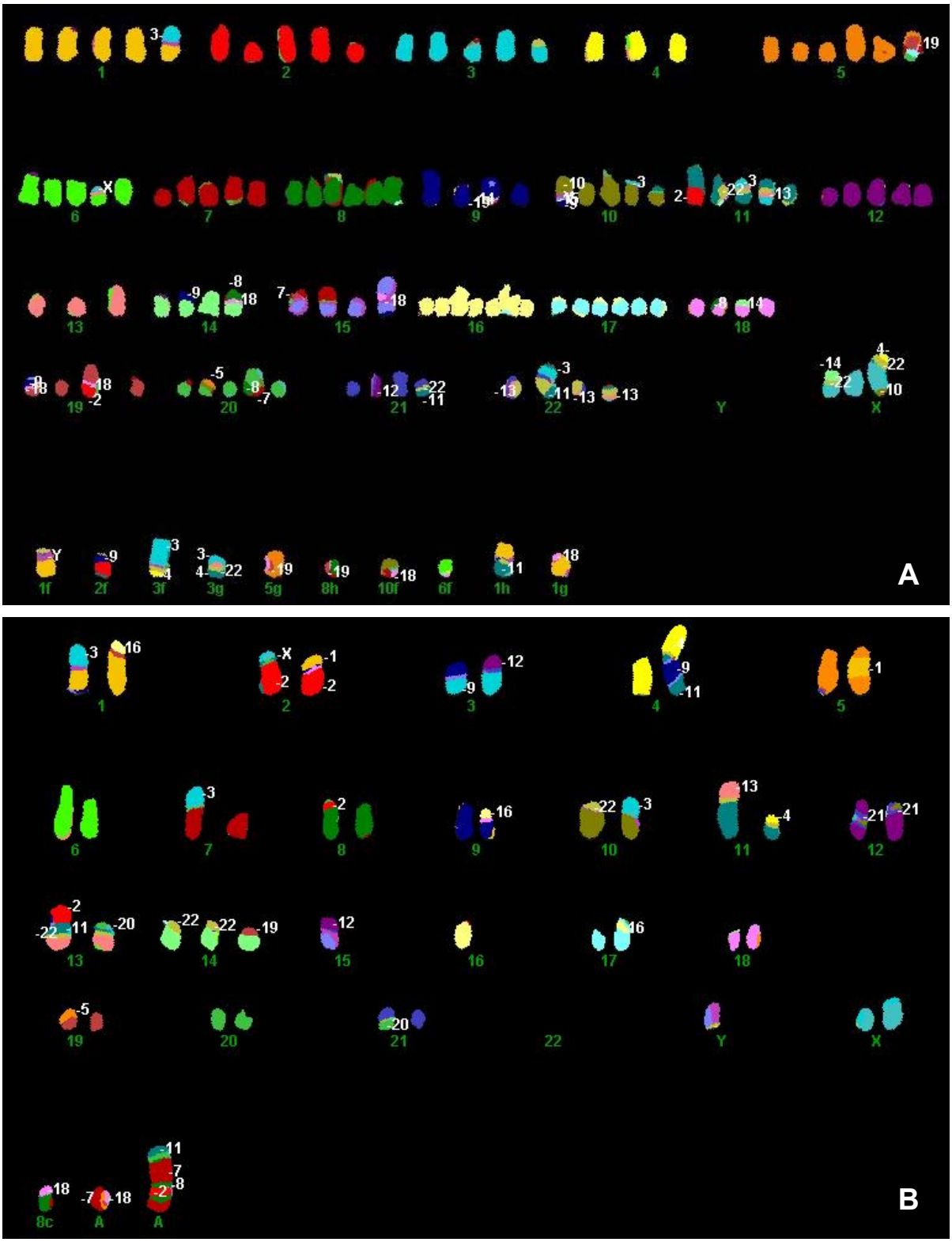

Figure 16. **A** WHCO5 with (1;3), t(1;19), t(2;9), t(3;22), t(5;19), t(9;19), t(9;14), t(2;11), t(3;11;13 ;22), t(8;14 ;18), t(7;15), t(2;19), t(3;11;22), t(4;10 ;22;X). **B** SNO with t(1;3), t(1;16), t(2;X), t(1;2), t(3;20), t(3;12), t(3;9), t(1;5), del 6, t(2;7;8;11;20), t(3;7), t(2;8), t(9;16), t(3;10), t(10;22), t(4;11), t(11;13;22), t(12;21), t(2;11;13), t(14;22), t(14;19), t(12;15), t(16;17), t(5;19), t(20;21), t(15;y), del X. Note that these images are the most representative karyotype out of 10 cells. Note the common t(1;3) marker of WHCO5 and SNO.

4.2 Analysis of *EGFR* gene amplification

4.2.1 Introduction

FISH was also performed on the cell lines for analysis of the epidermal growth factor receptor (*EGFR*) gene on chromosome seven. This gene is commonly over expressed in oesophageal carcinomas (Metzger *et al*, 2004) and in WHCO1, WHCO3 and SNO (Veale *et al*, 1989). The use of this probe (LSI EGFR SO/CEP7, Vysis Inc.) would help establish if the over expression is as a result of gene amplification in these cell lines. The probe is a combination of a probe for *EGFR* gene and a centromeric 7 probe; Cep 7 acts as a control for chromosome 7 copy number to establish true gene amplification as opposed to aneuploidy. The results are summarised in Table 9.

4.2.2 FISH results for *EGFR* gene amplification

100 interphase cells were analysed for each cell line as well as 10 metaphases.

WHCO1 showed aneuploidy for chromosome seven; approximately 5 to ten signals for both the centromeric probe and *EGFR* were seen in 100% of the cells (Figure 17A). That means that the *EGFR*/cep7 ratio is 1. In the metaphases of WHCO1 one could see that chromosome 7 copies were not all normal as reflected by their size (Figure 17B). WHCO3 had three copies of both chromosomes seven and the *EGFR* gene (*EGFR*/cep7 ratio is 1), seen in metaphase and interphase cells (Figure 18). The remaining cells had two or four copies. WHCO5 also has chromosome 7 aneuploidy so the *EGFR*/cep7 ratio is 1, with 4-7 centromeric seven and *EGFR* signals seen in 100% of cells (Figure 19A). Metaphases also showed that there are derivative chromosome seven copies (Figure 19B). WHCO6 had 3% of cells with an amplification of the *EGFR* gene. Two of these cells had mild amplification with the *EGFR*/cep7 ratio being

approximately 4 (Figure 20A) and one cell showed high amplification with the *EGFR*/cep7 ratio being approximately 6 (Figure 20B). However this is not significant, as 97% of the cells analysed had an *EGFR*/Cep7 ratio of 1 and no amplification was found in the metaphase cells analysed (Figure 21A and B). SNO had a significant 14% of cells showing mild to high amplification of *EGFR* where the *EGFR*/cep7 ratio was approximately 6 (Figure 22A). In three out of 10 metaphases analysed, the amplification could be seen down the length of the chromosome suggesting the formation of a homogenously staining region (HSR) (Figure 22B) the other seven metaphases all had a partial amplification (Figure 23). It was noted that the cells showing amplification were smaller in size. The remaining 86% of cells had aneuploidy with 5-8 signals for each probe.

Table 9. Summary of *EGFR* FISH results

OC Cell line	**Chromosome 7 copy number**	***EGFR* gene amplification**	***EGFR*/Cep3 ratio**
WHCO1	100% aneuploidy (5-10 copies	0%	1
WHCO3	100% aneuploidy (3-4 copies	0%	1
WHCO5	100% aneuploidy (4-7 copies	0%	1
WHCO6	97% aneuploidy (5-7 copies	3%	1
SNO	86% aneuploidy (5-8 copies	**14% high amplification**	**6 in the 14%**

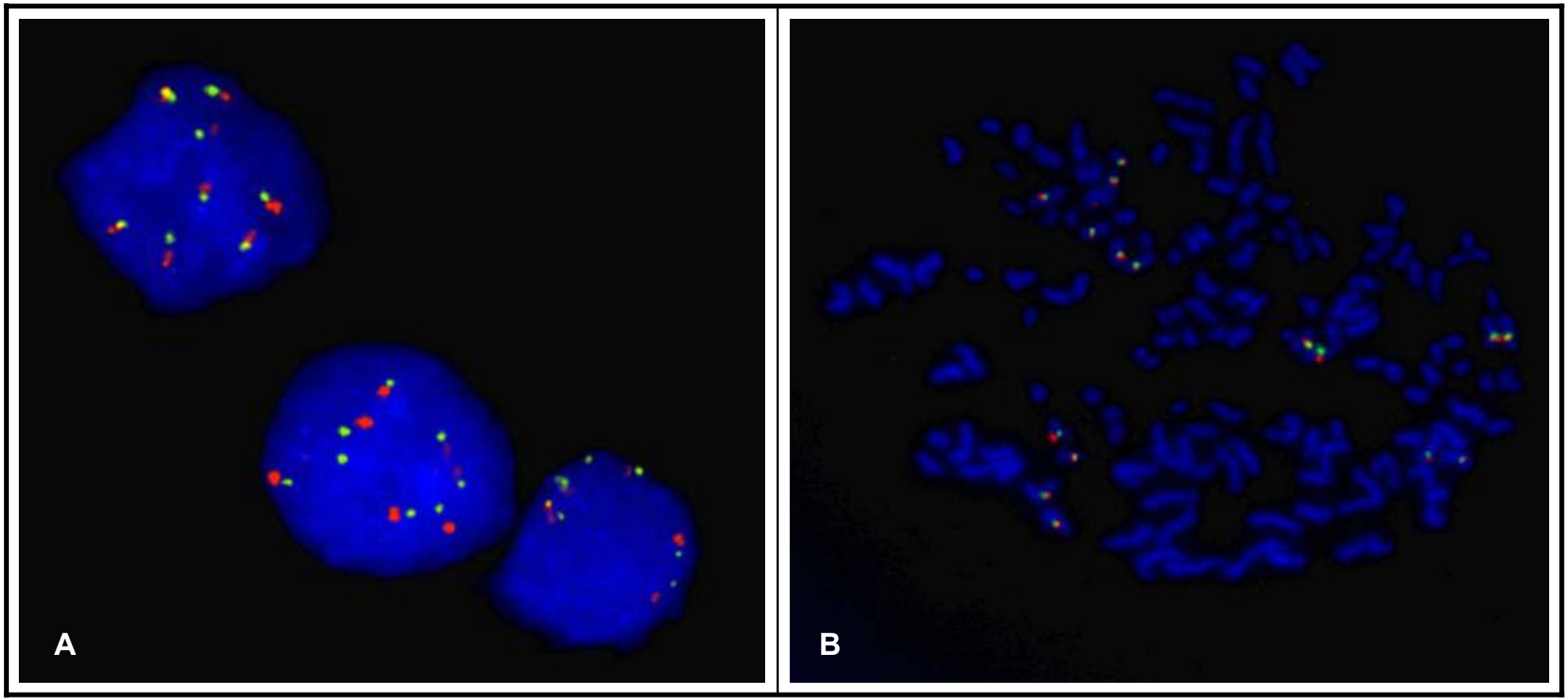

Figure 17. **A** Interphase cell from WHCO1 showing aneuploidy for chromosome seven, centromeric seven fluorescing green and *EGFR* gene fluorescing red. **B** Metaphase cell from WHCO1 showing multiple chromosome 7 copies.

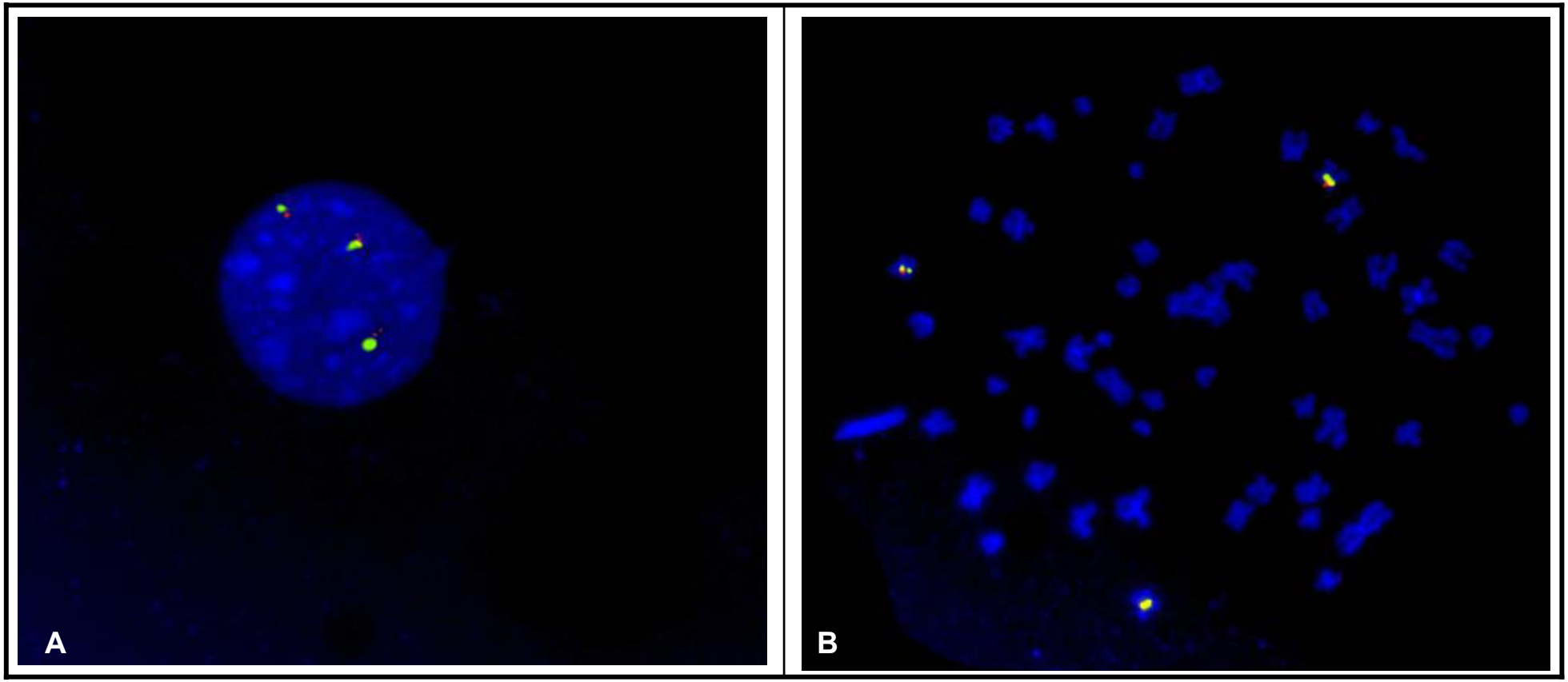

Figure 18. **A** Interphase cell from WHCO3 showing three copies of chromosome seven (green fluorescence) and three copies of *EGFR* gene (fluorescing red). **B** Metaphase cell from WHCO3 showing the three copies of chromosome seven and the EGFR gene.

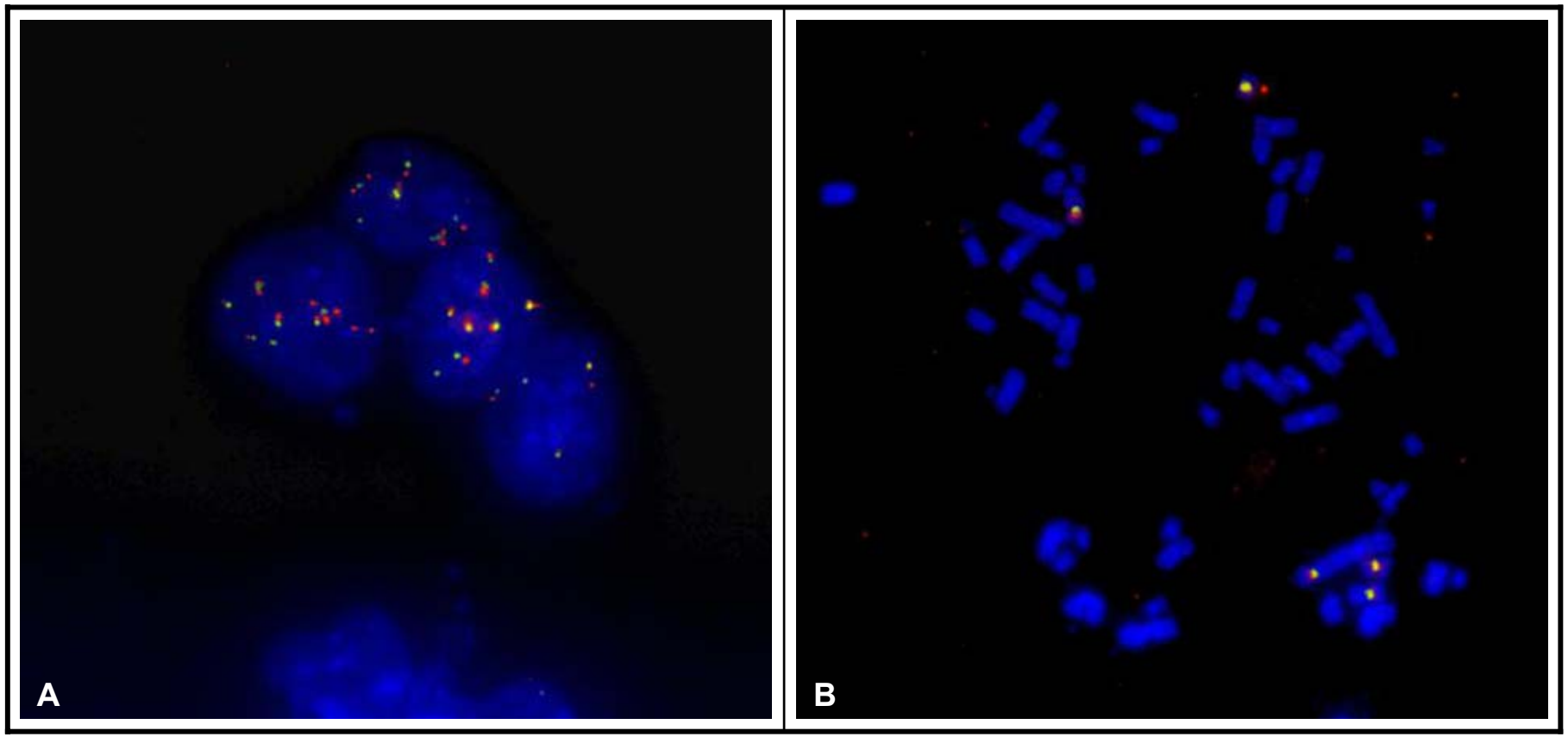

Figure 19. **A** Interphase cells in WHCO5 showing aneuploidy for chromosome 7. **B** Metaphase cell for WHCO5 showing chromosome 7 and derivatives of 7 (centromeric 7 fluorescing green and *EGFR* gene fluorescing red).

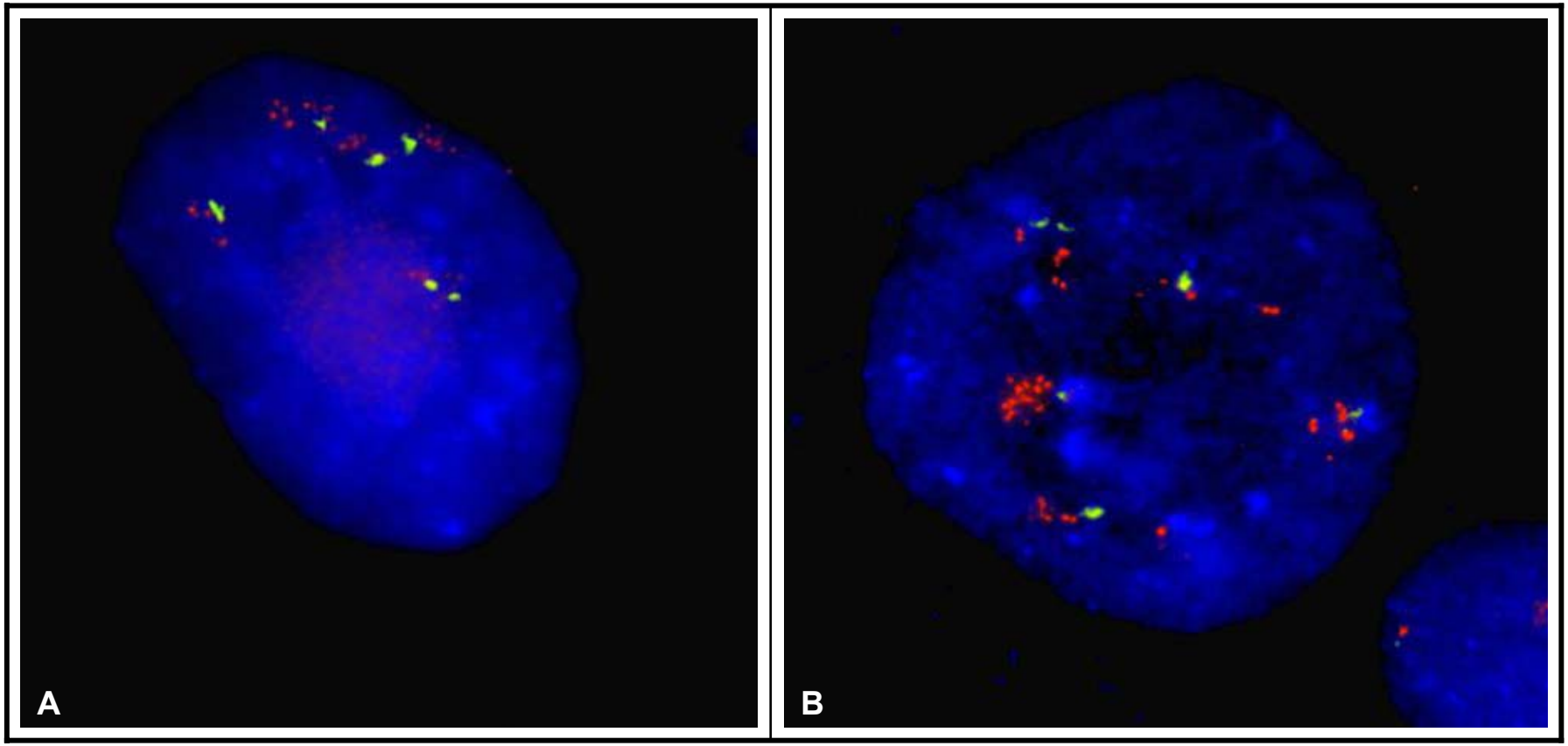

Figure 20. **A** WHCO6 interphase cell with mild amplification of the *EGFR* gene (Red fluorescence) where there is not quite more than twice the amount of red signals to green (centromeric 7). **B** WHCO6 interphase cell with high amplification of the *EGFR* gene.

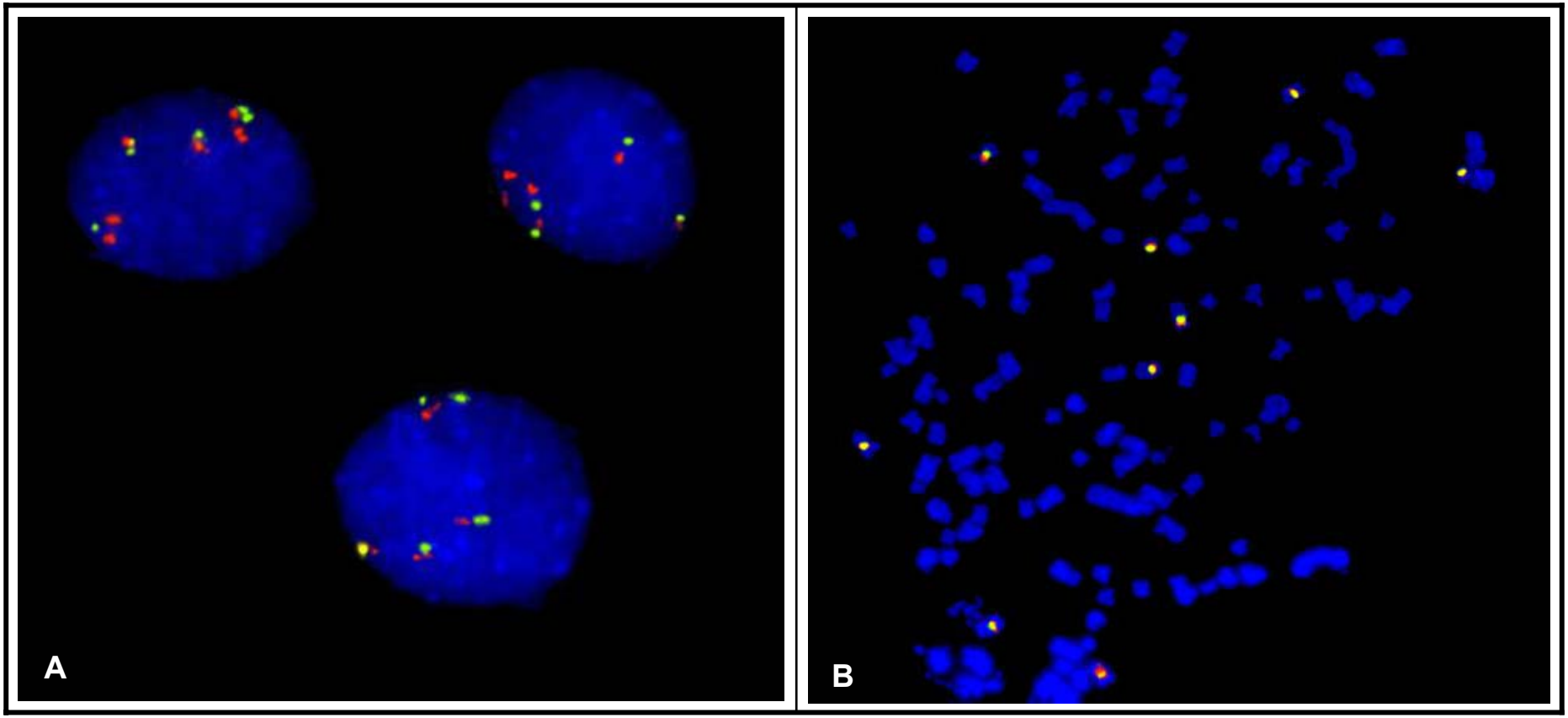

Figure 21. **A** WHCO6 interphase cell showing aneuploidy for chromosome 7(centromeric 7 fluorescing green, *EGFR* fluorescing red). **B** WHCO6 metaphase cell with aneuploidy of chromosome 7.

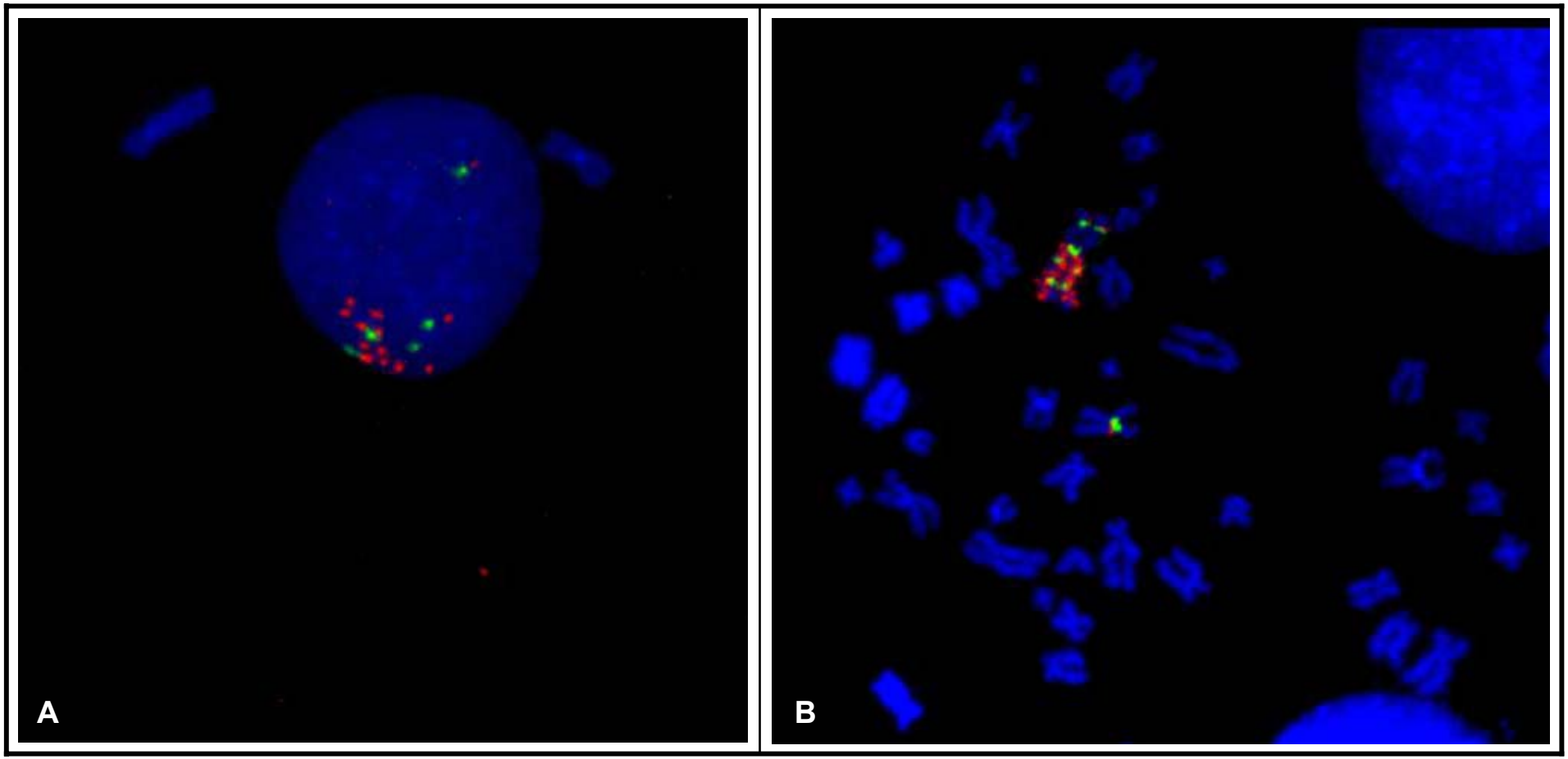

Figure 22. **A** Interphase cell from SNO with amplification of *EGFR*, red signals are more than twice the green (centromeric 7). **B** Metaphase showing the amplification of the EGFR gene (red signals) down the length of chromosome 7, the amplification unit seems to include the centromere (green signals), with the centromeric signal in triplicate along the chromosome.

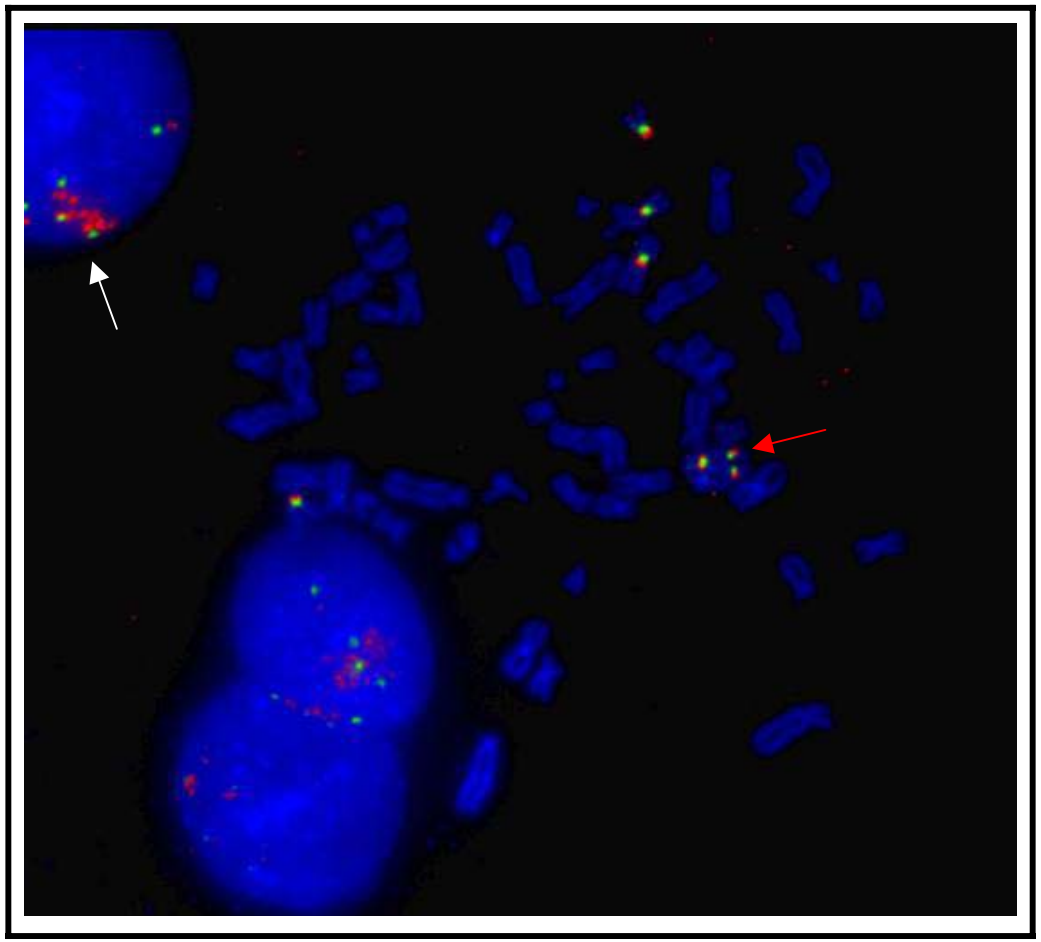

Figure 23. SNO, metaphase showing aneuploidy for chromosome 7 (centromeric 7 fluorescing green and *EGFR* fluorescing red) and partial amplification on one chromosome (red arrow) and a neighbouring interphase cell showing amplification of *EGFR* fluorescing red (white arrow).

4.3 Discussion

4.3.1 What is shown by M-FISH?

The common chromosomes involved in translocations in all of the cell lines were chromosomes 3, 8, 9, 12, 11, 13 and 22. This may correlate with some of the known affected genes in OC such as myc on 8q24, cyclin D1 on 11q13 and p16 on 9p21. Chromosomes 11 and 13 both have key oncogenes, cyclin D1 and the Retinoblastoma gene is on chromosome 13q14. Chromosome three was often involved in translocation and it would be valuable to map these translocations, specifically the t(1;3) common to WHCO5 and SNO, chromosome 3q gains may be important as 3q hosts the PK13CA and hTR oncogenes so it would be valuable to further investigate these loci. Chromosome 3q gains and 3p losses have been detected in OC samples by CGH (comparative genomic hybridisation), loss of 3p correlated with a poor prognosis (Kwong *et al*, 2004).

Chromosome 12 was often involved in the form of trisomy, there is CGH data suggesting involvement of chromosome 12p gains in oesophageal cancer progression and it may indicate a poor outcome (Kwong *et al*, 2004). It may be interesting to further define which parts of chromosome 12 are involved. Chromosome one was almost always involved in translocation, translocations were occurring in WHCO1, WHCO3, WHCO5, WHCO6 and SNO with various different partner chromosomes. Losses on 1p and gains in 1q have been detected by CGH; however there did not seem to be a correlation with clinical stage or disease progression (Kwong *et al*, 2004; Shiomi *et al*, 2003). Some of these breakpoints may be worthwhile analysing in greater detail. Chromosome 9 is highly involved in translocations and an important gene in the

literature, which may be involved in OC, is *p16* located on 9p. The chromosome 7 marker in SNO tied in with *EGFR* amplification unit seen in the FISH results for *EGFR* amplification. As far as the *FRA3B* and the *FRA16D* loci are concerned, chromosome three rearrangements were common and definitely could be involving this fragile site. Chromosome 16 did not display many rearrangements at all and does not seem to be displaying fragility of this region in keeping with *WWOX* expression seen in this study.

Our focus on characterising chromosomal rearrangements in this study was driven by the importance of detecting translocations in malignancies as these rearrangements pin point critical genes involved in the pathogenesis of malignancies. This is demonstrated in Ewing's Sarcoma, characterised by the t(11;22)(q24;q12) involving the FLI1 gene. This translocation is found in 80-90% of these tumours (Stegmaier *et al*, 2004). It is also demonstrated in the haematological malignancy, Chronic Myeloid Leukaemia, which is caused by the t(9;22) resulting in the BCR/ABL fusion transcript. These translocations activate oncogenic pathways, which propagates the malignancy. If key rearrangements could be detected in more malignancies such as OC perhaps more effective diagnostics and treatments may be developed.

No solid conclusions can be drawn from these complex results, however the results are useful as they have highlighted some genomic regions, which may be involved in OC and further support the rationale to further map and identify breakpoints. Further analysis will be made using various other techniques to establish what some of these abnormalities may mean; specifically the t(1;3) and t(3;22) breakpoints, which may

harbour a gene or genes at play in OC like the t(11;22) in Ewings sarcoma. Whether these events are primary or secondary abnormalities needs to be elucidated.

4.3.2 *EGFR* over expression

The over expression of the *EGFR* gene in WHCO1 and WHCO3 was not due to gene amplification. WHCO5 and WHCO6 did not show *EGFR* gene amplification either but SNO had amplification of the *EGFR* gene and this is likely to be paralleled by over expression of *EGFR*. DNA amplification of this region by itself did not seem to provide a selective growth advantage since they were in a small percentage, however it would be interesting to observe percentages in later passages. Over expression of *EGFR* in WHCO1, WHCO3, WHCO5 and WHCO6 had to be due to up-regulation of transcription by other mechanisms. It is still important to establish whether *EGFR* is over expressed in tumours as it is often associated with a poor prognosis (Khalil *et al*, 2003). With the development of the monoclonal antibodies directed at blocking these receptors as well as tyrosine kinase inhibitors targeting EGFR, this could be a promising factor for targeted therapy and assist in selecting therapy for patients with oesophageal cancer if over expression is detected. These results indicate that over expression of *EGFR* in OC may not always be due to gene amplification and in terms of diagnostic testing, EGFR over expression must be established perhaps not by FISH for *EGFR* amplification but other techniques such as immunohistochemistry for the detection of the EGFR protein.

CHAPTER FIVE – GENERAL DISCUSSION

5.1 Gene integrity within fragile sites and their role in oesophageal cancer

The cell lines WHCO1, WHCO3, WHCO6 and SNO were near diploid, extra Cep3 signals were seen with the BAC probes due to unbalanced rearrangements of chromosome three as evidenced by M-FISH. *FHIT* deletion status in SNO was evaluated relative to the near diploid status of the cell line, one copy of the gene was deleted and this was considered a full deletion of *FHIT* since two or more cep3 signals were detected. The FISH results for the remaining cell lines did not show deletions occurring within the locus with the probe system used in this study. However intragenic deletions are likely to be occurring in WHCO1, WHCO5 and WHCO6 where RT-PCR detected aberrant transcripts. This suggests that this particular probe combination is not sensitive in detecting all *FHIT* alterations but is still useful for the detection of translocations**.** Other BAC combinations may enable the detection of intragenic deletions, which may have escaped the BAC probe system used in these FISH experiments.

The translocation of chromosome three p near the *FHIT* locus in WHCO3 and the 1% break apart signals detected in WHCO1 and WHCO3 show that there is definite instability in the *FRA3B* region. The under representation of *FHIT* signals relative to the cep3 signals may still be indicative of hemizygous deletion equivalent and the lowered expression detected by RT-PCR may have biological consequences such as a growth advantage to these cells. This hypothesis has been supported by the finding that hemizygous deletion of *FHIT* results in a loss or reduction of Fhit protein expression in numerous human cancers (Ishii *et al*, 2001b).

The M-FISH results showed that chromosome three was involved in unbalanced rearrangements. Seen in conjunction with the FISH results, this suggests chromosome 3p or 3q unbalanced representation. In future studies, specific chromosome arm paints for 3p and 3q respectively, will determine which arm is present in these unbalanced rearrangements and will clarify whether this correlates with the under representation of *FHIT*, detected by FISH or if the *FHIT* locus is deleted by itself. Determining which chromosome three arm is involved in rearrangement and further mapping of translocation break points will also clarify the involvement of *FRA3B*, even where the *FHIT* locus is not affected, since the *FRA3B* site extends beyond the *FHIT* locus (Becker *et al*, 2002).

RT-PCR proved to be more sensitive than FISH, with the current probe design, in detecting *FHIT* alterations and three out of the five cell lines showed definite aberrant transcripts, WHCO5 and WHCO6 both expressed smaller products. SNO expressed a number of aberrant transcripts and did not express exon five, which is the most commonly deleted exon and is required for functional protein synthesis. SNO was thus expressing a non-functional Fhit protein.

The RT-PCR results also appeared to show down-regulation of the gene in comparison with controls, however this would have to be validated by a semi-quantitative RT-PCR comparing with controls having the exact starting RNA amount or by real time PCR using an external standard.

These cell lines all expressed *WWOX* and M-FISH did not reveal any significant rearrangements involving chromosome 16 indicating little involvement of this gene and the *FRA16D* site in these cell lines. *WWOX* could still be investigated in fresh tumour specimens due to the limitations of using cell lines for analysis of cancer.

It is thus concluded that these combined FISH and RT-PCR results showed that there might be a role for *FHIT* in the pathogenesis of oesophageal cancer in South Africa. Four of the five cell lines showed altered expression of *FHIT*. The limitations and draw backs of using cell lines for the analysis of cancer, such as the acquired changes occurring in cell culture, requires that this study be carried over to fresh tumour specimens to establish a definite role for *FHIT* in OC initiation or progression. Investigation of *FHIT* expression is under way in fresh tumour specimens.

5.2 M-FISH and *EGFR* gene amplification

The M-FISH has highlighted some chromosomal rearrangements, which are common to the five cell lines; these chromosomal rearrangements are worth looking at in more detail and may pinpoint genes, which are involved in OC development. Specifically the t(1;3) was common to WHCO5 and SNO as well as another translocation involving chromosomes 3 and 22 common to WHCO3 and WHCO5. These two common markers are very interesting, and the breakpoints on chromosome 1, 3 and 22 will be further investigated. The high involvement of chromosome 9 is also worth investigating as it was rearranged in four of the five cell lines (Summary in table 10). This study, being the first M-FISH study on OC cell lines in SA, has therefore highlighted some genomic regions, which should be analysed more intensively and should be examined in future studies on

fresh OC tumour specimens. The interesting marker chromosome 7, showing a triplicate banded pattern, detected in SNO was found to be an amplification unit involving the *EGFR* locus as well as chromosome seven centromere. The over expression of *EGFR* has been detected in SCC of the oesophagus in various studies. Chromosomal gains have also been shown using cDNA microarray techniques in the 7p12.13 region where the *EGFR* gene is situated (Metzger, 2004). A previous study on WHCO1, WHCO3 and SNO used ligand-binding assays to determine the number of *EGFR* receptors on the cell surface and showed that all these cell lines had over expression of this receptor (Veale, 1989). The *EGFR* gene was only amplified in SNO, the other cell lines had aneuploidy of chromosome seven and there seems to be unbalanced rearrangements of chromosome seven when analysing the M-FISH results. Mechanisms other than gene amplification might be responsible for *EGFR* over expression. These results show that other tests should be considered to assess EGFR over expression in OC, specifically if it is used in order to determine prognosis and treatment options for patients with OC in the future.

Table 10. Summary of results for FISH, RT-PCR and M-FISH. Important common abnormalities are highlighted

	WHCO1	**WHCO3**	**WHCO5**	**WHCO6**	**SNO**
***FHIT* LOCUS**	Deleted with respect to chr 3 copy number: *FHIT*/Cep3 ratio 0,5	Translocated and *FHIT*/Cep3 ratio of 1.	Deleted with respect to chr 3 copy number: *FHIT*/Cep3 ratio 0,5	Deleted with respect to chr 3 copy number: *FHIT*/Cep3 ratio 0,6	Deleted: *FHIT*/Cep3 ratio 0,4
***FHIT* EXPRESSION**	Normal and Aberrant transcript detected	Normal	Aberrant transcript only	Aberrant transcript only	Aberrant transcripts
***WWOX* EXPRESSION**	Normal	Normal	Normal	Normal	Normal
M-FISH common abnormalities	Trisomy 12	Trisomy 12 t(11;22), t(8;9;20), t(7;9;16;18), t(9;13;20), t(3;16;22),	t(1;3) t(2;9), t(9;19), t(9;14) t(3;11;13;22) t(3;11;22)	Trisomy 12 t(9;15)	t(1:3) t(3;9) t(11;13;22)
***EGFR* AMPLIFICATION**	Aneuploidy 7 *EGFR*/Cep7 ratio of 1	Aneuploidy 7 *EGFR*/Cep7 ratio of 1	Aneuploidy 7 *EGFR*/Cep7 ratio of 1	Aneuploidy 7 *EGFR*/Cep7 ratio of 1	Amplification *EGFR*/Cep7 ratio of 6

6. APPENDICES

APPENDIX A

Cell culture buffers

Complete medium

500ml RPMI 1640 medium (Sigma®)

10% 50ml Foetal Calf Serum (Sigma®)

1% Streptomycin-Penicillin (Highveld Biological)

PHA (Phytohemagglutinin) Stock

1mg PHA

1ml of SABAX water

Store at 4°C for one month

Methotrexate (MXT) Stock solution

0,1ml of MXT Lederle IV (Wyeth SA) (25mg/ml)

9,9ml of SABAX water

Store at 4°C

Methotrexate working solution

1ml of stock solution

9ml SABAX water

Store at -20°C

Thymidine Stock solution

4,8mg Thymidine (Sigma®)

10ml sterile medium

Thymidine working solution (100x dilution)

1ml thymidine stock solution

99ml medium

0,075M KCl

2,8g KCl (BDH)

500ml distilled water

Incubate at 37°C

Fixative

3 parts methanol

1 part glacial acetic acid (Merck® Laboratory Supplies)

Keep ice-cold

APPENDIX B

RNA buffers

10X FA buffer

200mM 3-[N-morpholino] propanesulfonic acid (MOPS) (Free Acid)

50mM sodium acetate

10mM EDTA

pH 7 with NaOH

Using DEPC water

1X FA buffer

100ml 10X FA buffer

900ml DEPC water

DEPC water

0,1ml DEPC (Diethyl pyrocarbonate, Sigma®)/ 100ml water

Shake vigorously

Incubate at 37°C for 12hours

Autoclave to remove DEPC

1% Denaturing agarose gel

0,51g Agarose (Whitehead Scientific)

50ml 1X FA buffer

0,54ml of 37% Formaldehyde (Merck® Laboratory supplies)

0,5µl Ethidium bromide

5X RNA loading dye

16 saturated aqueous bromophenol blue

80 500mM EDTA pH 8

720 37% formaldehyde

2ml 100% glycerol

3084 formamide

4ml 10X FA gel buffer

RNase-free water up to 10ml

APPENDIX C

LB top agar – per litre

10g Bacto® tryptone (DIFCO)

5g Bacto® yeast extract (DIFCO)

5g AAR® NaCl (SMM Instruments)

Autoclave

Frozen Stock cultures

Glycerol solution

65% Glycerol (vol/vol) (Merck® Laboratory Supplies)

0.1M $MgSO_4$ (Merck® Laboratory Supplies)

0.025M Tris.Cl, pH 8 (Merck® Laboratory Supplies)

TE (Tris/EDTA) buffer

10mM Tris HCl, pH 7,4 (Merck® Laboratory Supplies)

1mM EDTA, pH 8,0 (Merck® Laboratory Supplies)

2% Agarose gel

2g multi purpose agarose (GIBCO®)

100ml 1x TAE buffer

1µl Ethidium bromide

Heat until clear

Pours 3 gels

Bromophenol blue loading dye

0.1% Bromophenol Blue (Merck® Laboratory Supplies)

0.1M EDTA (Merck® Laboratory Supplies)

50% Glycerol

Make up volume with distilled water.

1xTAE buffer

40mM Tris-acetate (pH 7.6) (Merck® Laboratory Supplies)

1mM Na_2EDTA (Merck® Laboratory Supplies)

10X Nick translation buffer

0,5M Tris-HCl pH8.0 (Merck® Laboratory Supplies)

50mM $MgCl_2$ (Merck® Laboratory Supplies)

0,5mg/ml Bovine Serum Albumin (Boerhinger Mannheim)

0,1M β-mercaptoethanol

0.1ml β-mercaptoethanol (BDH)

14,4ml double-distilled water

10x nucleotide stock - SpectrumOrange

0.5mM dATP (Boerhinger Mannheim)

0.5mM dGTP (Boerhinger Mannheim)

0.5mM dCTP (Boerhinger Mannheim)

0.25mM dTTP (Boerhinger Mannheim)

0.25mM SpectrumOrange d-UTP (Vysis Inc.)

10x nucleotide stock – SpectrumGreen

0.5mM dATP (Boerhinger Mannheim)

0.5mM dGTP (Boerhinger Mannheim)

0.5mM dCTP (Boerhinger Mannheim)

0.25mM dTTP (Boerhinger Mannheim)

0.25mM SpectrumGreen d-UTP (Vysis Inc)

DNase I solution

3mg DNase I (Boehringer Mannheim)

0.5ml of 0.3M NaCl

0.5ml glycerol

Store at -20˚C

Hybridisation buffer

50% deionised formamide (Appendix C) (Saarchem)

2x SSC

10% dextran sulphate (Sigma®)

50mM sodium dihydrogen orthophosphate (Saarchem)

pH to 7,0 with disodium hydrogen orthophosphate (Saarchem)

Store at -20˚C

Hybridisation solutions

Denaturing buffer

35ml deionised formamide (Saarchem)

5ml phosphate buffer

5ml 20x SSC

5ml distilled water

pH to 7 with concentrated HCl

Deionised formamide

1 spatula full Analytical grade mixed bed resin AG 50-X8 (BioRad®) for every 100ml formamide (Saarchem)

Place on stirrer for 2 hrs

Filter with Whatman® No1 filter paper

Store at 4ºC

Phosphate buffer

Solution A: KH_2PO_4 (Saarchem) – 4,5g per 500ml, pH to 4,51

Solution B: Na_2HPO_4, $2H_2O$ (Saarchem) – 5,94g per 500ml, pH to 8,97

For 100ml add 41,3ml solution A and 58,7ml solution B

PH to 7.0

Autoclave

Wash buffers

50% formamide

20ml 20x SSC

80ml distilled water

100 ml formamide

pH to 7 with HCl

20x SSC

3M NaCl (SMM Chemicals)

0.3M sodium citrate (SMM Chemicals)

Adjust to pH 7.0 with concentrated HCl

Autoclave and store at room temperature

2X SSC with tween

2X SSC
0.05% Tween® 20 (Merck Laboratory Supplies)

DAPI (4', 6-diamino-2-phenylindole) stock solution

0,2mg/ml DAPI (Serva)

2x SSC

DAPI working solution

100 ml 2x SSC

100µl DAPI (0.2µg/ml)

DAPI wash solution

2x SSC: 5ml 20x SSC in 50ml

25µl Tween® 20 (Merck Laboratory Supplies) in 50ml

M-FISH buffers

2X SSC

10ml 20X SSC pH 7.0

90ml distilled water

10X PBS

80g NaCl

2g KCl

14,4g Na_2PO_4

2,4g KH_2PO_4

800ml distilled water

pH to 7,4 with HCl

Adjust to 1litre

1X PBS

20ml 10X PBS

180ml distilled water

0.4X SSC/0.3% tween

1ml 20X SSC pH 7

47,5ml distilled water

150µl Tween® 20 (Merck Laboratory Supplies)

Make sure pH is 7 and then adjust to 50ml

2X SSC/0.1% tween

5ml 20X SSC

42,5ml distilled water

50µl Tween® 20 (Merck Laboratory Supplies)

Make sure pH is 7 and adjust to 50ml

Vectashield with DAPI

42ng DAPI per ml of Vectashield (Vecta Laboratories)

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