DIFFERENTIALLY EXPRESSED GENES IN OESOPHAGEAL CANCER

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FRED ALEXANDER WAFULA WAMUNYOKOLI

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CERTIFICATE OF SUPERVISORS

In terms of paragraph eight of the "General regulations for the degree of Doctor of Philosophy", we as supervisors of the candidate FRED A. W. WAMUNYOKOLI, certify that we approve of the incorporation into this thesis material that has already been published or submitted for publication.

Prof M. Iqbal Parker
Division of Medical Biochemistry
Director, School of Biomedical Sciences
Faculty of Health Sciences

Hendricks

Dr Denver T. Hendricks
Division of Medical Biochemistry
Faculty of Health Sciences

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ABSTRACT

Oesophageal cancer (OC) is a leading cause of cancer death in the black population in South Africa. The lifetime risk of the disease amongst black males is 1 in 59. High incidence areas are the Transkei, Ciskei and KwaZulu-Natal where it is responsible for over 45% of all malignancies.

Cancer develops through a multistep process of genomic instability and clonal evolution. Several oncogenes, tumour suppressor genes and transcription factors have been shown to be altered in oesophageal cancer. Their role however, in the development and/or susceptibility to the development of oesophageal cancer is poorly understood.

The aim of this project is to identify candidate genes that are differentially expressed in oesophageal cancer patients with a view to understanding the development of oesophageal cancer. The ultimate objective of the project is to use this data to develop possible biomarkers for the disease.

In the first phase of the project, differential display reverse transcription polymerase chain reaction (DDRT-PCR) was carried out on both malignant and corresponding normal tissue of the same patient to identify candidate genes. Differentially expressed genes from DDRT-PCR were isolated, cloned, sequenced and identified by searching the GenBank database using BLAST software. Northern blot hybridization experiments corroborated the DDRT-PCR results which earlier identified five genes namely, (High mobility group binding protein 2A (HMGB-2A), Nasopharyngeal carcinoma transforming gene (Tx), Excision repair cross complementing gene 5 (ERCC), Fibulin-1D and N-Myc downstream regulated gene 1 (NDGR-1) to be differentially expressed. These genes have not been previously implicated in oesophageal cancer tumourigenesis.

In a separate study, microarray analysis using RNA isolated from tumour and corresponding normal oesophageal tissue of the same patient identified 370 differentially expressed genes. A sample of 13 genes was further analysed by Northern blotting experiments. The expression

levels of 3 (cdc25B, Integrin alpha 6 and MMP14) of the 13 genes were altered in the same manner in the tumour biopsy samples as observed in the microarray analysis.

Both the DDRT-PCR and microarray analysis identified candidate genes that have not been previously reported in the development of oesophageal cancer. These data provides a basis for further research into the relevance and possible role of these genes in the development of oesophageal cancer.

In the second phase of the project, immunohistochemical studies using antisera to a number of genes selected from DDRT-PCR and microarray analysis were carried out on a larger patient sample. The objective of this study was to determine the subcellular localisation and levels of expression the different proteins of these genes. Studies using antisera against fibulin-1D and NDRG-1 demonstrated the absence and abrupt change in subcellular distribution of fibulin-1D and NDRG-1 proteins, respectively, in dysplasia - a premalignant stage of oesophageal cancer. Furthermore, the levels of expression and subcellular partitioning of these proteins were closely associated with tumour grade. This suggests that these proteins may play an important role in oesophageal cancer tumourigenesis. In addition, these data strengthens the basis for the further investigation and possible consideration of these genes as potential biomarkers in the pathological diagnosis of oesophageal cancer.

CHAPTER ONE

1.0 INTRODUCTION

Cancer of the oesophagus (OC), like other cancers, is a multi-step process that passes through many different stages and may take several decades to manifest itself. The major problem in the management of this disease is the absence of early symptoms. Consequently, most patients are diagnosed during the rapidly progressing but short, late stage of the disease when there is very little that health workers can offer to the patient. The majority of patients, especially, those presenting with advanced disease (stage III lesions with evidence of extra-oesophageal spread) are treated with only a palliative intent, and the prognosis for these patients is poor, with a mean survival time of 3 to 4 months.

1.1 PATHOLOGY

Carcinoma of the oesophagus can be classified into several histological types. Squamous cell carcinoma (SCC) is the most common oesophageal malignancy accounting for between 50 to 60% of all oesophageal tumours worldwide. The location of the tumours in 50% of patients is in the middle third of the thoracic oesophagus, in the distal third of 30%, and in the proximal third in 10% of patients (Figure 1.1). The tumour is composed of sheets of polygonal or polyhedral cells demonstrating varying degrees of differentiation. The majority of tumours are moderately differentiated (Altorki *et al.*, 1992).

Figure 1.1

The regional distrubution of the principle subtypes of oesophageal carcinomas in the oesophagus.

The second most common type of oesophageal tumour is adenocarcinoma (ADC). This type of tumour accounts for 40 to 50% of all oesophageal malignancies worldwide. The location of the tumour in the majority of patients is in the distal third of the oesophagus. Most tumours are well differentiated and commonly appear as fungating or polypoid cells (Ellis *et al.*, 1983). Barretts oesophagus (BO), a pre-malignant condition that is closely associated with ADC, is caused by the chronic reflux of gastric acid into the oesophagus. This condition increases the risk of developing ADC by 30 to 40 fold through a multi-step process, from a metaplastic condition to invasive cancer (Spechler *et al.*, 1984).

Other minor and exceedingly rare malignancies include, small cell carcinoma (Tateishi *et al.*, 1974), malignant melanoma (Takubo *et al.*, 1983) and Leiomyosarcoma (Gaede *et al.*, 1978).

This thesis will focus primarily on SCC because it is the predominant oesophageal malignancy in South Africa.

1.2 EPIDEMIOLOGY

1.2.1 INCIDENCE

Oesophageal cancer (OC) commonly occurs in mid to late adulthood with an incidence peak between 50 and 70 years of age and rarely occurs in persons younger than 25. Mortality rates increase steadily with age, with a median age at death of 66 for men and 67 for women. The disease occurs predominantly in males, in whom the mortality rate is 4 to 6 times higher than females (Blott, 1994). The World Health Organization (WHO) estimated that in 1996, the number of deaths due to OC worldwide amounted to 450,000 out of a total 7.1 million cancer deaths, making OC the fifth most frequent cause of cancer deaths worldwide (WHO, 1997), and the fourth most common cancer in developing countries. More than 85% of the cases, particularly SCC, occur in developing countries (Day and Varghse, 1994), whereas ADC occurs mainly in developed countries (Kim *et al.*, 1997).

Squamous cell carcinoma is more prevalent in black populations than white populations, whereas the converse is true for ADC (Mannell and Murray, 1989). Although the reasons behind these patterns are unclear, the socio-economic status of the subjects (differences in health care, lifestyles and environmental factors) have been suggested as some of the contributory factors that may in part, account for the disparities in the patterns and distribution of the disease (Parkin *et al.*, 1992; Gammon *et al.*, 1997).

throughout, cancer of the oseophagus shows significant variability in incidence, with well-defined geographic and ethnic variations.

1.2.1.1 Americas

In the United States of America, the incidence of OC is approximately 6 per 100,000 people annually, with ADC accounting for about 50% of all cases (Blot and Fraumeni, 1982). ADC is the most common cancer among white patients, while SCC predominates in black patients (Blot *et al.*, 1993). Presently, OC is the second-leading cause of cancer death amongst black men under 55 years of age (Blot and Fraumeni, 1982).

In South America, countries with a high incidence of OC include, Brazil (Porto Alegre city), Uruguay and Argentina (Concordia province). The age standardized incidence rates (ASIR) for males per 100,000 people have been estimated to be 18.9, 11.9 and 17.5, respectively (Parkin *et al.*, 1997).

1.2.1.2 Europe

France has the highest incidence and mortality rates for OC in Europe, with an estimated combined incidence (SCC and ADC) of 26 per 100,000. The high incidence regions are Calvados, Somme and Haut-Rhin with ASIR in males of 22, 18 and 14.2 per 100,000 people, respectively. Other a high incidence areas are Western Scotland, Veneto region of Italy and the Basque region of Spain. These regions have an ASIR for males of 9.4, 9.7 and 9.6 per 100,000 people, respectively (Parkin *et al.*, 1997).

1.2.1.3 Asia

China has the highest incidence of OC in Asia. The national mortality rate from SCC is 19.6 per 100,000 deaths among men and 9.8 per 100,000 deaths among women, accounting for approximately 23% of all cancer deaths (Blot and Li, 1985).

The Northeastern part of Iran, especially, the Goubach, Maquandran and Gorgan provinces have a high incidence – up to 180 new cases per 100,000 people have been recorded. The former Soviet Union republics of Kazakhstan, Azerbaijan, Turkmenistan, Uzbekistan and Tajikistan form the central portion of the Asian OC belt where age standardized incidence rates of 85, 29, 111, 49 and 35, respectively, have been reported

(Duranceau, 1988). Clusters of high incidence areas have been reported from the Indian sub-continent, Sri-Lanka, Hong Kong and Singapore, particularly, among subjects of Chinese descent (Duranceau, 1988). The high incidence of OC among the Chinese descent population in Singapore and Hong Kong may suggest the prevalence of risk factors associated the either life style of these people or a probable genetic predisposition.

1.2.1.4 Africa

Population based cancer registries are important sources of information on the regional and worldwide incidence of cancer. In Africa, however, few countries have functioning registries that publish data regularly and provide mortality data of adequate quality to the World Health Organization (Sitas, 1998). Thus, most countries in Africa have accurate date published data on cancer incidence.

Cancer registration continues to be a difficult pursuit in Africa. Firstly, limited financial resources, lack of reliable sources of information within health-care delivery systems and the unavailability of reliable population data for estimating accurate incidence are only some of the major barriers to cancer registration. Secondly, the HIV/AIDS pandemic and re-emergence on a grand scale of some infectious diseases such as tuberculosis and malaria have resulted in minimal funding being allocated to cancer-control activities on the continent. Thirdly, the embracing of economic structural adjustment programmes currently underway in most African countries has resulted in the introduction or re-introduction of cost sharing programmes in the public health sector in order to cut costs. The consequences of this include dormancy of previously active cancer registries or the suspension of the establishment of new registries. These factors have affected attendance at medical institutions, particularly by the elderly, resulting in under-estimation or lack of the true incidence of the disease (Chokunonga *et al.*, 2000).

The incidence of OC in sub-Saharan Africa displays distinct patterns with the highest incidence belt present in the East, Central and Southern African regions. Black African populations inhabiting these areas are primarily Bantu speaking who have a common ancestry that is traceable to the area presently covered by Cameroon and the Democratic Republic of Congo. The Bantu populations traditionally eat cereal (maize, sorghum and millet) as their staple food. In contrast, the West African region – (Nigeria to the Gambia), has a lower OC incidence. The black African inhabitants within this region predominantly eat plantain and tubers such as yams and cassava as their staple food.

The above observations suggest that the incidence of OC in Africa may be influenced possibly in part, by genetic and/or nutritional factor(s) that are inherent in these two regions.

In the high incidence OC region of Africa, Zimbabwe has the highest incidence with age standardized incidence rates (ASIR) amongst black males 196 per 100,000 persons (Chokunonga *et al.*, 2000). Uganda has the second highest incidence of OC in Africa. The ASIR of OC among males has been estimated at 18 per 100,000 people (Parkin *et al.*, 1997). Further reports from clinicians suggest that the incidence of OC is high in countries along the east coast of the continent, particularly, Sudan, Ethiopia, Kenya, and Tanzania. These reports are based on the attendance records of cancer patients at the national referral hospitals of these countries.

As stated previously, in West Africa, OC is rare with the ASIR among males estimated at 1.7 per 100,000 people in Mali (Bayo *et al.*, 1990), 0.9 per 100,000 people in the Gambia (Bah *et al.*, 1990) and 0.6 per 100,000 in Guinea Conakry (Koulibay *et al.*, 1997).

South Africa has the third highest incidence of OC in sub-Saharan Africa. The ASIR is 11.6 and 4.9 per 100,000 for males and females, respectively (Sitas *et al.*, 1998). There is a marked variation in incidence between the different ethnic groups and also geographically within groups. Furthermore, squamous cell carcinoma is more prevalent in the black and mixed race (coloured) populations, while the white population predominantly develop ADC. These trends are in line with those observed internationally.

Oesophageal cancer is the third most prevalent cancer in males of all races (5.4% of all cancers), whereas, it is the most common cancer in black males (14.3%) with a lifetime risk of 1 in 59. In contrast, OC is ranked ninth with a lifetime risk of 1 in 164 amongst white males, a three-fold difference in risk. In mixed race and Asian males, OC ranks 4th and 7th respectively (Sitas *et al.*, 1998).

The high incidence regions of OC in South Africa are the Transkei and Ciskei areas in the Eastern Cape and Kwa-Zulu Natal provinces (Figure 1.2). A study of cancer incidence in four selected districts of the Transkei between 1985-1990 found OC to account for 46.5% of all malignancies in males (Makaula *et al.*, 1996).

Figure 1.2

A MAP SHOWING THE NINE PROVINCES OF SOUTH AFRICA. Provinces with a black cross represent areas with a high incidence of oesophageal cancer.

The reasons for the high prevalence of OC in these regions are not clearly understood, but aetiological factors such as tobacco use, alcohol abuse, nutritional deficiencies, xenobiotic contaminated foodstuffs and the frequent use of emmetics (induced vomiting by use of herbal concoctions) have been proposed to contribute significantly to the high incidence of OC in these regions. Some of the above factors will be discussed in detail in the next section.

Recent reports suggest that the incidence of OC may be higher in other provinces such as the Free State and Northern Provinces that have hitherto lacked either established or functioning cancer registries. For historical reasons, the National Cancer Registry in Johannesburg has not accurately accounted for cancer cases in the former "homeland" regions. In the current political dispensation more information is emerging regarding OC incidence, particularly, from those provinces that were thought to have very low or no OC cases.

1.2.2 AETIOLOGY

A large volume of literature suggests that numerous risk factors may play either a direct or indirect role in the aetiology of OC. There seems to be consensus though, that none of these factors singularly accounts for the high incidence in several high-risk areas, or for the variations in the frequency in distinct geographic areas.

Environmental factors have been suggested to play a predominant role in the development of OC, partly due to the variations in the incidence between and within geographical regions, gender and ethnic groups. Comparison of dietary and cultural habits in the different high incidence areas worldwide has shown little in common between the areas. It therefore seems likely that each high-risk area may have its own regional environmental factors contributing to the aetiology of the disease.

It has been proposed that oesophageal cancer is induced by prior damage to the oesophageal mucosa, followed by specific carcinogenic induction, the form of which may differ from region to region (Alberts, 1991). The oesophageal mucosa frequently comes into contact with environmental factors since its exposed location makes it an ideal route of entry for foreign, often, harmful agents, including chemical substances from tobacco smoking, alcohol, nutritional factors exposures and physical irritants. In addition, there are other factors such as infectious agents (e.g. viruses) that have been implicated in OC carcinogenesis.

1.2.2.1 Tobacco

Epidemiological and experimental studies have implicated tobacco smoking in the aetiology of up to a third of all cancers, including OC, lung, mouth and pharynx. The risk of developing OC is about five times higher among cigarette smokers than non-smokers, with the risk increasing to nearly ten fold in heavy smokers (Blot, 1994). Pipe smoking, a popular form of smoking amongst older people in the rural areas of South Africa has been strongly associated with an increased risk of OC (Segal *et al.*, 1988; Sumerak *et al.*, 1992).

Homegrown tobacco, which is widely used in pipe smoking in the Transkei, has been found to have a higher mutagenic potency than commercial tobaccos. Tobacco smoke is known to contain a wide variety of chemicals that have been shown to be highly mutagenic and carcinogenic in experimental animals and in humans (Ames *et al.*, 1995). In addition, the smoke contains oxidants that deplete the body's anti-oxidants (Duthie *et al.*, 1991). Tobacco tars and cigarette smoke contain, amongst other chemicals, polycyclic aromatic hydrocarbons (PAH's), N-nitroso compounds, epoxides, peroxy compounds and halo-ethers and tumour-promoting phenol and related compounds (van Duuren, 1968). Auerbach and workers demonstrated the most suggestive evidence for the role of tobacco as a carcinogen in the oesophagus. In autopsy examinations of the oesophagus of non-smokers, only 6.6% of the subjects showed some atypical nuclei in the basal layer of the oesophageal epithelium and none had carcinoma *in situ*. In contrast, 79.8% of smokers displayed atypical nuclei and 1.9% had carcinoma *in situ*. The oesophageal epithelium of smokers tended to be thick, reflecting a hyperplastic response (Auerbach *et al*, 1965).

The habit of chewing tobacco, betel nut or combinations of these with lime is common in India, Sri Lanka and Burma. It has been suggested that they contain constituents that cause chronic irritation, resulting in damage to the epithelium and thus stimulating hyperplastic and neoplastic changes of the epithelial cells (Warwick and Harington, 1973). This is thought to be partly responsible for the high incidence of cancer in the oral cavity and oesophagus in these countries.

1.2.2.2 Alcohol

The association of high alcohol consumption with OC is based on compelling epidemiological evidence that has demonstrated predisposition to multiple OC development due to heavy drinking (Morita *et al.*, 1994). The risk of OC in alcoholics is 3 to 4 folds higher than the risk in non-alcoholics (Yokoyama *et al.*, 1996b).

Case control studies in a number of countries such as Uruguay and France have shown a dose-response relationship between alcohol intake and the risk of OC (DeStefani *et al.*, 1990; Launoy *et al.*, 1997). The risk is highest among drinkers of hard liquor, lowest among beer drinkers and intermediate among wine drinkers. Studies in Northern China and Northwestern Iran however, have revealed that alcohol does not appear to be a significant factor contributing to the high incidence of OC in these countries. This suggests that different and or multiple factors other than alcohol may contribute to the aetiology of OC, depending on the locality (Parkin *et al.*, 1997).

Alcohol abuse, coupled with tobacco smoking has been shown to enhance OC risk in a synergistic rather than additive manner. Amongst individuals that are heavy smokers as well as heavy drinkers, the relative risk of developing OC increases 100-fold (Pottern *et al.*, 1981). Analysis of alcoholic beverages, especially, brews found in high incidence areas revealed that these beverages may contain contaminants such as N-nitroso compounds, mycotoxins, methans and tannins that are carcinogenic (IARC, 1988). In addition, the brewing of alcohol in areas with a high incidence of OC in South Africa has traditionally been carried out in cast iron containers. It is postulated that prolonged use of these containers may expose the home brew drinkers to potential carcinogens from the containers; but to date, no concrete evidence has been shown to support this hypothesis.

Ethanol *per se* is not carcinogenic; although it may act as a syncarcinogen or cocarcinogen (Lieber *et al.*, 1986). It is thought that alcohol acts as a solvent, interacting and increasing the penetration of other carcinogens into the basal cell layers in the oesophagus. Secondly, heavy drinking often leads to a poor nutritional status of drinkers. Heavy alcohol consumption interferes with absorption and metabolism of vitamins such as folate, β-carotene and vitamin A, possibly leading to increased risk of cancer development (Ahmed *et al.*, 1994). Thirdly, metabolism of alcohol is associated with increased production of free radicals that have been implicated in carcinogenesis and may promote tumourigenesis in OC (Mufti *et al.*, 1993).

1.2.2.3 Nutritional factors

The majority of OC patients, whether from Asia, Africa, Europe or the Americas, come from the poorer class of these societies.

A poor diet, comprising mainly carbohydrates with a low intake of animal protein, fresh fruits and green vegetables, has consistently been associated with an increased risk of OC in both high and low incidence areas (Jaskiewicz *et al.*, 1987b). A diet rich in fresh fruits and vegetables on the other hand, has been shown to confer some protection against OC (Notani and Jayant, 1987).

Populations with high incidence of OC, such as in China, Iran and South Africa, have mostly maize and wheat as staple foods. The general dietary intake of micronutrients such as beta-carotene, vitamin B, vitamin C, magnesium, zinc and certain minerals are low in these areas (van Rensburg, 1981). In this context, it has been proposed that a deficiency of certain dietary nutrients can result in an added insult or a poorer defense mechanism for the oesophageal mucosa. Additional evidence comes from research using rats, where a zinc deficient diet leads to alterations that include hyperkeratosis and hyperplasia of the oesophageal mucosa. In addition, the incidence of oesophageal tumours is higher and the lag time for induction of these tumours is shorter in zinc deficient experimental animals (Duranceau, 1988). Vitamin A levels in the body play an important role in the stability of the oesophageal mucosa. Vitamin A and zinc help to maintain the integrity of the epithelium, thus vitamin A depletion is associated with a decrease in RNA and DNA synthesis in epithelial cells, while keratinization, mitosis and metaplastic changes increase significantly. Riboflavin and other vitamin B types are essential for maintaining the integrity of the squamous epithelium in the oesophagus and Riboflavin deficiency has been shown to increase the mitotic rate of the oesophageal mucosa in baboons (Duranceau, 1988).

A high intake of maize in combination with high alcohol consumption and tobacco smoking seem to increase the risk of OC. In an Italian study, the high intake of maize was found to be a risk factor in heavy drinkers and smokers. A deficiency of essential micronutrients, in addition to a high maize/wheat diet and tobacco and alcohol abuse might increase the risk of OC several-fold. Malnutrition and deficiencies may set the stage for OC development by creating an oesophageal mucosa and submucosa with poor defense mechanisms that is easily subject to chronic irritation and inflammation. The vulnerable mucosa has defective repair mechanisms and premalignant changes easily occur, with a chronologic evolution towards chronic oesophagitis, atropy, hyperplasia, dysplasia, resulting in carcinoma *in situ*.

1.2.2.4 Xenobiotics

The human body is constantly exposed to harmful compounds usually lipophilic in nature that are readily metabolized into water-soluble metabolites by detoxifying enzymes and are excreted. In addition, the body may be exposed to xenobiotic compounds that are metabolized to highly reactive intermediates that form DNA adducts that are potential carcinogens. Both categories of compounds are detoxified by the same xenobiotic-detoxification system in the body and in the event of a long-term exposure to these compounds or a malfunction of this system; the intermediates may accumulate and lead to increased risk of developing cancer.

Xenobiotic metabolizing enzymes are functionally divided into two groups that complement each other. The phase I enzymes include, amongst others, the Cytochrome P450 superfamily (CYPs) members that generally activates the metabolite by adding a functional group to the xenobiotics by oxidation, reduction or hydrolysis reactions. Phase II, which is the detoxification phase, involves the conjugation of the phase I products with water-soluble cellular compounds such as glutathione, acetyl CoA or glucuronic acid, rendering the metabolites water soluble and easily excretable. Phase II is catalysed by conjugating enzymes such as glutathione-S-transferases (GSTs), N-acetyltransferases (NATs), sulpho-transferases and epoxide hydrolases. Examples of compounds metabolized by this system include benzo[*a*]pyrenes, benzo[*a*]anthracene, nitroso compounds, epoxides and lactones. Occasionally, xenobiotics are activated into highly reactive products that can react covalently with cellular macromolecules such as nucleic acids, proteins and lipids to covalent adducts.

Some genetic polymorphisms in xenobiotic-metabolizing enzymes that result in altered activities of the enzymes have been established as major determinants of host-specific chemical susceptibility to carcinogenesis (Puga *et al.*, 1997). A genetically determined increase in phase I mono-oxygenase activity or a decrease in conjugation (phase II), may result in an increased formation of activated carcinogens that are able to form DNA adducts (Hemminki, 1990). The extent of accumulation and persistence of DNA adducts, however, is further dependent on the DNA repair mechanisms of the cell. If the damage to DNA persists, or is not repaired properly, it may lead to a genetic mutation (Umar and Kunkel, 1996). For the un- or miss-repaired DNA, damage may to lead to the clonal expansion of cells with a selective growth advantage if the mutation or damage was in a critical gene (proto-oncogene or tumour suppressor gene) and the gene's function is altered. Nimura *et al.*, (1997), reported a significant association between the inducibility polymorphisms of CYP1A1 and CYP2E1 and the null genotype of the detoxifying GSTM1. Heavy smokers were found to be at a higher risk of OC with the presence of a CYP2E1 polymorphism (Lin *et al.*, 1997). Polymorphisms in GSTπ and NAT2 genes that are associated with decreased levels of detoxification were found to be associated with an increased risk of OC (Morita *et al.*, 1997; van Lieshout *et al.*, 1999).

One way by which the DNA reactive compounds are introduced into the body is via the oesophagus. The human oesophagus expresses several xenobiotic-metabolizing enzymes and consequently has the capacity to activate chemical carcinogens into reactive DNA binding metabolites. Genetic polymorphisms and alterations in these metabolizing enzymes are likely to predispose one to OC (Morita *et al.*, 1997).

Acetaldehyde generated from alcohol metabolism is eliminated by aldehyde dehydrogenase 2 (ALDH2), the low-K_m form of ALDH (Bosron and Li, 1986). A single base pair difference in exon 12 of the gene that encodes the enzyme ALDH2*1/2*2 results in a catalytically inactive isoenzyme (Hsu *et al.*, 1986; Yoshida *et al.*, 1991). Subjects defective, in these enzymes show an increased risk of developing OC. This defect has been shown to be prevalent amongst people of Asian descent (Crab *et al.*, 1989; Yokoyama *et al.*, 1996a).

1.2.2.5 Human papilloma virus

Human papilloma virus (HPV) is a member of the papovavirus family. This is a family of closed circular double-stranded DNA viruses consisting of over 73 different HPV genotypes (Zur Hausen *et al.*, 1994). Certain genotypes of HPV such as 16, 18, 31 and 32 have been strongly implicated in the development of cervical cancers. The involvement of HPV in the development of oesophageal cancer remains poorly understood.

Studies have generated conflicting data with regards to the presence of HPV in OC. The differences might be due to differences in susceptibility to oesophageal HPV infection in different populations, or to variations in sensitivity of techniques used for detecting the virus (Sur and Cooper, 1998). The pooled data of high-risk areas show a range in the presence of HPV in OC from 13 to 63%, with the overall incidence of about 22% (Lam, 2000).

The predominant HPV genotypes reported in OC include of HPV 6, 11, 16 and 18. Recently, some putative new HPV types DL231, 347, 369, 428 and 436 have been detected in OC (De Villiers *et al.*, 1999; Lavergne *et al.*, 1999) but the malignant potential of these putative new HPV types remains unknown.

Recent studies have shown that the low risk HPV 11 is the most common form of HPV in OC tumours in the Transkei region of South Africa. The presence of HPV 39 also adds to the mystery surrounding the role of HPV in OC (Matsha *et al.*, 2002).

It is possible that HPV is important in the pathogenesis of OC in high incidence areas; however, the low overall prevalence suggests that other risk factors, (which may have synergistic actions with HPV) rather than HPV alone might be important in the pathogenesis of OC.

1.2.2.6 Mycotoxins

In parts of South Africa, epidemiological evidence suggests that oesophageal cancer may be associated with fungal-infected maize (Marasas *et al.*, 1979). Some mycotoxins, such as aflatoxins, sterigmatocystin, penicillic acid, luteoskyrin, cyclothlorotin and patulin, are clearly carcinogenic in experimental animals (Jaskiewicz *et al.*, 1987a; Gelderblom *et al.*, 2001). This underscores the potential role of mycotoxins in oesophageal tumourigenesis.

Contamination of corn (*Zea mays*) and other foodstuffs with fungi has been shown to be substantially higher in areas that have a high incidence as compared to areas with low incidence of OC. In the Southwestern districts of the Transkei (which is an OC hotspot) in South Africa, for example, corn kernels, are highly contaminated with the fungus, *Fusarium*. The main species of *Fusarium* recorded in these regions were *graminearum*, *moniliforme* sheldon and *moniliforme* variety *subglutinaus* (Marasas *et al.*, 1979). Further studies on the agronomy of the soils in the above regions revealed zinc and molybdenum deficiencies. Deficiency of the later mineral is known to increase the susceptibility of maize to a variety of fungal infections, especially, *Fusarium moniliforme*. *Fusarium* is thought to contribute to the development of OC via a number of ways. First, it promotes the formation of nitrosamines, which are potential carcinogens and secondly, the fungus produces fumonisins B_1 and B_2, mycotoxins that exhibit cancer-promoting activities (Li *et al.*, 1980; Yang, 1980). Despite intensive investigation into the role of *Fusarium*-derived mycotoxins in the development of OC in South Africa the role of the mycotoxins remains unclear. There have been interesting reports that *Fusarium* contains compounds that can form DNA adducts, although the identity of these compounds is presently unknown and this observation awaits confirmation.

1.2.2.7 Familial factors

Evidence of genetic susceptibility to OC has come from a number of studies. In one such study in a high incidence area in China, adjustment for known environmental risk factors did not affect the risk of family history (Hu *et al.*, 1992). In another study, an autosomal recessive mendelian inheritance pattern was found in 4% of the population examined (Carter *et al.*, 1992). However, the evidence from these studies is weak and identification of the responsible gene(s) would allow a better assessment of the relative contribution of genetic susceptibility and environmental factors.

Tylosis is a hereditary disorder involving hyperkeratosis of the palms and soles and is inherited as an autosomal dominant trait. In a study performed in England, tylotic family members were shown to have a high rate of OC, while there were no cases of OC among the non-tylotic relatives. These results suggested that the tendency to develop OC might be genetically linked. A recent study has shown that 90% to 95% of patients with tylosis is at risk of developing SCC of the oesophagus by the age of 65 (Marger and Marger, 1993). Genotype pedigree analysis of a tylosis family has located the tylosis OC gene to the chromosomal region 17q23-qter, but the involvement of this gene in sporadic OC remains to be determined. There is no evidence of a published study on the involvement of the tylosis gene in the development of OC in South African patients. It may therefore be of interest to determine the importance of this hereditary predisposition to the development of OC in SA.

1.2.2.8 Other factors

Several factors have been linked to the development of OC. In some high incidence areas of China, Brazil and Iran, people customarily eat food and drink beverages that are at temperatures of 60-88°C. These foods and beverages are thought to inflict a frequent thermal insult to the oesophagus, consequently leading to continuous injury of the oesophageal epithelium. This injury possibly facilitates the development cancer of the oesophagus (Ghadirian, 1987; Victoria *et al.,* 1987). Studies in Iran have found that wheat flour is contaminated with silica-like fibres, while hot chilli and shrimp with uncut spines are regularly consumed in high-risk areas of India. These factors are thought to induce chronic trauma of the oesophagus, stimulating epithelial proliferation and consequently predisposing individuals to OC (Duranceau, 1988).

In high incidence areas of China and Iran, individuals regularly consume large amounts of pickled vegetables and fruits. Chemical analysis of these foods have revealed significant levels of nitroso compounds, benzo[*a*]-pyrenes and polycyclic aromatic hydrocarbons. These compounds are thought to act as promoters of OC development.

Achalasia and oesophageal diverticula are conditions in which stasis occurs in the oesophageal lumen and have been associated with increased incidence of SCC of the oesophagus (Wychulis *et al.*, 1971). Chewing of tobacco snuff, betel juice and opium pipe residues (which contain pyrolysates and certain alkaloids) are common in high incidence areas in Iran and South Africa. The constituents in the above have been shown to have mutagenic properties. In addition, these compounds may damage the oesophageal epithelium and stimulate hyperplastic and neoplastic changes in oesophageal epithelial cells (Cook-Mozaffari *et al.*, 1979).

The above factors provide interesting clues on the aetiology of OC. However, there is only indirect evidence of their potential importance, since there is no causal relationship. It is therefore imperative for us to identify potential carcinogens and tumour promoters in each high-risk area, and to further investigate the exact role-played by each contributory causal agent in the process of carcinogenesis.

In spite of the well-documented potential of risk factors in the predisposition to oesophageal cancer, there is a poor understanding of the molecular mechanism involved in the development of OC. It therefore important that we understand these mechanisms in order to develop better diagnostic, therapeutic and prognostic measures that will contribute positively to the management of oesophageal cancer.

To date a number of factors and genes have been implicated in the development of oesophageal cancer. The majority of these factors have been investigated using *in vitro* systems that involve the use of cancer cell lines, while cohort studies using few patients have been undertaken. Nevertheless useful information on the probable molecular mechanisms that may in part account for the development of oesophageal cancer has been revealed. In addition, important molecular targets for therapeutic, diagnostic and prognostic purposes have been identified or proposed. Many of these genes and/or factors show differential expression during the different phases of tumourigenesis, thereby making them worthy targets in the management of oesophageal cancer.

The section below will highlight some of the previously characterised and postulated alterations and aberrations of pertinent molecular and biochemical pathways whose malfunction initiate and/ or promotes tumourigenesis. This section will be discussed with a bias to oesophageal cancer with emphasis on the alteration of these genes and factors in squamous cell carcinoma of oesophageal cancer.

1.3 MOLECULAR BASIS FOR CELLULAR TRANSFORMATION IN OESOPHAGEAL CANCER

1.3.1 Integrated circuit of the cell

A fundamental difference between cancer cells and normal cells is the unregulated growth displayed by neoplastic cells. Under normal circumstances, cell growth is tightly controlled by a complex network of signalling pathways that converge on the regulatory elements of the cell cycle.

In most cancer cells, this circuitry is "re-programmed" resulting in functionally altered gene expression. The consequence of this alteration is disruption of a delicately balanced equilibrium maintained by components of the signalling pathway. This ultimately leads to cellular de-regulation and dysfunction that may result in neoplasia (Hanahan and Weinberg, 2000).

1.3.2 Development of cancer through a multistage process.

Cancer development is recognized as a multi-step process during which the cell accumulates a number of genetic alterations in a range or variety of molecular pathways resulting in aberrant morphological and functional characteristics. The type and temporal sequence of genetic changes differ in different target cells and tissues (Kinzler and Vogelstein, 1996; Montessano *et al.*, 1996).

The process of carcinogenesis is postulated to begin with an initiation phase during which the target cell is exposed to for example, carcinogenic chemicals, viruses and ionizing radiation. This phase is followed by a promotion phase, which takes longer than the initiation phase and appears to be reversible in the early stages. The promotion phase is characterized by clonal expansion of dysplastic cells. A conversion phase follows the promotion phase during which one or more proto-oncogenes are activated, while tumour suppressor genes are inactivated. The process of carcinogenesis is completed by a progression phase during which there is tumour formation (Harris, 1988).

1.3.3 Acquired characteristics of cancer cells.

Oncology research over several decades has successfully demonstrated that most and perhaps all types of human cancers share a common number of molecular, biochemical and cellular characteristics that manifest themselves as tumourigenesis progresses. These characteristics can be categorized into six essential alterations in cell physiology that individually or collectively define malignant growth. They are; (1) independence or self-sufficiency in sensitivity to growth signals, (2) insensitivity to known growth inhibitory (anti-growth) signals, (3) ability to evade apoptosis, (4) tendency to have limitless replicative potential, (5) ability to promote and sustain angiogenesis and (6) the capacity to invade adjacent tissues and metastasize into distant tissues and organs (Hanahan and Weinberg, 2000).

The particular order and temporal sequence in which each character is acquired vary widely both among tumours of the same type and certainly between tumours of different types. Moreover, in certain tumours a specific genetic event may singularly contribute only partially to the acquisition of a single characteristic, while in others, this event may aid in the simultaneous acquisition of several distinct characteristics. These characteristics confer one or more growth advantages that lead to the progressive conversion of a normal cell into a neoplastic one (Hanahan and Weinburg, 2000).

Although conceptually each characteristic may autonomously promote tumourigenesis, it is plausible that these characteristics act synergistically and cooperatively regardless of the divergent molecular pathways ultimately resulting in neoplasia. There is increasing evidence suggesting that transformed cells frequently “conscript” non-transformed cells to produce factors that augment tumourigenesis (Werb, 1997; Kinzler and Vogelstein, 1998; Olumi *et al.*, 1999).

The sections below will attempt to describe and discuss the spectrum of molecular events involved in cellular transformation with a primary emphasis on oesophageal cancer in the context of the six essential hallmarks listed above. As noted above, some of the molecular alterations discussed may result in the acquisition of more than one of the hallmarks identified above thus complicating assignment to a particular category.

1.3.3.1 Acquired characteristic 1: Self-sufficiency in growth signals.

Growth signals participate in critical cellular functions such as signal transduction, DNA transcription and ultimately cell division. These signals are transmitted into the cell by transmembrane receptors that bind to distinctive classes of signalling molecules such as diffusible growth factors, extracellular matrix components and cell-to-cell adhesion molecules. Tumour cells are thought to generate many of their own growth signals thereby reducing their dependence on stimulation from their normal tissue microenvironment. This independence disrupts a critically important homeostatic mechanism that normally operates to ensure proper behaviour by the various cell types (Hanahan and Weinberg, 2000).

Analysis of the molecules involved in the generation, transmission and translation of the growth signals shows alterations in the expression levels of the key components at all hierarchical levels involved in growth stimulation. This hierarchical network includes growth factors, growth factor receptors, signal transducers and nuclear factors.

1.3.3.1.1 Growth factors.

Growth factors cause cells in the resting (G_o) phase to enter and proceed through the cell cycle. The *INT-2* and *HST-1* genes both encode growth factors of the fibroblasts growth factor (FGF) family and is also known as FGF-3 and FGF-4, respectively. In oesophageal cancer, co-amplification of *INT-2* and *HST-1* has been observed in 30 to 50% of primary tumours and appears to be correlated with a higher probability of metastases and aggressive behaviour of the disease (Tsuda *et al.*, 1989; Kitagawa *et al.*, 1991; Wagata *et al.*, 1991). Overall, there is insufficient clinical data, besides the extensive studies in cell lines, about the role of growth factors in OC.

1.3.3.1.2 Growth factor receptors.

The most important growth factor receptor in this group is the Epidermal Growth Factor Receptor (EGFR or HER-1) and its close homologues the erb-B family (erb-B-1, erb-B-2, erb-B-3 and erb-B-4).

In SCC and ADC, amplification but not re-arrangement of EGFR has been shown to vary between 15 to 30% of OC cases (Hollstein *et al.*, 1988; Lu *et al.*, 1988; al Kasspooles *et al.*, 1993). Japanese studies have noted that the 5-year survival rate of OC patients was much lower if there was over expression of erb-B-1, while erb-B-1 over expression is associated with minimal response to chemo/radiotherapy (Ozawa *et al.*, 1989; Mukaida *et al.*, 1991; Yoshida *et al.*, 1993; Itakura *et al.*, 1994; Hirai *et al.*, 1998). There is therefore much evidence in support of erb-B-1 as a marker for poor prognosis in OC.

The expression of erb-B-2 in OC ranges from 0 to 38% (Mori *et al.*, 1987; Chang *et al.*, 1992; Suwanagool *et al.*, 1993; Shiga *et al.*, 1993; Suo *et al.*, 1995; Ikeda *et al.*, 1996; Hardwick *et al.*, 1997), but the number of cases described is small. From these studies no significant association between the levels of expression and the grade, histology or prognosis of the tumours has been noted. It is possible that erb-B-2 over-expression may play a role in oesophageal carcinogenesis, but the value and importance of erb-B-2 as a useful prognostic marker in OC is yet to be determined.

Cell surface receptors have been identified as targets of deregulation during tumourigenesis. Receptor over-expression would enable cancer cells to become hyper-responsive to ambient levels of growth factors that normally would not trigger proliferation (Fedi *et al.*, 1997). Recent studies have suggested that cancerous cells probably switch the types of extracellular matrix receptors they express, thereby favouring ones that transmit pro-growth signals (Lukashev and Werb, 1998; Giancotti and Ruoslahti, 1999).

1.3.3.1.3 Signal transducers

An important group of genes involved in signal transduction is the *ras* gene family. The *ras* oncogene family is frequently mutated in many cancers, for example, ADC of the lung, colon and pancreatic tumours (Montessano *et al.*, 1996). Hollstein *et al.*, (1988) and Victor *et al.*, (1990) have found no mutations in the ras family members in neoplasms of the oesophagus. However, three other studies revealed over expression of the *ras* protein in OC (Lam *et al.*, 1995; Ono *et al.*, 1994; Ruol *et al.*, 1990). It is thought that the over expressed of this protein may be related to higher proliferative state of cancer cells compared with normal cells. The overall findings suggest that *ras* mutations are unlikely to be significant in the pathogenesis of OC.

compared with normal cells. The overall findings suggest that *ras* mutations are unlikely to be significant in the pathogenesis of OC.

1.3.3.4 Nuclear factors

There are several nuclear factors that have been reported to play an important role in tumour progression. The best-studied family is the *myc* oncogene, which is reportedly over expressed and amplified in many types of cancer. This family comprises of c-*myc*, N-*myc* and L-*myc*. The c-myc gene encodes a nuclear phosphoprotein, *p62* that binds to DNA after dimerization with max protein. The gene product is required for cell proliferation (for entry into G_1 from G_0), differentiation and possibly DNA replication. Activation of the c-*myc* gene occurs in a variety of human tumours and may contribute to tumour progression in both early and late stages of the disease (Schirer and Peltenburg, 1993). There is however, limited data on c-*myc* gene alteration in OC. The few published studies have reported c-*myc* to be amplified in 14 to 25% of OC cases (Lu *et al.*, 1988; Esteve *et al.*, 1993). In a similar study, the *myc* protein was present in both the cytoplasm and nucleus of advanced OC tumours (Miyazaki *et al.*, 1992). In spite of the probable importance of c-*myc* in tumourigenesis, more studies are required to establish its importance in OC pathogenesis.

1.3.3.2 Acquired characteristic 2: Insensitivity to anti-growth signals.

For cancer cells to proliferate, they must develop mechanisms that evade or switch off anti-proliferative signals. Many of these signals are conveyed via the circuitry involved in the cell cycle specifically the G_1/S checkpoint. The anti-growth circuit is disrupted in the majority of human cancers (Dyson *et al.*, 1989; Fynan and Reiss, 1993; Zuo *et al.*, 1994).

Many tumour suppressor genes (TSG) and nuclear factors belonging to the oncogene families control the expression of proteins that control the cell cycle at the G_1/S and G_2/M checkpoints. They interact with each other and control the rate of cell-cycle progression. This plays an important role in the switch between cell proliferation and differentiation with the main control lying in the progression from G_1 to S phase of the cell cycle (Pardee, 1989). Cyclins and cyclin dependent kinases (cdks) regulate passage through the cycle by regulating phosphorylation of the *Rb* protein. *Rb* protein binds to transcription factors of the E2F family that are released upon phosphorylation. The E2F transactivated genes are essential for entry into S-phase and DNA replication. This process can be inhibited by protein products of TSGs such as *p15, 16, 21, 27* and *53,* and stimulated by c-myc protein.

1.3.3.2.1 Alteration of genes involved in cellular proliferation

CDKN2A also referred to as *MST1, CDK41, INK4A* or *CDKN2* encodes the protein *p16.* This gene is thought to contribute to carcinogenesis by de-regulating cell-cycle progression through G_1. Mori *et al.,* (1994) have reported somatic mutations in 14 out of 27 oesophageal cancer patients from Japan. Other studies have shown variations ranging from 0 to 86% in both exons 1 and 2 depending on the methods used to detect these alterations (Busatto *et al.*, 1998; Muzeau *et al.*, 1997; Shamma *et al.*, 1998; Xing *et al.*, 1999 and Yang *et al.*, 1997). The loss of *p16* expression has been associated with poor survival and correlates with cyclin D1 expression (Takeuchi *et al.*, 1997; Yang *et al.*, 1997 and Bussato *et al.*, 1998). From the limited data, it appears that loss of *p*16 expression has a prognostic role in OC. However, most of the data is derived from homozygous rather than heterozygous deletions. Hence, further studies need to be carried out on the latter deletions to authenticate the prognostic role and value of *p16* in OC.

WAF1 gene also referred to as *CIP1, CAP20, PIC1 or SD1* encodes the protein *p21*. This protein is thought to play an important role in tumourigenesis as it is directly up-regulated by wild type *p53* and is thus considered to be a downstream effector of *p*53 induced G_1 arrest. Studies in Japan, China and Hong Kong have shown that the incidence of *p*21 mutations in OC ranges from 27 to 85 % in OC patients. Most of these studies however, have used very small sample sizes (Hirai *et al.*, 1998; Ohashi *et al.*, 1997; Seta *et al.*, 1998; Toh *et al.*, 1997; Yang *et al.*, 1997; Lam *et al.*, 1999). The *KIP1* gene encodes the *p27* protein. This protein is one of the cyclin-dependent kinase inhibitors that blocks progression from G_1 to S phase by binding cyclin D1-CD4 and or cyclin E-CDK2, thus inhibiting their activity. In a study of 66 patients, Shamma *et al.*, (1998) found loss of *p27* expression in OC patients.

The *p53* gene is the best analysed of all genes involved in OC. Mutations in *p53* appear to be an early event in the development of OC. In the case of SCC, *p53* mutations and/or elevated *p*53 protein have been detected in the basal layer of normal or dysplastic oesophageal mucosa (Bennett *et al.*, 1992; Gao *et al.*, 1994; Sarbia *et al.*, 1994; Mandard *et al.*, 1997). Although these findings are suggestive, they do not constitute direct evidence that these alterations and the associated pathologies are precursors of SCC. There is enormous literature on the prevalence of *p*53 aberrations in OC and the incidence of genetic alterations range from 10 to 85%, with the frequency of mutations appearing to be higher in high incidence areas. The majority of *p*53 mutations in OC are localized within four evolutionary conserved domains of the gene, exons 5 through 8. To date no mutations have been reported in exons 1 to 4 in OC. In the literature, only one mutation was found in exon 10 (codon 342) in a Canadian study, while in a similar Japanese study, a single mutation was found in exon 11 (codon 342) (Casson *et al.*, 1998; Wagata *et al.*, 1993).

In ADC, mutations are distributed throughout exons 5 to 8 with hot spots at codons 175 (exon 5), 248 and 273 (exon 8), while in SCC they are prevalent in codon 175 (exon 5) and 270 (exon 8) (Hollstein *et al.*, 1996). Although mutations at codon 175 are frequent in SCC (8%), mutations at codon 248 or 273 amount to only 3% of all point mutations while mutations at codon 270 (4%) in SCC are virtually non-existent in ADC (Hollstein *et al.*, 1996). Many of the *p*53 mutations are point mutations and in SCC, the predominant type of mutation is an A to T transition representing more than 30% of all mutations. In ADC, *p*53 mutations show a very high frequency (60%) of G: C to A: T transitions at CpG dinucleotides (Montesano and Hainaut, 1998). Mutations in SCC are indicative of the involvement of exogenous carcinogens such as tobacco, alcohol and nutritional components. Transitions at CpG dinucleotides are considered as the hallmark of mutations occurring spontaneously through hydrolytic deamination of 5-methylcytosine (Montesano and Hainaut, 1998).

In European and North American patients, a much higher prevalence of *p*53 mutations has been noted in smokers than in non-smokers (Wagata *et al.*, 1993) while In India, consumption of chillies has been positively associated with *p*53 protein over-expression (Gaur *et al.*, 1997).

Retinoblastoma (*Rb)*, encodes a nuclear phosphoprotein – *pRb* that is involved in cell cycle regulation. *pRb* negatively regulates the cell cycle at the G_1/S transition. Most *Rb* mutations are deletions, insertions or point mutations. In OC patients, the frequency of *Rb* alterations has ranged from 0 to 100% (Jiang *et al.*, 1993; Shamma *et al.*, 1998; Yang *et al.*, 1997; Boynton 1992). In most cases, *Rb* alterations were detected in a third to half of the OC cases. In addition, in many of these studies, cyclin D1 over-expression is associated with over expression of *Rb*, and *p16* (Cheety and Cheety, 1997; Roncalli *et al.*, 1998; Yang *et al.*, 1997;Busatto *et al.*, 1998). These findings are consistent with the molecular relationship described between cyclin D1, *p*16 and *Rb* proteins. This suggests that alterations in the expression levels of the *Rb* protein may play a significant role in the development of oesophageal cancer.

1.3.3.3 Acquired characteristic 3: Ability to have limitless replicative potential.

All human cells carry an intrinsic autonomous program that limits their multiplication via telomeres and an enzyme that maintains them, telomerase. This probably explains why, despite uncoupling the growth program of a cell from the signals in its environment is not sufficient to generate macroscopic tumours. Malignant cells have evolved mechanisms that have successfully breached the cells defense against limitless replication. They have achieved this by de-regulating the expression telomerase.

Telomeres - several thousand short TTAGGG base pair repeat elements protect chromosomal ends from recombination and loss. The unprotected chromosomal ends participate in end-to-end chromosomal fusions yielding karyotypic disarray, which leads to the “crisis” state. This state is characterized by massive cell death, although occasionally for every 10^7 cells one variant emerges that has the acquired ability to multiply without limit – immortalization (Wright *et al.*, 1989; Counter *et al.*, 1998). The second function is to resolve the "end replication problem" which involves the progressive and potentially catastrophic shortening of the 3' end of DNA during replication. These telomeres are progressively shortened during each cell cycle due to the inability of the DNA polymerase to completely replicate the 3’ ends of chromosomal DNA during each S phase. The latter problem can only be overcome by telomerase making a DNA copy of its own antisense sequence and fusing it to the 3' terminus of the chromosome (Blackburn, 2000). These telomeres are progressively shortened during each cell cycle due to the inability of the DNA polymerase to completely replicate the 3’ ends of chromosomal DNA during each S phase.

Furthermore, the role of telomerase in immortalized cells has been demonstrated *in-vitro* by ectopically expressing the enzyme in cells, where it conferred unlimited replicative potential onto a variety of normal early passage, pre-senescent cells (Bodner *et al.*, 1998; Vaziri and Benchunol, 1998).

Several research groups have measured human telomerase RNA (hTR) expression and correlated the enzyme activity with pathological stage and postoperative survival. Usselmann *et al.*, (2001) found no relationship between hTR activity and the advancement of oesophageal/gastro-oesophageal junction adenocarcinomas (Usselmann *et al.*, 2001). Similarly, comparing telomerase activity between oesophageal carcinoma biopsies and normal oesophageal mucosae, Takubo *et al.*, (1997) found no significant difference in the enzyme activity between the carcinomas and normal tissues.

hTR may be a target for novel therapeutic drugs, however, from the limited published data on telomerase activity in OC, telomerase expression alone has not been demonstrated to be significant in oesophageal cancer.

1.3.3.4 Acquired characteristic 4: Ability to evade apoptosis.

Current research evidence indicates that the apoptotic program is latent in virtually all cell types. Once triggered by a variety of physiological signals, the program proceeds in a precisely choreographed series of steps that are all accomplished within 30 to 120 minutes (Wyllie, 1980).

The apoptotic machinery can be broadly divided into two components - sensors and effectors. The sensors monitor the cell's status and activate the death pathway in response to abnormalities such as DNA damage, signalling, survival factor inefficiency or abrogation of cell-matrix and cell-cell adherence - based signals (Ishizaki *et al.*, 1995; Giancotti and Ruoslahti, 1999; Evan and Littlewood, 1998). The effectors of apoptosis include caspases, tumour necrosis factor receptor and the Bcl2 family.

It is possible that virtually all cancer cells harbour alterations that enable evasion of apoptosis. Evidence from studies in mouse models, cultured cells and analysis of biopsy samples in human cancers, suggests that acquired resistance to apoptosis is a major characteristic of perhaps all types of cancer. Successful evasion of apoptosis facilitates malignant transformation and can be achieved via a number of strategies. Probably most common target is the best-known pro-apoptotic regulator, *p*53. *p*53 - the "guardian of the genome" has been shown to be highly mutated in OC and other cancers. Impaired or loss of p53 function also means that it may not perceive signals evoked by other abnormalities such as hypoxia or oncogene hyper-expression (Levine, 1997).

The Bcl2 family of proteins are the most important regulators of apoptosis. They consist of at least 15 proteins with either pro-apoptotic effects (e.g Bax, Bak, Bid, Bim) or anti-apoptotic effects (e.g Bcl-2, Bcl-xl, Bcl-w) (Linette *et al.*, 1996; Mazel *et al.*, 1996). Studies have shown Bcl-2 over-expression to range from 32 to 74% in oesophageal cancer patients with pre-invasive lesions and poorly differentiated tumours (Sarbia *et al.*, 1998).

1.3.3.5 Acquired characteristic 5: Ability to invade other tissues and to metastasize.

Deregulation of cell adhesion and motility is an important hallmark of cancer, enabling neoplastic cells to migrate to distant secondary sites where at least initially, nutrients and space are not limiting factors for cell proliferation.

Both invasion and metastasis involve not only a change in tissue microenvironment but also activation of extracellular proteases. Despite the poor understanding of the genetic and biochemical determinants and regulation of tissue invasion and metastasis, the processes are envisaged to take place via a series of sequential interactions. The first step involves the release of normal cellular contacts within the primary tumour. This is followed by invasion through the underlying basement membrane, destruction of and passage through the extracellular matrix (ECM) and eventual penetration into minor blood or lymphatic vessels circulation to distant sites is followed by trans-endothelial extravasation and movement through the ECM with colonization and growth at a secondary site (Hart, 1996).

Several classes of proteins involved in cell adhesion and motility are altered in cells possessing invasive or metastatic capabilities. The section below will briefly highlight some of the important metastasis suppression genes, cell adhesion molecules, matrix degrading proteases and their inhibitors and factors involved in the migration of metastatic cells.

1.3.3.5.1 Metastasis genes

Non-Metastasis 23 (*NM23*) gene also called *NME1* is thought to be an important in metastasis suppression. This poorly studied gene is thought to achieve this by regulating cell functions via microtubules involved in mitotic spindle formation and cell locomotion.

Alternatively, the protein could be thought to regulate cell signal response via physical association with G proteins (Backer *et al.*, 1993). In a small study using only 32 patients, Patel *et al.*, (1997), found only 27% of the OC tumour biopsies to stained positively for nm23. Patients who were nm23 negative had a significant reduction in overall survival rate. Further studies however, are required to confirm the true role of nm23 in OC pathogenesis.

1.3.3.5.2 Cell adhesion molecules

Cell adhesion molecules are known to regulate cell-cell and cell-matrix interactions (Katz *et al* 1991; Streit *et al.*, 1996). These molecules are important in the development and homeostasis of all tissues as well as pathologic conditions such as inflammatory disease, infections and malignancies. They affect the metastatic potential of malignant tumours by serving as both positive and negative modulators and may direct tumour cells to particular tissues, allowing them to attach in those tissues, migrate across the vessel wall and grow at the secondary site. Cell adhesion molecules can be broadly categorized into several families and include cadherins and integrins.

Cadherins are calcium-dependent cell-cell adhesion transmembrane glycoproteins. E-cadherin is one of the most important adhesion molecules expressed by epithelial cells and suppresses invasion and metastasis. Loss of E-cadherin function in the majority of epithelial cancers has been attributed to mutational inactivation, transcriptional repression or proteolysis of the extracellular domain of the protein. Constitutive expression of E-cadherin in cultured cancer cells and in transgenic mouse models suppressed tumour invasiveness and metastasis underscoring the role of this gene in tumourigenesis (Christofori and Semb, 1999). Desmosomal glycoprotein, a major adhesive component of the desmosome and catenin are other subtypes of this family. Reduction of E-cadherin, desmosomal glycoprotein and catenin expression has been significantly associated with tumour de-differentiation, infiltrative growth, lymph node metastasis, hematogenous recurrence and poor prognosis in OC (Kadowaki *et al.*, 1994; Tamura *et al.*, 1996; Sanders *et al.*, 1998; Kimura *et al.*, 1999; Nakanishi *et al.*, 1997; Natsugoe *et al.*, 1997; Nakano *et al.*, 1998).

1.3.3.5.3 Proteins involved in degradation of the extracellular matrix

During metastasis, cancer cells must detach and cross several ECM barriers. The first is the basement membrane (BM). The BM consists of a scaffold of collagen onto which the other components of the BM such as laminin and proteoglycans are assembled (Martinez-Hernandez and Amenta, 1983). No single protease can account for all the proteolysis that occurs during invasion and metastasis. Hence several proteases have been shown to play an important role in the proteolytic process. These include matrix metalloproteinases (MMPs), plasminogen activators, cathepsins and heparanases.

MMPs belong to a superfamily of proteases that can cleave virtually any component of the ECM thereby facilitating cell migration. They belong to zinc-containing endopeptidases that are synthesized as inactive zymogens and are historically divided into collagenases, gelatinases, stromelysins and matrilysins on the basis of their specificity for ECM components. Alternatively, they can be divided into eight distinct structural groups, five of which are secreted and three of which are membrane-bound (MT-MMPs) (Reviewed by Egelbald and Werb, 2002).

In addition to the ECM components, other known substrates of MMPs are insulin-like growth factor binding protein (Manes *et al.*, 1997) and perlecan (Whitelock *et al.*, 1996) that are cleaved to release IGFs and FGFs, respectively. Other substrates are E-cadherin (Noe *et al.*, 2001), CD44 (Kajita *et al.*, 2001) and the EGFR family (Codony-Servat *et al.*, 1999 and Vecchi *et al.*, 1998). Consequently, MMPs have been implicated in regulating various cell behaviours such as cancer-cell growth, differentiation, angiogenesis and immune surveillance (Reviewed by Egelbald and Werb, 2002).

The expression of MMPs 1, 2, 3, 7 and 9 has been extensively studied by immunohistochemical methods in OC (Shima *et al.*, 1992; Porte *et al.*, 1998; Murray *et al.*, 1998; Adachi *et al.*, 1998). Studies have revealed that the expression of these MMPs was generally detected in at least half of OC samples.

MMP activities are tightly regulated by endogenous inhibitors such as 2-macroglobulin (Sottrup-Jensen *et al.*, 1989) and Thrombospondin-1 (Bein and Simons, 2000; Rodrigues-Manzaneque *et al.*, 2001). The best-studied endogenous MMP inhibitors are tissue inhibitors of metalloproteinases (TIMPs) that reversibly inhibit MMPs. To date this class of inhibitors consists of four members namely, TIMP1, 2, 3 and 4.

Several studies have been carried out to elucidate the temporal and spatial expression patterns of TIMPs in normal, hyperplastic and neoplastic cells. TIMP 1, 2 and 3 mRNA are over-expressed in endometrial adenocarcinomas, especially in the less differentiated carcinomas (Maatta *et al.*, 2000). Similarly, elevated levels of TIMP1 significantly correlated with the presence of lymph node metastasis, development of distant metastasis and early death from breast carcinoma, while TIMP2 over-expression was associated with development of distant metastasis of the same disease (Ree *et al.*, 1997). A study on the expression of TIMP1 and 3 in oesophageal adenocarcinoma observed that the majority of tumours over-expressed TIMP1 and 3. Tumour samples over expressing TIMP1 show a significantly higher frequency of lymph vessel invasion, vascular vessel invasion, lymph node metastasis, advanced-stage disease and poor prognosis than low-expression cases (Mori *et al.*, 2000).

Plasminogen activator (PA) is another important protease involved or implicated in ECM degradation during metastasis. This serine-specific protease converts inactive plasminogen to active plasmin that degrades collagen type IV, fibronectin, fibrin, laminin and also activates latent collagenases. Two types of PAs are produced, tissue type (t-PA) and urokinase type (u-PA). The primary function of t-PA is to dissolve blood clots whereas u-PA is involved mainly in the degradation of tissue. The total u-PA activity is influenced by its inactive zymogens and the amount of PA inhibitors (MacDonald and Steeg, 1993).

The role of u-PA in metastases has been examined using anti-uPA antibodies, which was shown to inhibit invasion and metastasis (Ossowski and Reich, 1983, Hearing *et al.*, 1988, Ossowski *et al.*, 1991). Furthermore, administration of u-PA (Kodama and Tanaka, 1978; Tanaka *et al.*, 1981) results in enhanced metastasis whereas administration of a specific inhibitor of uPA (amiloride) reduced the formation of metastatic colonies in rats (Kellen *et al.*, 1988).

The expression of the u-PA, its inhibitor - plasminogen activator inhibitor type 1 (PAI-1) and urokinase-type plasminogen activator receptor - (uPAR) have been analyzed in several OC studies. In a study using resected adenocarcinoma tumour specimens, uPA and PAI-1 levels were elevated 16 and 14 fold, respectively compared to the controls (Nekarda *et al.*, 1998; Hewin, 1996). A recent study found a 5.0, 3.6 and 5.4 fold increase in uPA, uPAR and PAI-1 mRNA levels in SCC of the oesophageal cancer, respectively, compared to the normal control tissue (Tang *et al.*, 2001).

Cathepsins are a family of aspartate proteases consisting of four members; cathepsin B, D, H and L (Duffy, 1996). They degrade extracellular matrix proteins such as laminin, elastin, fibronectin, proteoglycans and collagen (Lah *et al.*, 1989; Mason *et al.*, 1986). Cathepsin B activity is elevated in a variety of human cancers and has been correlated with aggressive tumour behaviour, early relapse and shorter survival in breast cancer (Gabrijelcic *et al.*, 1992), colorectal cancer (Campo *et al.*, 1994) and lung cancers (Sukoh *et al.*, 1994). In OC, high extracellular expression of cathepsin B protein was found in 72.5% of the oesophageal adenocarcinoma specimens studied (Hughes *et al.*, 1998). A similar study found a variation in the expression levels of cathepsin L, with over-expression in 58% of primary oesophageal adenocarcinomas and 33% of squamous-cell OC (Cassons *et al.*, 1997). Expression of cystatin B, a known inhibitor of cathepsin L was found to be markedly decreased in OC tumour tissue compared with that of the corresponding normal tissue. This decrease correlated with lymph-node metastasis in the cases studied.

1.3.3.5.4 Proteins involved in cell migration

In order to complete the invasion and metastasis process, tumour cells must migrate after modifying the ECM. Several motility factors, components of the ECM and growth factors have been reported to induce tumour cell motility. Examples of such factors that stimulate tumour cell motility are migration-stimulating factor (MSF), autocrine motility factor (AMF), autotaxins (ATX), and scatter factor (SF).

Migration stimulating factor stimulates the migration of normal fibroblasts. The mechanism of MSF has not been determined, but it may stimulate fibroblast migration indirectly by altering ECM structure (Schor and Schor, 1987; Schor *et al.*, 1988).

Autocrine motility factor is a cytokine produced by various tumour cells that stimulate their *in vitro* motility. It mediates its effect via cell-cell surface receptors that are localized at the leading and trailing edges of motile cells (Nabi *et al.*, 1992).

Autotaxin (ATX) is an autocrine motility stimulating protein that stimulates both random and directional migration of melanoma cells through a G-protein receptor mediated (Stracke *et al.*, 1992). Evidence of an association between ATX, cancer development and metastasis has previously been demonstrated. For example, in hepatocellular carcinoma (HCC), intrahepatic metastasis, vascular invasion and poor differentiation were more frequently noted in HCC patients with high ATX expression than in patients with low expression (Zhang *et al.*, 1999). In studies using cell lines, ATX mRNA levels were found to be elevated in non-small-cell-lung cancer cell lines (Yang *et al.*, 1999).

Although motility factors have been shown to be involved in cell motility both *in vitro* and *in vivo,* there is little information with regards to their alteration and possible role in oesophageal cancer.

1.3.3.6 Acquired characteristic 6: Ability to sustain angiogenesis.

Angiogenesis has become an important prognostic indicator for cancer progression and an increasingly attractive therapeutic target. The importance of angiogenesis has been further strengthened by numerous studies that have positively correlated the vascular density of tumours with disease progression, metastasis and tumour survival.

The regulation of tumour angiogenesis is thought to be by both pro-angiogenic and anti-angiogenic factors. The balance of these factors constitutes an "angiogenic switch". Whether new blood vessels are formed or not depends on the delicate equilibrium between the pro and anti-angiogenic factors (Folkman, 1995; Pepper, 1997).

The ability to induce and sustain angiogenesis seems to be acquired in discrete steps during tumour development, via an angiogenic switch from vascular quiescence and is thought to proceed via two phases, pre-vascular and vascular. The pre-vascular phase is characterized by a balance between the rates of cell proliferation and apoptosis. The vascular phase is initiated when the pro-angiogenic factor effects override the anti-angiogenic factor effects. The latter step is characterized by exponential tumour cell growth (largely due to a decrease in the rate of apoptosis) as well as tissue invasion and the hematogenous spread of tumour cells. The transition from pre-vascular to the vascular phase is referred to as the angiogenic switch.

The principle tumour pro-angiogenic regulator is vascular endothelial growth factor (VEGF) (Leung *et al.*, 1989), the expression of which is enhanced by insulin-like growth factor I and II, and IL-6 (Goad *et al.*, 1996; Kim *et al.*, 1998; Cohen *et al.,* 1996). Another important pro-angiogenic factor is basic fibroblast growth factor (bFGF) (Gospodarowicz *et al.*, 1979).

Evidence for the importance of inducing and sustaining angiogenesis in tumours is now overwhelming. For example, anti-VEGF antibodies are able to impair neovascularization and growth of subcutaneous tumours in mice (Kim *et al.*, 1993), while a dominant interfering version of VEGF receptor 2 (fik-1) also inhibited neovascularization and growth of sub-cutaneous tumours in nude mice (Millauer *et al.*, 1994).

Tumours appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and inhibitors (Hanahan and Folkman, 1996). This is achieved via three ways. The first involves altered gene expression that favours increased expression of pro-angiogenic factors such as VEGF/ bFGFs. The second strategy would be the down regulation of anti-angiogenic factors, for example, thrombospondin-1 (Singh *et al.*, 1995; Volpert *et al.*, 1997). The third possibility would be an alteration in the regulation of proteases that control the availability and levels of both pro and anti-angiogenic factors.

Increased vascularity is now associated with a poor prognosis in breast (Weidner *et al.*, 1991), lung (Macchiarini *et al.*, 1992), prostate (Weidner *et al.*, 1993), cervical (Smith-Maclune *et al.*, 1994) and colon carcinomas (Takahashi *et al.*, 1995). Conversely, angiogenesis is an increasing attractive therapeutic target. A number of inhibitors such as steroids and heparin (Crum *et al.*, 1985), laminin peptides (Sakamoto *et al.*, 1991), minocyclin (Tamargo *et al.*, 1991) have been shown to inhibit tumour angiogenesis. The important question, however, especially with regard to the development of efficacious and novel antiangiogenic based therapeutic interventions is whether different types of tumour cells use the same or distinct molecular pathways to activate the angiogenic switch.

Studies in OC have shown that VEGF expression is significantly higher in the tumour cells of patients with distant metastasis. In addition, the survival rate of patients with elevated VEGF expression was significantly worse than that of patients with normal VEGF expression (Inoue *et al.*, 1997).

In a similar study Tanigawa *et al.*, (1997) found that high VEGF protein levels were associated with well-differentiated tumours, deep invasion, blood vessel invasion, high incidence of distant metastasis and poor prognosis. The potential of VEGF to contribute to tumour growth is further strengthened by immunohistochemical data that demonstrates a positive association between the presence of VEGF in OC cells and enhanced tumour extension and greater neovascularization (Kitadai *et al.*, 1998).

The limited studies on tumour angiogenesis in OC clearly show that the high level of VEGF in tumours is an indicator of poor prognosis.

In summary the above discussion dwells on a very small number of genes that are thought to play an important role(s) in the development and progression of oesophageal cancer. These genes are differentially expressed during the different phases of the disease and their expression has important implications in tumourigenesis. Several of the genes can be seen to have overlapping functions that impact on tumourigenesis meaning their classification in terms of function is more academic rather than definitive.

In spite of the broader picture of their involvement in cancer, their actual mechanisms of action still remain poorly understood in oesophageal cancer. In addition, few case studies and few patient numbers have led to general conclusions about their possible role in oesophageal cancer. Further studies need to be undertaken to fully understand the actual role of these genes in OC tumourigenesis. In pursuit of this goal, it is hoped that more genes that impact on OC development will be revealed.

The scope of this thesis limits the number of genes that can be adequately described under the molecular basis of OC. Consequently, there are genes that were not discussed in this section because they have either been poorly characterized or investigated not only in OC but also in other cancers. This does not reduce their importance in tumourigenesis but rather act as an incentive for their further characterization and investigation into their possible role in tumourigenesis.

1.4 CURRENT PROBLEMS IN OESOPHAGEAL CANCER

The events during the natural progression of cancer of the oesophagus remain poorly understood. The disease is thought to occur over a long period of time, possibly over 20 years. During this period, the disease is essentially clinically latent. However, the patient experiences mild but definite symptoms related to swallowing as the disease progresses. These symptoms are transient in nature and cause no immediate concern to the patient. They are distinctly different from those caused by oesophagitis in being related only to the discomfort experienced during food intake. During the clinical stages II and III, the disease progresses with increasing rapidity. The patient presents with progressive dysphagia, loss of weight and retrosternal pain. A 25% five year survival or a reasonable degree of palliation may still be expected in most cases. However, patients with stage IV have an average life expectancy of less than 3 months (Huang, 1988).

In order to assess the degree of aggressiveness of the disease, histopathologic classification, grading and staging of resected tumours have been standardised into a TNM staging system. This system of classifying tumours emphasizes depth of oesophageal wall invasion by the primary tumour (T), nodal involvement (N) and distant metastasis (M) (Sobin and Witterkind, 1997).

Several therapeutic approaches are currently being employed in the management of OC. These include surgery, radiotherapy and chemotherapy. At present surgical resection remains the main stay of OC treatment. Survival of patients who undergo oesophagectomy is stage dependent; 68 to 85 % of stage 1and II, while only 15 to 28 % of stage III patients who undergo oesophagectomy survive. A very small percentage of patients will achieve long-term survival after surgical resection because most patients are diagnosed when the disease is very advanced (Roth and Putman, 1994).

Radiotherapy and brachytherapy have been used for decades, especially in patients who are intolerant to chemotherapy. The results of radiotherapy delivered with curative intent remain disappointing. Brachytherapy has been used as a boost to external beam radiotherapy but they are now both used with palliative intent (Smalley *et al.*, 1994).

Management of OC using single or multiple chemotherapeutic agents has been in existence for a long time. Cisplatin has proven to be the most effective component of any chemotherapeutic regimen for treatment of SCC and ADC. A combination of Cisplatin and 5 Fluorouracil is considered to be the standard regimen for patients with SCC (Ajani, 1994).

The above therapeutic approaches have yielded modest success because most patients present themselves when the disease is highly advanced. Consequently, the prognosis of OC remains very poor and the average 5-year survival rate remains less than 10%.

1.4.1 Biomarkers

Elucidation of the sequence of molecular alterations during the process of carcinogenesis will define the most useful markers that are potentially beneficial in the early detection of cancer and intervention with novel preventative or therapeutic measures. In addition, such studies will also identify intermediate to end point markers that can be used in cancer incidence and mortality studies (Shaw and Mulshine, 1994).

The advantages of detecting circulating tumour markers include the limited harm or clinical invasion of the patient. Secondly, is the ability to use highly specific monoclonal antibodies in immunoassays? Thirdly, use of highly sensitive radio, enzyme, fluorescent or luminescent probes that detect up to a picogram per ml of protein and finally, the possibility of the development and availability of commercial detection kits.

On the other hand, the disadvantages of detecting would be the enormous cost of automation to achieve a high number of patients during screening. This is in the light of the fact that OC is basically a disease of the "developing countries". Developing countries are currently grappling with health budgets that are financially highly constrained. Secondly, a high number of circulating tumour cells 10^5 to 10^6 are required to achieve more accurate results. Thirdly, successful screening is highly dependent on tumour marker expression; synthesis, release, catabolism, excretion as well as blood supply to the tumours.

Another cause for worry is that, most tumour markers are not tumour-specific but may be elevated transiently or often maintained in the low pathologic range in different or counterpart benign diseases of the tumour in question. This leads to low specificity. It has also been shown that a tumour marker relevant for a specific tumour may only be partially expressed and synthesized, but not released or incorrectly elevated in serum because of altered catabolism (e.g. liver insufficiency) or excretion (e.g. renal sufficiency) or not elevated because of insufficient blood supply. Lastly, most tumour markers may not be organ-specific and are found to be elevated in several other diseases.

1.4.2 Types of markers

The process of carcinogenesis is dynamic and exact boundaries are largely artificial and academic. A promising biomarker should measure a critical aspect of the process of carcinogenesis and possibly be used in a variety of applications.

A marker could be a susceptibility, exposure, intermediate, early detection, prognosis, clinical or pathological marker. Each of these applications reflects a specific use of a biomarker and requires context-specific validation. Many markers may have more than one useful application. Host susceptibility markers may be used to define high-risk populations for selective screening, early detection markers to identify subjects for intervention studies, intermediate end-point markers to determine the success of an intervention, while molecular markers can be further subdivided depending on tumour site, genetics and differentiation status of the tumour (Birrer *et al.*, 1994).

It is therefore imperative for us to employ a panel of independent markers to improve the tight correlation between the precise sequence of events in carcinogenesis. For example, do these markers disappear with treatment or removal of an offending agent (e.g. cigarette smoke) or do they reappear after treatment is stopped. Conversely, do they persist despite therapy and resolution of classical morphological abnormalities? The value of a useful marker should be assessed based on its ability to detect and monitor the development of cancer. This may in addition include its suitability to link the interactions between intervention therapies and the molecular and biochemical changes associated with the development of cancer (Birrer *et al.*, 1994).

1.5. IMPORTANCE OF THIS STUDY

Successful development and implementation of effective early detection strategies will depend partly on the identification and characterization of molecular and biochemical markers that are sensitive and specific indicators cancer development.

Traditional diagnostic methodologies such as cytology washings and brush cytology have been extensively used to detect OC at very early stages of the disease. However, because of the prohibitive cost and logistical problems, routine mass cytology and endoscopic screening have restricted these applications to high-risk areas and more so to health institutions in urban areas. However, most citizenry in the third world countries are basically rural based, where health infrastructure is very poor and generally inaccessible.

Considerable energy has been expended in the development of methods that are more efficacious in the screening and detection of OC, but this had limited success. To date there is no satisfactory diagnostic marker available for predicting the likelihood of premalignant or neoplastic changes in oesophageal cancer. Molecular biology techniques offer a new and exciting approach that may improve the early detection, staging, treatment and prognosis of OC. Previous studies have shown, for example, that serum carcinoembryonic antigen (CEA) levels are often elevated in patients with tumours of the gastrointestinal tract. Preliminary results have demonstrated that reverse transcription polymerase chain reaction (RT-PCR) can detect and identify mRNA specific for CEA in lymph nodes of patients before histological evidence of metastasis is present (Luketich *et al.*, 1998).

Techniques such as differential display RT-PCR and cDNA microarray analysis essentially identify genes that are either over or under expressed in a particular tissue. These methodologies could identify candidate genes whose expression profiles will help identify high-risk individuals in the population at risk of developing OC. In principle these techniques should complement other histological methods such as DNA flow cytometry and immunohistochemistry to allow for more effective and accurate early detection of genetic abnormalities in high-risk populations.

This project hopes to identify candidate genes that will be of significant importance and value in the establishment of a panel of biomarkers that can contribute positively towards the management of oesophageal cancer. Against the above background, this study will use molecular biology approaches to identify and determine the function(s) of candidate genes that are differentially expressed in malignant tissue compared to their corresponding normal oesophageal mucosa from OC patients.

CHAPTER TWO

DIFFERENTIALLY EXPRESSED GENES

2.1 DIFFERENTIAL DISPLAY REVERSE TRANSCRIPTION PCR

2.1.1 OVERVIEW

All phases of life of an organism including development, maintenance, immunity, injury and senescence are characterized by differential gene expression.

In 1992, Liang and Pardee developed a powerful new technique called differential display of eukaryotic mRNA (also referred to as differential display reverse transcription PCR or DDRT-PCR) to facilitate the detection and isolation differentially expressed genes in target tissues. This technique offers unprecedented sensitivity, reproducibility, speed and the possibility to compare and analyse patterns of gene expression by creating "RNA fingerprints" representing subpopulations of expressed transcripts. Visual comparison of these fingerprints across gel lanes readily identifies any differentially expressed transcripts. The technique has been used to simultaneously identify and isolate transcripts that are either up regulated or down regulated from multiple samples in a variety of biological systems.

Numerous modifications and refinements of the principles of DDRT-PCR aimed at enhancing the usefulness of the technique have been suggested and incorporated over the years (Bauer *et al.*, 1993; Liang *et al.*, 1993; Liang and Pardee, 1995 and Wan *et al.*, 1996).

Differential display represents a powerful approach that can be used to identify differential expression of novel genes in pathological conditions. This chapter describes the use of DDRT-PCR to identify genes that are differentially expressed in oesophageal cancer.

A schematic representation of the experimental procedures involved in DDRT-PCR is summarized in figure 2.1

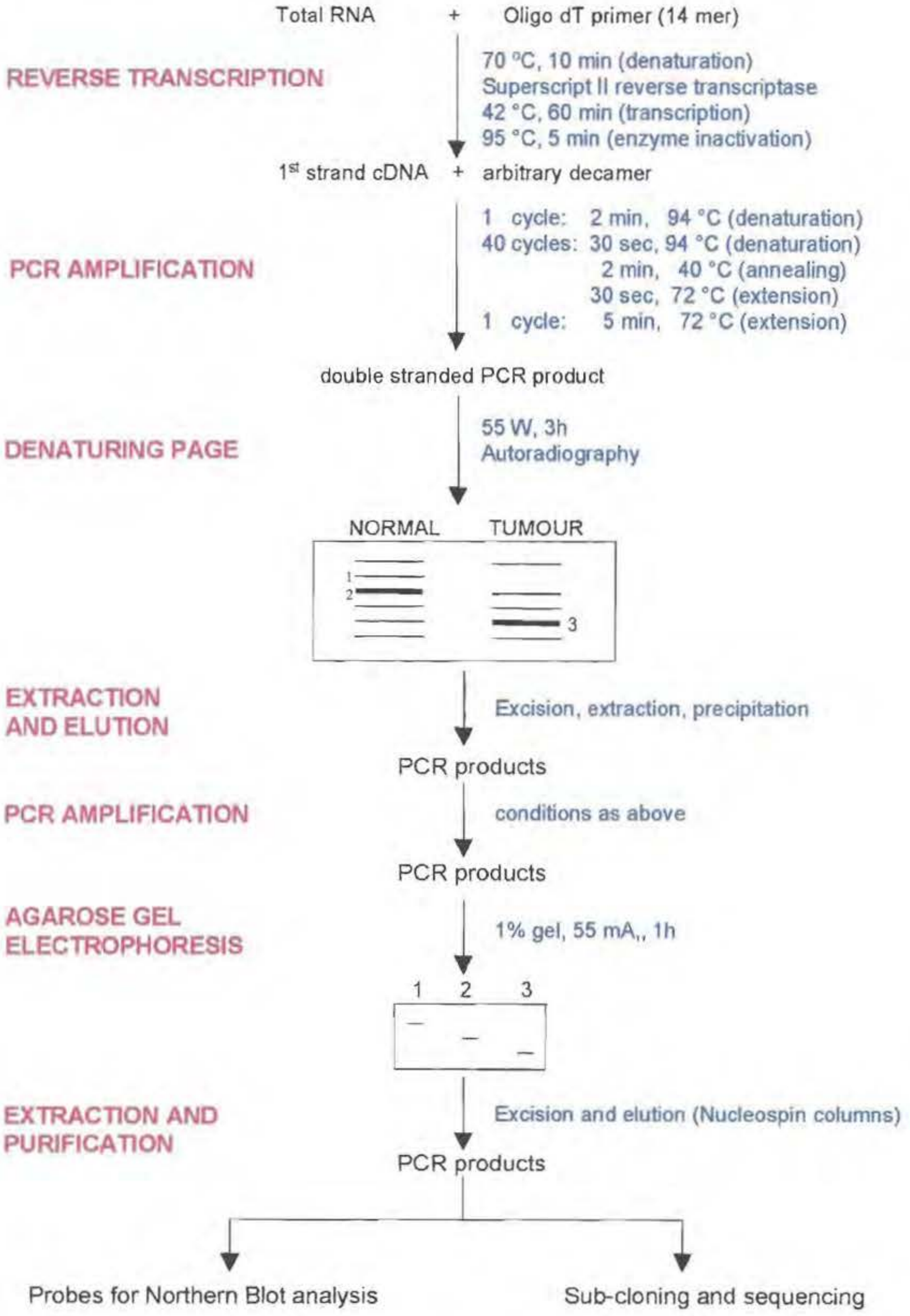

Figure. 2.1

A schematic representation of DDRT-PCR. Total RNA was reverse transcribed as described in section 5.1.6. The first strand cDNA was subsequently PCR amplified using the protocol in section 5.2. The resulting PCR products were electrophoresed on denaturing PAGE and genes identified as differentially expressed genes were recovered, eluted and re-amplified by PCR. The PCR products were used in subsequent experiments.

2.1.2 RESULTS

2.1.2.1 OESOPHAGEAL CANCER BIOPSIES AND CELL LINES

Four primary squamous cell carcinoma tumours and adjacent normal mucosa from different patients were used. In addition, a single adenocarcinoma tumour biopsy was incorporated in this study for purposes of comparison.

The samples were obtained from the oesophageal cancer clinic of the Cardiothoracic Surgery Department at Groote Schuur Hospital, Cape Town. Briefly, the biopsies excised from the patient during endoscopy were snap frozen in liquid nitrogen and thereafter stored at –70 °C until used. Each specimen was histologically analysed prior to its use in these studies (Table 2.1).

The SCC samples were obtained from black Africans and mixed ancestry patients. Mixed ancestry (coloured) refers to a heterogeneous group of mixed ancestry including, San, Khoi, Malaysian, Indonesian and Javanese and Western European origin. There is no evidence from the clinical history records that the patients, with the exception of the ADC patient, received brachytherapy, radiotherapy or chemotherapy prior to their surgery.

Human oesophageal cancer cells of the KYSE series (70 and 520), were kindly donated by Yutaka Shimada from the University of Kyoto, Japan (Shimada *et al.*, 1992), while the WHCO (Witwatersrand Human Carcinoma of the Oesophagus) series (1, 5 and 6) were kindly donated by Alan Thornley of the University of Witwatersrand, South Africa (Table 2.2). In addition, The Breast cancer cell line MDA-MB-231-5 acquired from American Type Culture Collection (ATCC, USA) was used as a control in DDRT-PCR experiments (Table 2.2)

Table 2.1

PATIENTS USED IN THESE STUDIES. The table below is a summary of some of the clinical and phenotypic characteristics of the patients whose tumour and normal biopsies were used in these studies.

PATIENT NUMBER	GENDER	AGE	PATHOLOGY	RACE
1	Male	53	SCC	Black
2	Male	50	SCC	Mixed ancestry
3	Female	62	SCC	Black
4	Female	53	SCC	Mixed ancestry
5	Male	53	ADC	White

Table 2.2

THE KYSE AND WHCO CELL LINES USED IN THESE STUDIES. The table below is a summary of some of the characteristics of the cell lines (Shimada *et al.*, 1992; Thornley, personal communication).

CELL LINE	CELL DIFFERENTIATION STATUS OF THE PRIMARY TUMOUR
KYSE 70	Poor
KYSE 520	Moderate
WHCO 1	Moderate
WHCO 5	Moderate
WHCO 6	Moderate

2.1.2.2 RNA ISOLATION

Total cellular RNA was isolated from the biopsies and cell lines using the GITC method as described in section 5.1.1. The quality and yield of RNA obtained from the patient biopsies varied from patient to patient. These variations could mainly be attributed to the handling of the biopsy specimens after surgery and prior to their snap freezing in liquid nitrogen. A long lapse time between tissue excision and snap freezing may contribute significantly to the degradation of RNA due to the actions of intracellular RNAses. In addition, the low yield of RNA from the adjacent normal tissue samples is due to the small biopsy sizes that were collected because of ethical and medical considerations. Large biopsies may favour not only the spread of the disease but also the possibility of developing opportunistic infections due to laceration. The variations in the quality and quantity of both the normal and tumour RNA that was isolated therefore limited the number of representative patient samples that could be used in these studies. The RNA samples used in these studies are shown in Figure 2.2

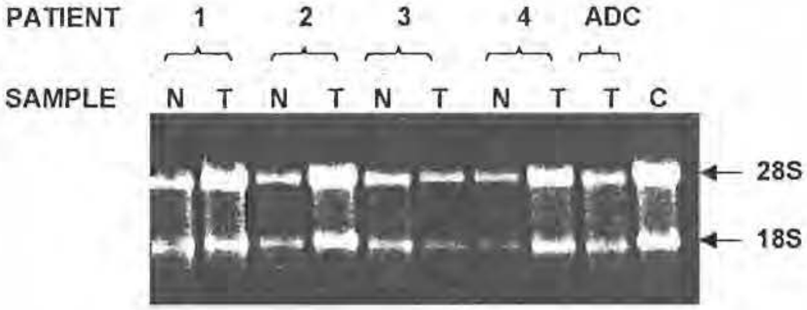

Figure 2.2

RNA SAMPLES USED IN DDRT-PCR EXPERIMENTS. Total cellular RNA was isolated from normal (N) and corresponding tumour (T) biopsies, and the MDA-MB- 231-5 breast cancer cell line (C) using the guanidine isothiocyanate (GITC) method as described in section 5.1.1. After DNAse treatment 1µg of each sample was electrophoresed on a formaldehyde containing 1 % agarose gel. The gel was washed several times in 0.5 M ammonium acetate to remove excess formaldehyde and subsequently stained in ethidium bromide. After staining the gel was visualized under Ultraviolet light (UV) and photographed. RNA from a tumour biopsy of a patient with oesophageal Adenocarcinoma (**ADC**) were electrophoresed alongside the RNA samples from patients with SCC.

2.1.2.3 DDRT-PCR

A total of 15 DDRT-PCR experiments were carried out using a combination of 3 sets of degenerate oligo dT (T_{12}MV) and 5 sets of arbitrary decamer (OPA) primers (Table 2.3). These primer combinations have previously been used in studies to identify differentially expressed genes in breast cancer (Liang *et al.*, 1993).

Table 2.3

PRIMERS USED IN DDRT-PCR EXPERIMENTS. The decamer primers and degenerate anchored oligo dT primers used in the DDRT-PCR experiments (Liang *et al.*, 1993).

PRIMER	**SEQUENCE**
OPA 16	5' AGC CAG CGA A 3'
OPA 17	5' GAC CGC TTG T 3'
OPA18	5' AGG TGA CCG T 3'
OPA 19	5' CAA ACG TCG G 3'
OPA 20	5' GTT GCG ATC C 3'
Degenerate oligo dTA	5' TTT TTT TTT TTT (G/A/C) A 3"
Degenerate oligo dTC	5' TTT TTT TTT TTT (G/A/C) C 3'
Degenerate oligo dTG	5' TTT TTT TTT TTT (G/A/C) G 3'

DDRT-PCR was performed as described in sections 5.1.6 to 5.4. A fragment was considered to only as differentially expressed if differential expression between the tumour and normal sample was observed in at least 2 patients. This was done by visually assessing of the intensity of each band across the patient profiles by at least two independent observations. Examples of denaturing PAGE autoradiographs considered to have differentially expressed genes are shown in figures 2.3 to 2.7.

The number of differentially expressed genes varied with the combination of primers used in the DDRT-PCR experiments. For example, a combination of the oligo dTA and OPA 20 (figure 2.3), and oligo dTG and OPA 19 (figure 2.7) primer sets yielded more differentially expressed genes than the oligo dTC and OPA 17 (figure 2.5) and oligo dTG and OPA 16 (figure 2.6) primer combinations. These observations suggest that the OPA 16 and 17 primers are "weaker" primers compared to the OPA 19 and 20 primers because the former produce more low intensity bands and blank lanes that result in fewer differentially expressed bands. This does not necessarily mean that the primers OPA 16 and 17 are unable to identify rare mRNA subpopulations, rather the predominance of few intense, sharp bands that do appear in an all-or-none fashion could possibly result in fewer true positive bands arising from experiments that use OPA 16 and 17 primers as the arbitrary primers. Consequently, a larger repertoire of arbitrary primers is required to amplify as many rare mRNA subpopulations as possible.

A tumour sample (without its corresponding normal sample) from an ADC patient was included in the DDRT-PCR as an internal control. The differential gene expression pattern exhibited by the ADC tumour sample was very similar to the expression patterns of the tumour samples from the SCC patients in all the DDRT-PCR experiments (figures 2.3 to 2.7). This suggests that the primer sets used in these experiments amplify similar mRNA species that are expressed in both ADC and SCC pathological subtypes of oesophageal cancer.

In DDRT-PCR using oligo dTG and the arbitrary decamer 19, RNA from breast cancer cell MDA-MB-231-5 was used as a control. This was because of the inadequate amount of tumour sample from the ADC patient.

Genes identified as differentially expressed on the DDRT-PCR autoradiographs were excised from the dried gels and the DNA extracted as section 5.4. The recovered DNA was PCR re-amplified using the protocol in section 5.2.1. The total number of fragments recovered and PCR re-amplified is summarised in Table 2.4.

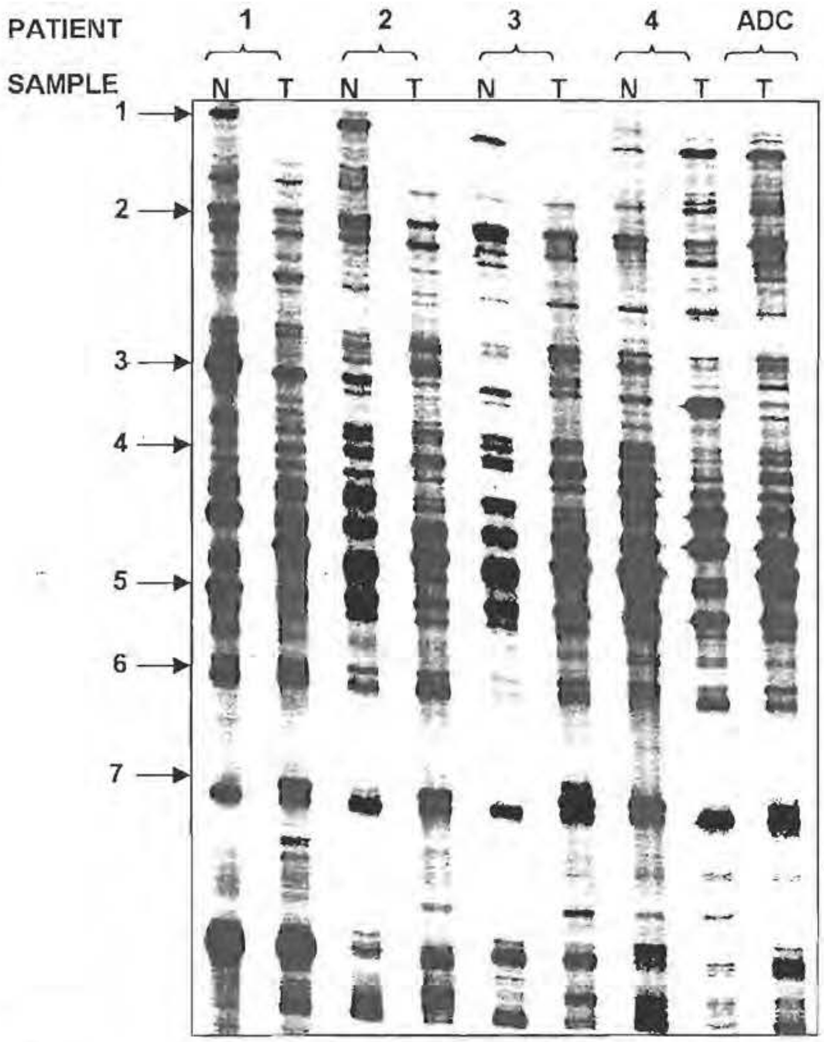

Figure 2.3

DDRT-PCR USING OLIGO dTA AND OPA 20 PRIMERS. 1µg Total cellular RNA was reverse transcribed using a 14 mer degenerate oligo dTA primer as described in section 5.1.6. The first strand cDNA was subjected to PCR in the presence of a 10 mer arbitrary primer (OPA 20), oligo dTA primer and α- [^{32}P] dCTP. The resulting PCR products were electrophoresed on a denaturing 6% polyacrylamide gel containing 7M urea. After which, the gel was dried under vacuum and subsequently exposed to X-ray film overnight. The differentially expressed genes selected for further analysis are shown by arrows **1** to **7**. **N**; normal tissue, T; tumour biopsy, **ADC**; adenocarcinoma tumour biopsy (control).

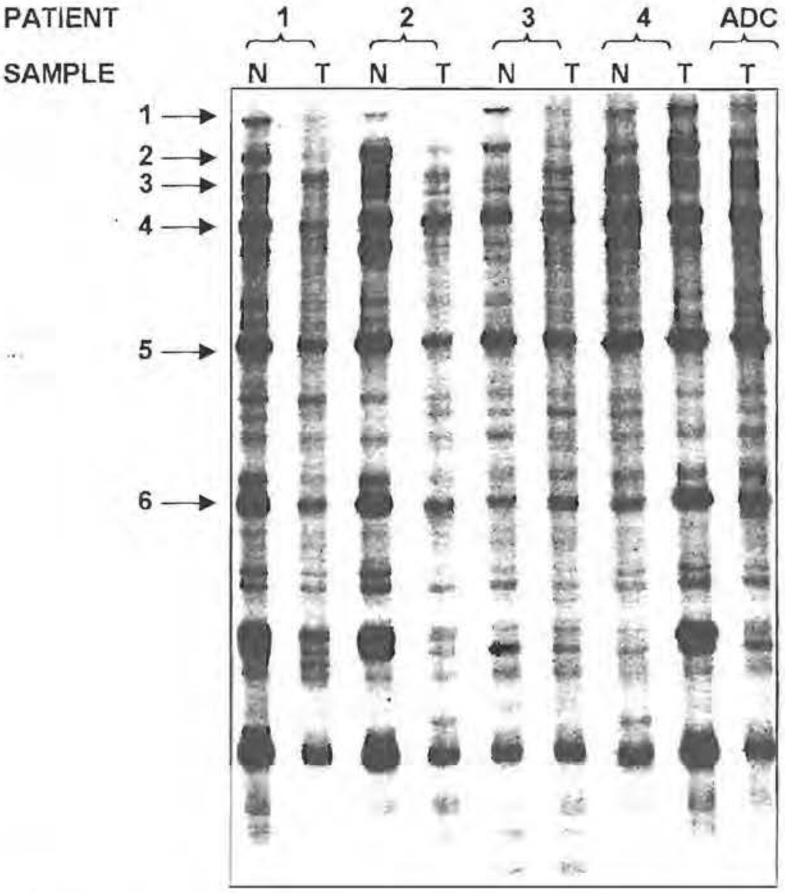

Figure 2.4

DDRT-PCR USING OLIGO dTC AND OPA 16 PRIMER SETS. RNA (1 µg) was reverse transcribed using a 14 mer degenerate oligo dTC primer. PCR was carried out using the first strand cDNA in the presence of a 10 mer arbitrary primer (OPA16) as described in the legend of figure 2.3. Differentially expressed genes selected for further analysis are shown by the arrows **1** to **6**. **N**; normal tissue, **T**; tumour biopsy, **ADC**; adenocarcinoma tumour biopsy.

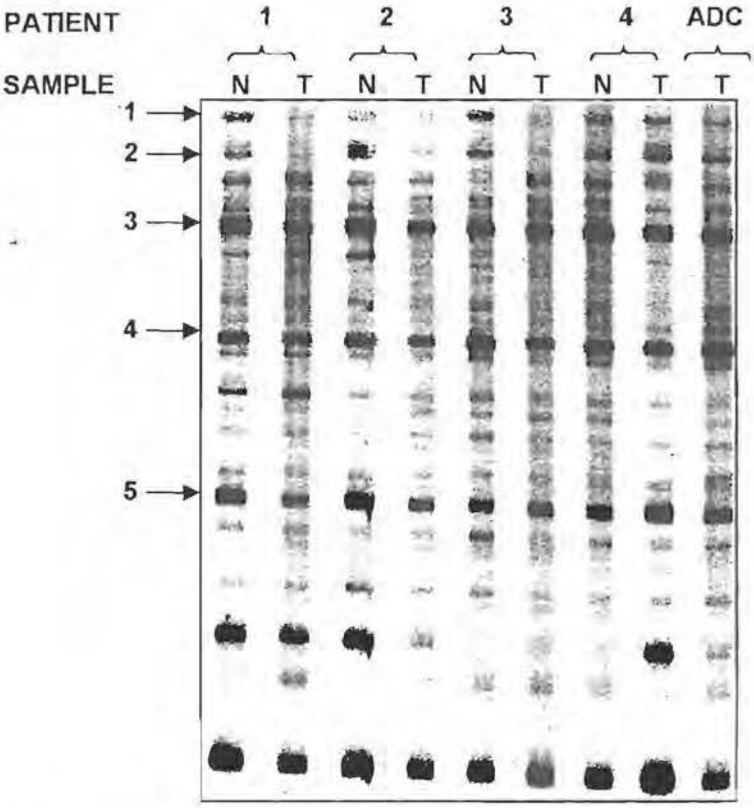

Figure 2.5

DDRT-PCR USING OLIGO dTC AND OPA 17 PRIMERS. Total cellular RNA was reverse transcribed using a 14 mer degenerate oligo dTC primer. Second strand synthesis was carried out by PCR using the the first strand cDNA in the presence an arbitrary decamer (OPA 17) as described in section 5.2.1. The differentially expressed genes are shown by arrows **1** to **5**. **N**; normal tissue, T; tumour biopsy and **ADC**; adenocarcinoma tumour biopsy.

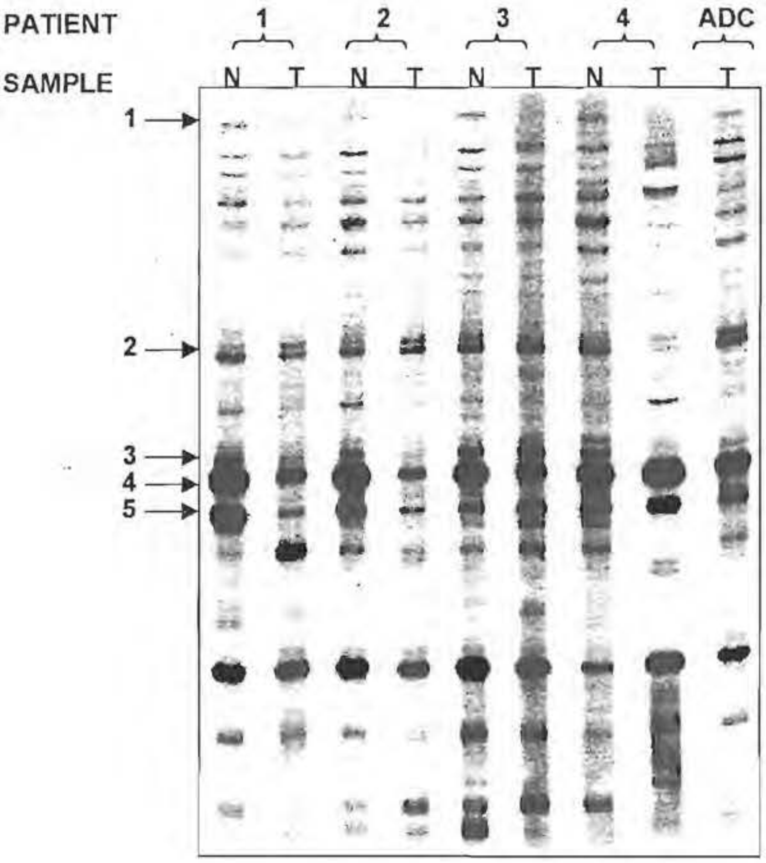

Figure 2.6

DDRT-PCR USING OLIGO dTG AND OPA 16 PRIMERS. Total RNA was reverse transcribed using a 14 mer degenerate oligo dTG primer as described in section 5.1.6. This was followed by PCR which incorporated the use of the former primer and a 10 mer arbitrary decamer (OPA 16). The products were analyzed as described in the legend to figure 2.3. Differentially expressed genes selected for further analysis are shown by arrows **1** to **5**. **N**; normal tissue, **T**; tumour biopsy, **ADC**; adenocarcinoma tumour biopsy.

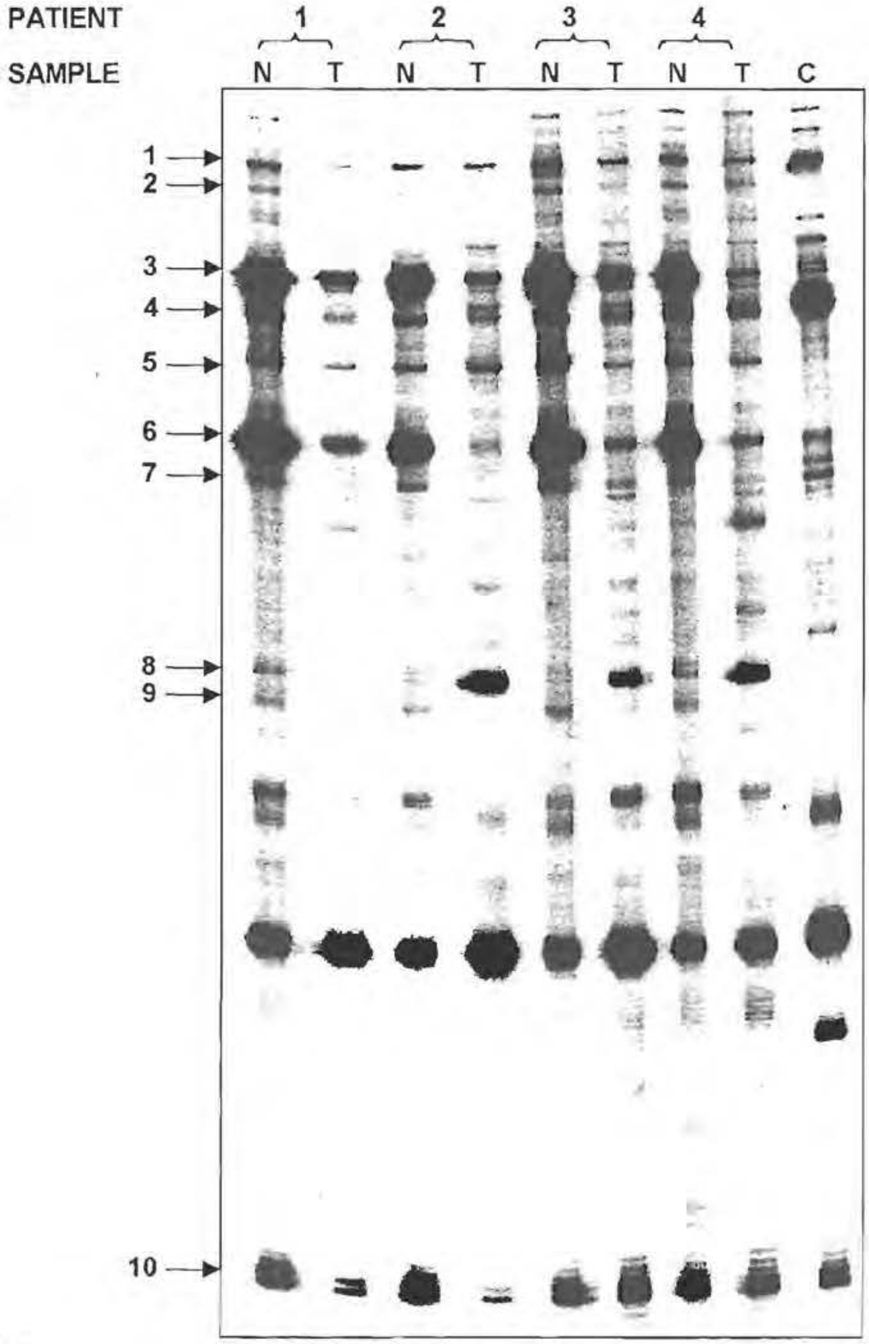

Figure 2.7

DDRT-PCR USING OLIGO dTG AND OPA 19 PRIMERS. Total RNA was reverse transcribed using a 14 mer oligo dTG. PCR for second strand cDNA synthesis was performed in the presence of a 10 mer arbitrary decamer (OPA 19) and oligo dTG primers as described in the legend of figure 2.3. The selected differentially expressed genes are shown by arrows **1** to **10**. **N**; normal, T; tumour and **C**; MDA-MB-231-5 breast cancer cell line (control).

Table 2.4

TOTAL NUMBER OF FRAGMENTS ISOLATED FROM DDRT-PCR GELS USING DIFFERENT DEGENERATE PRIMERS. The total number of fragments excised and eluted (section 5.4) from the dried denaturing PAGE gels using the different degenerate primers. A total of 50 fragments were isolated from DDRT-PCR using different combinations of both the degenerate and arbitrary primers.

Degenerate oligo dT primer	**Number of fragments**
A	13
C	18
G	19

An aliquot (5µl) of the re-amplified DNA was electrophoresed on a 1.5% agarose gel to ascertain the success of the DNA extraction and the PCR re-amplification processes. The remaining re-amplified products were stored at –20 °C for subsequent studies. Examples of PCR re-amplified fragments from different DDRT-PCR gels are shown in Figures 2.8 to 2.14.

The fragments 20A5 (band 5, using OPA 20 and oligodTA primers) corresponding to the tumour tissues in DDRT-PCR gel (figure 2.3) were recovered and re-amplification. These fragments were over expressed in the tumour compared to the normal tissue in DDRT-PCR. The molecular weights of the fragments were determined from a standard curve using the 1 Kb plus DNA ladder (Life Technologies). PCR amplification yielded a ~250 base pair fragment (figure 2.8). Similarly, 4 fragments corresponding fragments 20A7 (band 7, using OPA 20 and oligodTA primers) were recovered and PCR re-amplified. These fragments were predominantly expressed in the tumour specimens of 3 of the 4 patients examined. Re-amplification of these fragments using the same primer sets yielded DNA with ~200 base pairs (figure 2.9).

PCR re-amplification of fragments 16C2 (band 2, DDRT-PCR using OPA 16 and oligodTC primers) that are over expressed in normal tissues yielded ~700 base pair cDNA fragments. (figure 2.10). Similarly, re-amplification of fragments 17C4 (band 4, DDRT-PCR using OPA17 and oligodTC primers) yielded a 350 base pair cDNA fragment (figure 2.11). This band was under-expressed in the tumour specimens of three (patients 1,2 and 3) of the four samples matched patients.

Fragments 16G4 (band 4, DDRT-PCR using OPA 16 and oligodTG), 19G6 (band 6, DDRT-PCR using OPA19 and oligodTG) and 19G10 (band 10, DDRT-PCR using OPA 19 and oligodTG) were under-expressed in the tumour samples of 3, 4 and 2 patients, respectively, out of the four patients. Upon PCR re-amplification, these fragments yielded cDNA of molecular weight ~320, 300 and 170 base pairs, respectively (figures 2.12, 2.13 and 2.14).

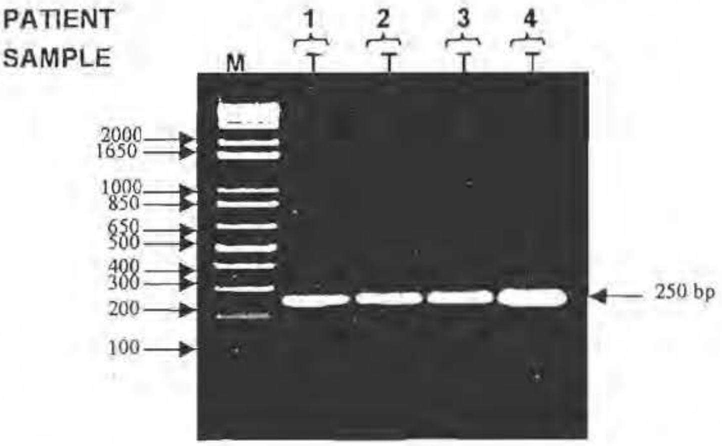

Figure 2.8

PCR RE-AMPLIFIED 20A5 DNA FRAGMENT. Fragments 20A5 (band 5 from DDRT-PCR carried out using OPA 20 and oligo dTA primers, figure 2.3) were excised from the dried denaturing polyacrylamide gel. The DNA was extracted using boiling water and ethanol precipitated in the presence of glycogen as a carrier. The recovered DNA was re-amplified by PCR using the conditions outlined in section 5.2.1. The products DNA (5µl) were electrophoresed on a 1.5 % agarose gel, the gel stained in ethidium bromide (0.25 µg/ml) and visualization under ultraviolet light (UV). A 1 Kb DNA ladder (500 ng) (Life Technologies) was used as the marker. The size of the re-amplified products was estimated to be 250 base pairs. Abbreviations: M; DNA ladder, T; tumour biopsy.

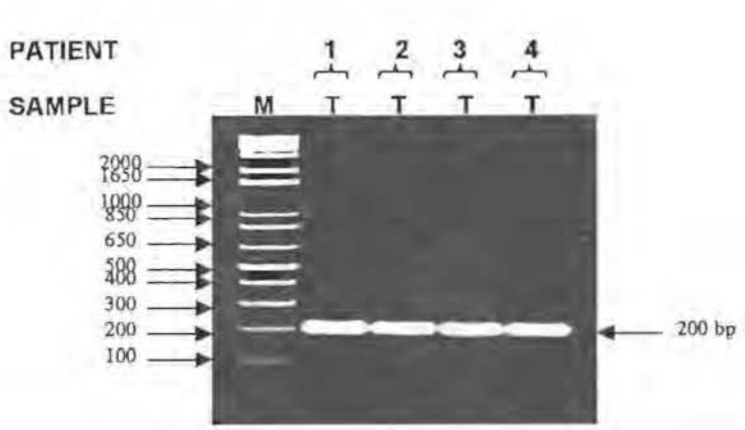

Figure 2.9

PCR RE-AMPLIFIED 20A7 DNA FRAGMENTS. Fragments 20A7 (band 7 from DDRT-PCR performed using OPA 20 and oligo dTA primers, figure 2.3) were recovered as described in section 5.4 and re-amplified by PCR. The products were subsequently electrophoresed in a 1.5% agarose gel as described in the legend of figure 2.8. The size of the electrophoresed DNA fragments was estimated to be 200 base pairs. Abbreviations: M; 1Kb DNA ladder, T; tumour sample.

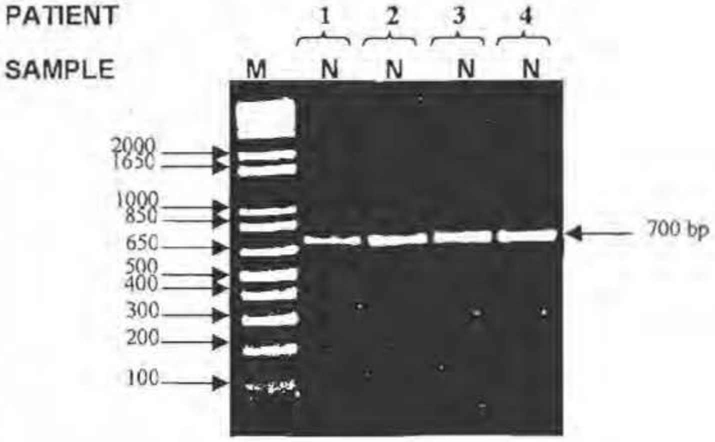

Figure 2.10

PCR RE-AMPLIFIED 16C2 DNA FRAGMENT. Fragments 16C2 (band 2 from DDRT-PCR carried out using OPA 16 and oligo dT C primers, figure 2.4) was recovered from the denaturing polyacrylamide gel and re-amplified by PCR as described in section 5.2.1. The PCR products were electrophoresed on a 1.5 % agarose gel as decribed in the legend to figure 2.8. The estimated size of the electrophoresed DNA fragments is 700 base pairs. Abbreviations: M; 1 Kb plus DNA ladder N; normal biopsy sample.

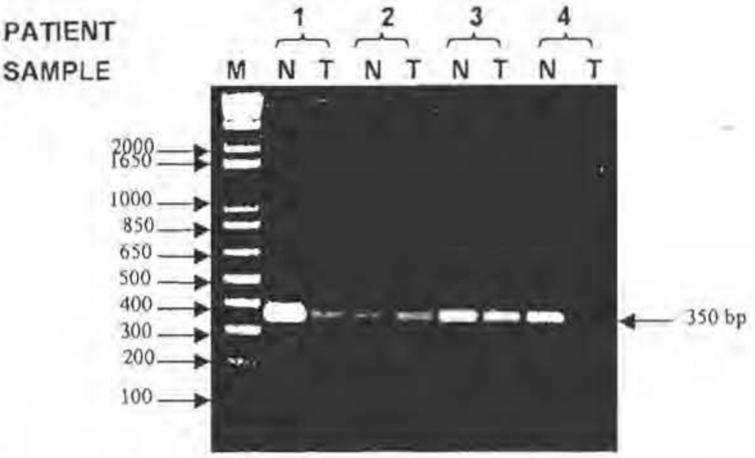

Figure 2.11

PCR RE-AMPLIFIED 17C4 DNA FRAGMENT. Fragments 17C4 (band 4 from DDRT-PCR carried out using OPA 17 and oligo dTC primers, figure 2.5) were excised from the gel, eluted, re-amplified by PCR and the products electrophoresed on a 1.5% agarose gel as previously described in the legend of figure 2.8. The size of the re-amplified products was estimated to be 350 base pairs. Abbreviations: M; 1 kb DNA ladder, N; normal tissue sample, T; tumour sample.

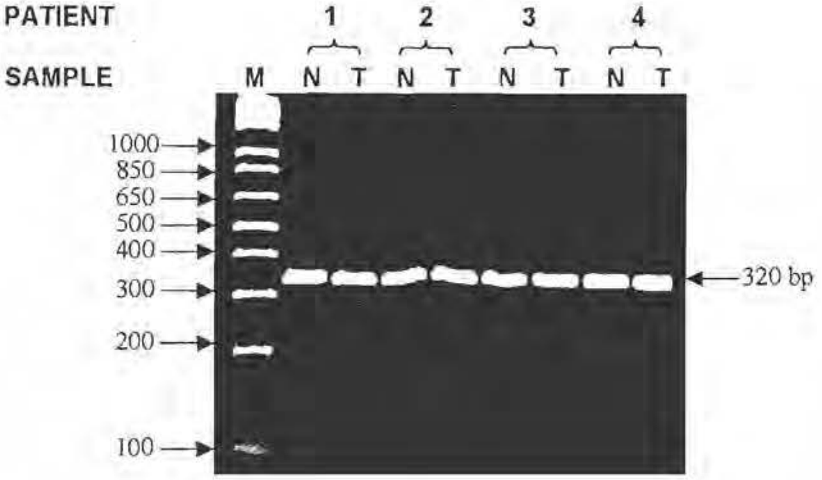

Figure 2.12

PCR RE-AMPLIFIED 16G4 DNA FRAGMENT. Fragments 16G4 (band 4 from DDRT-PCR performed using OPA 16 and oligo dTG primers, figure 2.6) were recovered, re-amplified by PCR and resulting products electrophoresed on a 1.5% agarose gel as described in section 5.4.2. The size of the electrophoresed products was estimated to be 320 base pairs. Abbreviations: M; 1 Kb DNA ladder, N; normal tissue sample, T; tumour sample.

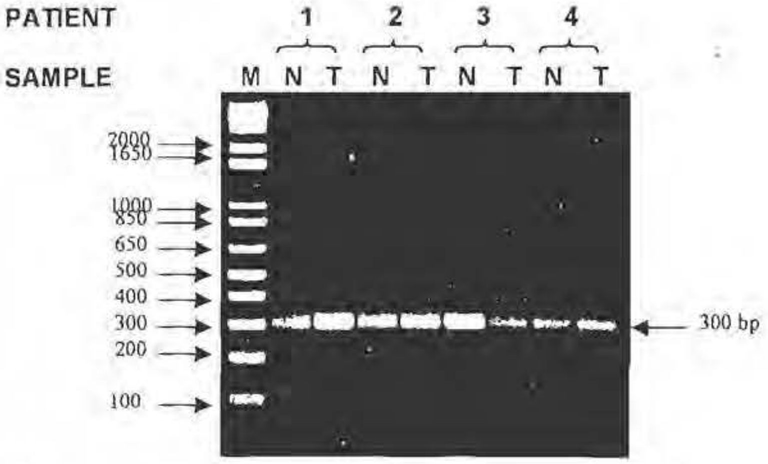

Figure 2.13

PCR RE-AMPLIFIED 19G6 DNA FRAGMENT. Fragments 19G6 (band 6 from DDRT-PCR performed using OPA 19 and oligo dTG primers, figure 2.7) were recovered, eluted, re-amplified by PCR and electrophoresed as described in sections 5.2, 5.3 and 5.4. The size of the fragments was estimated to be 300 base pairs. Abbreviations: M; 1 Kb DNA ladder, N; normal sample and T; tumour tissue.

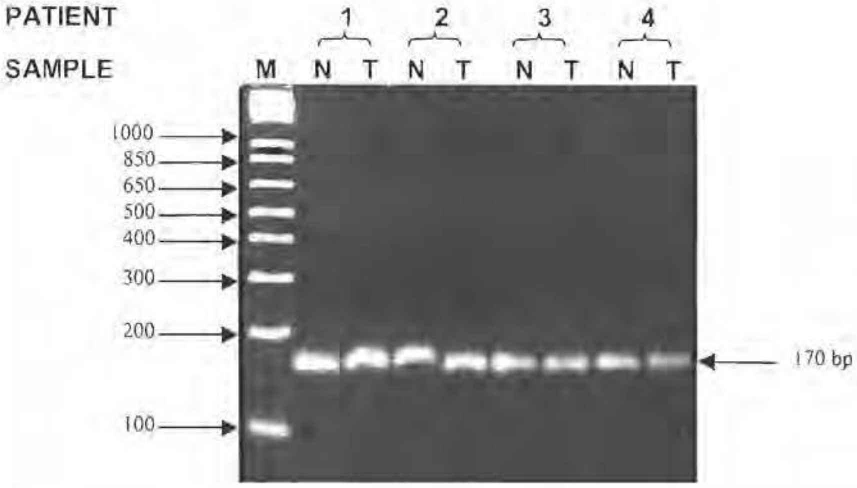

Figure 2.14

PCR RE-AMPLIFIED 19G10 DNA FRAGMENT. Fragments 19G10 (band 10 from DDRT-PCR performed using OPA 19 and oligo dTG primers, figure 2.7) were recovered, eluted and subsequently re-amplified by PCR as described in the legend to figure 2.8. The re-amplified products were electrophoresed on a 1.5% agarose gel, stained in ethidium bromide and thereafter visualized under UV light. The size of the electrophoresed DNA was estimated to be 170 base pairs. Abbreviations: **M**; 1 Kb DNA ladder **N**; normal tissue and **T**; tumour tissue.

2.1.2.4 CLONING AND SEQUENCING

The PCR re-amplified fragments were cloned into pGEM-T-easy vector using the T-A cloning technique as described in section 5.5.2 according to the instructions of the manufacturer.

The procedures of ligation, transformation, plating of cells and picking of positive colonies were carried out as described in sections 5.5. The presence of correct inserts in each colony was determined by PCR amplification using the flanking sequences. Clones with a molecular size corresponding to the size of the previously PCR amplified cDNA were selected and grown in 5ml Luria-Bertani (LB) broth. A 500μl aliquot of the culture was archived as glycerol stock as described in section 5.5.4, while the remainder was used for DNA isolation and sequencing.

Plasmids isolated from each clone with the correct insert size were sequenced using the dideoxy chain termination method (Sanger *et al.*, 1977) as described in sections 5.6. The sequencing reactions were separated on a denaturing 6% polyacrylamide gel containing 7 M urea as described in section 5.3.1. After electrophoresis, the gel was processed and the sequence read manually. The sequences were compared to sequences in Genbank and other databases using Blast software (Altschul *et al.*, 1990).

The results of the Blast analysis were categorized into 3 groups. The first group was referred to as "unknowns without a match". These were sequences that could not be matched to any sequences deposited in GenBank. The second group was referred to as the "unknown genes with a match". These sequences corresponded to database sequences that were poorly characterized such as ESTS (expressed sequence tags), image clones, Bac and Yac clones. The third group were "known genes with a match". This group comprised of fragment sequences with a homology greater than 80%. The biological functions of these genes were either partially or fully characterized. The distribution of the genes into the 3 categories is summarized in Figure 2.15.

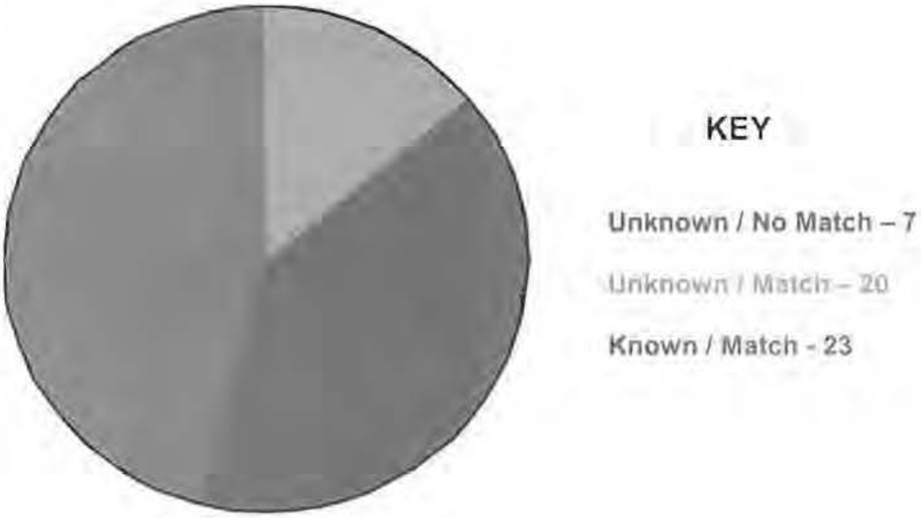

Figure 2.15

THE DISTRIBUTION OF GENES SEQUENCED FROM DDRT-PCR. The genes from DDRT-PCR were sequenced as described in section 5.6. The sequences of these genes were matched to homologous sequences within the database using BLAST software. Each sequence was categorized into one of the 3 groups namely, unknown/no match, unknown/match and known/match. The number in each bracket is the total number of genes in that particular category.

2.1.2.5 SELECTION OF GENES FOR FURTHER ANALYSIS BY NORTHERN BLOTTING

A set of criteria was developed and applied in selecting candidate genes that were further screened as truly differentially expressed genes. These genes were analysed further by Northern blot analysis. The first criterion was to select genes that were classified as "unknown/no match". This was based on the premise that these genes may be novel since; their sequences do not correspond to the database sequences. This was therefore an opportunity to identify previously unknown genes. The second criterion was to select representative fragments from the "unknown/match" group. This group of genes mainly comprised ESTs and IMAGE clones whose sequences have been deposited in the databases. These genes are poorly characterised in terms of their biological function. We selected a representative Image clone whose sequence homology was greater than 80% to its corresponding homologous sequence in the database. Finally, we selected representative fragments that had a homology of greater than 80% compared to their corresponding database homologues from the "known/match" category. These genes had previously been characterised biologically. Most the genes selected from this class had been investigated in other cancers, especially using cell lines rather than human tumour specimens. This provided an important opportunity to investigate the expression levels and patterns of these genes in oesophageal cancer and based on their documented function, deduce their possible role in tumourigenesis of oesophageal cancer. A total of 16 genes were further analysed by Northern blot hybridization. Of the 16 genes selected, 7, 1 and 8 genes were from the unknown/no match, unknown/match and known categories, respectively. All the selected genes were down regulated in the tumour samples with the exception of the Nasopharyngeal carcinoma-transforming gene and two "unknown/ no match" fragments 16A1 and 2 (Table 2.5).

2.1.2.6 NORTHERN BLOT ANALYSIS

Northern blot analysis was carried out using normal and tumour RNA as described in section 5.1.5. The total radioactivity counts per minute (cpm) for each sample was quantitated using the Instant Imager 2024 electronic autoradiograph (Parkard). The final counts of each sample were computed as a ratio of the sample to the control after using the following formulae;

$$\frac{\text{Total counts of test sample} - \text{background of the membrane}}{\text{Total counts of control sample} - \text{background of the membrane}}$$

Similarly, Northern blot hybridization was carried out using 1μg RNA from oesophageal cancer cell lines WHCO1, 5, 6 and KYSE 70 and 520. A normal oesophageal biopsy was used as a control because of the lack of suitable cells that would be used as a control in these experiments. The use of normal biopsies has its limitations because of the heterogeneity of the cell populations contained within the biopsy that fails to totally represent the biological conditions in the normal oesophagus.

Table 2.5

GENES SELECTED FOR FURTHER ANALYSIS BY NORTHERN BLOT HYBRIDIZATION. A summary of the expression patterns of the differentially expressed genes selected for Northern blot analysis. Abbreviations: **HMBGB** (High mobility group protein 2A), **Tx** (Nasopharyngeal carcinoma transforming gene), **ERCC** (Excision repair cross complementing 5 gene), **NDRG** (N-myc downstream regulated 1 gene), ↑ (approximately ≥ 2x up regulation of gene by visual inspection), ↓ (approximately ≥ 2x down regulation of gene by visual inspection).

FRAGMENT	**ACCESSION NUMBER**	**GENE**	**DDRT-PCR (Tumour)**
20A5	EmbAL035420.15	*HMGB*	↓
20A7	GbAF178581.2	*TX*	↑
16C2	GbL20046.1	*ERCC-5*	↓
16C3	GbM38690.1	*CD9/MRP1*	↓
17C4	EmbX60111.1	*CD9/MRP1*	↓
16G4	GbAD126110	*FIBULIN*	↓
19G5	GbBC007960.1	Image clone	↓
19G6	GbAF019226.1	D2-2 protein	↓
19G10	EmbX92845.1	*NDRG*	↓
16A1	unknown	unknown	↑
16A2	unknown	unknown	↑
16A3	unknown	unknown	↓
20A2	unknown	unknown	↓
20A3	unknown	unknown	↓
16G2	unknown	unknown	↓
16G3	unknown	unknown	↓

The expression patterns of 4 of the 16 genes analysed by Northerns were found to be in the same direction as those observed from DDRT-PCR. 1 gene exhibited a variable expression pattern in the 4 tumour specimens examined, while 11 genes exhibited no signal despite repeating the experiments three times on different days with freshly labelled probe (Table 2.6).

Table 2.6

EXPRESSION PATTERN OF SELECTED GENES AFTER NORTHERN BLOT ANALYSIS. The expression patterns of 4 of the 16 genes were altered in a similar fashion as previously observed in DDRT-PCR. 1 gene exhibited variable expression while 11 genes showed no signal. Abbreviations: ↓ (down regulation), ↑ (up regulation).

FRAGMENT	**GENE**	**DDRT-PCR (tumour)**	**NORTHERN BLOTS (tumour)**
20A5	*HMGB*	↓	variable
20A7	*TX*	↑	↑
16C2	*ERCC*	↓	↓
16C3	*CD9 / MRP1*	↓	No signal
17C4	*CD9 / MRP1*	↓	No signal
16G4	*FIBULIN*	↓	↓
19G5	Image clone	↓	No signal
19G6	D2-2 protein	↓	No signal
19G10	*NDRG*	↓	↓
16A1	unknown	↑	No signal
16A2	unknown	↑	No signal
16A3	unknown	↓	No signal
20A2	unknown	↓	No signal
20A3	unknown	↓	No signal
16G2	unknown	↓	No signal
16G3	unknown	↓	No signal

2.1.3 Discussion

2.1.3.1 High mobility group protein 2A (HMGB 2A)

High mobility group proteins also referred to as high mobility group binding proteins (HMGB) are highly charged non-histone chromosomal proteins which comprise of four members, namely HMGB-1-1 or 2, HMGB-14/-17, HMGB-1 (Y) (Enick and Bustin, 1985) and HMGB-3 (West *et al.*, 2001). These proteins are present in all eukaryotic cells and have a relatively well-conserved primary structure (Bustin and Reeves, 1996). They mainly serve as architectural components that influence different levels of chromatin structure (Enick and Bustin, 1985) in addition to binding to transcription factors that regulate gene expression (Obourn *et al.*, 1993). The HMGB-2 family is made up of 2 members; HMGB-2A and HMGB-2B. HMGB-2A has been mapped to chromosome Xq28.

High mobility group protein 2A (HMGB-2A) was marginally under expressed in the tumour specimens of 2 of the 4 patients (figure 2.16). In patients 3 and 4, HMGB-2A was down regulated by factors of 1.3 and 1.4, respectively, while there was insignificant change in expression levels in patients 1 and 2. These results are similar to the DDRT-PCR results in which only two (patients 1 and 2) of the four patients exhibited an under expression of HMGB 2A in the tumour samples compared to the corresponding normal samples (figure 2.3 band 5).

There is no evidence in the literature that explicitly refers to the differential expression of HMGB-2A in cancer or tumour cell lines. This is the first study that demonstrates the expression levels of HMGB-2A in oesophageal cancer tumour tissue and its corresponding normal biopsy. Most studies have investigated the expressions of the HMBG-1 family. Previous studies have revealed that HMGB-2 is a DNA binding protein that binds to moderately bent DNA in the minor groove at regions of flexibility containing A/T rich dinucleotide sequences. This binding stabilizes the bent conformation of DNA promoting the formation of a high order nucleoprotein complex, which comprises of DNA protein kinase and other transcription factors such as octamer transcription factors1, 2 and 6 (Zwilling *et al.*, 1995), *p53* (Jayaraman *et al.*, 1998), (Obourn *et al.*, 1993), 1L-4 (Klein-Hessling *et al.*, 1996), IL 2 (John *et al.*, 1995) and IFN-β (Thanos and Maniatis, 1992). In essence, HMGB-2 is an important building block that augments DNA transcription. It is therefore expected that HMGB-2 would be up regulated in cancer cells that are highly proliferative in nature. The results from both DDRT-PCR and Northern blots show a reverse trend to the above explanation.

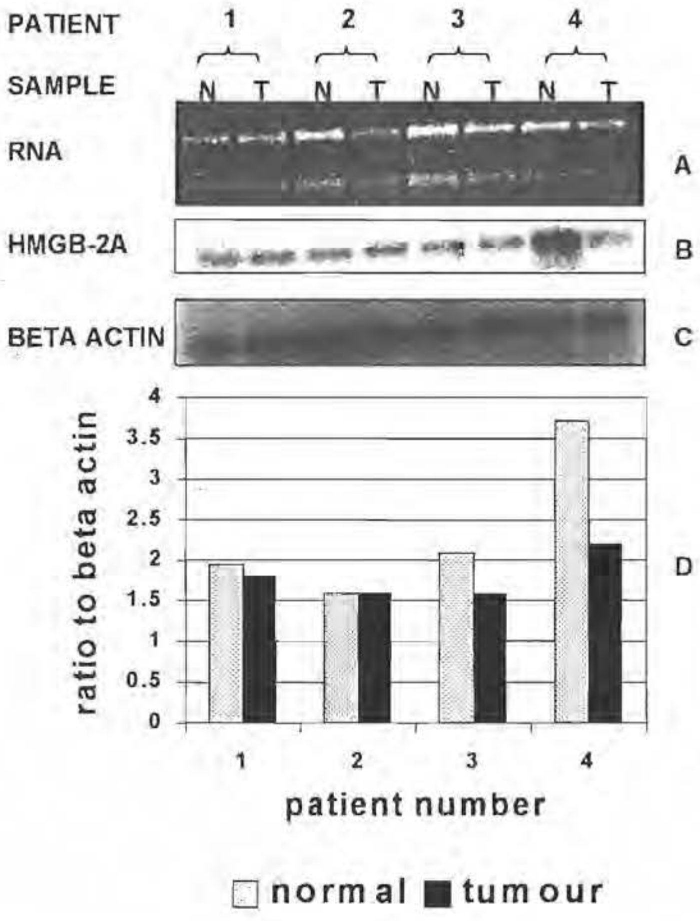

Figure 2.16

EXPRESSION OF HIGH MOBILITY GROUP PROTEIN 2A (HMGB-2A) IN NORMAL AND TUMOUR BIOPSIES. 1µg of total RNA from normal (**N**) and corresponding tumour (T) from four different patients were electrophoresed and stained with ethidium bromide as described in section 5.1.4. The ethidium stained gel is shown under UV illumination in panel (**A**). This gell was used in Northern blot experiment as described in section 5.1.5. HMGB-2A probe was radiolabelled and hybridized to the nytran membrane as described in section 5.7. The membrane was exposed to X-ray film overnight (**B**) and subsequently analyzed for total radioactivity using the InstantImager 2024 electronic autoradiograph (Parkard). Beta actin was used as an internal control (**C**). The final counts of each sample were computed after subtracting the background intensity of the blot from the total counts and dividing by the final counts (total – background) of control as described in section 2.1.2.6 (**D**). The HMGB-2A gene was shown to have variable expression in the 4 patients.

Previous studies have shown that although separate genes encode HMGB-1 and HMGB-2, the two proteins are closely related with an 82% amino acid sequence identity. In addition, HMGB-1 and HMGB-2 have been found to be functionally interchangeable (Boonyaratanakornkit *et al.*, 1998). It is probable that HMGB-2A is down regulated in cancer of the oesophagus compared to the other HMGB family members. This may be an indication that the expression of HMGB proteins is variable in different cancers. In spite of the down regulation of HMGB-2A, there is probably a homeostatic up regulation of HMGB-1 that ultimately maintains the HMGB1/2 complex with respect to stimulating DNA binding and transcriptional activity. Although these studies did not assay for the expression levels of HMGB1 in the tumour verses normal samples it is possible that this homeostatic maintainance accounts for the sustained high transcriptional activity that is observed in tumours with a low expression of HMGB-2A.

These results therefore create a basis further investigation into the expression levels and patterns of not only HMGB-2A but also of other family members in the development of oesophageal cancer. Since this gene family is an important scaffold for transcription it may be a potentially important therapeutic target in cancer management.

2.1.3.2 Nasopharyngeal carcinoma transforming gene

Nasopharyngeal carcinoma transforming gene (Tx) was first identified by cloning of DNA from the Chinese nasopharyngeal carcinoma (NPC) cell line, CNE2, into non-transformed promotion sensitive mouse JB6 (P+) C141 cells (Cao *et al.*, 1991).

The Tx gene was marginally up regulated in patients 1, 3 and 4 (figure 2.17). This up regulation ranged from 1.2, 1.5 and 1.3 fold for patients 1, 3 and 4 respectively. Conversely, there was a down regulation of Tx in patient 4. This result is questionable because of the unequal loading of the normal verses the tumour RNA samples. The unequal loading may account for the false result obtained. Overall however, these results are in agreement with the DDRT-PCR results (figure 2.3 band 7) which show that Tx was up regulated in 3 of the 4 patients.

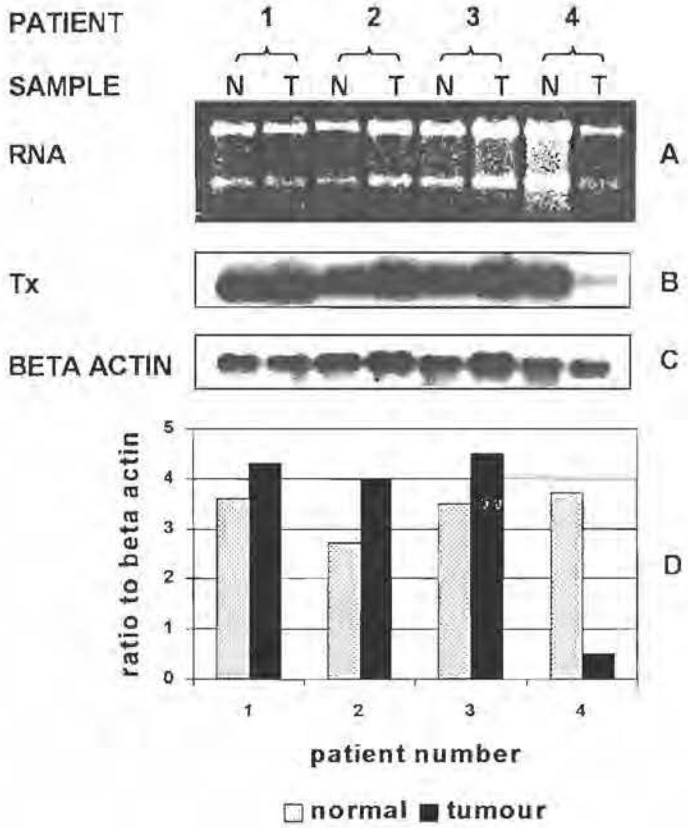

Figure 2.17

EXPRESSION OF NASOPHARYNGEAL CARCINOMA TRANSFORMING GENE (Tx) IN NORMAL AND TUMOUR BIOPSIES. Total cellular RNA (1µg) from normal (**N**) and corresponding tumour (**T**) from four different patients (**A**) were used in Northern blot experiments as described in sections 5.1.4 and 5.1.5. The α- [^{32}P] dCTP radiolabelled Tx probe was hybridized to the membrane as described in section 5.7. After several washes in SSC buffers, the membrane was exposed to X-ray film overnight (**B**), followed by analysis for total radioactivity using the InstantImager. Beta actin was used as an internal control (**C**). The final radioactive counts (**D**) of each sample were computed as described in section 2.1.2.6. The Tx gene was shown to be marginally over expressed in the tumour specimens of 3 of 4 patients (1, 2 and 3).

There is little information in the literature on the functional and biological characteristics of the Tx gene. A previous study found the Tx gene to have no homology to either the ras family of genes (Ha, Ki and N) nor to human promotion sensitive genes such as src, myb, jun, fos, raf and EGFR (Cao *et al.*, 1991).

This is the first study that shows Tx to be differentially expressed in oesophageal cancer. The up regulation of this gene though marginal in the tumour biopsies, suggests that this gene may play an important role in tumourigenesis. This role can only be speculative at the moment considering the current limited information on this gene, however it may be a proto-oncogene as its expression level is virtually nil in all the normal samples of DDRT-PCR (figure 2.3 band 7). Alternatively, it may be an important cell-signalling factor (growth factor, growth factor receptor, signal transducer or even a nuclear factor) that is downstream of the well-characterised oncoprotein signalling pathways. This factor is probably "switched" on during tumourigenesis and promotes and sustains progressive transformation of a normal cell into a neoplastic one. Further studies such as transfections of the gene into normal cells need to be carried out to decipher the probable biological role of Tx in tumourigenesis.

2.1.3.3 Excision repair cross complementing gene 5

Living organisms have developed a complex network of repair mechanisms to deal with spontaneous and environmentally induced structural DNA alterations. One such mechanism is the highly conserved nucleotide excision repair (NER) pathway (Bootsma and Hoeijmakers, 1993). The NER pathway removes a wide range of DNA lesions including UV light induced lesions. To date approximately 30 proteins are known to be involved in the well-characterized multi-step reaction in mammalian NER (de Laat *et al.*, 1999). The first step of the process leads to lesion recognition and incision of the damaged strand on each side of a lesion. A 24-32 mer oligonucleotide is removed, followed by the synthesis of new DNA using the complementary strand as the template and ligation.

In humans, this repair pathway involves the Xeroderma pigmentosum (XP) proteins and associated factors of which 8 have been isolated and characterized (de Laat *et al.*, 1999). The equivalent of the XP genes in rodents are the excision repair cross-complementing rodent repair deficiency genes (ERCC), while in yeast they are the RAD family of genes.

ERCC-5 (XPG) gene has been localized to chromosome 13q33 (Samec *et al.*, 1994) and is organized into 15 exons and 14 introns. This gene encodes an acidic protein of 1,196 amino acids (Scherly *et al.*, 1993). To date 6 alternatively spliced XPG mRNA isoforms have been identified.

The ERCC-5 gene was down regulated the tumour samples of patients 2, 3 and 4 by factors of 1.3, 1.4 and 1.5, respectively (figure 2.18). On the other hand, there was an up regulation of ERCC-5 in tumour sample of patient 1 compared to the normal control. These results overall are congruent to the DDRT-PCR result (figure 2.1.4 band 2). The up regulation of the ERCC-5 in patient number 1 is perhaps an indication that this gene is not 100% altered in a similar fashion in all tumours. Alternatively, the up regulation may be a tumour grade dependent. Thus depending on the grade of the tumour and the "route" followed to acquire the cancer phenotype, the ERCC-5 gene will exhibit differential expression that is reflective of these characteristics.

The protein encoded by XPG has several functions. First, the protein has both single strand specific endonuclease and 5'→3' exonuclease activities, which enable XPG to cleave a variety of artificial DNA substrates, including DNA bubbles, sprayed arms and stem-loops (O'Donovan *et al.*, 1994a; Habraken *et al.*, 1994). Secondly, it has a structural function in the assembly of the NER DNA-protein complex as it interacts with 3 key components of this system namely, TFIIH (core transcription repair factor II H) which catalyses open formation around the DNA lesion and facilitates the repair complex assembly, RPA (replication protein A) which stabilizes the opened DNA complex and positions other nucleases. Lastly, XPG interacts with proliferating cell nuclear antigen (PCNA), a factor involved in DNA repair synthesis. These interactions are important in the stability of the NER multi-protein complex and may perhaps facilitate "cross talk" between incision and gap filling stages of the NER machinery (O'Donovan and Wood 1993; Scherly *et al.*, 1993; O'Donovan *et al.*, 1994b).

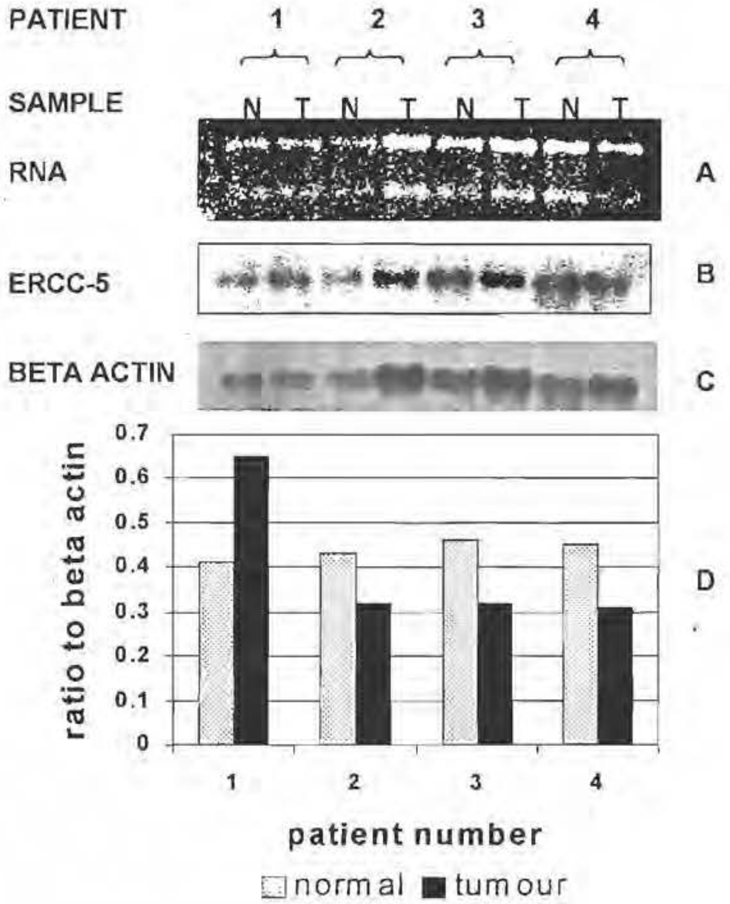

Figure 2.18

EXPRESSION OF EXCISION REPAIR CROSS-COMPLEMENTING (ERCC-5) GENE IN NORMAL AND TUMOUR BIOPSIES. RNA from normal (N) and corresponding tumour (T) from four different patients (A) were used in Northern blot experiments as described in 5.1.5. Radiolabelled ERCC-5 probe was hybridized ($2x10^6$ cpm/ml) to the membrane in the presence of ULTRAhyb buffer. After several washings the membrane was exposed to X-ray film overnight (B). The membrane was further analyzed for total radioactive counts. Beta actin was used as a control (C). The final total radioactivity counts of each sample was determined as described in section 2.1.2. (D). The ERCC-5 gene was found to be marginally under expressed in the tumour samples of 3 patients (2, 3 and 4), but significantly up regulated in the tumour sample of patient 1.

A down regulation of ERCC-5 would adversely impair the DNA repair mechanism of the cell. The most important consequence of this would be the accumulation of DNA lesions such as adducts and UV induced lesions. If the damage to DNA persists particularly, if it occurs in a proto-oncogene or tumour suppressor gene, this may lead to an altered function of the gene resulting in a clone with selective growth advantage. The consequence of this would be the development of a neoplastic cell that has the potential to clonally expand into cancerous cells. The down regulation of ERCC-5 in both the cell lines and tumour tissues may therefore play an important role not only in initiation of neoplasia but also in the promotion and sustenance of cellular characteristics that promote the development of cancer.

These results are the first to show a deregulation of ERCC-5 in oesophageal cancer and provide an important basis for the further investigations into the possible deregulation of other closely factors involved in NER pathway. It would be interesting to determine whether these factors are deregulated in a similar fashion as ERCC-5 and how this deregulation relates to the initiation, promotion and maintenance of tumourigenesis in not only oesophageal cancer but also in other cancers.

2.1.3.4 Fibulin 1-isoform D

The fibulins are a class of extracellular matrix and blood glycoproteins that currently constitute five members, namely, fibulin 1 and 2 (Argraves *et al.*, 1989; Pan *et al.*, 1993; Balbona *et al.*, 1992), fibulin 3 (S1-5/EFEMP1) (Lecka-Czernik *et al.*, 1995), fibulin 4 (MBP1/H411/EFEMP2) (Gallagher *et al.*, 1999), and fibulin 5 (EVEC/DANCE) (Kowal *et al.*, 1999).

The fibulin family of proteins is extensively found in basement membrane and connective tissue, especially elastic fibers (Roark *et al.*, 1992). They bind to the extracellular matrix (ECM) proteins such as fibronectin, laminin, nidogen, endostatin, tropoelastin and fibrinogen (Balbona *et al.*, 1992; Pan *et al.*, 1993; Tran *et al.*, 1995; Sasaki *et al.*, 1999).

Multiple forms of fibulin 1 proteins, namely, A, B, C and D exist. They differ in their C-terminal regions and are produced through the process of alternative splicing of their precursor RNA (Argraves, 1999). The fibulin 1 isoform D is identical in its first 566 residues to the other isoforms (A-C) however, it has a unique 137 amino-c-terminal segment encoded by alternatively spliced portion of its transcript (Tran *et al.*, 1997).

FIBULIN 1-D message transcript was significantly under expressed in all the tumour samples. This under expression was by factors of 2.6, 2.1, 1.7 and 8.75 for patients 1, 2, 3 and 4, respectively (figures 2.19). These results are in agreement with the previously observed data in DDRT-PCR experiments (figure 2.1.6 band 4). These results reveal a more than 50% decrease in the expression of fibulin-1D in 75% of the patients examined by Northern analysis. This is the first study to document the expression levels of *FIBULIN-1D* mRNA in oesophageal cancer.

In our study, *FIBULIN-1D* mRNA was down regulated in all the oesophageal cancer cell lines compared to the normal oesophageal epithelium used as a control (figures 2.20). The gene was down regulated by factors of 1.4, 1.3, 2.3, 3.5, and 4.1 for WHCO1, WHCO5, WHCO6, KYSE 70 and KYSE 520 cell lines, respectively. There are no studies in the literature that have documented fibulin-1D expression in oesophageal cancer cell lines. This study however is in agreement with previously published data that has reported a down regulation of fibulin-1 in other cancers cell lines (Roark *et al.*, 1995; Qing *et al.*, 1997).

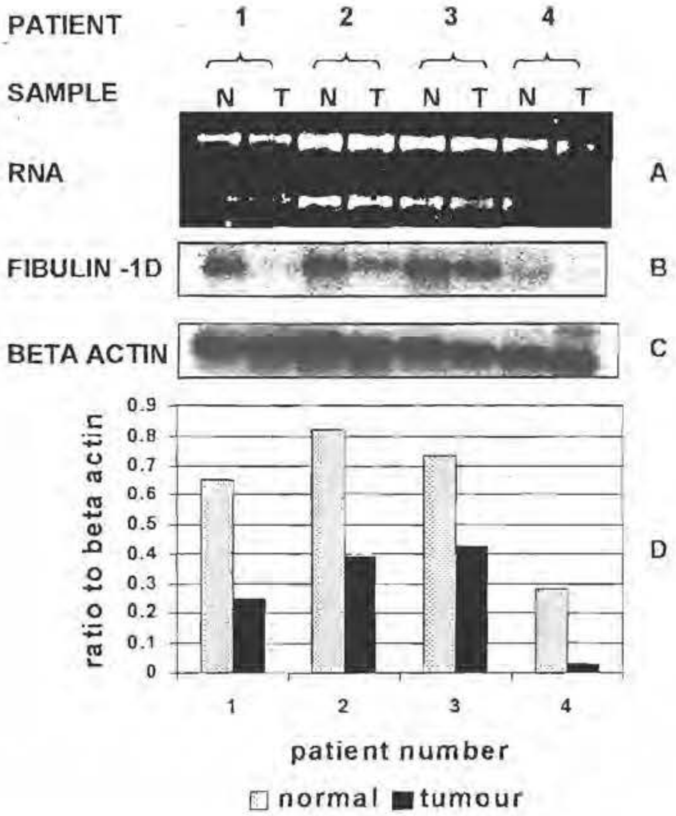

Figure 2.19

EXPRESSION OF FIBULIN ISOFORM-1D GENE IN NORMAL AND TUMOUR BIOPSIES. RNA from normal (N) and tumour (T) specimens of four different patients (**A**) was used in Northern blot experiments as described in sections 5.1.4 to 5.1.5. The radiolabelled Fibulin -1D probe was hybridized to the membrane overnight. After several washes in SSC, the membrane was exposed to X-ray film overnight (**B**). Beta actin was used as an internal control (**C**). The final radioactive counts of each sample were determined as described in section 2.1.2.6 (**D**). The Fibulin-1D gene was significantly under expressed in the tumour samples of all the patients examined.

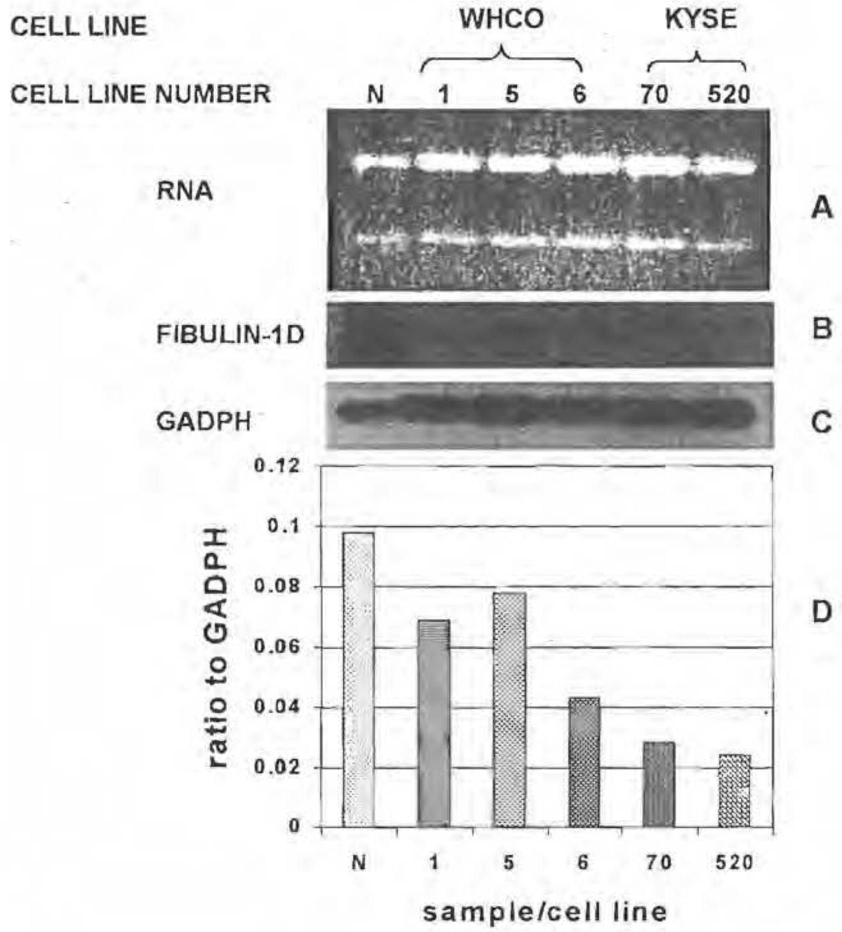

Figure 2.20

EXPRESSION OF FIBULIN-1D GENE IN OESOPHAGEAL CANCER CELL LINES. RNA from cell lines and a normal oesopahgeal tissue (**N**) were used in Northern blot analysis (**A**) as described in sections 5.1.4 and 5.1.5. $\alpha[^{32}P]$ dCTP labelled Fibulin-1D probe was hybridized to the membrane overnight. After several washes in SSC, the membrane was exposed to autoradiographic film (**B**). GADPH was used as an internal control (**C**). Total radioactivity counts for each sample were computed and plotted (**D**). Fibulin-1D gene was significantly under expressed in all the 5 cell lines compared to the normal oesophageal tissue.

The association of fibulin with ECM components has lead to the suggestion that it plays a leading role in several important cellular and tissue events such as embryonic development and organogenesis, hemostasis and thrombosis, fibrinogenesis, tissue homeostasis and tissue remodeling (Argraves, 1999). Additional studies have shown that fibulins are indeed involved in modulating cell morphology, growth, adhesion and motility (Gallagher *et al.*, 1999; Nakamura *et al.*, 1999; Argraves, 1999).

Recently, fibulin 1 was shown to inhibit cell adhesion and motility *in-vitro* in a wide range of cancer cell lines. The underlying mechanism is poorly understood however; it is thought that this is accomplished by suppressing the motility promoting activity of fibronectin. In addition, these suppressive effects are independent of integrin α_4 interactions with fibronectin and also of the sulphation of GAG moieties on cell surface proteoglycans (Twal *et al.*, 2001). Further studies have shown that constitutive over expression of fibulin 1 in tumour cells delays tumour formation *in-vivo* and suppress anchorage-independent cell growth, motility and invasion *in-vitro* (Qing *et al.*, 1997; Hayashido *et al.*, 1998).

The implication of Fibulin-1D down regulation would therefore be very important in cancer invasion and metastasis. The foregoing events are essentially reliant on the degradation of factors involved in cell adhesion and proteins involved in the attachment of the cell to the basement membrane. An under expression of fibulin would first, deregulate the cell's behaviour by abrogating cell-to-cell interaction and secondly "loosen" the cell attachment to the basement membrane. These alterations would enhance the "severance " of the cell from the basement membrane by proteolytic enzymes and possibly augment the motility of the "free" cell into the surrounding stroma. Fibulin-1D down regulation has not been previously reported in oesophageal cancer or in oesophageal cancer cell lines. Based on previously published data, these results are highly suggestive that fibulin-1D plays an important role in tumourigenesis. These results therefore provide an important basis to further investigations (using cell transfection studies) into whether constitutive expression of fibulin-1D directly correlates with the regulation of other molecules involved in cell-to-cell adhesion and how this impacts on the growth of OC cell lines. The findings of such a study could shed more light on the possible temporal order of the de-regulation of important cell-to-cell adhesion molecules and also identify candidate targets for biomarkers and therapeutic intervention.

2.1.3.5 N-myc down regulated gene 1

N-myc down regulated 1 protein (NDRG-1) also referred to, as RTP/DRG1/Cap43/rit42/TDD5/NDR1 is a family member of the GTP-binding proteins comprising NDRG-1, 2, 3 and 4. Studies have demonstrated that *NDRG* family of genes is downstream of *N-MYC* and is also down regulated by the same (van Belzen *et al.*, 1997; Kalaydjieva *et al.*, 2000 and Zhou *et al.*, 2001). These closely related proteins share a high homology between them (Kalaydjieya *et al.*, 2000). They are widely expressed in human (van Belzen *et al.*, 1997), mouse (Li and Trueb, 2000), *Xenopus* (Kumar *et al.*, 1993), *Drosophila* (Sommer *et al.*, 1994), *C.elegans* (Wilson *et al.*, 1994) and yeast (Hudson *et al.*, 1993). These proteins harbour the five characteristic motifs (G1 to G5) that are believed to interact with GTP (Li and Trueb, 2000).

N-MYC downstream-regulated gene 1 (*NDRG-1*) was down regulated in the tumour samples of 3 patients (figure 2.21). This down regulation was by factors of 2.8, 1.5 and 1.4 in patient 1, 2 and 3, respectively. There is a marginal down regulation in patients 2 and 3 compared to patient 3. This suggests that the *NDRG-1* is differentially expressed in different tumours and these differences may be tumour grade dependent. Alternatively, the percentage contamination of the tumour cells by non-cancerous cells probably increase the amount of *NDRG-1* thus accounting for the subtle difference in the expression levels of *NDRG-1* between the different tumour samples. The marginal down regulation results observed in Northern blot confirm the gene expression data observed in the DDRT-PCR experiments (figure 2.7 band 10). Conversely, the tumour sample of patient 4 exhibited an up regulation of *NDRG-1*. The reason for this observation is presently unknown.

The precise molecular and cellular function(s) of *NDRG-1* are still largely unknown. Several studies however, have demonstrated that NDRG protein levels are modulated by various conditions. For example, *NDRG-1* is up regulated by agents such as mercaptoethanol, tunicamycin (Kokame *et al.*, 1996), nickel components (Zhou *et al.*, 1998), DNA damaging agents and certain stages of the cell cycle (Kurdistani *et al.*, 1998), synthetic retinoids (Piquemal *et al.*, 1999) among others. In addition, the gene is up regulated under conditions such as differentiation (van Belzen *et al.*, 1997), activation of *p53* (Kurdistani *et al.*, 1998, Yu *et al.*, 1999), and hypoxia (Salnikow *et al.*, 2000). Other pathways, such as the peroxisome proliferator-activated receptor α and retinoid X receptor pathway and the histone deacetylation pathway are thought to be probable targets of NDRG1 action (Guan *et al.*, 2000).

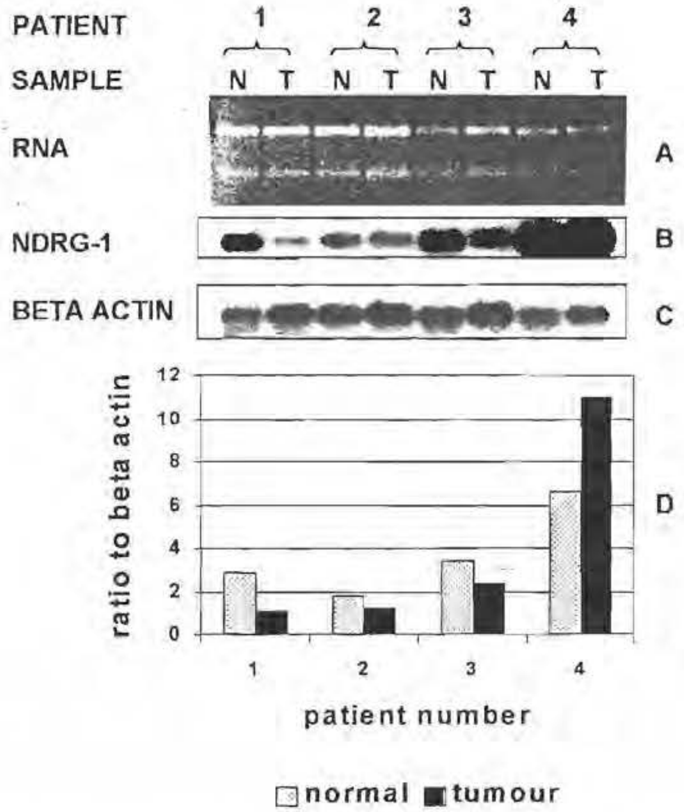

Figure 2.21

EXPRESSION OF N-MYC DOWN REGULATED GENE 1 (NDRG-1) IN NORMAL AND TUMOUR BIOPSIES. Total RNA from normal (**N**) and corresponding tumour (**T**) from four different patients (**A**) was used in Northern blot experiments as described in sections 5.1.4 and 5.1.5. $\alpha[^{32}P]$ dCTP labelled NDRG-1 probe was hybridized to the membrane overnight, washed severally in SSC and finally exposed to X-ray film overnight (**B**). Beta actin was used as an internal control (**C**). The final radioactive counts of each sample were calculated and presented in form of a bar graph (**D**). The NDRG-1 gene was marginally under expressed in the tumour samples of 3 patients (1, 2 and 3), while it was significantly up regulated in the tumour sample of patient number 4.

The above observations suggest that *NDRG-1* gene is involved in proliferation/differentiation programme of the cell. Though the importance and role of *NDRG-1* in tumourigenesis remains largely circumstantial, the most compelling evidence comes from studies that have shown that it interacts specifically with the SCL/TAL1 oncogene product. SCL/TAL1 is a basic-helix-loop-helix transcription factor involved in cell growth and differentiation. Usually, SCL/TAL1 is located in the nucleus but during some phases of the cell cycle, it translocates into the cytoplasm where it may interact with NDRG-1 protein. SCL/TAL1 is required for normal development of hematopoietic cells and disruption or of the corresponding gene seems to be responsible for the onset of T-cell leukemia (Pulford *et al.*, 1995).

The down regulation of the *NDRG-1* gene has been shown previously in metastatic colon cancer tissues and their cell lines (Guan *et al.*, 2000; Nimmrich *et al.*, 2000), MCF-7 mammary carcinoma cell lines and U937 myelomonocytic cell line (Piquemal *et al.*, 1999). These results are in general agreement with our results although the published data does not state the actual factors or percentage alterations by which *NDRG-1* is down regulated. Conversely, *NDRG-1* gene is up regulated during differentiation of colon epithelium cells *in vitro* and *in vivo* (van Belzen, 1997) although no percentage alterations are stated. Based on the above observations it is probable that *NDRG-1* gene is an important target in tumour pathogenesis. This is further strengthened by the fact that *NDRG-1* gene is down regulated by the *N-MYC* proto-oncogene. The role of the *N-MYC* gene in other cancers such as, small cell carcinoma of the lung (Wong *et al.*, 1986), Retinoblastoma (Lee *et al.*, 1984) and Wilms (Nisen *et al.*, 1986) is well described, however, the opposite is true in oesophageal cancers. It would therefore be of interest to determine the expression levels of *N-MYC* in oesophageal cancer vis-à-vis *NDRG-1* protein and investigate whether constitutive expression of NDRG-1 could impact on cell growth and differentiation. Secondly, the *NDRG* genes share a high homology with alpha/beta hydrolases (Shaw *et al.*, 2002). These enzymes constitute a large protein superfamily that catalyzes a diverse range of reactions. Is it probable that the NDRG family of proteins have an enzymatic function that is crucial in the generation of a polypeptide that is an important effector of either cellular signaling or proliferation? These are but some of the questions that have arisen from this study and stimulate further investigations into the probable role of NDRG-1 protein in tumourigenesis.

2.1.3.6 Unconfirmed genes

2.1.3.6.1. CD9 / MRP1

CD9 also referred to *MRP1* encodes the protein *p24*. This protein belongs to the transmembrane-4 superfamily (Rubinstein *et al.*, 1996; Wright and Tomlinson, 1994). This protein is a 24-27 kDa glycoprotein that contains an extracellular N-glycosylated domain that may function as a cell-surface-anchored receptor (Lanza *et al.*, 1991; Rubinstein *et al.*, 1994).

The precise physiological functions of the protein remain unknown, however it is possible that it participates in signal transduction, antigen presentation, cell proliferation, adhesion or motility (Rubenstein *et al.*, 1996; Wright and Tomlinson, 1994; Lanza *et al.*, 1991; Rubinstein *et al.*, 1994).

Recent studies of human cancers demonstrated the relationships between reduced expression of CD9 and aggressive behaviour of the tumour, especially, lymph node metastasis. Examples of such cancers are lung adenocarcinoma (Higashiyama *et al.*, 1995) breast cancer (Miyake *et al.*, 1996), colon cancer (Mori *et al.*, 1998) melanoma (Radford *et al.*, 1995) and pancreatic cancer (Guo *et al.*, 1996).

DDRT-PCR results show a down-regulation of CD9 in the tumour samples of 3 patients (figure 2.5). In spite of having attempted on 3 separate occasions to detect the mRNA of this gene by Northern blot, no significant signal was achieved from Northern blot analysis. This failure could reflect the low levels of the mRNA in the samples examined. The use of techniques such as quantitative real time-PCR rather than Northern blot analysis may be more sensitive in quantitating this message.

2.1.3.6.2 Image Clone

DDRT-PCR identified a fragment that was consistently down regulated in the tumour tissues (figure 2.7 band 5). Sequence analysis did show that this fragment displayed a high homology to a poorly described image clone (Accession number GbBC007960). Northern blot analysis results were negative for this clone suggesting that the mRNA levels may be expressed in low levels in the tissues examined. With the ever-increasing information on genetic make-up of different organisms, it is hoped that this image clone will be clearly identified and further characterized in the not distant future.

2.1.3.6.3 D2-2 protein

The D2-2 protein is a protein that is highly expressed in the thyroid and moderately in the pancrease, adrenal cortex, testis and small intestines. In tumourigenic cells, it is found to be highly expressed in glioblastomas, and prostrate cancer cell lines while in tumour tissues, high expression levels have been seen in breast carcinoma. Conversely, it is under-expressed in B-cell lymphoma and meningioma. The proteins' deduced 80 amino acid sequence does not share identity with any known protein sequences in the various databases. D2-2 is highly expressed in the brain and heart of 20-week fetuses but its expression subsequently drops to low levels in adults. This differential expression has led suggestions that D2-2 may play a role in the development of these organs (Sehgal *et al.*, 1997)). No radioactive signal could be obtained by Northern blot analysis and we can therefore conclude that the level of mRNA is too low for detection and that probably real time PCR may be more informative.

2.1.3.6.4 No match genes

Differential display represents a powerful approach for identifying differential expression of novel genes in disease conditions. DDRT-PCR identified 7 fragments that had no homologous sequences in the databases. These genes were further analyzed by Northern blot analysis to confirm their differential expression. In spite of 3 attempts to screen for their expression, no mRNA signals were observed. These cDNA fragments will in future be used to screen different cDNA libraries to determine whether they are homologous to any known genes.

2.1.3.7 Use of cell lines to determine differential expression of genes

Cell lines have been the preferred choice of biological material to study differential gene expression. This is because of the homogeneity of cell populations and the easy to manipulate cell culture conditions. We endeavored to study whether the genes we had identified to be differentially expressed in tumour tissue were similarly differentially expressed in oesophageal cancer cell lines. The major drawback for these experiments was the lack of a suitable control that would effectively represent the normal epithelial cells of the oesophagus. Consequently, we used normal human oesophageal biopsies though this raises questions as to their suitability as appropriate controls. This study however, was able to establish a pattern between fibulin-1D expression in the different oesophageal cancer cell lines and the human tumour biopsies. *FIBULIN-1D* was down regulated in both the OC cell lines and tumour tissues. No success was achieved in relating the gene expression patterns and levels of *HMGB Tx*, *ERCC* and *NDRG* in the OC cell lines to those observed in the tumour tissue.

2.1.3.8 Limitations of DDRT-PCR

Several researchers have successfully applied DDRT-PCR in the past to discover differentially expressed genes. Many molecular biologists however, are abandoning the technique because of the increasing frustration and failure of the technique, principally because of the following criticisms.

2.1.3.8.1 Questionable ability to detect rare mRNA

Approximately 99% of the 20,000 to 30,000 mRNA species present in a given cell are rare ($^1/_{20,000}$ to $^1/_{70,000}$) (Wan *et al.*, 1996). To detect these rare species the technique recommends the titration (in the 0.2 μg total RNA typically used) of each RNA sample since non-reproducible bands in duplicate reactions are presumed to originate from rare mRNAs species that are not present in sufficient quantities to be amplified to detectable levels (Mou *et al.*, 1994; Liang *et al.*, 1993). Bertoli *et al.*, (1995) specifically addressed the issue of rare mRNA detection using a computer simulated differential display model. They demonstrated that many more bands were generated in DDRT-PCR than were visualized on a gel. The rationale is that template mRNA present at higher levels at the start of the reaction would be amplified to detectable levels more quickly because dNTPs become limiting. In addition, they would be preferentially displayed over less abundant transcripts. This therefore essentially limited the number of rare transcripts that could be detected by this technique.

The inefficiency of 10 mers as primers has been identified as a second factor limiting the sensitivity of DDRT-PCR. It has been argued that DDRT-PCR is unable to display the vast majority of rare mRNAs regardless of the primer design and combinations employed. Instead, it is suggested that increased sensitivity would be accomplished by using longer, more selective and efficient primers at higher annealing temperatures (Bertoli *et al.*, 1995).

2.1.3.8.2 High percentage of false positives.

The most resounding criticism against DDRT-PCR has been the very high percentage of false positives (Liang and Pardee, 1995; Galindo *et al.*, 1997). A false positive result is when the candidate band on the DDRT-PCR gel ultimately yields a hybridization signal in both or neither of the compared sources in a Northern blot. The possible reasons for this are contamination of RNA with residual DNA from the isolation procedure (Liang and Pardee, 1995). Secondly, the generation of DDRT-PCR artifacts that eventually led to false positives. This has been attributed to contaminating DNA, experimental variability and the inherent design of DDRT-PCR, using low stringency conditions in the presence of short primers (Zhao *et al.*, 1996). These studies however, minimized the possibility of DNA contamination as a source of interference in our assay by treating our RNA with DNAse prior to the RT-PCR steps. Thirdly, is the aspect related to redundancy (cloning the same cDNA more than once), this is mainly due to mismatching, "weak" primers (some arbitrary primers do not contribute to amplification in combination with an oligo dT primer) and is also governed by the molar ratio between the oligo dT and arbitrary primer (Galindo *et al.*, 1997).

2.1.3.8.3 Cumbersome verification of putative positives.

The verification of putative positives obtained by DDRT-PCR is time consuming and requires large quantities of RNA whereas the initial stages can be completed in a few days or weeks, the cloning of candidate bands to generate DNA for sequencing and/or probes for the final verification by Northern hybridization in cumbersome (Galindo *et al.*, 1997).

2.1.3.8.4 Limited usefulness of information

The main concern raised is that amplification products identified as candidate differentially expressed mRNA do not usually comprise the complete coding regions of genes for subsequent cloning and manipulation (Galindo *et al.*, 1997). This can be overcome by using 5' rapid amplification of cDNA ends (5' RACE) a technique that offers the possibility to obtain full-length cDNA from DDRT-PCR amplification products (Frohman *et al.*, 1988).

2.1.4 Conclusion

In spite of the shortcomings of DDRT-PCR, Northern hybridization positively identified and corroborated the DDRT-PCR results that identified five genes to be differentially expressed in squamous cell carcinoma of the oesophageal cancer. These genes were, *HMGB*, *TX, ERCC (XPG), FIBULIN* and *NDRG*. They have not been previously described in OC tumourigenesis however; they have been investigated in other cancers, predominantly in cell lines rather than human tumour specimens.

Antibodies to fibulin-1 and NDRG-1 proteins were acquired to enable further studies (immunohistochemistry) to be carried out to ascertain expression profiles of these genes in a larger patient sample. From these studies we hope to answer basic questions such as how the expression of these genes correlate with the clinicopathological parameters such as tumour grade, stage of the disease of oesophageal cancer patients.

Table 2.7

SUMMARY OF THE VARIOUS GENES ANALYSED BY NORTHERN BLOT ANALYSIS IN BOTH HUMAN BIOPSIES AND OC CELL LINES. 17 genes were analyzed by Northern blot analysis to confirm whether they were differentially expressed as previously visualized in DDRT-PCR. Of the 17, 4 genes were differentially expressed in the same fashion in DDRT-PCR as in Northern blot analysis using human biopsies. *FIBULIN-1D* was the only gene that was differentially expressed in a similar manner in DDRT-PCR and Northern blot analysis using both human biopsies and OC cell lines.

FRAGMENT	**GENE**	**DDRT-PCR (Tumour)**	**NORTHERN (Tumour)**	**OC CELL LINES**
20A5	*HMGB*	↓	Variable	Inconclusive
20A7	*TX*	↑	↑	Inconclusive
16C2	*ERCC*	↓	↓	Inconclusive
16C3	*CD9*	↓	No signal	Not done
17C4	*CD9*	↓	No signal	Not done
16G4	*FIBULIN*	↓	↓	↓
19G5	Image clone	↓	No signal	Not done
19G5	D2-2 protein	↓	No signal	Not done
19G10	*NDRG*	↓	↓	Inconclusive
16A1	No Match	↑	No signal	Not done
16A2	No Match	↑	No signal	Not done
16A3	No Match	↓	No signal	Not done
20A2	No Match	↓	No signal	Not done
20A4	No Match	↓	No signal	Not done
16G2	No Match	↓	No signal	Not done
16G3	No Match	↓	No signal	Not done

In addition, we anticipate being able to visualize the cellular compartments that stain for these proteins and how these proteins distribute or re-distribute during progression of the disease. These results should provide an indication as at what stage of the disease the genes are down regulated.

In light of the shortcomings of DDRT-PCR, a second molecular biology approach cDNA microarray was used concurrently to add to the assessment of genes that are differentially expressed in oesophageal cancer.

2.2 cDNA MICROARRAY ANALYSIS

2.2.1 OVERVIEW

Classical histopathological parameters and clinical criteria have traditionally been used to classify the different types of cancers and describe their pathogenesis. Treatment of patients has been based on various criteria such as tumour stage, spread (including metastases), patient age and the degree to which cancer cells resemble their normal counterparts. Although a combination of clinical and histopathological parameters are currently used to assess a patients' risk profile, these prognostic variables are indeed rough approximates of the underlying cellular and molecular variations within the complex and multistep process of tumourigenesis. These molecular variations are a reflection of the differential gene expression that characterises the transformation of a normal cell into a neoplastic one. Identification of differentially expressed genes therefore provides useful information about the transformation process, in addition to identifying relevant target genes that can be of diagnostic, prognostic and therapeutic importance in the management of cancer. Secondly, the data generated from differential gene expression is important in the classification of human cancer, as increasingly informative and clinically relevant information based on more detailed molecular analysis of tumours is unveiled.

At present, oncology research has clearly shown that molecular heterogeneity within individual cancer categories is evident in the variable presence of the numerous chromosomal abnormalities such as translocations and deletions of tumours suppressor genes (Alizadeh *et al.*, 2000).

Recent technical and analytical advances make it practical to identify and quantify the expression of thousands of genes in parallel using cDNA microarrays (Schena *et al.*, 1995). The advent of microarrays to analyse DNA, RNA or proteins has identified numerous genes that differentially expressed in cancer and also helped to refine the classification of cancer to levels hitherto unattainable by classical methodologies. Microarrays are able to identify and categorise genes that can be used to define different cell populations based on the measurement of differential gene expression patterns (De Risi *et al.*, 1996). cDNA representing thousands of genes are immobilised onto glass slides/chips or membranes to which labelled (radioisotope or fluorescent) cDNA from normal and cancer cells is applied to determine gene activity. The gene-expression data is analysed using statistical methods with the aim of identifying gene clusters that correlate with the histopathological or clinical data of the patient. In addition, this information provides important clues on the biochemical and molecular changes taking place during the tumourigenesis process. This can be linked to the clinical and histopathological parameters of the disease thus providing important information as to the relationship between alterations of gene activity vis-à-vis the development and progression of tumourigenesis.

To date, a number of malignancies have been analysed by microarrays. Examples of cancers studied so far are oesophageal cancer (Lu *et al.*, 2001), breast cancer (Perou *et al.*, 1999), colon cancers (Hernandez, 2000), ovarian cancers (Ono *et al.*, 2000) and prostate cancers (Xu *et al.*, 2000). Molecular profiling or gene-expression profiling has several advantages in revolutionizing cancer management. First, it will help to identify a small number of marker genes that can be used to stratify patients into molecularly relevant categories which will improve the precision and power of cancer classification and hence diagnosis. Secondly, molecular profiling will possibly provide new avenues for assessing the efficacy of various treatments. Thirdly, gene expression profiling should provide a panel of molecular/biochemical markers whose expression can allow a more predictive and accurate prognostic assessment of patients. Lastly, microarrays could identify genes and pathways that really matter in the tumourigenesis process thereby revealing new targets for therapy (Berns, 2000; Alizadeth *et al.*, 2000; Caldas and Aparicio, 2002).

In summary, cDNA microarray analysis is a useful tool to discover genes frequently involved in neoplasia and provide novel clues to diagnosis, early detection and intervention of SCC of oesophageal cancer.

This section will discuss how cDNA microarray analysis was used to identify differentially expressed genes in normal and corresponding tumour samples of SCC of oesophagus. A schematic representation of cDNA glass microarray analysis is shown in figure 2.2.1.

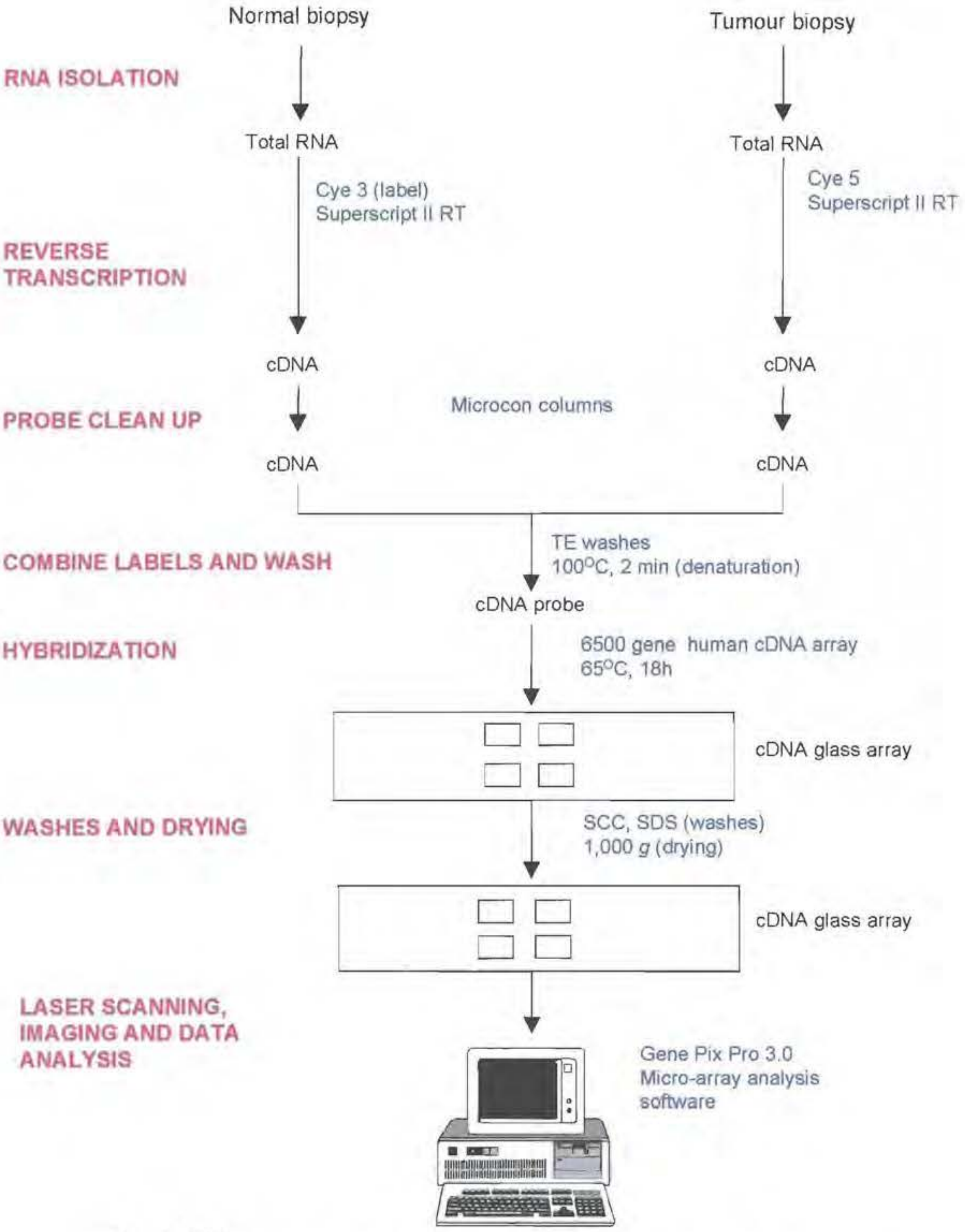

Figure 2.22

A SCHEMATIC REPRESENTATION OF MICROARRAY ANALYSIS.

representation of microarray analysis. Total RNA from patient samples was reverse transcribed into cDNA in the presence of different fluorophore labels as described in section 5.9.1. The labelled cDNA was pooled and washed severally in TE and used as a probe. The labelled probe was hybridized onto a cDNA glass array as described in section 5.9.3. The array was subsequently analyzed for differentially expressed genes using GenePixPro 3.0 software.

2.2.2 RESULTS

2.2.2.1 Oesophageal cancer biopsies

Two squamous cell carcinoma tumour biopsies and their corresponding normal mucosa from two Black patients were used in these studies (Table 2.8). The clinical histories of both patients revealed that neither of them received brachytherapy, radiotherapy nor chemotherapy prior to their surgery.

2.2.2.2 RNA isolation

Total cellular RNA was isolated from the four specimens using the TRIzol method (Chomczynski and Sacchi, 1987). The quality and quantity of the isolated RNA was determined by spectrophotometry ($_{A260/280}$) and formaldehyde agarose (1%) gel electrophoresis (28S/18S ratio).

Table 2.8

PATIENTS USED IN cDNA MICROARRAY STUDIES. The table below is a summary of some of the clinical and phenotypic characteristics of the patients used in the array studies.

NUMBER	GENDER	AGE	PATHOLOGY	STAGE	RACE
1	Male	60	SCC	3	Black
2	Female	62	SCC	3	Black

2.2.2.3 cDNA MICROARRAY ANALYSIS

2.2.2.3.1 Samples

Microarray analysis studies were carried out at the National Cancer Institute (NCI), National Institutes of Health (NIH), USA. Previous studies at NCI had demonstrated that a minimum of 40 μg total RNA per sample was required for successful fluorescent labelling and microarray analysis (Hendricks and Birrer, personal communication). In addition, each experiment was carried out in duplicate to ascertain the reproducibility of the results.

The low yield of RNA from the normal biopsies necessitated that both normal biopsies RNA were pooled. The same was done for the tumour specimens RNA. This was carried out in order to use equal amounts of both tumour and normal RNA in the experiments. In addition, because of the low yield of normal RNA, reciprocal labelling was carried out on both sets of the pooled RNA to check for reproducibility of results.

2.2.2.3.2 Labelling and probe clean-up

The normal and tumour RNA samples from the 2 patients were pooled in the same ratio and reciprocally labelled using Cy5-AP3-dVTP and Cy3-AP3-dVTP (Amersham Life Sciences), and the probes separated from the unincorporated label as described in sections 5.9.1 and 5.9.2.

2.2.2.3.3 Hybridization

The clean tumour and normal probes were pooled for each reciprocal labelling experiment i.e. Cy5 (tumour) was pooled with Cy3 (normal) probe, while Cy5 (normal) probe was pooled with Cy3 (tumour) as described in section 5.9.2.

Each pooled set of probes was hybridized to human gene chip contained 6,500 genes (Advanced Technology Centre, NIH, USA) as described in section 5.9.3 These genes were classified into several categories including, proto-oncogenes and oncogenes tumour suppressor genes, growth factors, signalling pathways and receptors, housekeeping genes, KIAA protein genes and ESTs (Expressed Sequence tags) among others.

2.2.2.3.4 Scanning of fluorescence intensities

The intensity of Cy3 and Cy5 signals were analysed using a scanner (Axon instruments, USA) and GenePix Pro 3.0 microarray analysis software (Axon, instruments). In addition, the fluorescence intensities were normalized and corrected to account for background bias on the array using the GenePix Pro 3.0 microarray analysis software.

2.2.2.3.5 Identification of under expressed and over expressed genes

Fluorescence intensities in the Cy3 and Cy5 channel for each spot was extracted into an Excel file and the Cy3/Cy5 ratio calculated. Fairly conservative criteria were used to identify differentially expressed genes based on criteria previously established at the Advanced Technology array facility at the NIH. The following criteria was used:

(a) Spots with a mean Cy3/Cy5 ratio ≥ 1.7 were considered over expressed.
(b) Spots with a mean Cy3/Cy5 ratio ≤ 0.6 were considered under expressed.
(c) Spot intensities had to be ×2 above background for consideration.
(d) All spots were visually inspected to exclude artefactual signals (e.g. small specks and scratches of fluorescence unrelated to the applied spots and badly applied DNA spots).

The selected spots (genes) from either Cy3 or Cy5 intensity "channels" were indexed based on the ratio of their mean intensities. The sorted lists (Cy3 and Cy5) were merged and final list sorted on ratio of means. Genes from the final sorted list were grouped into 4 categories based on their intensities.

(a) High intensity/high mean ratio cluster of genes.
(b) High intensity/low mean ratio cluster of genes.
(c) Low intensity/high mean ratio cluster of genes.
(d) Low intensity/low mean ratio cluster of genes.

The rationale of these groupings was to select genes from each category, carry out Northern blot analysis and compare the ratio of expression both from the microarray and Northern blot analysis in order to determine whether the spots (genes) were truly differentially expressed.

2.2.2.3.6 Microarray results

The first array was successful as analysed by the scanner. However, the reciprocal array failed due to poor incorporation of the Cy5 fluorescent label. Consequently, only the results of the first array were used for subsequent analysis. A total of 370 genes were found either to be up or down regulated on the array of which 10 genes were selected for Northern blot analysis (Table 2.9) based on the perceived role of the gene in tumourigenesis and carcinogenesis in general. Representative genes involved in apoptosis, cell cycle regulation, xenobiotic metabolism, cell adhesion, metastasis, growth factors, genes, anti-oxidants and transcription factors were selected (Table 2.9)

2.2.2.4 Probes for Northern blot analysis

A probe for each of the selected genes was acquired in form of cDNA cloned into the pT7T3D-pac plasmid (Pharmacia) from Research Genetics (USA). The clones were transfected into competent XL1 blue *E.coli* cells and the inserted cDNA sequences were verified as described in section 5.6. All the cDNA sequences of the selected genes with the exception of CDC 25, CYP- P450 , INTEGRIN and INSULIN-LIKE GROWTH FACTOR did not correspond to the published image consortium sequences of the cDNA immobilized on the array. Consequently, specific primers were designed to the CDC 25B, CYP- P450 , INTEGRIN $\alpha 6$ and INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 3 to generate specific PCR probes using DNA from serum of normal control human subjects (figure 2.23). The PCR products were cloned into pGEM-T easy vector and sequence verified as described in section 5.6 before their use as probes for Northern blot analysis.

Table 2.9

GENES SELECTED FOR NORTHERN BLOT ANALYSIS. A summary of the different genes and their expression levels in tumour relative to normal biopsies. The genes above were selected for further analysis by Northern blot hybridization. Abbreviations: **CDC 25** (cell division cycle 25B), **CYP P450** (Cytochrome P450 family 4A class 11), **FGFR** (Fibroblast growth factor receptor 2), **GR** (Glutathione reductase), **ILGF** (Insulin–like growth factor binding protein 3), **MMP** (Matrix metalloproteinase 14), **SCY20-LARC** (small inducible cytokine subfamily A and liver activation regulated chemokine), **TIMP** (Tissue inhibitor of matrix metalloproteinase 2), ↓ and ↑ (decreased and increased expression, respectively), Image number refers to the clone ID number registered at the IMAGE consortium (www.ncbi.nlm.nih.gov).

GENE	IMAGE NUMBER	PROBABLE FUNCTION (S)	MEAN ratio EXPRESSION (TUMOUR: NORMAL)
CASPASE	323500	Apoptosis	0.56↓
CDC 25	786067	Cell cycle	1.85↑
CYP P450	129082	Xenobiotic metabolism	1.84↑
FGFR	809464	Growth factor	1.69↑
GR	460538	Anti-oxidant	0.75↓
INTEGRIN	141395	Cell adhesion	2.11↑
ILGF	1407750	Cell cycle	6.15↑
MMP	270505	Cell adhesion / metastasis	2.69↑
SCYA20-LARC	927027	Chemokine	10.49↑
TIMP	325072	Metastasis	0.72↓

2.2.2.5 Northern blot analysis

Northern blot analysis was carried out using total cellular RNA (1μg) for each sample as described in section 5.1.4 and 5.1.5. The probes used in these experiments were generated as described in section 5.5. Briefly, the plasmids with the correct (confirmed sequence) inserts were grown up in LB broth, purified and the inserts cut from the plasmids by restriction nuclease digestion. The inserts were isolated, purified and quantitated by agarose gel electrophoresis (figure 2.24).

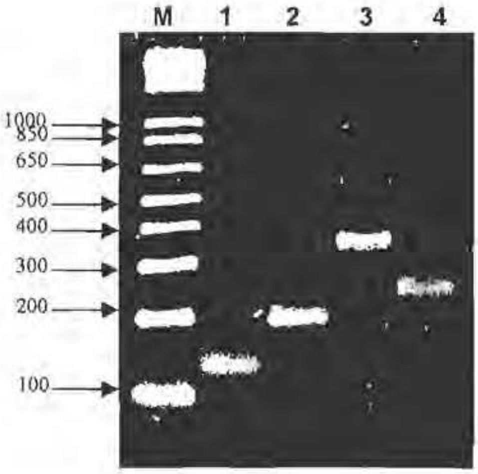

Figure 2.23

GENES SELECTED FROM MICROARRAY ANALYSIS WHOSE PROBES WERE GENERATED BY PCR AMPLIFICATION. Cytochrome P-450 4A11 (**1**), Integrin α_6 (**2**), Insulin-like growth factor binding protein 3 (**3**), and cdc 25B (**4**) cDNA gene probes for Northern blot hybridization experiments were generated by PCR amplification using primers specifically designed to each gene as described in sections 5.2.4 to 5.2.7. The products of PCR amplification were electrophoresed on a 1.5% agarose gel. The gel was stained with ethidium bromide and visualized under UV light. The size of the PCR products were verified by comparing the molecular of the PCR product with the estimated weights of the PCR product size from the primer design protocol. Abbreviation: **M**; 1 kb DNA ladder.

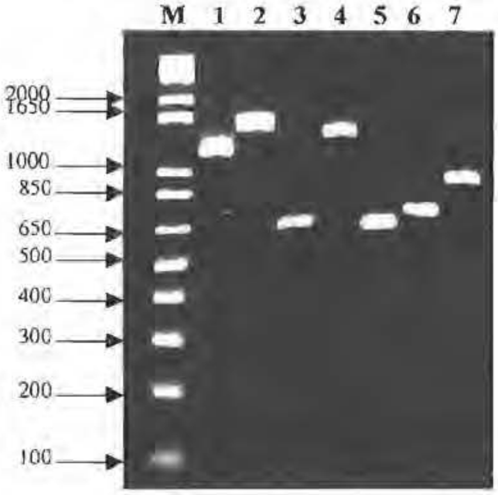

Figure 2.24

GENES USED AS PROBES FOR NORTHERN BLOT ANALYSIS. Genes selected from microarray analysis were used as probes in Northern blot experiments. These genes were acquired in form of clones (cloned into pT7T3D-pac vector [Pharmacia]) from Research Genetics. The clones were transfected into competent XL 1 blue *E.coli* cells using the calcium phosphate method as described in the section 5.5. Plasmid with correct insert sizes were sequence verified as described in section 5.6 and subsequently digested by using Eco RI and HIND III (Promega) restriction enzymes as described in section 5.5.7. The inserts were purified by agarose gel electrophoresis as described in section 5.4.3 and 5.4.2. The molecular weights of the inserts ranged from 650 to 1500 base pairs. Abbreviations: **M** (1Kb plus DNA ladder), **1** (Caspase 6), **2** (Fibroblast growth factor receptor 2), **3** (Glutathione reductase), **4** (Matrix metallo-proteinase 14), **5** (Tissue inhibitor of metallo-proteinase 2), **6** (Tissue inhibitor of metallo-proteinase 2), **7** (Small inducible cytokine sub-family A and Liver activation regulated chemokine).

The results of Northern blot hybridization experiments are summarized in Table 2.10 The expression patterns of *CDC 25, INTEGRIN*, and *MMP* in all the patients tested were similar to the results observed in microarray analysis. Glutathione reductase expression was flipped (opposite) to those of the microarray analysis. The remaining 6 genes failed to give a signal (Table 2.10).

Table 2.10

EXPRESSION PATTERNS OF GENES IN NORTHERN BLOT ANALYSIS. A summary of the results of the expression patterns and levels (fold changes) of the different genes that were selected from microarray and further analysed by Northern blot analysis. ↑ (Up regulated) and ↓ (Down regulated).

GENE	MICROARRAY (tumour)	NORTHERN BLOTS (tumour)
CASPASE	↓ (0.56)	No signal
CDC 25	↑ (1.85)	↑ (4 to 9)
CYP P450	↑ (1.85)	No signal
FGFR	↑ (1.69)	No signal
GLU.REDUCTASE	↓ (0.75)	↑ (1.4 to 1.9)
INTEGRIN	↑ (2.12)	↑ (2 to 6)
ILGF	↑ (6.15)	No signal
MMP	↑ (2.69)	↑ (1.4 to 3.3)
SCYA20-LARC	↑ (10.49)	No signal
TIMP	↓ (0.72)	No signal

2.2.3 Discussion

2.2.3.1 cdc 25B

The activities of various cyclin-dependent kinases (cdK) and cyclin complexes that regulate the different cell cycle transitions influence the progression of the cell cycle (Morgan, 1995). These kinases are regulated by both binding to regulatory proteins and by phosphorylation.

cdc25 was one of the first regulatory components of the eukaryotic cell cycle machinery to be isolated. In humans, cdc25 phophatases are a multigene family comprising of cdc25A, B and C (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Nagata *et al.*, 1991). The members share approximately 50% homology at the amino acid sequence level. The highest homology is found within the catalytic domain, while the N-terminal domains are very different suggesting that these phosphatases have different functions in cell cycle regulation.

cdc25B consists of multiple alternately spliced forms cdcB1, B2 and B3, of which cdc25B2 and cdc25B3 are expressed at detectable levels of protein (Baldin *et al.*, 1997b; Forrest *et al.*, 1999).

The microarray data shows that the cdc 25B is up regulated by a factor of 1.8. This factor was determined by comparing the fluorescence intensity value of the Cy3 channel (tumour sample) (889) to the intensity value for the Cy5 channel (510) (normal sample). Further analysis of the expression levels of cdc 25B mRNA by Northern blot analysis using human biopsies show that the cdc 25B gene is up regulated by a factor of between 4 and 9 in all the tumour specimens compared to the corresponding normal oesophageal mucosae (figure 2.26). The results of the Northern blot analysis confirm the microarray data however, the former shows a higher up regulation factor than the latter suggesting that the cdc 25B is probably up by a factor higher than 1.8. The variation in the up regulation of the cdc25B gene may be indicative of the differences in the de regulation of the cell cycle in the cells of different grades. Based on the Northern blot results, we can infer that selecting "spots" (genes) with Cy3/C5 ratios of greater than 1.8 will positively identify genes that display significant differences in RNA expression.

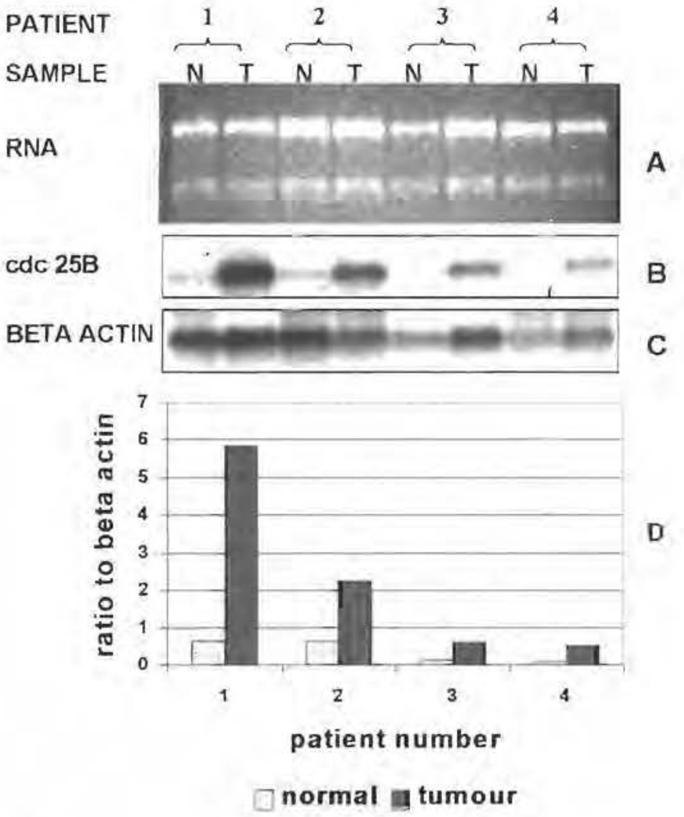

Figure 2.25

EXPRESSION OF CELL DIVISION CYCLE 25B (cdc 25B) GENE IN NORMAL AND TUMOUR BIOPSIES. Total RNA from normal (**N**) and corresponding tumour (**T**) from four different patients (**A**) was used in Northern blot experiments as described in sections 5.1.4 and 5.1.5. $\alpha[^{32}P]$dCTP Radiolabelled probe was hybridized to nitrocellulose membrane using ULTRAhyb buffer. After several washings, the membrane was exposed to X-ray film overnight (**B**). Beta actin was used as an internal control (**C**). The final counts of each sample were calculated as described in section 2.1.2.6 (**D**). The cdc 25B gene was significantly over expressed in all the tumour samples.

These results are consistent with previous studies that have shown an over expression of cdc25B in different types of cancer. For example, cdc25B is over expressed in breast (Galaktionov *et al.*, 1995), lung (Wu *et al.*, 1998), head and neck (Gasparotto *et al.*, 1997), squamous cell carcinoma of the oesophagus (Nishioka *et al.*, 2001) and human oesophageal squamous cell carcinoma cell lines (HKESC-1 and HKESC-2) (Hu *et al.*, 2001).

cdc25B functions at the G_2/M transition in cell cycle. Though its role is not fully understood it is has been shown that the phosphatase accumulates rapidly as the cells enter G_2 phase and is then rapidly destroyed as cells exit mitosis by cyclin A/ cdc2-dependent phosphorylation mechanism (Gabrielli *et al.*, 1996; Baldin *et al.*, 1997a; Forrest *et al.*, 1999).

cdc 25B may also be responsible for the activation of cyclin A/ cdk2 in early G_2 phase (Goldstone *et al.*, 2001) and also initiate the activation of cyclin B/ cdc2 complex, possibly in the cytoplasm at prophase by removal of the inhibitory phosphatases thereby triggering the entry of the cell into mitosis (Nishijima *et al.*, 1997; De Souza *et al.*, 2000).

The over expression of cdc25B would essentially ensure that the cell is rapidly ushered into the mitotic phase consequently stimulating cell proliferation. This is primarily achieved by cdc25B maintaining a sufficient pool of active cyclins A and B complexes.

Further research has shown that cdc25A and B are potential human oncogenes since they are able to transform primary mouse fibroblasts in co-operation with mutated Ha-ras or loss of *Rb* (Galaktionov *et al.*, 1995). Therefore cdc25B deregulation will stimulate and promote tumourigenesis. These results not only corroborate previously published data on cdc25B but also support the importance of this gene in tumourigenesis.

2.2.3.2 Integrin α_6

Integrins are a family of structurally, immunochemically and functionally related cell-surface heterodimeric receptors, which mediate cell migration and adhesion (Hynes, 1987). They are composed of non-covalently linked α and β sub-units. To date, over 20 family members have been described. The diversity of integrins is expanded further by alternative splicing of the mRNA encoding some of the integrin sub-units, posttranslational modifications and interactions with other cell-surface and intracellular molecules (Green *et al.*, 1998; DeMelker and Sonnenberg, 1999).

Each integrin sub-unit contains an extracellular domain, a transmembrane region and a cytoplasmic domain (Hynes, 1992). There is little homology between the cytoplasmic domains of the α sub-units except for seven conserved amino acids (Demelker and Sonnenberg, 1999).

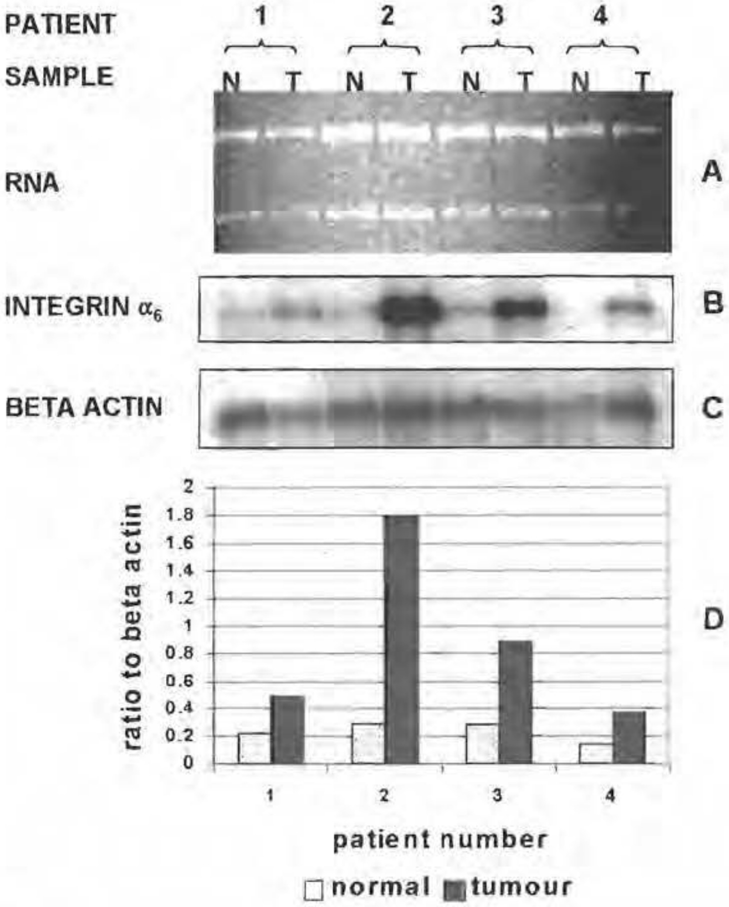

Figure 2.26

EXPRESSION OF INTEGRIN α_6 IN NORMAL AND TUMOUR BIOPSIES. RNA from normal (**N**) and corresponding tumour (**T**) from four different patients (**A**) was used in Northern blot experiments as described in section 5.1.4 and 5.1.5. The radiolabelled Integrin probe was hybridized to the membrane overnight. This was followed by several washes in SSC buffers and exposure to X-ray film overnight (**B**). Beta actin was used as an internal control (**C**). The final counts per minute of each sample were calculated as described in section 2.1.2. (**D**). The Integrin α 6 gene was significantly over expressed in the tumour specimens of all the patients examined.

The microarray data shows that integrin α_6 gene is up regulated by a factor of 2 in the tumour sample (Cy3 intensity of 1102) compared to the normal sample (Cy5 intensity of 560). Similarly, Northern blot analysis revealed that integrin α_6 was up regulated in all the tumour specimens (by a factor of between 2 and 6) compared to the corresponding normal tissues (figure 2.26). The Northern blot results confirm the microarray data, in addition to revealing the differential up regulation on integrin α_6 in the different patients. This suggests that the integrin α_6 gene is differentially up regulated in tumours of SCC of the oesophagus. This has not been previously demonstrated, however, it is probable that the gene expression is tumour grade dependent

The results from the microarray analysis and Northern blot analysis are in agreement with previously published data on integrin α_6 expressions in other cancers such as SCC of larynx, cervix and lung (Kimmel and Carey, 1986; Costantini *et al.*, 1990).

A hallmark of integrins is the ability of individual family members to recognise and bind multiple ligands. This repertoire of ligands includes a large number of extracellular matrix proteins such as collagens, fibronectins; laminins. The number and diversity of these ligands reflect the primary function of integrins in cell adhesion to extracellular matrices (Plow *et al.*, 2000).

The role of integrins in carcinogenesis is multifaceted as they have been implicated in an array of roles including cellular differentiation, proliferation, prevention of apoptosis, angiogenesis, matrix degradation and cellular adhesion (Jones *et al.*, 1997; Sheppard, 1996; Agrez *et al.*, 1994; Wechsel *et al.*, 1999; Li *et al.*, 1998; Brooks *et al.*, 1994a; Brooks *et al.*, 1994b; Brooks *et al.*, 1996). Examples of integrin involvement in these processes are exemplified by the constitutive expression of $\alpha_6\beta_4$ in breast carcinoma cells that promotes invasion through matrigel (Shaw *et al.*, 1997) while induction of integrins β_3 (Filardo *et al.*, 1995) in melanoma cell line increases their metastatic potential. Additional studies have shown that integrin $\alpha_v\beta_3$ is strongly expressed at the invasive front of malignant melanoma cells and angiogenic blood vessels (Brooks *et al.*, 1994). This suggests that integrins play an important role in invasion and angiogenesis. On the other hand, the binding of integrins to ECM ligands initiates several pro-survival mechanisms to prevent apoptosis that essentially promotes tumourigenesis (Hood and Cheresh, 2002).

An up regulation of integrin α_6 in oesophageal cancer therefore is indicative of the probable importance of this molecule in oesophageal cancer in terms of supporting cellular signalling, matrix degradation and remodelling, cell survival during invasion and metastasis. This however, may not be universally applicable in all cancers; for example, reduced expression of integrin α_1, α_6, β_1, and β_4 has been associated with the formation of neoplasms in breast epithelial tissue (Mizejewski, 1999). The question arising from this observation is whether the activities of integrins are cancer tissue or cancer cell type specific?

These results add a new member to the spectrum of cancers that exhibit deregulation of integrins while shedding more light on the expression patterns of integrin α_6 in various OC cell lines. This information not only strengthens the cause for using integrins as potential targets in the development of therapeutic interventions but also underscores their importance as candidate biomarkers for the various tumourigenesis phases.

2.2.3.3 Matrix metalloproteinase 14

Matrix metalloproteinases (MMPs) are a family of enzymes that are responsible for degradation of virtually all components of the ECM. To date, over 28 mammalian MMP family members divided broadly into 2 groups; either soluble (or secreted type) and membrane-type MMPs (MT-MMPs) have been described (Reviewed by Egelblad and Werb, 2002).

Membrane-type MMPs (MT-MMPs) are tethered to the plasma membrane either through a transmembrane domain (Sato *et al.*, 1994; Cao *et al.*, 1995) or a glycosylphosphatidyl mositol anchor (Itoh *et al.*, 1999; Kojima *et al.*, 2000).

MMP14 (MT1-MMP or MT-MMP1) is probably the best-studied MT-MMP. It is expressed as 63kDa protein on the surface of on a number of a number of cells such as macrophages (Sato *et al.*, 1997), endothelial cells (Harada *et al.*, 1998) and invasive cancer cells (Seiki, 1999).

The microarray data show an up regulation of MMP 14 by a factor of 2.6 (Cy3 intensity, 647; Cy5 intensity, 243). Similarly, Northern blot analysis results are in general agreement with the microarray data. The tumour specimens of 3 of the 4 patients studied displayed an up regulated of MMP14 by a factor of between 1.4 and 3.3 (figure 2.27). This range is within the factor of 2.6 previously recorded in microarray analysis suggesting that MMP 14 is differentially up regulated in tumour tissues. The MMP 14 expression levels in patient number 4 were unaltered. This suggests that perhaps MMP 14 is not altered in the tumour samples of this particular patient.

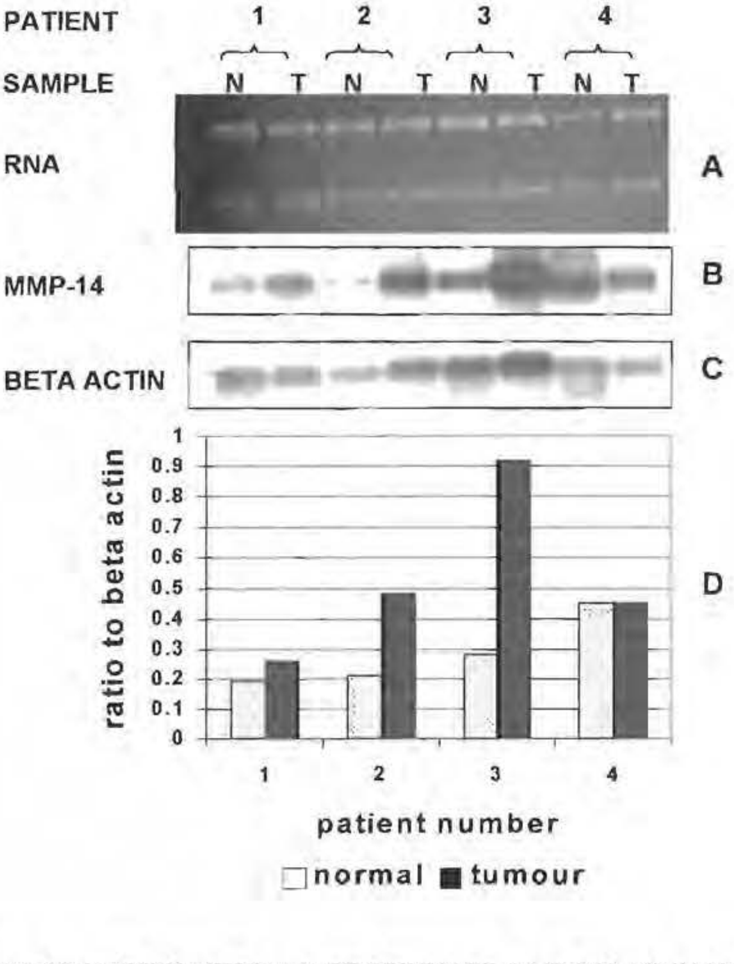

Figure 2.27

EXPRESSION OF MATRIX METALLO-PROTEINASE 14 (MMP-14) IN NORMAL AND TUMOUR BIOPSIES. RNA from normal and corresponding tumour from 4 different patients (**A**) was used in Northern blot experiments as described in sections 5.1.4 and 5.1.5. The radiolabelled MMP 14 probe was hybridized to the nytran membrane overnight an thereafter washed several times in SSC buffers before exposure to X-ray film (**B**). Beta actin was used as an internal control (**C**). The final counts of each sample were calculated as described in section 2.1.2.(**D**). The MMP 14 gene was over expressed in the tumour specimens of patients 1, 2 and 3 while its expression was unaltered in tumour sample of patient number 4.

The above results are in line with previously published data on the expression of MMP 14 in cancer cell lines. For example, MMP14 is up regulated in head and neck, vulvar, lung, breast, colon and liver carcinomas (Sato *et al.*, 1994; Okada *et al.*, 1995; Harada *et al.*, 1998; Johansson *et al.*, 1999).

The role of the MMP family has been shown to be multi-faceted. They are involved in a wide range of activities including cell growth and differentiation, apoptosis, migration and invasion among others (Nagase and Woessner, 1999).

The up regulation of MMP14 is not unexpected because this can be related to important cancer characteristics such as metastasis. Before the latter can take place, the invading cell must degrade the ECM components so as to facilitate their movement across the ECM. The ECM is composed of different types of molecules that not only serve to attach the cell to the basement membrane but also are also important in cell signalling.

MMP14 has been shown to degrade some of these components. For example, it cleave collagen types I, II and III, fibronectin, laminin1 and 5. These are important structural components that anchor the cells to the basement membrane. Secondly, MMP14 cleaves cell receptors that are important in cell-to-cell interactions such as CD44 and integrin α_v. This therefore facilitates the detachment of the invading cells from their neighbours and surrounding matrix (Egelblad and Werb, 2002). Thirdly, MMP14 activates other MMPs such as proMMP2 (gelatinase A) (Sato *et al.*, 1994) and proMMP13 (pro-collagenase 3) (Knauper *et al.*, 1996). The activation of these multiple proteinase cascades promotes the degradation of additional ECM components and therefore augments invasion and metastasis. This link has been previously reported (Nakahara *et al.*, 1997; Tsunezuka *et al.*, 1996).

Recent studies have implicated MMP14 in the regulation of angiogenesis (Kolb *et al.*, 1997; Kim *et al.*, 2000). An up regulation of MMP14 therefore is likely to promote angiogenesis, which is an important pre-requisite for the establishment and proliferation of the newly metastasised cells in their new location.

The results of MMP14 expression are in line with previously published observation and therefore reiterate the importance of MMP14 in the development of cancer of the oesophagus.

2.2.3.4 Glutathione reductase (GR)

Glutathione and its related enzymes play a vital role in the detoxification pathways of a wide variety of compounds including xenobiotics, drugs and oxidative stress products.

The key glutathione related enzymes involved in the defense against the reactive intermediates of dioxygen reduction are superoxide dismutase (SOD) catalase (CT), glutathione peroxidase (GP_x) and glutathione reductase (GR_x). The glutathione anti-oxidant system of the cell prevents the formation of hydroxyl radicals. Catalase and GP_x convert hydrogen peroxide to water and O_2 in the presence of reduced glutathione (that is converted is the oxidized form). Superoxide dismutase which converts superoxide radicals (O_2-) into H_2O_2 and is protected against inactivation by CT and GP_x. Reciprocally; SOD protects GP_x against inhibition by O_2 (Blum and Fridovich, 1985).

GR_x is principally responsible for the maintenance of the reduced glutathione (GSH) pool. This enzyme essentially regenerates reduced glutathione from the oxidized form; hence GR_x is critical in allowing a constant redox balance of glutathione.

Molecular biology studies have suggested that oxygen derived free radicals may inflict damage on proteins and DNA. For example, hydroxyl radicals generated from degradation of hydrogen peroxide have been shown to cause mutagenic damage to DNA by hydroxylation of the bases of DNA, especially, guanine to form 8-hydroxyguanine (Floyd, 1990). This mutagenic event causes a change in base pairing from a G:C base to a 8 hydroxylgluanine: adenine pair, which may eventually lead to a substitution of G by T in the subsequent progeny strand causing a G to T transversion (Kasai and Nishimura, 1991).

The GR_x gene was up regulated by a factor of 1.4 to 1.9 in 3 of the tumour samples compared to the corresponding normal specimens. These results are in contrast to those earlier observed from microarray analysis that revealed a down regulation of GR_x in the tumour samples by a factor of 0.75 (table 2.9).

The microarray results show a down regulation of GR_x in tumour compared to the normal sample. These results support previously published data that has shown appreciable decreases in the activities of GP_x, GR_x and glutathione-S-transferase in cervical carcinomas compared to their corresponding normal tissues (Balasubramaniyan *et al.*, 1994).

Although these studies did not assay for the levels of GSH, the microarray results suggest that GSH level is decreased. Previously published work shows that indeed GSH levels are low in OC patients compared to controls (Hunter *et al.*, 1992; Levy *et al.*, 1999). This makes sense given that glutathione related enzymes require GSH to perform their anti-oxidation and detoxification functions. A down regulation of GR_x and a subsequent reduction in GSH would suggest that glutathione related enzymes are principally non-functional and thus render the cancer cells unprotected against oxyradicals. Such a situation could promote the development of genetic mutations and genomic instability.

The Northern blot results however contradict the microarray results. The results of the former demonstrate a general up regulation rather than a down regulation of GR_x in the tumour samples. An up regulation of GR_x has been previously reported in cancer tissues. For example, in lung cancer tissues whereas there is a lower level of GSH in neoplastic tissue compared to normal the converse are true for glutathione-associated enzymes (Saydam *et al.*, 1997).

It is probable that an up regulation of GR_x may indeed be a reflection of important metabolic changes or detoxification processes that accompany the promotion and/or the progression of cancer. It is therefore plausible that GSH is synthesized *de novo* rather than from the recycling of oxidised glutathione (Perquin *et al.*, 2000). This proposal is strengthened by previous reports that have shown an up regulation of enzymes such as α-glutamyl transferase and α-glutamylcysteine sythetase that provide important precursors in the synthesis of glutathione. The up regulation of the above enzymes has also been linked to the increased resistance of cancer cells to drugs such as cisplatin (Godwin *et al.*, 1992; Tipnis *et al.*, 1999). The up regulation of such rate limiting enzymes could therefore lead to elevated *de novo* synthesis of GSH. Whether the GR_x gene is up regulated by this alternative mechanism in this situation is a matter of conjecture. Lastly, tumour cell populations are heterogeneous in grade. Although cancer cells may have a poor redox status as previously observed, it is probable that this is not a general phenomenon rather it depends on the grade of the cancer cell. Some cells may have therefore developed an adaptive process to synthesize GSH in order to circumvent the poor redox status. The predominance of the latter population of cell types may partly account for the results observed in Northern blot analysis.

2.2.3.5 No signal genes

An important draw back of the microarray analysis was that the results were based on a single successful experiment. Although this raises the question of reproducibility, the Northern blot experiments successfully confirmed the differential expression of 3 genes from the microarray data. The expression levels were altered in a similar way in the Northern blot hybridization experiments as in the microarray analysis.

A significant number of genes analysed by Northern blot analysis did not show a signal after 2 independent hybridization experiments. This was unexpected given that some of the genes for example; SCY20-LARC was over expressed by more than a factor of 10 in the tumour compared to the normal sample in the microarray analysis. There are several reasons for these negative results. First reason is the microarray data was based on a single successful experiment. Consequently it is likely that many of the differentially expressed genes may have been artefacts. Several experiments would have minimized this by "optimising" truly differentially expressed genes.

Secondly, the tumour tissues from the biopsies used in these experiments were not micro-dissected from normal tissue. The consequence of this is the presence of contaminating normal tissue, none-cancerous cells and a high percentage of heterogeneous populations of tumour cells. This would probably prevent the observation of subtle changes in the expression levels of different genes.

Thirdly, although Northern blot analysis is an easily accessible and affordable technique for evaluating mRNA expression, it is not sensitive enough in detecting subtle changes in mRNA expression levels. Real time PCR would offer greater sensitivity in detecting these changes, especially, in the light of the limiting quantities of the starting material.

Fourthly, the fact that the microarray analysis could not repeated several times was a major handicap in definitively identifying differentially expressed genes. This can be attributed to the severe limitation in the number of patient samples studied and number of experiments carried out. This therefore was a major stumbling block in identifying and eliminating possible "artefacts" or false positives that may have emerged in the final sort list of genes that were further investigated by Northern analysis.

Finally, the DNA sequence of some of the image clones acquired from Research Genetics (cdc 25B, CYP P-450 4A11, integrin α_6 and insulin-like growth factor binding protein 3) did not correspond to the registered sequence of the clone in the IMAGE consortium (www.ncbi.nlm.nih.gov). The personnel at the array centre of ATC, NIH, acknowledged this problem and consequently advised on sequence verification of the clones prior to their use as probes for Northern blot analysis. This raises questions about the quality of the array and more specifically the accuracy of the image clones used on the array. In light of the above revelations, the validity of the results of some of the "no signal" genes is questionable. In order to verify these results, specific primers to these genes need to be synthesized and the expression levels and patterns of these genes determined by more sensitive techniques such as real time PCR.

2.2.3.6 Use of cell lines to determine gene expression

This study undertook to study the gene expression levels and patterns within oesophageal cancer cell lines of some of the genes previously shown to be differentially expressed in microarray analysis. A major drawback of these experiments was the unavailability of cells that could be used as a suitable control or the equivalent of the normal squamous epithelium of the oesophagus. Consequently, normal human oesophageal biopsies were used as the control. These tissues however, were not micro dissected to specifically isolate the epithelial cells. This therefore raises questions as to their suitability in representing normal epithelial cells. Experiments carried out using cell lines and the normal tissue biopsies were inconclusive in determining the differential expression patterns and levels of the genes in cell lines.

2.2.4 Disadvantages of microarray analysis

Gene expression profiling using cDNA microarrays has become a highly popular technique for high throughput screening of genes in various pathological conditions. This technique however, remains inaccessible due to the prohibitive cost of its installation, operation, maintenance and management.

Secondly, the success of microarray heavily depends on the reverse transcription of mRNA. As discussed previously, it is still debatable as to whether the majority of rare species of mRNA are amplified as efficiently as the abundant ones. It is therefore possible that some species of mRNA are not efficiently amplified and hence are not detected.

Thirdly the choice of ready-made microarray chips is an important consideration. In spite of our pursuit to identify differentially expressed genes in OC, it is of uttermost importance to prudently select arrays depending on the aim of the study. As earlier stated, cancer is a multistage disease in which genes are differentially regulated and expressed. The choice of the array should take into account the types or possible types of genes vis-à-vis the grade and stage of the tumour. It is possible that genes that may be relevant in SCC of oesophageal cancer are indeed irrelevant in other squamous cell carcinomas such as cervix or lung. In addition, since cancer tissues contain various types of cells such as mesenchymal cells, blood cells and vessels and cells involved in inflammation, the gene expression profiles in micro-arrays will inevitably reflect gene profiling of these cells as well. Thus interpretation of data needs to be handled with care so as not to exaggerate the possible involvement of genes that may possibly have very little to do with classification or development of oesophageal cancer.

2.2.5 Conclusion

Microarray analysis identified several genes as differentially expressed in OC. Northern blot analysis corroborated these findings. Furthermore, some of these genes were altered in a similar “direction” and level in the Northern blot analysis as in the microarray.

Previous studies have found some of these genes such as MMP14, cdc25B and integrin α_6 to be differentially expressed in other cancers and also to play an important role in tumourigenesis

These studies successfully identified differentially expressed genes in oesophageal cancer using microarray analysis and reiterate the probable importance of these genes in oesophageal cancer. In addition, there are several genes that were not confirmed to be differentially expressed using Northern blot analysis. The expression levels of these genes need to be verified and the genes further evaluated for their importance in OC using *in vitro* based studies.

Table 2.10

EXPRESSION PATTERNS OF THE VARIOUS GENES ANALYSED BY NORTHERN BLOT HYBRIDIZATION EXPERIMENTS. Summary of the expression patterns of the different genes that were selected from microarray analysis and further verified by Northern blot analysis. Abbreviation: ↑ (up regulation of gene), ↓ (down regulation of gene).

GENE	MICROARRAY (T)	NORTHERN (T)
CASPASE	↓	No signal
CDC 25	↑	↑
CYP P-450	↑	No signal
FGFR	↑	No signal
GLU. REDUCTASE	↓	↑
INTEGRIN	↑	↑
ILGF	↑	No signal
MMP	↑	↑
SCYA20-LARC	↑	No signal
TIMP	↓	No signal

Table 2.10

EXPRESSION PATTERNS OF THE VARIOUS GENES ANALYSED BY NORTHERN BLOT HYBRIDIZATION EXPERIMENTS. Summary of the expression patterns of the different genes that were selected from microarray analysis and further verified by Northern blot analysis. Abbreviation: ↑ (up regulation of gene), ↓ (down regulation of gene).

Antisera to cdc25B, MMP14 and integrinα_6 were acquired and used in immunohistochemical studies. These experiments seek to ascertain the expression patterns of these genes in a larger patient sample. The data obtained from these studies will be correlated with the expression levels of the genes, disease progression and patient's survival.

Chapter 3 will attempt to delve into the use of immunohistochemical techniques to understand gene expression and its relationship to cellular location. This will be linked to OC progression and patient survival to further understand the impact of the distribution of these genes on cancer progression.

Chapter 3

Immunohistochemistry

3.1 Overview

Immunohistochemistry is an extremely useful immuno-detection technique to identify and localize antigens or tissue (cellular) constituents. It is an important tool in tissue pathology that allows for the precise examination of aspects of cell function and their relationship to cell and tissue morphology. This technique has not only improved our knowledge in understanding disease processes but also their diagnosis. The advantage of this technique in diagnostic applications is its sensitivity. It is able to reveal small amounts of antigen with minimal background staining; it is reliable, reproducible, cost effective, safe, and versatile and can be applied to virtually all types of tissues and tissue preparations. This chapter will discuss the use of immunohistochemistry to study the cellular localisation and levels of expression of cdc25B, fibulin-1D, integrin α_6, MMP14 and NDGR-1 vis-à-vis the development and progression of oesophageal SCC.

3.2 Patient samples

Formalin fixed paraffin-embedded oesophageal resections of 70 patients were used. These resections were carried out in the Department of Cardiothoracic Surgery, Groote Schuur Hospital between the years 1983 and 2000. The ages of these patients ranged from 23 to 80 years with a mean age of 53.3 ± 10.1 years. 50 males and 20 female patients were used. The number of patients used in the study was restricted by the lack of sufficient numbers of embedded tissue blocks. The clinico-pathological data of these patients are summarised in Table 3.1. Two independent pathologists confirmed the histopathologic classification using the World Health Organization (WHO) classification system and the TNM classification (WHO, 1999). The pathologic staging was classified using the International Union against Cancer (UICC) criteria (Sorbin *et al.*, 1997). The suitability of each section was assessed by analysing the hematoxylin/ eosin stained sections and thereafter matching each slide to its identical paraffin block. The selected blocks were sectioned into 2μM thick sections using a microtome as described in 5.10.3. A small sample of 5 patients with dysplasia, 8 patients with oesophagitis 6 patients with Barretts oesophagus and 10 metastatic lymph nodes were included in this study for the purpose of comparison.

CHARACTERISTIC	**NUMBER OF PATIENTS**
Histological stage	
0	1
1	4
2A	33
2B	6
3	25
4	0
Tumour grade	
Carcinoma *in situ*	1
Well differentiated	11
Moderately differentiated	47
Poorly differentiated	11
Survival	
Alive	11
Lots to follow up	12
Deceased	47
Race	
Black	26
Mixed ancestry	36
Caucasian	8
Gender	
Males	50
Females	20
Other oesophageal conditions	
Dysplasia	5
Barretts oesophagus	6
Oesophagitis	8

Table 3.1

SOME CLINICO-PATHOLOGICAL CHARACTERISTICS OF THE PATIENTS USED IN IMMUNOHISTOCHEMICAL STUDIES. A total of 70 patients were used in these studies. These patients underwent oesophageal resection at the Groote Schuur hospital in Cape Town from the years 1983 to 2000.

3.3 Results and discussion

3.3.1 Fibulin-1D staining

Paraffin embedded sections of 50 patients from table 3.1 were selected for this study because of insufficient antibody was available for all the sections. Prior to staining, the blocks and tissue sections were treated as described in section 5.10.4. Polyclonal goat anti-fibulin 1 (Santa Cruz Biotechnology) was used as the primary antibody. The tissue sections were stained in 2 batches using the automated DAKO universal stainer (DAKO) and IHC software (DAKO) as described in section 5.10.4. The secondary antibody was detected using Diaminobenzene (DAB) as the chromogenic substrate. After counter staining in hematoxylin, the slides were observed under an Olympus BHS microscope (Zeiss). The photographs of the hematoxylin stained slides were taken under various original objective magnifications using the Axiophot microscope (Zeiss) and Lieca Image Manager version 2.0 software. Each test slide contained a section of normal oesophageal epithelium for comparison with the tumour. In addition, human skin tissue sections were incorporated in each staining batch as a positive control. For the negative control the incubation with antibody was omitted. To control for the variability in the staining efficiency of the sections between the two batches, 5 randomly selected non-stained duplicate slides from the first batch were stained in the second batch of slides. There was no statistically significant variation (results not shown) in the staining intensity between the duplicate slides stained in both. It can therefore be assumed that there was no significant variation difference between the 2 batches of staining indicating that the staining procedure had been optimised appropriately. The sebaceous glands of the skin were used as the positive control stained for fibulin-1 (figure 3.1), an observation previously been reported by Roark *et al.*, (1995).

3.3.1.1 Normal oesophageal squamous epithelial cells

Fibulin-1 was localised in both the membrane and cytoplasm of maturing or differentiating cells of the normal epithelial squamous cells of the oesophagus (figure 3.2), whereas, the basal layer of the normal epithelium failed to stain for fibulin-1. This is the first report on fibulin-1 expression in oesophageal tissues, although the immunolocalization of fibulin-1 has been previously reported in the cells of other organs such the lungs, skin, brain, cervix, heart and kidney (Roark *et al.*, 1995). The failure of the basal layer to stain for fibulin-1 suggests that this protein is synthesized mainly by differentiating and maturing cell types rather than undifferentiated cells in normal oesophageal epithelium. It is therefore probable that this gene is only actively transcribed in oesophageal epithelium.

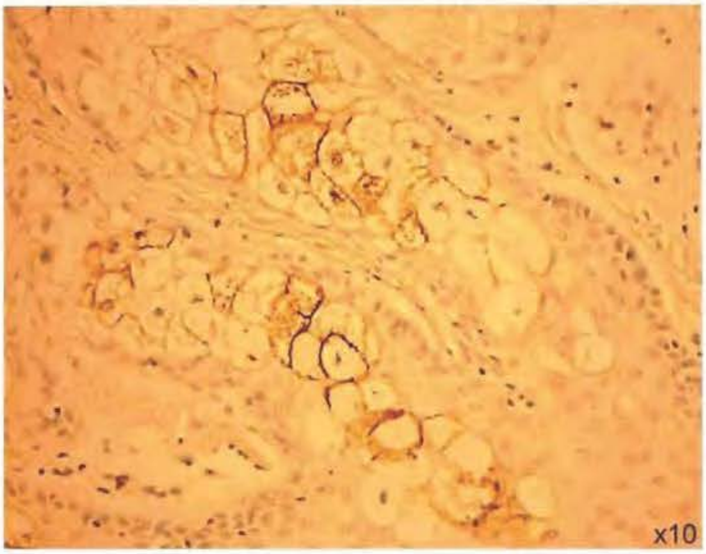

Figure 3.1 Immunohistochemical stain of fibulin-1 in sebaceous gland of the skin. The sebaceous glands stained positively for fibulin-1. (x10 of the original magnification).

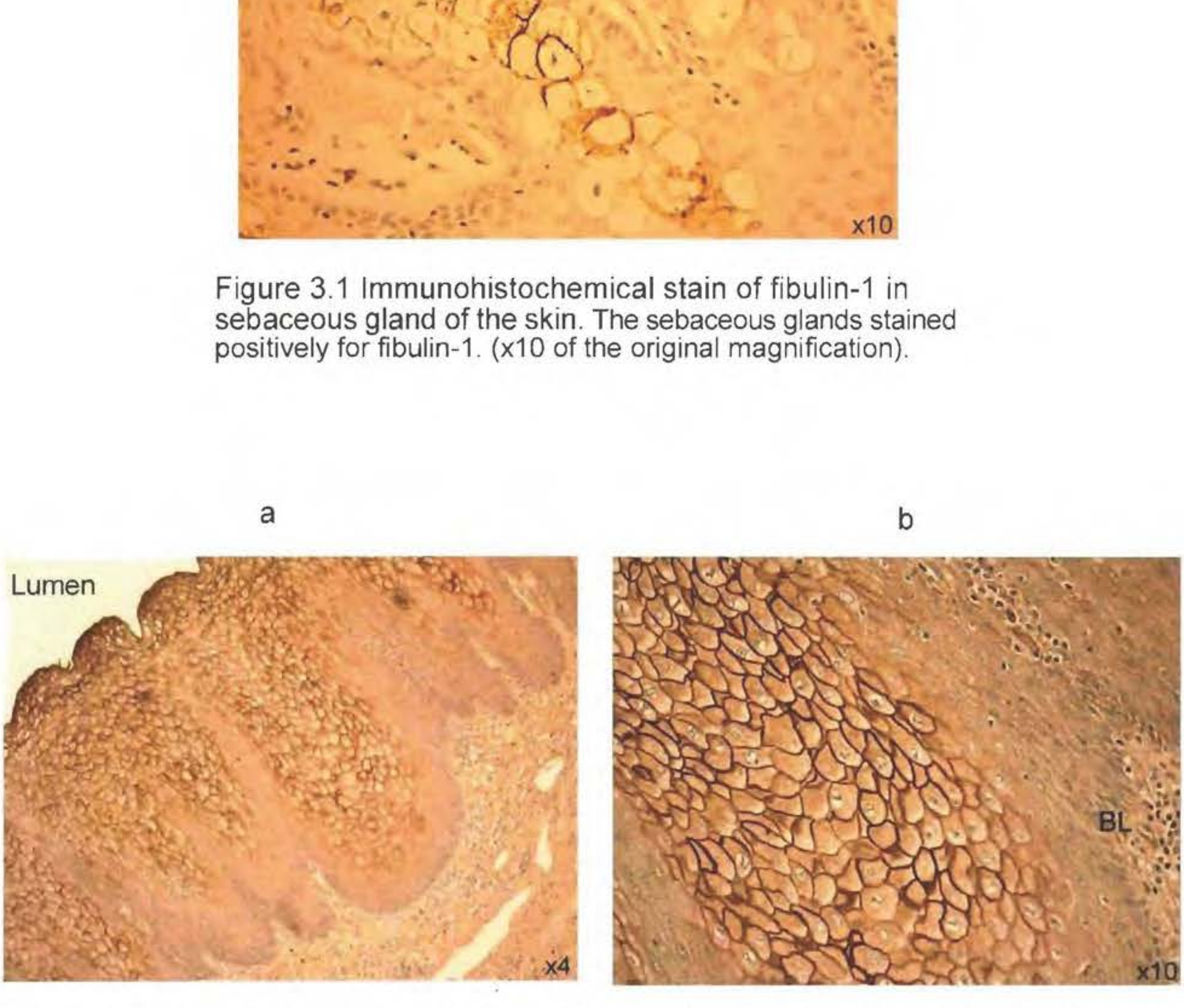

Figure 3.2 Normal squamous epithelium of the oesophagus. The cytoplasm and membrane of the maturing epithelial cells stained positively for fibulin-1 (figure 3.2b), while the basal layer of the normal epithelium did not stain for fibulin (figure 3.2b). (figures 3.2a and b at x4 and x10 of original magnification, respectively). Abbreviations: BL (Basal cells), Lumen (Lumen of oesophagus).

3.3.1.2 Dysplasia

The fibulin-1 stain in was absent in dysplastic cells (figure 3.3 a and b). The staining pattern of dysplastic areas resembled the pattern exhibited by the basal layer of the normal oesophageal epithelium (figure 3.2b). This is the first report on the staining pattern of fibulin-1 in dysplasia. Dysplasia is a condition characterised by the basal layer cells proliferating without maturation. During there proliferation the dysplastic gradually reduces the amount normal epithelium. Thus dysplasia may be either low grade or high grade depending on the amount of normal epithelium remaining. This study found a consistent absence of fibulin-1 expression in all grades of dysplasia. The absence of fibulin-1 in all different grades of dysplasia is perhaps an indication that the fibulin gene is "switched off" very early in dysplasia and maintained in this latent state in all the grades of dysplasia. Secondly, the similarity in the staining patterns of fibulin-1 in both the dysplastic cells and the basal layer of the normal epithelium cell suggests that the factor(s) regulating expression of fibulin-1 in both cell types are essentially the same and operate to down regulate fibulin-1 production in undifferentiated cell types. The regulation of fibulin-1 expression has not been previously described.

3.3.1.3 Invading cancer cells

Invading cancer cells displayed reduced fibulin-1 stain (figure 3.4) compared to the normal oesophageal epithelium. This pattern closely resembles that obtained in dysplastic cells. This implies that Fibulin-1 is down regulated in invading cancer cells, perhaps in a similar fashion as that in dysplastic cells.

3.3.1.4 Well and moderately differentiated cancer cells

Well-differentiated (figure 3.5b) and moderately differentiated (figure 3.6b) tumour cells stained positively for fibulin. There was however, a reduction in the intensity of the stain in both the well and moderately differentiated cells compared to the normal epithelial cells (figure 3.2b). Moderately differentiated tumours generally exhibited less fibulin-1 staining as compared to well-differentiated tumours.

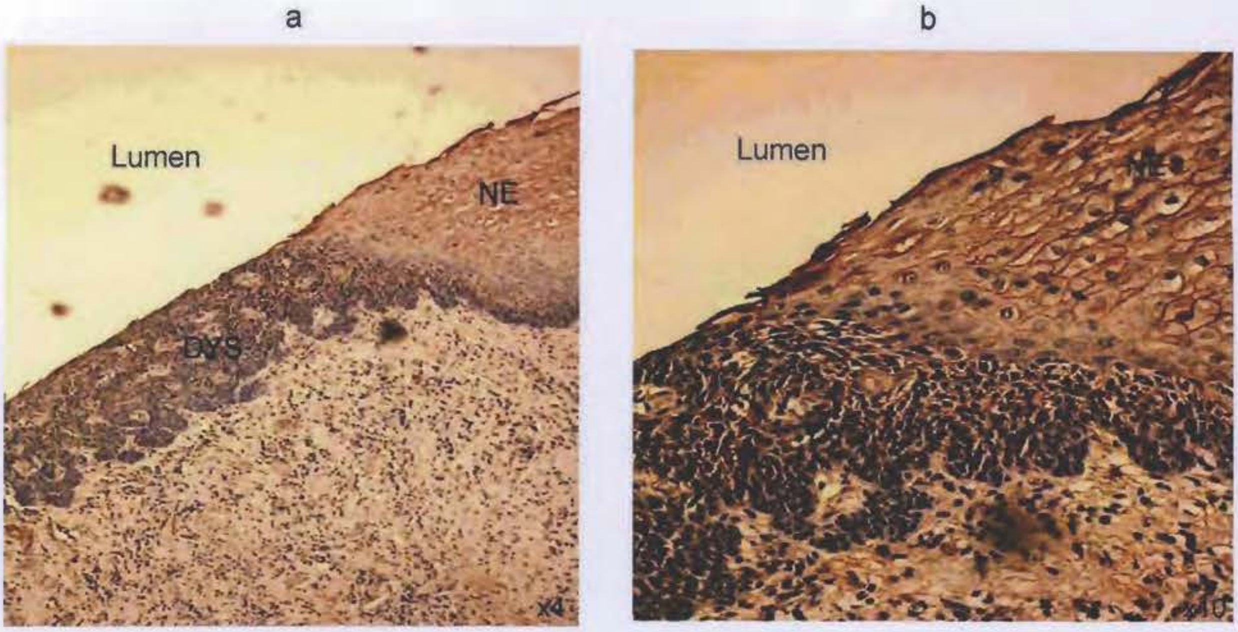

Figure 3.3 Dysplasia of the oesophagus. Dysplastic cells adjacent to normal squamous epithelial cells (figure 3.3a). The dysplastic cells failed to stain for fibulin-1 (Figure 3.3b). (figures 3.3a and b at x4 and x10 of original magnification, respectively). Abbreviations: Lumen (lumen of the oesophagus), DYS (dysplastic cells), NE (Normal epithelial cells).

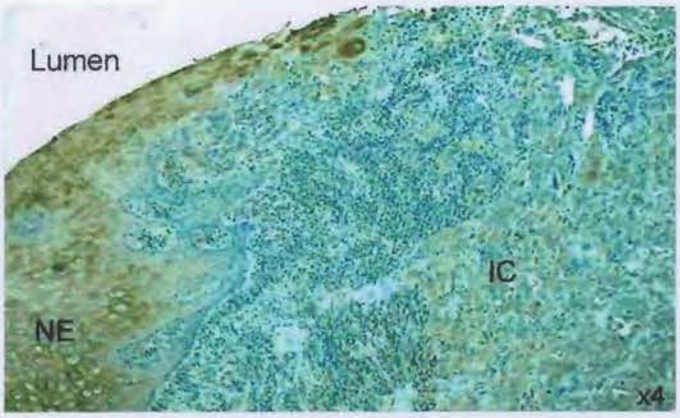

Figure 3.4 Squamous cell carcinoma at early phase of invasion. The invading cancer shows reduced amounts of fibulin-1 compared to the normal epithelial cells. Abbreviations: NE (normal epithelium) , IC (invading cancer growth), Lumen (Lumen of the oesophagus). (x4 of the original magnification).

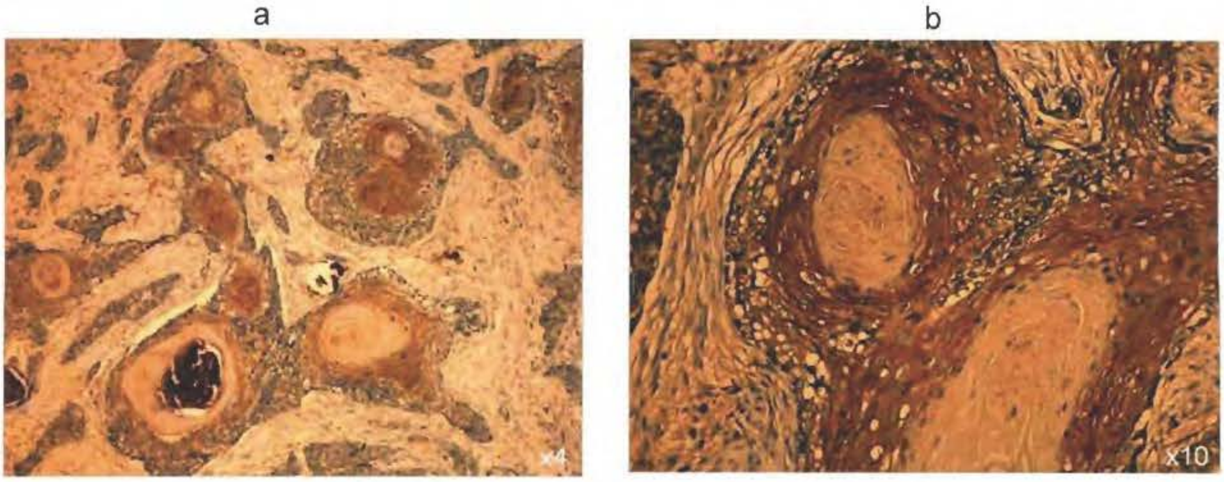

Figure 3.5 Well differentiated squamous cell carcinoma. Fibulin-1 was localised in the membrane and cytoplasm of well-differentiated cells. (figures 3.4a and b at x4 and x10 of the original magnification, respectively).

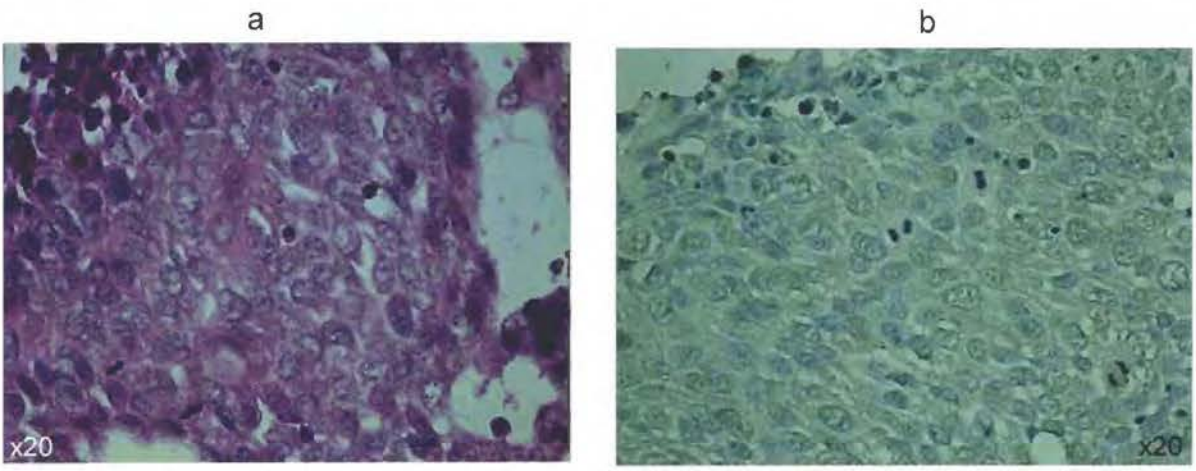

Figure 3.6 Moderately differentiated squamous cell carcinoma. The intensity of the fibulin-1 stain of the moderately differentiated cells was reduced compared to the normal epithelial cells. Figure 3.5a (hematoxylin/eosin stain), figure 3.5b (immunohistochemical stain of fibulin). (x20 of the original magnification).

Lower levels of fibulin-1 observed in these cells suggest that the fibulin-1 gene is partially down regulated in these cancers. It is expected that the well-differentiated cells might produce more fibulin-1 than moderately differentiated tumour cells because the former though cancerous, mimic the characteristics of the normal squamous cells epithelial cells of the oesophagus. However, both well and moderately differentiated cell types usually undergo cell remodelling involving changes in cell morphology, growth, adhesion and motility influenced by tumourigenesis promoting agents or conditions. The deregulation of these processes may in part account for the lower production of fibulin-1. The low expression of fibulin-1 observed in these studies is confirmed by previously published data that demonstrates that a low expression of fibulin-1D not only enhances tumour formation but also invasive capacity of in cell lines (Qing *et al.*, 1997).

3.3.1.5 Poorly differentiated and metastatic cancer cells

Poorly differentiated tumours (figure 3.7b) displayed little or no fibulin-1 stain as compared to the well-differentiated tumour cells (figure 3.5b). Similarly, metastatic cells in the lymph nodes (figure 3.8) exhibited a weaker intensity in the fibulin-1 stain compared to well-differentiated tumour cells (figure 3.5b). These results suggest that there is a halt in fibulin-1 production in poorly differentiated cancer cells. The consequence of this down regulation is alteration in the interaction of fibulin-1 with other important components of the ECM. These interactions are important in cell signalling that may act via the ECM. In addition, perturbation of these interactions interferes with the formation and stabilization of the ECM structures. This will favour tumourigenesis by promoting and cell migration activities and tumour growth as has been earlier reported (Qing *et al.*, 1997; Hayashido *et al.*, 1998; Twal *et al.*, 2001).

3.3.1.6 Other cell types

There was no change in the intensity of the fibulin stain in severe oesophagitis (figure 3.9b). Based on the small sample size examined in these studies, these results suggest that in spite of inflammation, the regulation of the fibulin-1 gene is not adversely altered. It probably requires more than inflammation to dysregulate the fibulin-1 gene in oesophagitis. It can therefore be inferred that although fibulin-1 production is deregulated in OC tumourigenesis, it is not an early event. This however needs to be confirmed using a larger sample of patients. Fibulin-1 stain was observed in the epithelium of the 6 normal Barretts oesophagus sections stained (figure 3.10b).

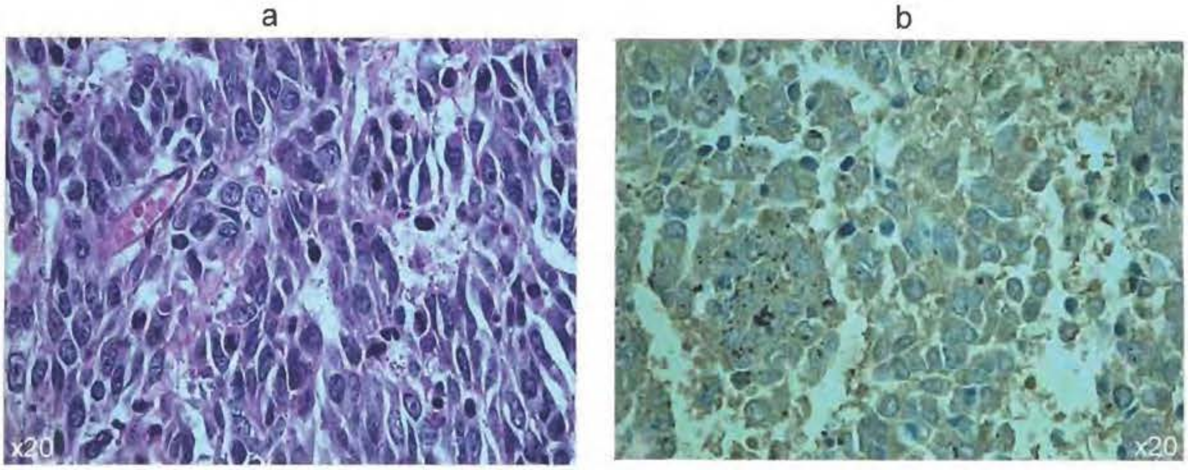

Figure 3.7 Poorly differentiated squamous cell carcinoma. Poorly differentiated cells show reduced staining for fibulin-1 compared to the normal epithelium. Figure 3.7a: (hematoxylin/eosin), b (immunohistochemical stain of fibulin). (x20 of the original magnification).

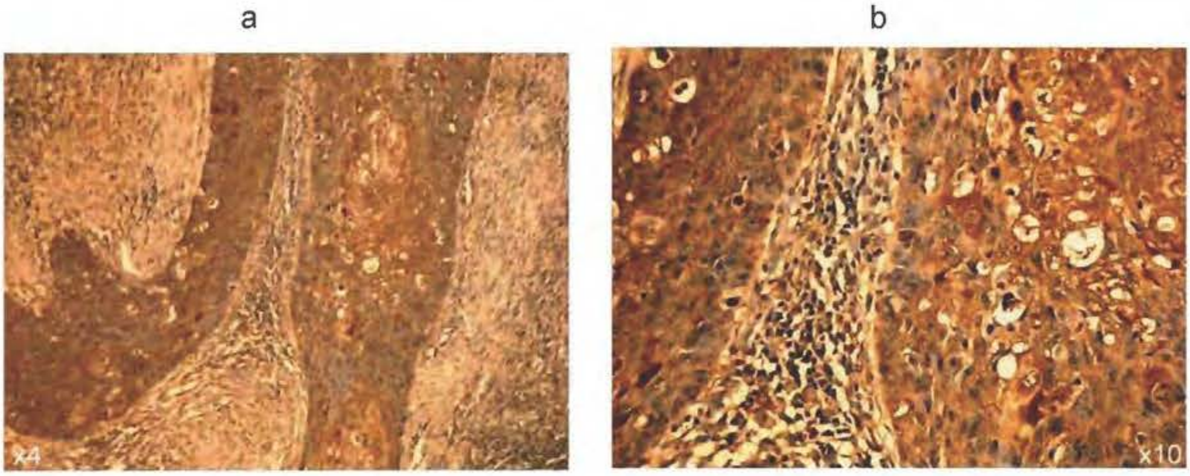

Figure 3.8 Metastatic well differentiated squamous cell carcinoma. Well-differentiated cells in the lymph node show a similar staining pattern of fibulin-1 as seen in cancer at the primary site. (figures 3.8a and b at x 5 and x10 of the original magnification, respectively).

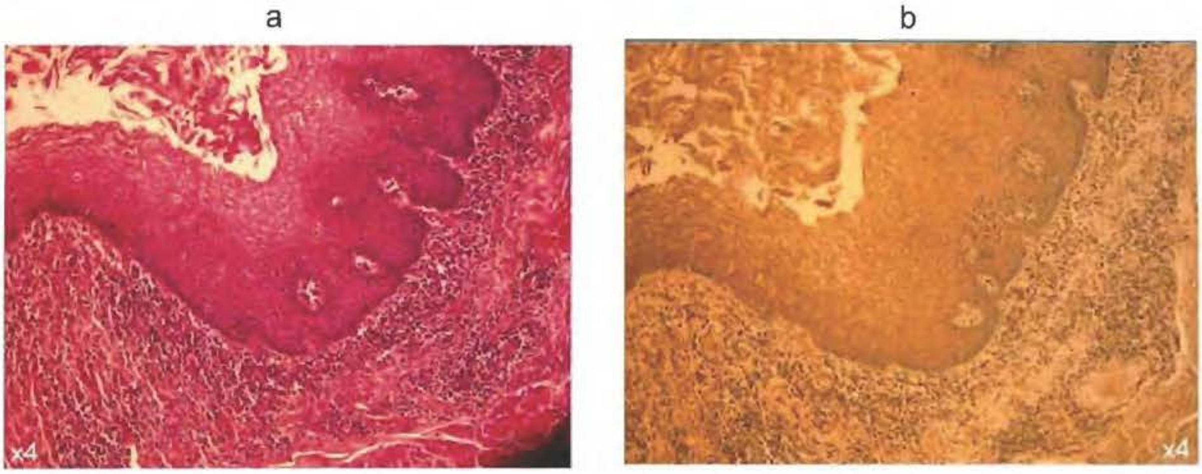

Figure 3.9 Severe oesophagitis. Severe oesophagitis displays a similar staining pattern of fibulin-1 as seen in normal oesophageal squamous epithelium. Figures 3.9a (hematoxylin/eosin), b (immunohistochemistry of fibulin). (x4 of the original magnification).

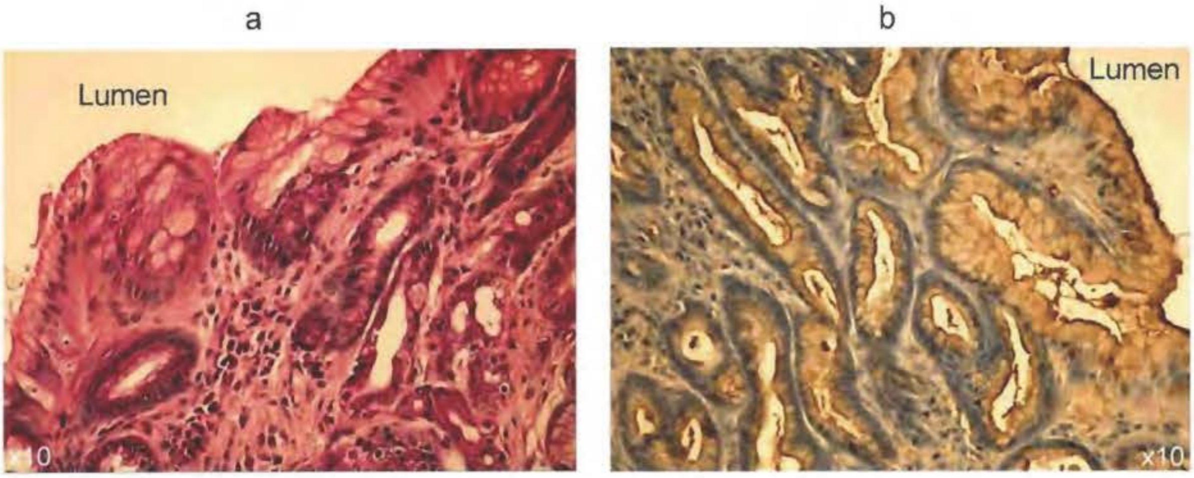

Figure 3.10 Barretts oesophagitis. The cells displayed luminal staining of fibulin-1. Figure 3.10a (hematoxylin/eosin stain), b (immunohistochemistry of fibulin-1). (x10 of the original magnification). Abbreviation : Lumen (Lumen of the oesophagus).

3.3.1.7 Quantitation of fibulin stain

The criteria used for quantitating the fibulin-1 stain included the staining intensity and percentage of cells stained. A range of 1 to 3 was used for classifying the intensity (amount) of fibulin-1 stain. A value of 0 indicated absence of stain; 1, weak stain; 2, moderate stain and 3, intense stain of the tumour area. In addition, the numbers of cells staining (quantity) for fibulin-1 were recorded according to the following classification. Of less than 25% of the cells stained, was given a score of 0; 25-50% staining a score of 1; 50-75% staining a score of 2 and a score of 3 was given to over 75% of the cells staining positive for fibulin-1. Five fields were selected randomly and scored under the x10 magnification objective for the above characteristics. Two independent people of whom one was a pathologist carried out the scoring. In addition, the scoring was carried out blind with regards to the grade of the tumour and the stage of the disease. The above parameters were individually scored in all the five fields and recorded for analysis.

3.3.1.8 Statistical analysis

The data from the section 3.3.1.7 was analysed using the statistical package, Statistica. Previously it has been shown that data (immunohistochemical) of this nature usually has a high variation because of the small patient sample and the inherent differences in the data due to the wide differences in the clinico-pathological parameters of the patients. Consequently the logarithms of the totals rather than the raw totals of the parameters are used to minimise this variations. The means of the logs of the total score were computed for both categories that were analysed.

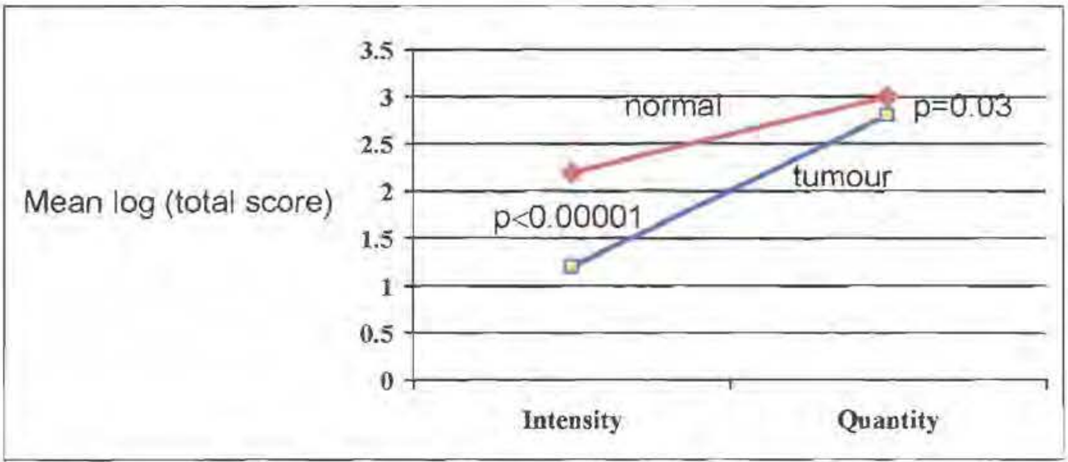

Figure 3.11

Relationship between the intensities and quantities of fibulin-1 staining in both the tumour and normal cells. The intensities of fibulin-1 and the number of cells staining in both the tumour and normal specimens were computed as described in section 3.3.1.7. There was a statistically significantly ($p< 0.00001$) lower amount of fibulin-1 expressed in the tumour cells compared to the normal cells. All tumour cell types (well, moderate, poorly differentiated and metastatic cancer cells) were evaluated in the tumour category.

From the graphical representation (figure 3.11), the difference in the total number of cells staining for fibulin-1 in both the tumour and normal is statistically significant (p=0.03). Between the 2 groups it can be concluded that there a fewer number (quantity) of tumour cells staining for fibulin-1 compared to the normal cells. Secondly, there is a statistically very significant (p< 0.0001) reduction in the total amount (intensity) of fibulin-1 produced in the tumour cells compared to the normal cells. These results corroborate the earlier findings in DDRT-PCR (figure 2.6).

There is a difficulty in interpreting these results given that fibulin-1 expression was absent in the all the dysplasia samples examined. However, there was expression of fibulin-1 in both the well and moderately differentiated carcinoma cells albeit a lower level compared to the dysplastic. The reason for this change is difficult to explain given that dysplasia is a precursor of neoplasia. It would therefore be expected that fibulin expression levels remain reduced in the well and moderately differentiated carcinomas compared dysplastic cells. This suggests that the expression levels of fibulin are dependent on the stage of tumour differentiation rather than the stage of the disease.

The importance of fibulin-1 down regulation has been discussed previously in the earlier sections. To reiterate this, down regulation contributes positively to processes that favour tumourigenesis. This study therefore provides important basis for further investigations into ascertaining the probable time during which fibulin-1 expression becomes deregulated during tumourigenesis and whether this deregulation occurs concomitantly with the deregulation of other important cell adhesion molecules.

These results suggest the possible value of fibulin-1 as marker for dysplasia. There is however a need to identify factors that are involved in the dysregulation of fibulin in order to understand their interaction and probably dissect the molecular pathways involved. This will identify a panel of molecules whose altered expression may assist in distinguishing and identifying different cell types or grades. Secondly, additional studies will be enlightening in terms of understanding the molecular basis of the disease and also the probable progression of the disease.

3.3.2 NDRG-1 staining

Paraffin embedded sections of 70 patients were selected for analysis. The blocks were sectioned, fixed, deparaffinized, rehydrated and blocked for endogenous peroxidase as described in section 5.10.4. Antigen retrieval was carried out by pressure-cooking as described in section 5.10.5. Polyclonal Rabbit anti-NDRG-1 was used as the primary antibody. Dr Therese Commes of the University of Montpellier, France, kindly donated this antibody. This antisera has been previously used to detect NDRG-1 in different cell lines (Piquemal *et al.*, 1999). The tissue sections were stained in 2 batches using the automated DAKO universal stainer and IHC software (DAKO) described in section 5.10.5.

Each test slide contained a section of adjacent normal oesophageal squamous epithelium that served as an internal control for the tumour tissue. In addition, tissue sections of human placenta membranes were incorporated in each batch as a positive control. The negative control was not stained for antibody. Similar measures to those taken during the staining of the slides for fibulin-1 were undertaken to control for variability in staining of the sections between the two batches. There was a statistically insignificant difference in the staining patterns and intensity of between the 2 sets of randomly selected slides. It can therefore be concluded that there was little variability in the staining between the 2 staining batches. The placenta membranes exhibited both cytoplasmic and nuclear staining as has been previously reported by van Belzen *et al.,* (1997) (figure 3.12).

3.3.2.1 Normal oesophageal squamous epithelial cells

The basal layer of the normal epithelium of the oesophagus exclusively exhibited cell membrane staining (figure 3.13d). The maturing cells from the basal layer exhibited pure nuclear staining and a blush of cytoplasmic staining as they matured towards the surface of the epithelium (figure 3.13a). Maturing cells did not exhibit mixed (cytoplasm and nuclear) staining. In addition, there was an abrupt change in the cellular location of the protein from purely membranous to exclusively nuclear as the cells mature from the basal layer towards the surface (figure 3.13d).

This pattern of staining has not been previously described. The mutually exclusive cellular localization of the NDRG-1 stain indicates that the protein partitions preferentially in different cell compartments in the course of cellular maturation. This may be suggestive of the protein having different functions during cellular differentiation and maturation. This is supported by earlier reports that the protein shuttles between the nucleus and the

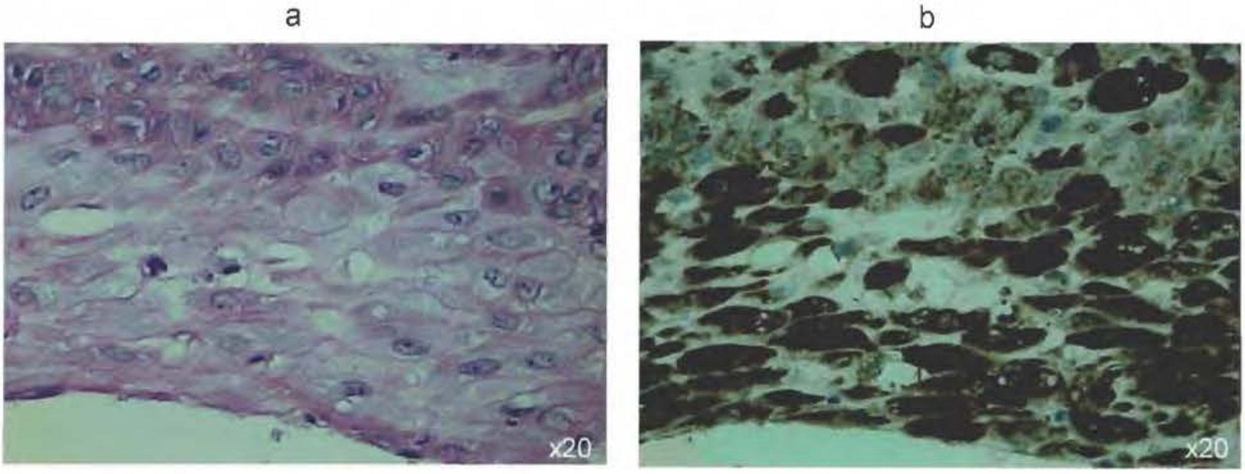

Figure 3.12 Placental membranes. NDRG-1 stain was localised in the cytoplasm and nucleus of the epithelial cells of the placenta membranes. Figure 3.12a (hematoxylin/eosin), b (immunohistochemistry of NDRG-1). (x20 of the original magnification).

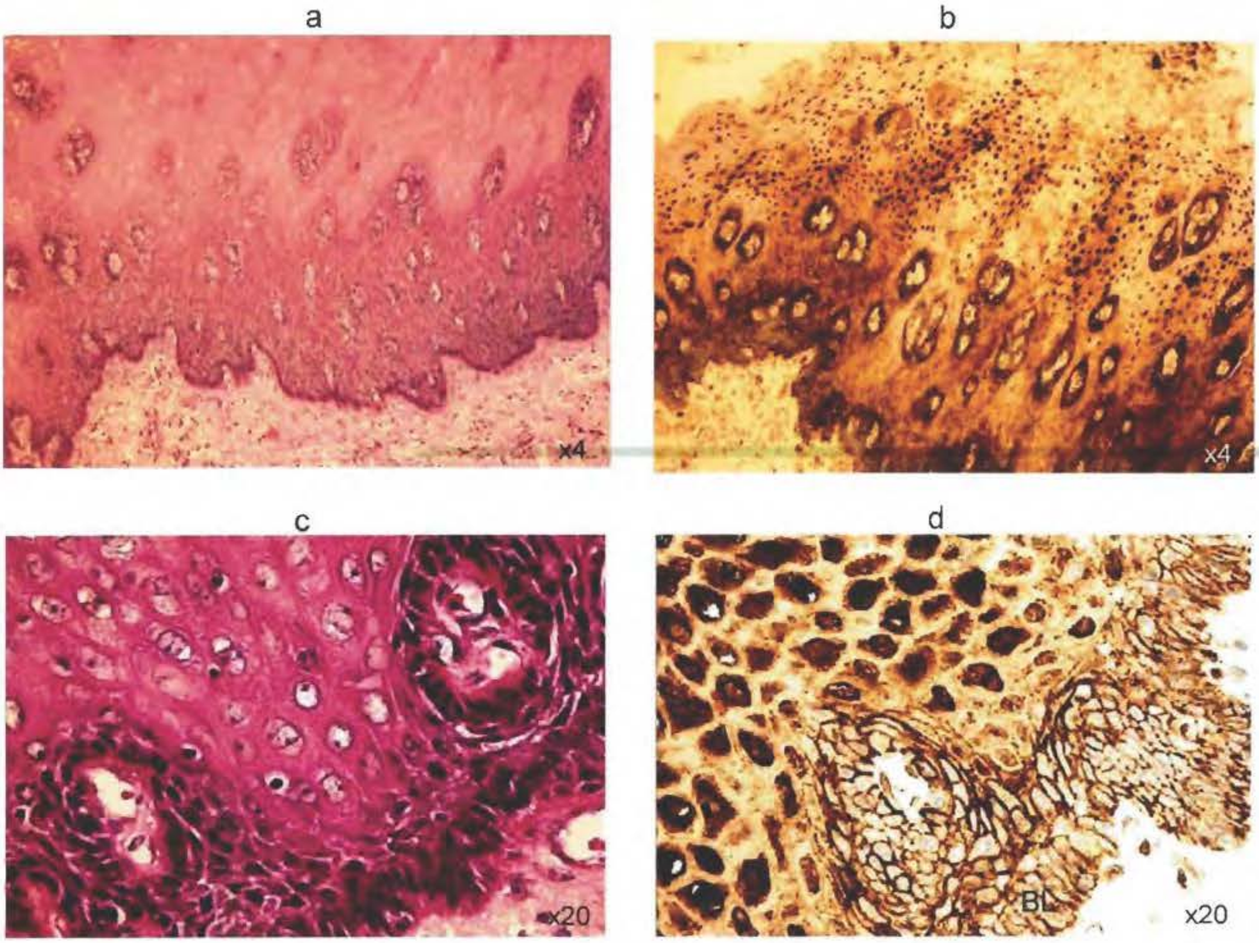

Figure 3.13 Normal squamous epithelium of the oesophagus. The NDRG- stain was localised exclusively in the cytoplasm and nucleus of the maturing cells (figure 3.13a), while the basal cells display exclusive membrane staining of NDRG-1 (figure 3.13d). Note the abruptness from exclusive membrane in the basal cells to exclusive nuclear localisation of NDRG-1 in the maturing cells of the epithelium. Figures 3.13a and c (hematoxylin/eosin), while b and d (immunohistochemistry of NDRG-1). Figures 3.13a and b (x4 of the original magnification) while c and d (x20 of the original magnification). Abbreviation: BL (Basal cells).

cytoplasm (Pulford *et al.*, 1995). It is interesting to consider the conditions that determine this differential partitioning. It is interesting to consider that the factors or conditions that determine this differential partitioning. It is known that NDRG-1 is phosphorylated (Li and Treub, 2000) but it is not known if the phosphorylation status of NDRG-1 affects it partitioning? It has been previously reported that NDRG-1 is down regulated by N-myc proto-oncogene (Shimono *et al.*, 1999). Based on the staining pattern and intensity and intensity it can be inferred that N-myc is switched off as the cells mature from the basal membrane towards the surface.

3.3.2.2 Dysplasia

There was exclusive membranous staining in different of dysplasia (figures 3.14)). This pattern is identical to the one typified by the basal layer of the normal oesophageal epithelium. The transition in the staining pattern from the typical membranous in basal cells to the nuclear and cytoplasmic characteristic in the maturing cells of the normal epithelium to the exclusively membranous staining in dysplasia has not previously been reported in cancer. Secondly, this membranous staining pattern was evident in all sections that contained both normal epithelium and dysplastic areas. Dysplasia is characterised by basal layer cells exhibiting proliferation without accompanying maturation. It is inviting to speculate that N-myc is up regulated and in turn down regulates NDRG-1 expression in dysplastic cells, although other factors could conceivably also regulate the expression of NDRG-1. Since the different grades of dysplasia displayed the same staining pattern and cellular partitioning of NDRG-1, this is probably indicative that N-myc up regulation is maintained throughout the different grades of dysplasia resulting in low NDRG-1 protein. This observation supports previously published data that has shown that constitutive expression of N-myc is strongly linked to increased proliferative rates in neuroblastoma cells (Lutz *et al.*, 1996). The preferential partition within the membrane suggests that the "shuttling" property of the protein is inhibited in dysplasia. The factors and underlying mechanism influencing this unique partitioning are unknown.

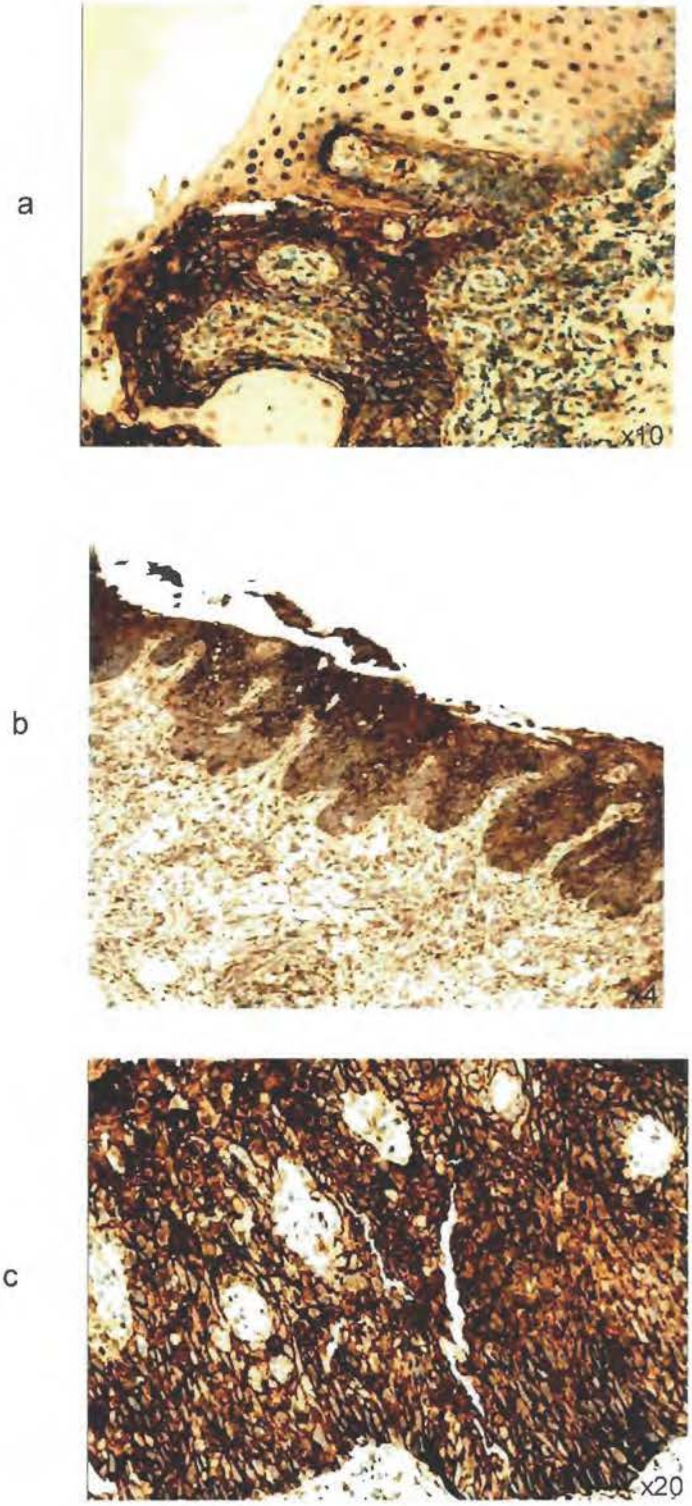

Figure 3.14 Dysplasia of the oesophagus. *Dysplastic cells display predominantly membrane* staining of NDRG-1. Figure 3.14a, b and c are x20, x10 and x5 of the original magnification.

3.3.2.3 Well differentiated cancer cells

The well-differentiated tumours predominantly exhibited membrane, cytoplasmic and mixed (cytoplasmic and nuclear) staining (figure 3.15d). Exclusively nuclear staining was not observed in the sections studied. The latter observation was different to the typical nuclear staining shown by the maturing cells and differentiated cells of the normal epithelium. In addition, the keratin pearls and calcified areas typically observed in well-differentiated squamous cell carcinoma failed to stain for NDRG-1 in all the sections examined (figure 3.15d).

This type of staining has not been previously described in oesophageal cancer. Well-differentiated cancer cells although exhibiting cancer cell characteristics such as deregulated keratin secretion essentially mimic the normal epithelial cells. For example, they have some degree of gradient maturation and have intracellular bridges. The question is what factors influence this differential regulation. Secondly, how are these factors regulated, especially in well-differentiated cancer cells? The total absence of purely nuclear staining suggests that cellular partitioning and possibly the functioning of the NDRG-1 protein and possibly the functioning of NDRG-1 protein is possibly altered in tumour tissue.

In spite of these cells secreting keratin to form pearls, no NDRG-1 was observed in the keratin confirming that NDRG-1 remains an intracellular protein even in neoplastic cells.

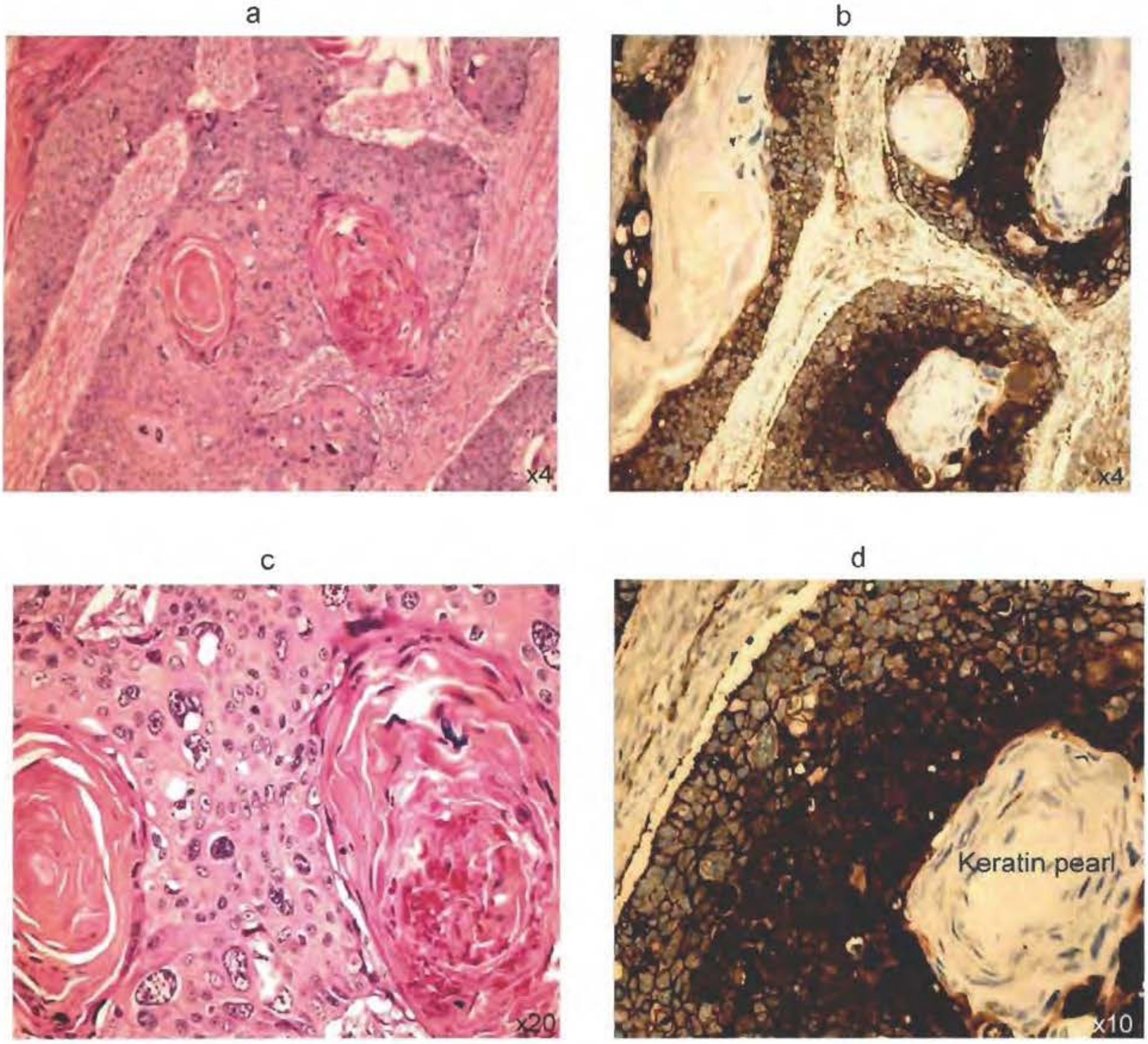

Figure 3.15 Well differentiated squamous cell carcinoma. The well-differentiated cells exhibited a mixed type of staining (membrane, cytoplasm plus nuclear localisation). The membrane staining pattern of the well-differentiated cells (figure 3.15d) was similar to that exhibited by the basal cells of the normal epithelium (figure 3.15c). Note the keratin pearls fail to stain for NDRG-1. Figure 3.15a (hematoxylin/eosin) while b, c and d (immunohistochemistry of NDRG-1). Figures 3.15a and b (x4 magnification), c (x20 magnification) and d (x10 of the original magnification).

3.3.2.4 Moderately differentiated cancer cells

Moderately differentiated tumours exhibited a similar staining pattern to well differentiated tumours. In addition, some cells exhibited mixed (cytoplasmic and membrane) staining (figure 3.16b). Moderately differentiated cancer cells are essentially an "intermediate" grade between the well and poorly differentiated ones. Moderately differentiated cells are expected to display characteristics exhibited by both well and poorly differentiated cell types. It is likely, however, that the *N-MYC* gene is still differentially expressed thus leading to the differential expression of NDRG-1 as shown by the staining patterns of the protein.

3.3.2.5 Poorly differentiated cancer cells

Poorly differentiated tumour cells exhibited nearly exclusive membrane staining (figure 3.17b). This staining pattern was similar to the NDRG-1 staining of the basal layer of the normal epithelial membrane and dysplastic regions staining. In addition, most of the poorly differentiated tumours failed to stain for NDRG-1. This staining pattern and expression level of NDRG-1 is suggestive that N-myc is up regulated and in turn virtually shuts down NDRG-1 expression. This probably explains why some of the poorly differentiated tumour failed to stain for NDRG-1.

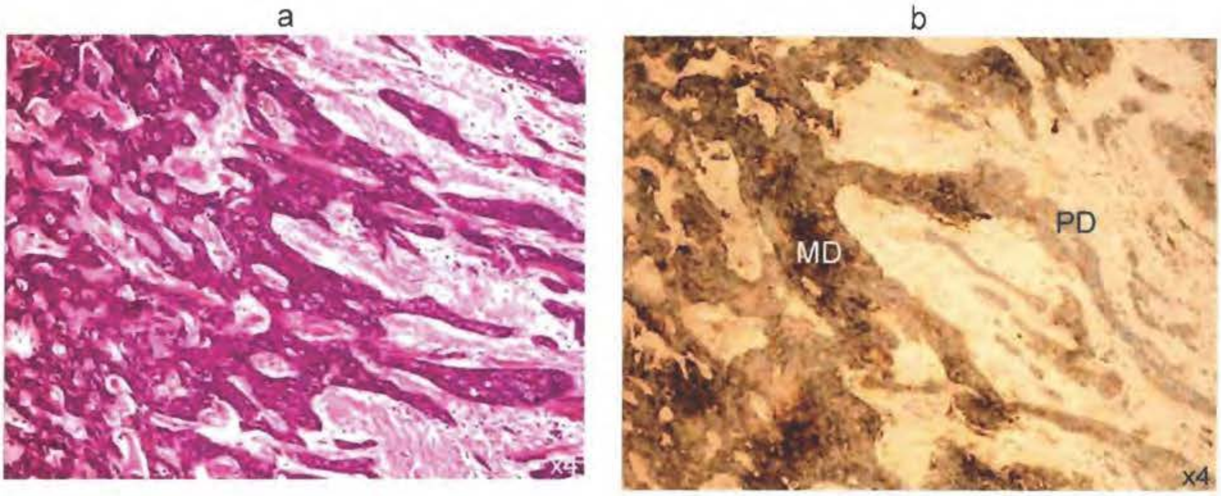

Figure 3.16 Moderately differentiated squamous cell carcinoma. Moderately differentiated cells (MD) stain more intensely for NDRG-1 than their adjacent counterpart poorly differentiated cells (PD). Figure 3.16a (hematoxylin/eosin), b (immunohistochemistry of NDRG-1). (x4 of the original magnification).

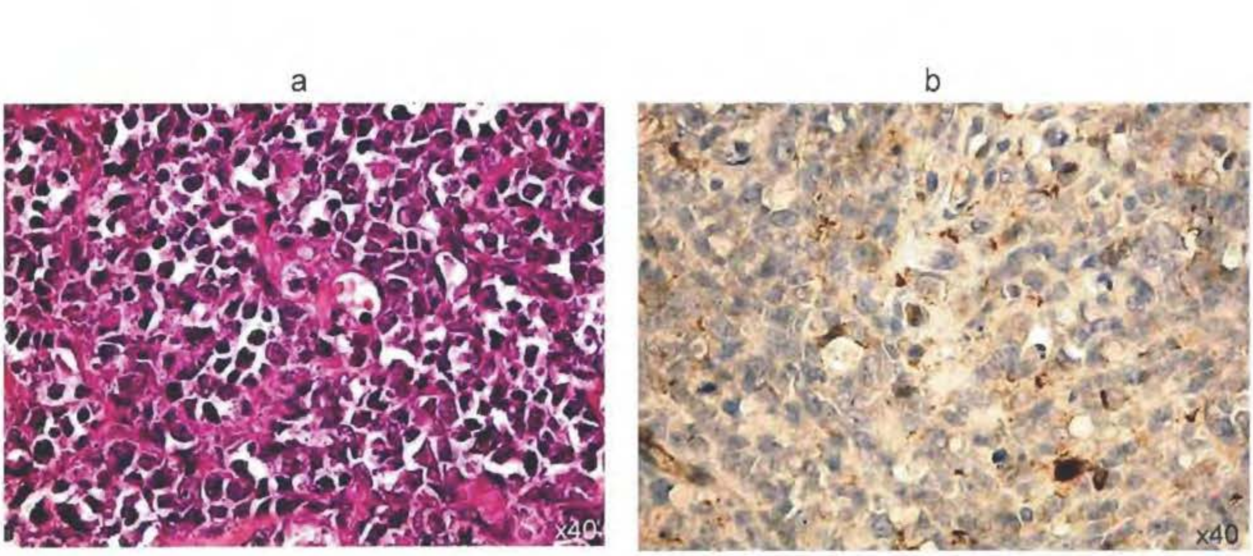

Figure 3.17 Poorly differentiated squamous cell carcinoma. The poorly differentiated cells exhibited minimal or absence of NDRG-1 stains. Figures 3.17a (hematoxylin/eosin), b (immunohistochemistry of NDRG-1). (x20 of original magnification).

3.3.2.6 Other cell types

All the metastatic tissue sections examined exhibited a similar staining pattern to well differentiated tumours (figure 3.18b) despite the small sample size of the sample used in the study. The cells displayed cell membrane, cytoplasmic and mixed (cytoplasmic and nucleus and/ or cytoplasmic and membranous) but no exclusive nuclear staining.

Tissue sections of mild oesophagitis displayed an identical staining pattern to the normal oesophageal epithelium. The maturing cells of the severe oesophagitis, (figure 3.19b) however, displayed a more prominent and intense membranous staining than in the normal epithelium (figure 3.13d). Earlier results (figure3.13b) did show the absence of membrane staining in the maturing cells of the normal epithelium. If severe oesophagitis is a precursor of mild dysplasia, the NDRG-1 staining pattern of severe oesophagitis does to some extent support that statement. The prominent membranous stain shown by the latter is probably indicative that the normal epithelium is in a "transient phase" towards a dysplastic one. More patients, however, need to be studied in order to establish the validity of this transition between normal epithelium and severe oesophagitis.

The membrane and cytoplasm of the normal epithelium of Barretts oesophagus (figure 3.20b) stained for NDRG-1, whereas areas of dysplasia (figure 3.20d), the membrane and cytoplasm failed to stain for NDRG-1 protein. The NDRG-1 stain observed in the dysplastic sections is probably due to the protein being "pushed" out of the cell due to the enlargement of the nucleus, rather than it being secreted through normal mechanisms. The NDRG-1 stain was able to distinguish between dysplastic and “normal” Barretts epithelium. The absence of nuclear staining suggests that the NDRG-1 gene is down regulated in a similar fashion as in dysplasia of squamous cells of the oesophagus.

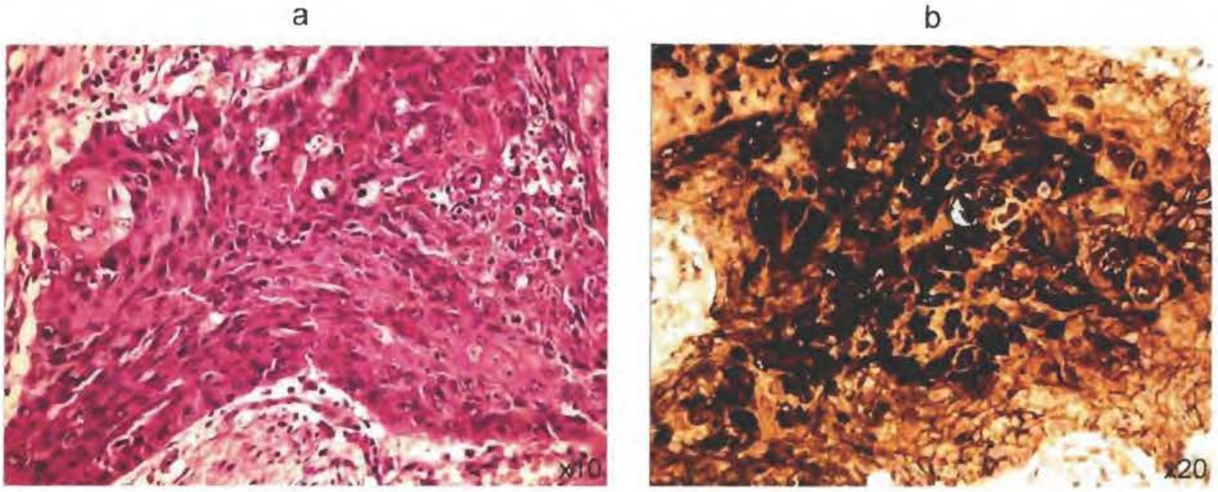

Figure 3.18 Metastatic squamous cell carcinoma. The metastatic cells show a mixed NDRG-1 staining pattern similar to their counterparts at the original site. Figure 3.18a (hematoxylin/eosin), b (immunohistochemistry of NDRG-1). Figure 3.18a and b are x10 and x20 of the original magnification, respectively.

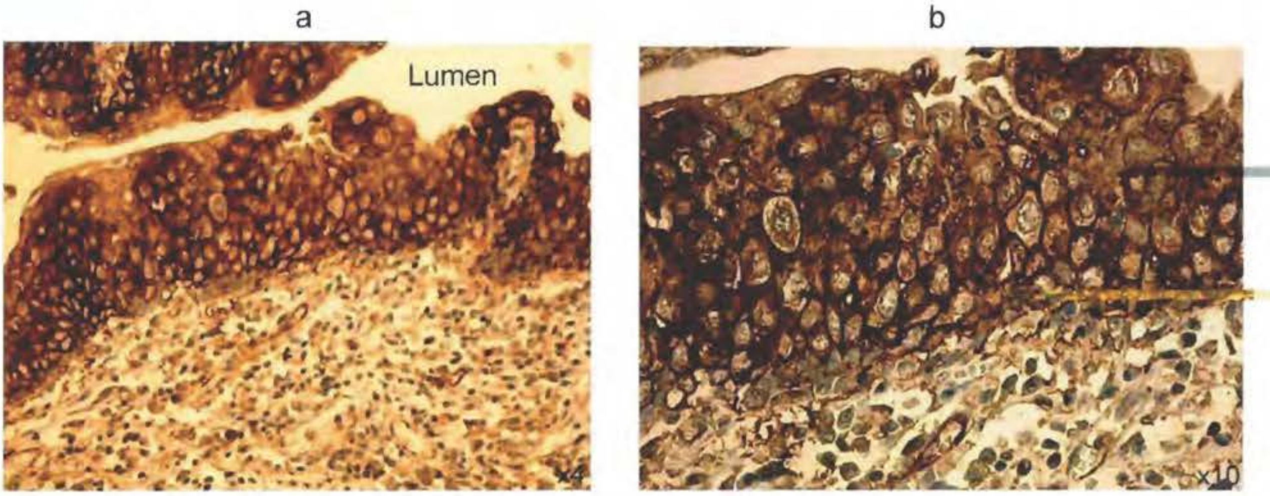

Figure 3.19 Severe oesophagitis. The epithelial cells in severe oesophagitis display intense NDRG-1 staining pattern that is mainly membranous compared to their normal epithelial counterparts. Figure3.19a (hematoxylin/eosin), b (immunohistochemistry of NDRG-1). Abbreviation: Lumen (Lumen of the oesophagus). Figure 3.19 a and b are x4 and x10 of the original magnification, respectively.

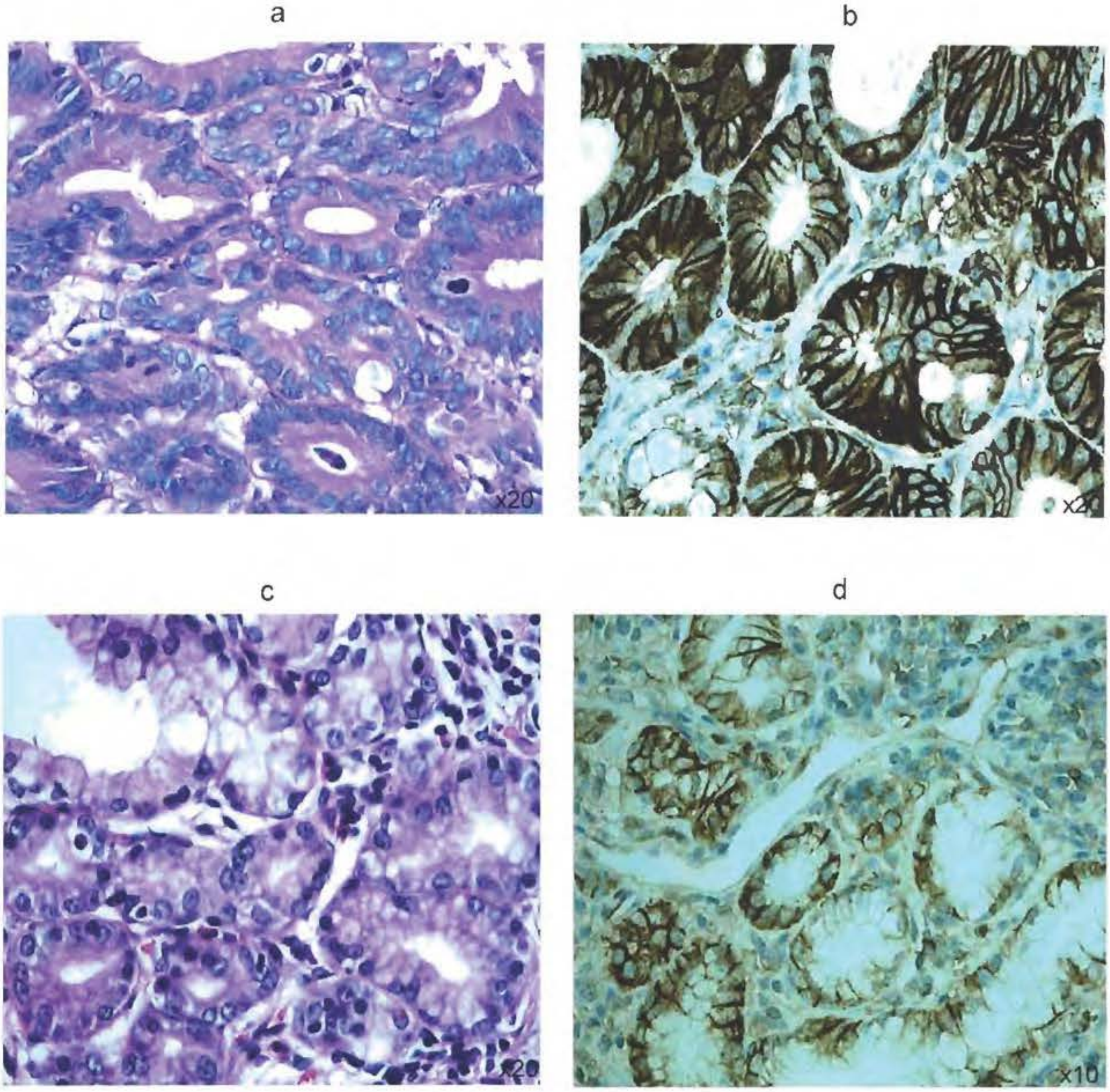

Figure 3.20 Barretts oesophagitis. The cells display mainly membranous stain (figure 3.20b) while Barretts oesophagitis associated with inflammation shows a mainly luminal staining pattern (figure 3.20d). Figures 3.20a and c (hematoxylin/eosin), b (immunohistochemistry of NDRG-1). Figures 3.20a, b and c (x20 of the original magnification), while d (x10 of the original magnification).

Figure 3.21
SUMMARY OF THE STAINING PATTERNS AND CELLULAR LOCATIONS OF NDRG-1 IN THE DIFFERENT CELL TYPES. The table below is a summary of the different NDRG-1 staining patterns and intensities in the different types of cells. Membrane localisation is observed in all cell types, exclusive membrane staining is seen in dysplasia and poorly differentiated cell types while mixed type of staining are found in well and moderately differentiated cell types. The intensity of the stain decreases from well to poorly differentiated cell types. Abbreviations: – (absence), + (presence).

Epithelial status	**Membrane**	**Cytoplasm**	**Nucleus**	**Intensity of NDRG-1 stain**
Normal	+	+	+	high
Dysplasia	+	–	–	high
Well differentiated	+	+	+	high
Moderately differentiated	+	+	+	High / moderate
Poorly differentiated	+	–	–	low

3.3.2.7 Quantitation of NDRG-1 stain

Criteria were developed for quantitating the immunoreactivity of NDRG-1 using a score range of 0 to 3. A value of 0 indicated 0-25% of tumour area stained; +1, 25-50% stained; +2, 50-75% stained and +3 more than 75% stained. Similarly for non-stained or non-expressing cells, less than 25% of tumour area unstained was given a score of 0; 25-50% unstained area, a score of 1; 50-75% unstained area a score of 2 while more than 75% unstained area a score of 3. Five fields were selected randomly and scored for the above characteristics. This was carried out under the x10 magnification objective. Two investigators of whom one was a pathologist performed the scoring. The evaluation was carried out blind with respect to tumour stage and grade.

In addition to the above criteria, the cellular localisation of the stain was evaluated. The stain was categorised according to the site of localisation in the cell; as membranous, cytoplasmic, nuclear or mixed (membrane and cytoplasm and/or cytoplasm and nucleus). Each compartment was scored individually in all the five fields and recorded for analysis.

3.3.2.8 Statistical analysis

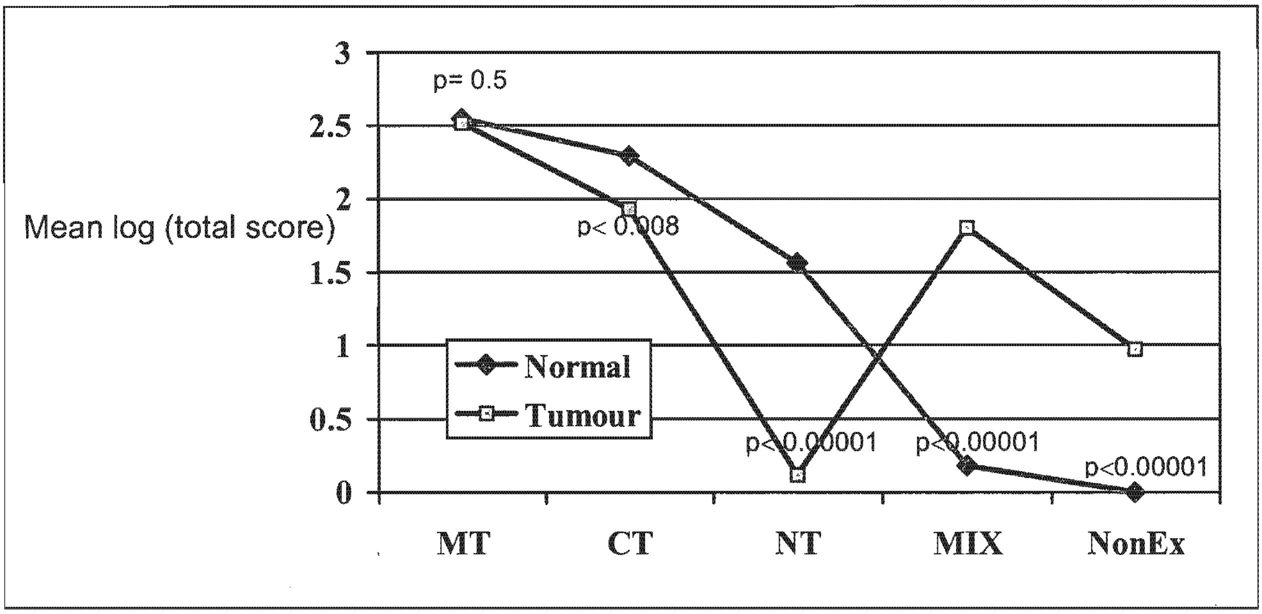

Figure 3.22. The relationship between cellular localisation of NDRG-1 stain in tumour and normal cells. The locations of the NDRG-1 stain were computed for tumour and normal cells. There were statistically very significant differences in the NDRG-1 locations between the normal and tumour cells with regard to exclusive nuclear stain, mixed stain and the none-expressing cells. Abbreviations: MT (total membrane), CT (total cytoplasm stain), NT (total nuclear), MIX (total mixed cellular loaction), NonEx (total cells not expressing NDRG-1).

The summary of the data in figure 3.22 shows a clear relationship between cellular localization and tissue type. All cell types, including the basal layer cells, displayed membrane staining. This was supported by the statistical data that shows that there is no significant difference between the tumour and normal tissues. This implies that membrane staining is not an effective discriminatory cell compartment for distinguishing normal from tumour cells. Cytoplasmic staining was observed in all the cells with the exception of dysplastic cells. There were however, varying degrees of stain within the different cells. This was further supported by the statistically significant ($p<0.008$) difference between the normal and tumour specimens. Exclusive nuclear stain was virtually absent in the tumour specimens. These observations suggest that on the basis of exclusive nuclear staining, one can reasonably differentiate a normal oesophageal squamous cell from a cancerous one. Mixed staining was predominantly observed in the tumour rather than in the normal cells as supported by the statistical analysis. It can be inferred that mixed type of staining would be useful in distinguishing normal from tumour cells. Most cells that failed to stain were tumour rather than normal cells. This suggests that the normal cells, regardless of their differentiation status, will always stain whereas the same is not true for tumour cells.

The results of NDRG-1 stain show that it is possible to distinguish dysplasia and poorly differentiated tumour cells from normal and well-differentiated cancer cells. The protein expression fails to explicitly distinguish the different grades of cancer from the normal cells particularly in the early phases of the disease. The differences are only observed at a rather advanced stage of the disease when there is little medical help that can be offered to the patient. In spite of these draw backs the distinctive pattern of NDRG-1 staining is an indication that events at the molecular level are not necessarily congruent with the morphological characteristics of the cell. Caution therefore needs to be taken when relating molecular changes and phenotypic changes. The question is whether molecular changes are a better indicator of cancer progression compared to morphological changes. Shouldn't these two levels of characterisation complement rather than be used independently as endpoints in determining and categorising diagnosis and prognosis? This should be a long-term goal of oncology research if better management of cancer is to be achieved.

This project did not assay for the levels of N-myc in the either the oesophageal cancer cell lines or tissues. Based on the literature, N-myc has been extensively implicated in the regulation of NDRG-1. It is of significance to draw some analogy on the probable relationship between N-myc expression vis-à-vis the levels of NDRG-1 in oesophageal cancer.

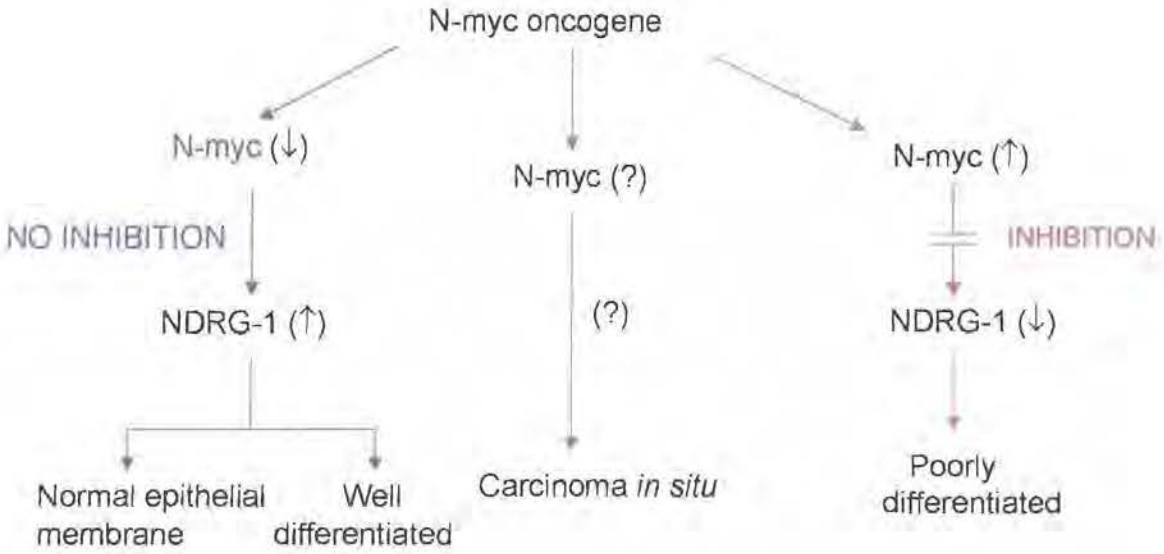

Figure 3.23. The possible relationship between N-myc and NDRG-1 status in tumourigenesis. The above schematic representation illustrates the probable relationship between N-myc expression vis-à-vis NDRG-1 staining patterns between the different cell types. An up-regulation of N-myc favours the staining pattern observed in poorly differentiated tumours while a down-regulation favours the patterns observed in normal oesophageal epithelium and well differentiated tumour cells.

Based on the above model, it is probable that when N-myc is down regulated, NDRG-1 is up regulated. Consequently, the NDRG-1 exhibits nuclear, cytoplasmic or mixed cellular partitioning. These staining patterns are prevalent in the normal oesophageal epithelium as well in the well and moderately differentiated cell types. On the other hand when N-myc is up regulated it inhibits NDRG-1 synthesis and the protein preferentially is localised in the membrane of the cell as exhibited by dysplastic and poorly differentiated cells. The question is why *N-MYC* is suddenly down regulated in a "poorer" grade of cancer (well differentiated) when it was initially up regulated in a milder grade (dysplasia)? Further studies are required to establish the relationship between *NDRG-1* regulation and *N-MYC* expression. It is possible that NDRG-1 is not entirely regulated by N-myc but by other pathways too. This may in part explain why there is dramatic change in the staining patterns between the different cell types that cannot be entirely explained by changes in *N-MYC* gene expression.

3.3.2.9 Relationship between patient survival and clinico-pathological characteristics

The most important end point of this project is to ascertain the probable relationship between patient survival, tumour grade and/or stage of the disease and the staining of the different proteins. Establishment of links between the above parameters has important implications in the management of oesophageal cancer, especially with respect to early screening, diagnosis and possibly prognosis.

Overall survival rate was determined and defined as the interval between surgery and June 1st 2002. The end point for this variable was either death, still alive or date last seen at the oesophageal clinic (lots to follow up LTF). Overall survival curves for univariate analysis were assessed using the Kaplan-Meier method (Kaplan and Meier, 1958).

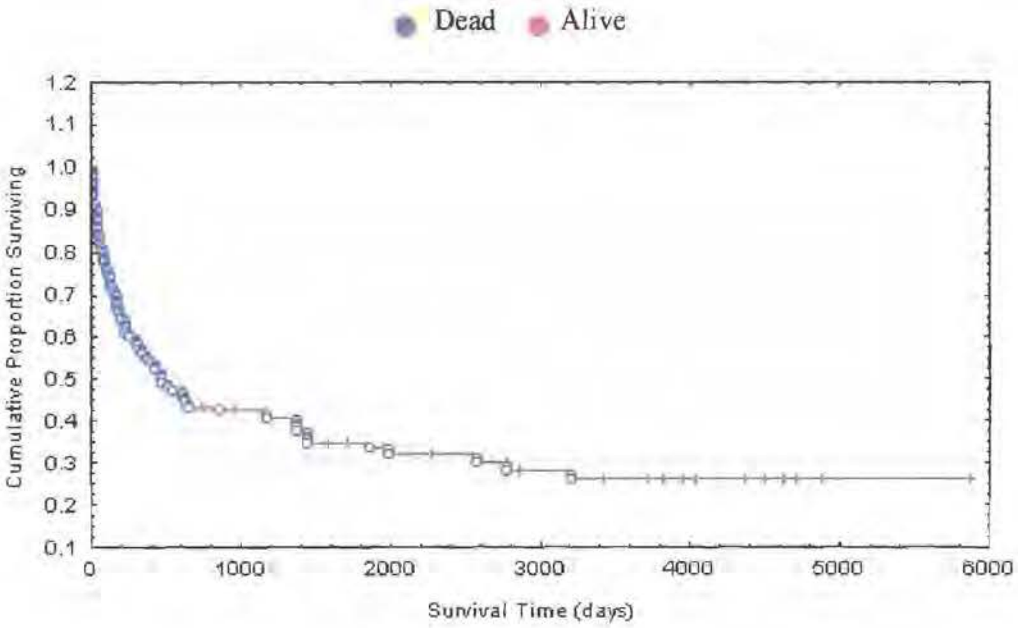

Figure 3.24

The overall survival of the patients used in immunohistochemical studies.
A total of 70 patients were incorporated in this study. The patients were of different stages of the disease. This analysis was carried out as described in section 3.3.3

The overall survival curve was constructed using days rather months of survival (figure 3.24). From the curve it can be seen that close to 60% of the patients died within the first 800 days (2 years) after visiting the oesophageal clinic. Very few patients survived longer than 2000 days (5.5 years). These results conform to previously published data that shows that oesophageal cancer patients have a very poor prognosis. This is based on the fact that most patients seek medical attention when they have advanced disease and there is little that medical practitioners can offer to improve their prognosis.

It was also interesting to establish the relationship between the stage of the disease and survival. Kaplan-Meier curves of survival verses stage of the disease (figure 3.25) were therefore plotted. Most patients with stage 3 disease died earlier than the other stages. The better survival of patients with stage 2B compared to those with stage 2A is unusual. This can be explained by the bias in the number of stage 2B patients compared to stage 2A patients. We managed to incorporate only 6 stage 2B compared to 25 of stage 2A patients due to the unavailability of resected oesophageal samples from stage 2B patients. In addition, there is no curve for stage O patients because only 1 histological sample was available for this study. dysplastic, well and poorly differentiated cases as compared to the moderately differentiated cases. Overall these results show a good correlation between the stage of the disease and survival. These trends are expected as previous data on oesophageal cancer show that people with the advanced disease have a poorer prognosis than those with an early stage.

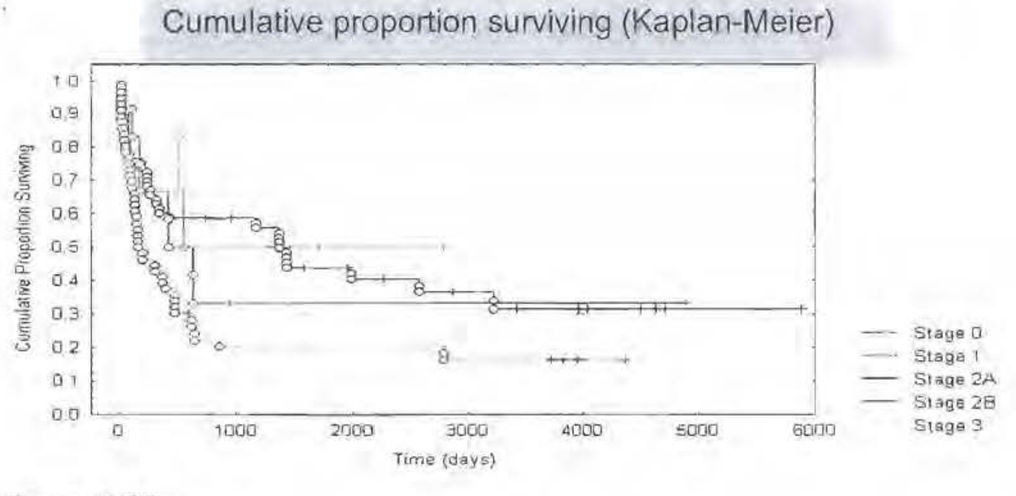
Cumulative proportion surviving (Kaplan-Meier)

Figure 3.25

The relationship between stage of disease and survival of the patient.70 patients were used to determine the relationship between stage of the disease and survival as described in section 3.3.3

Survival curves based on grade were not constructed because of the biased representation of the different grades.

We attempted to correlate the intensity of fibulin-1 stain with survival. There was however, no statistically significant relationship between stain and survival. In addition, there was no statistically significant relationship between the cellular partitioning of the NDRG-1 protein and survival. The reason for this lack of relationship is because of the very small patient sample size used in the study.

3.3.2.10 False positives

This study endeavoured to minimize the possibility of having false positives. This was achieved by quenching the endogenous peroxidase enzyme present in the tissues using 1% hydrogen peroxide (section 5.10.4). Although this diminshes the effects of the endogenous enzyme, it does not eliminate its activity completely. The negative control slides (slides incubated without the antibody) gave insignificant background staining after the chromogenic substrate diaminobenzene (DAB) was added. This proved that the effects of the endogenous peroxidase were minimal in both the fibulin-1 and NDRG-1 immunohistochemistry experiments.

3.3.2.11 False negatives

A major problem of using archived tissue is that the antigen epitopes of some proteins are less stable with increase in storage time. The consequence of this is that we are likely to see many sections that are false negatives. This is true for sections that are stored for long periods of time under inappropriate conditions such as high ambient temperatures and humidity. If this phenomenon occurs, the results should be reflected consistently in both the control and test samples. These inconsistencies were not observed when both fibulin-1 and NDRG-1 antibodies were used. There was however, a significant difference between the number of non-staining cells in the tumour tissue and normal control when anti-NDRG-1 was used. If indeed there was a significant loss of antigen epitopes of the NDRG-1 protein, this should have been reflected by having a similar percentage of cells both in tumour section and normal control failing to stain for NDRG-1. The significant difference observed between the 2 tissue types suggests that the high presence of non-NDRG-1 expressing cells in the tumour tissue is true rather than an artefact.

3.3.2.11 Immunostain for cdc25B, integrin α_6 and MMP14

These antibodies failed to produce any tangible staining. This was despite numerous attempts using various antigen retrieval methods as described in the literature. These failures are attributed to the fact that these antibodies were only developed for frozen rather than paraffin embedded sections. Secondly, it has been suggested that paraffin embedding of tissues destroy the antigen epitopes of proteins such as integrin α_6.

3.4 Conclusions

Significant success was achieved using NDRG-1 and fibulin-1 antisera. This was in spite of integrin α_6, cdc25B and MMP14 stains failing to yield tangible results. The immunohistological staining patterns and the levels of expression of NDRG-1 and fibulin-1 correlated positively with the cell type. This gives a glimmer of hope that these proteins can be used further investigated to determine their roles in tumourigenesis of cancer of the oesophagus.

Chapter 4

Conclusions

This study successfully identified genes that are differentially expressed in oesophageal cancer. Northern blot analysis confirmed the earlier results of DDRT-PCR and microarray analysis that identified several differentially expressed genes such as *HMGB, ERCC (XPG)*, nasopharyngeal carcinoma transforming gene (*TX*), *FIBULIN, NDRG, CDC25, INTEGRIN* and *MMP*. Most of these genes have been shown to play very important roles in the progress of tumourigenesis in other cancers although some have not been previously described in oesophageal cancer tissues or cell lines. For example, *FIBULIN, NDRG, ERCC* and *TX* genes have not been previously described in oesophageal cancer. This is the first study to document the expression levels and patterns of these genes in oesophageal cancer. Based on the differences in their expression patterns and levels, it is hoped these studies will stimulate further investigation into their possible role in other cancers including oesophageal cancer. On the other hand, *CDC25, INTEGRIN*, and *MMP* expression levels and patterns have previously been described in OC. This study confirmed the differential expression of these genes in tumour tissue as previously described. This study therefore supports the importance of these genes in tumourigenesis, especially in oesophageal cancer in South Africa.

This study confirmed the versatility of DDRT-PCR and microarray analysis. In addition, it demonstrated the ability of these techniques to screen and identify novel and poorly characterised genes that perhaps play an important role(s) in cancer development.

Several genes were differentially expressed but their expression levels could not be confirmed by Northern blot analysis. Most of these genes have poorly characterised biological functions. It is therefore prudent to confirm their differential expression in tumour biopsies and also establish whether they are similarly expressed in oesophageal cancer cell lines and other cancer cell lines. Some of these genes may be novel in terms of their importance in tumourigenesis.

A number of genes were found to be differentially expressed in both the microarray and DDRT-PCR but their signals were not detectable by Northern blot analysis. Further studies using more sensitive techniques such as real time PCR need to be employed to accurately determine whether the low abundance ("no signal") transcripts are indeed differentially expressed. This will further enlarge the spectrum of genes that have been shown to differentially expressed and stimulate further research into their molecular and biochemical role(s) in tumourigenesis.

Genes such as nasopharyngeal carcinoma transforming gene are poorly described in oesophageal cancer and other cancers. Further studies using *in vitro* and *in vivo* systems are required to investigate and elucidate the possible role of this gene in tumourigenesis.

The immunohistochemistry studies clearly demonstrated a significant difference ($p< 0.00001$) in the amount of fibulin-1 produced in the tumour and normal samples. Fibulin-1D was down regulated in tumour tissue specimens as seen by DDRT-PCR experiments. These results were corroborated by immunohistochemical studies. Furthermore, fibulin-1 was able to differentiate dysplastic and poorly differentiated tumour cells from the normal cells. Although these results need to be confirmed in a larger patient sample, they provide an important basis for the further investigation into the possible role of fibulin-1 in tumourigenesis.

This is the first published study that reports unequivocally on the relationships between NDRG-1 protein expression and cellular localisation vis-à-vis tumour grade. The NDRG-1 protein stain pattern correlated with the grade of the tumour. This was exemplified by the distinct differences in the cellular localisation of the protein as the grade of the tumour decreased. These distinct staining patterns overwhelmingly suggest that NDRG-1 protein is a useful marker for distinguishing some of the tumour grades, in addition to differentiating normal oesophageal squamous cells from tumour cells. A large patient sample in form of biopsies and cytological brushings are required to validate these results. This should form a basis for the evaluation of this gene as a candidate biomarker in the mass screening of high-risk populations.

In summary this study has enriched our understanding of the possible molecular basis of oesophageal cancer. It has clearly shown that several genes from divergent molecular pathways are deregulated in oesophageal cancer. This clearly shows the importance of integrating different molecular and biochemical pathways in order to better understand the process of tumourigenesis. Several question however arise from these studies. First, is when and how are these genes deregulated during OC tumourigenesis. Secondly, is whether these deregulations are important in the progress of oesophageal cancer or simply a consequence of cancer development? Thirdly is whether the process of tumourigenesis could be reversed, slowed down or remain unaffected if the deregulation of these genes is reversed? These are but a few of the questions that arise from thesis. The unveiling of these genes therefore gives impetus for their further investigation in other systems such as cell culture. It is hope that from such studies some of these questions can be answer. This should give us a better insight into the different types of molecular pathways and the possible mechanisms of action of the genes involved in the development and progression of oesophageal cancer.

CHAPTER 5

MATERIALS AND METHODS

5.1 Isolation and analysis of RNA

5.1.1 Isolation of RNA from tissues and cells

Total RNA was isolated based on the GITC method described by Chomczynski and Sacchi, (1987). The tissue samples (used in DDRT-PCR) archived at –80°C were removed and maintained in liquid nitrogen. The samples were transferred into pre-weighed Nunc tubes (Greiner labortechnik) and reweighed. The weighed tissue was transferred into a 10 ml Falcon tube (Becton Dickinson Labware) containing denaturing solution (section 5.11) (ratio of denaturing solution: tissue, 1ml; 100mg) and homogenized (maximum speed, 1min, 4°C) using the Ultra Terrax homogenizer (Junke Kunkle). Thorough mixing by inversion followed. On the other hand, cancer cell lines grown to confluency in a T-75 flask (Greiner Labortechnik) were rinsed (x2) with ice-cold PBS solution followed by the addition of 2mls of denaturing solution at 4°C. The cell lysate was passed through the pipette 10 times to completely lyse the cells. 200μl of 2M Sodium Acetate (pH 4.0) was added to either of the homogenates followed by vigorous shaking to completely dissociate the nucleoprotein complexes. 1ml of water-saturated phenol was added either to the cell lysate or the tissue homogenate. The mixture was thoroughly mixed by vortexing. 0.2mls of 49:1 chloroform/isoamyl alcohol was added, the mixture, mixed thoroughly and the suspension incubated for 15min at 4°C. Thereafter the suspension was centrifuged (10,000 *g*, 20min, 4°C) in a Beckman JA 20 rotor (Beckman). The upper aqueous phase was transferred by pipetting into a fresh Falcon tube and the RNA precipitated by addition of 1 volume of absolute isopropanol and incubation for 30min at -20°C. Thereafter the sample was centrifuged (10,000 *g*, 10min, 4°C). The superantant was aspirated off and the pellet dissolved in 300μl of denaturing solution. RNA was precipitated using isopropanol as described above. The RNA pellet was obtained by centrifugation (10,000 *g*, 10min, 4°C) and subsequently washed in 75% ethanol by centrifugation (10,000 *g*, 10min 4°C) and air-dried. The extracted RNA was dissolved in a suitable volume (50 to 100μl) of DEPC-treated water. An aliquot (5μl) of the RNA was quantitated by spectrophotometry $A_{260/280}$, and thereafter its integrity determined by gel electrophoresis using agarose 1% formaldehyde gels. RNA was stored in aliquots of 1μg at -80°C until required for further analysis.

5.1.2 Isolation of RNA using TRIzol reagent

An alternative to the use of the GITC method above, total RNA (for samples used in Microarray analysis) was isolated from the tissue samples using TRIzol reagent (Life Technologies) as per the instructions recommended by the manufacturer.

5.1.3 Removal of contaminating DNA from RNA

5µg of RNA was digested using 5U of RNase free DNase (Promega) in the presence of 1x RNase-free DNase buffer and 5U of RNasin (Promega) in a total reaction volume of 10µl. The reaction mixture was incubated for 30min at 37°C. After incubation, the reaction volume was made up to 50µl using DEPC-treated water. The RNA was extracted by adding 50µl of phenol/chloroform (3:1) vortexing and thereafter micro-centrifuged (13,000 *g*, 2min, 4°C) to separate the phases. The upper phase was transferred into a clean tube. Thereafter 5µl of 3M sodium acetate (pH 5.2) and 200µl of 100% ethanol were added. The mixture was incubated for 30min at -70°C to precipitate the RNA. The mixture was subsequently centrifuged (13,000 *g*, 10min, 4°C) and the pellet washed with 500µl of 70% ethanol. The air-dried RNA pellet was dissolved in 15µl of DEPC-treated water and quantitated by spectrophotometry A_{260}.

5.1.4 Electrophoresis of RNA on agarose formaldehyde gels

Horizontal 1% (w/v) agarose formaldehyde gels were prepared by boiling 0.4g of agarose in 27 ml of sterile distilled water and 4ml of a 10x RNA running buffer; these proportions are adequate for the preparation of a minigel. The agarose was allowed to cool to ~ 60°C and 8.5 ml of formaldehyde was added (NB: at this stage, it was important to work in the fume hood to avoid formaldehyde fumes). The agarose formaldehyde solution was poured into gel casting tray and allowed to set at room temperature in the fume hood. 1µg of RNA was mixed with 3.3µl of sterile DEPC treated distilled water, 1.5µl of 10x RNA running buffer, 7.5µl of formamide, 2.7µl of formaldehyde and 1.5µl of RNA loading buffer (section 5.11) in a final volume of ~26µl. The RNA mixture was heated at 55°C for 10min and loaded onto the agarose formaldehyde gel. Electrophoresis was carried out at 30mA in 1x RNA running buffer. The buffer was mixed at 30min time intervals. The electrophoresed gel was washed in 3 changes (each 20min) with 0.5M ammonium acetate to remove the formaldehyde. This was followed by staining in ethidium bromide (0.25µg/ml) and destained in several changes of 0.5M ammonium acetate until the 28S and 18S bands could be visualised on the UV box in the absence of any background fluorescence.

5.1.5 Northern blotting onto nylon membranes

After visualisation of the 28S and 18S RNA bands under UV light, the RNA was soaked in 10X SSC for 20 minutes. The RNA was transferred overnight by blotting onto Hybond-N membrane (Amersham) or Nytran membrane (Scleicher and Schuell) in 10x SSC using the sandwich method of Sambrook *et al.,* (1989). After the transfer, the gel was stained in ethidium bromide and viewed under UV light to ascertain the success of the transfer. The membrane was rinsed in 6x SSC and the excess liquid allowed to evaporate at room temperature. The RNA was fixed onto the membrane (with the RNA side up) by UV cross-linking using a Spectrolinker, XL-1000 UV crosslinker (Spectronics Corporation). The membrane was stored between Whatman 3MM paper at 4°C until required.

5.1.6 Reverse transcription of total RNA

Reverse transcription was set up by adding the following to a DEPC-treated microfuge tube; 1μg RNA, 1μM (final) oligo dT (A, C or G) and DEPC-treated H_2O to make a final volume of 12μl. The mixture was heated for 10min at 70°C and then quickly chilled on ice for 5min. The contents were collected by brief centrifugation (12,000 *g*, 10sec, 4°C). Thereafter, 5μl of (10x) first strand buffer (Life Technologies), 2μl of dithiothreitol (10mM) and 1.6μl of dNTPmix (20μM) (Takara) were added and the contents mixed by gently pipetting up and down (x5). The mixture was incubated for 10min at 42°C. 1μl (200U) of Superscript II reverse transcriptase enzyme (Life Technologies) was added; the mixture was pipetted gently up and down (x5) and incubated for 1h at 42°C. The enzyme was inactivated by heating 95°C, 5min. The contents were cooled on ice and collected by centrifugation (13:000 *g*, 5min, 4°C).

5.2 PCR AMPLIFICATION PROTOCOLS

5.2.1 PCR reaction set-up for DDRT-PCR

10x Amplification buffer (Takara)	-	2μl
2.5μM 4dNTP mixture (Takara)	-	1.6μl
25mM $MgCL_2$ (Takara)	-	1.5μl

2μM Arbitrary decamer (0.2μM final) - 2μl
20μM OligodT primer (1μM final) - 1.6μl
cDNA - 2μl
5U/μl Taq DNA polymerase (Takara) - 0.2μl
Distilled water - 9.1μl
TOTAL VOLUME - 20 μL

PCR thermal cycling conditions

1 cycle:	2min	94°C	(denaturation)	
	30 sec	94°C	(denaturation)	40 cycles
	2min	40°C	(annealing)	
	30sec	72°C	(extension)	
1 cycle:	5min	72°C	(extension)	
Final step:	indefinitely	4°C	(hold)	

PCR carried out in a Hybaid Omnigene PCR machine (Hybaid).

5.2.2 Beta actin

Reaction mixture

Amplification buffer (x10) - 2μl
dNTP mix (2.5mM) - 1.6μl
MgCL$_2$ (25mM) - 1.2μl
Forward primer (50pmol final) - 0.7μl
Reverse primer (50pmol final) - 1.3μl
DNA - 1μl
Taq DNA Polymerase (5U/μl) - 0.2μl
Water - 12μl

Exon	Primer pair	PCR conditions	Fragment size
5	**F**-5' TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA 3' **R**-5' CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG 3'	94 : 2min (initial denaturation) 94 : 1min 53 :1min 35 cycles 73 : 1min 73 : 2min (extension)	630 bp

5.2.3 Glyceraldehyde-3-phosphate dehydrogenase

Reaction mixture

Amplification buffer - 2μl
dNTP (2.5mM) - 1.6μl
$MgCL_2$ (25mM) - 1.2μl
Forward primer (5μM) - 2μl
Reverse primer (5μM) - 2μl
cDNA - 2μl
Taq polymerase (1u) - 0.2μl
Water - 9μl

Exon	**Primer pair**	**PCR conditions**	**Fragment size**
3	**F**-5'ACG TTG GTC GTA TTG GG 3' **R**-5' AGG GAT GAT GTT CTG GAG AG 3'	94 : 2min (initial denaturation) 94 : 30sec 60 : 2½min } 30 cycles 74 : 45sec 74 : 5min (extension)	599 bp

5.2.4 Integrin α6

Reaction mixture constituted as for GADPH

Exon	**Primer pair**	**PCR conditions**	**Fragment size**
3 Trans. 2	**F**-5'CAT GTG ACC GAT ATG 3' **R**-5' AGT AGC TGC TAC ACC TTG 3'	94 : 2min (initial denaturation) 94 : 30sec 50 : 1min } 25 cycles 72 : 30sec 72 : 10min (extension)	210 bp

Abbreviation: Trans (RNA Transcript)

5.2.5 cdc25B

Reaction mixture constituted as for GADPH

Exon	Primer pair	PCR conditions	Fragment size
1 Trans. 1	F-5'CTT CGG AAC ATC ACC AAC TC 3' R-5' GCT TCC ATG GCA TCT TGA 3'	94 : 2min (initial denaturation) 94 : 30sec, 51 : 1min, 72 : 30sec } 25 cycles 72 :10min (extension)	270 bp

5.2.6 Insulin like growth factor binding protein 3

Reaction mixture constituted as for GADPH

Exon	Primer pair	PCR conditions	Fragment size
1	F-5'TGA CTC TGC TGG TGC TGC TC 3' R-5' CTG ACG GCA CTA GCG TTG AC 3'	94 : 2min (initial denaturation) 94 : 30sec, 55 : 1min, 72 : 30sec } 25 cycles 72 : 10min (extension)	390 bp

5.2.7 Cytochrome P450 4A11

Exon	Primer pair	PCR conditions	Fragment size
1	F-5'TGA GTG TCT CTG TCC TGA GC 3' R-5' TTG AGC AGC CAC TGC CTA 3'	94 : 2min (initial denaturation) 94 : 30sec, 51 : 1min, 72 : 30sec } 25 cycles 72 : 10min (extension)	160bp

Reaction mixture constituted as for GAPDH

5.3 Denaturing polyacrylamide gel electrophoresis

5.3.1 Denaturing gel mix set up and electrophoresis

A denaturing gel 6% was constituted as follows; addition of 15ml of a 40% Acrylamide/Bisacrylamide stock (section 5.11), 42g urea, 10ml TBE (x10) and 50ml of distilled water. Gentle warming solubilized the urea. The solution was made up to 100ml degassed and filtered by vacuum. 500μl of 10% Ammonium persulphate and 50μl TEMED were added to the gel.

The gel was cast into 45cm long glass plates and left to stand overnight. The gel was pre-electrophoresed at 55W until it attained a temperature of 50°C as indicated by the temperature strip. 3.5μl of the PCR product with 2μl formamide loading buffer was heated for 2min at 80°C before immediately loading. The gel was electrophoresed at 55W for approximately 3h until the xylene cyanol dye run to 5cm of the bottom.

5.3.2 Processing of electrophoresed gels

The glass plates were separated and a piece of Whatman 3MM filter paper placed over the gel without trapping bubbles between the filter paper and gel. The gel was covered with Saran (plastic) wrap and dried for 30min under vacuum with 60°C heat using a gel drier (Hoefer Scientific Instruments). The dried gel was aligned to the x-ray (Agfa) film by stapling and exposed overnight at room temperature with or without screens depending on its radioactivity.

5.3.3 Development of x-ray film

The x-ray film was removed from the gel and immersed into developing solution (section 5.11) (Agfa) and developed for 1min by gentle rocking and then washed under running tap water. This was followed by immersion into fixer solution (Agfa) for 1min. Finally the film was rinsed thoroughly under running tap water and left to dry completely in an oven set at 20°C.

5.4 PROCESSING OF DIFFERENTIALLY DISPLAYED GENES

5.4.1 Recovery of differentially displayed DNA

The x-ray film was aligned with the gel. Each gel slice attached to Whatman 3MM filter paper was excised out using a fresh scalpel blade and placed into a fresh microfuge tube with 100μl of distilled H_2O. The gel was incubated for 10min at room temperature followed by boiling for 15min at 100°C. The tube was centrifuged (12,000 *g*, 2min, 4°C). The supernatant was pipetted into a clean tube. 10μl of 3M sodium acetate (pH5.2), 5μl of (10mg/ml) glycogen and 400μl of absolute ethanol was added to the supernatant. The mixture was incubated (30min, -70°C) and centrifuged (12,000 *g*, 10min, 4°C). The pellet was washed with 500μl of 85% ethanol, air dried and dissolved in 10μl TE (pH8).

5.4.2 Analysis of DNA by electrophoresis on agarose gels

1-2% (w/v) agarose was dissolved in 1X TBE buffer, the agarose was dissolved by boiling in a microwave, after cooling to ~50°C, ethidium bromide (3µl of a 10mg/ml solution) for a minigel) was added and the gel cast into horizontal gel casting trays. The gel was allowed to set. DNA mixed with DNA loading buffer (section 5.11) was loaded and electrophoresis performed at 50 mA.

5.4.3 Extraction of DNA from agarose gels

Electrophoresd DNA fragments were recovered using the Nucleospin extraction columns (Macherey-Nagel). Electrophoresed DNA fragments were excised out of the gel using fresh scalpels as visualized under short length UV light. The gel slices were minced using the blades and transferred into a fresh preweighed 1.5ml microfuges tubes. After weighing, 300µl of NT1 buffer was added for each 100mg of agarose gel. The samples were incubated for 10 min at 50°C with brief vortexing every 2min. The dissolved gels were transferred into fresh Nucleospin extract columns and centrifuged (8,000 *g*, 1min, 20°C). The flow through was discarded and the trapped DNA washed twice with 600µl of NT3 buffer (12,000 *g*, 1min, 20°C). The extraction columns were centrifuged (12,000 *g*, 2min, 20°C) to remove residual NT3. The Nucleospin extract column was placed into a clean 1.5ml microfuge and 50µl of elution buffer (NE) added. The columns were left to stand for 2mins and thereafter centrifuged for (12,000 *g*, 2min, 20°C). The extracted DNA was concentrated from 50µl to 20µl by using a Savant SVC100 speedy-vac. The success of the extraction was verified by electrophoresis of an aliquot (2µl) of the extract on a 2% agarose gel.

5.5 CLONING

5.5.1 Preparation of competent cells

XL1-Blue cells, were inoculated into 5ml of Luria-Bertani (LB) broth and grown overnight at 37°C with shaking at 200rpm (New Brunswick Scientific Co. Inc.). The overnight culture was used to inoculate 20ml LB in a sterile 50ml centrifuge tube giving a starting optical density$_{600nm}$ (OD$_{600nm}$) of about 0.1 as determined on a Beckman DU 650 Spectrophotometer (Beckman). Cells were allowed to reach their exponential growth phase (OD$_{600nm}$ of 0.4-0.6) at 37°C with shaking at 200rpm, and pelleted at 2 000rpm in a bench top centrifuge (Beckman Model TJ-6 Centrifuge) for 10min at 4°C. The pellet was gently re-suspended in a 10x packed cell volume (PCV) with ice-cold 50mM $CaCl_2$ solution and incubated for 2h. The cells were obtained by centrifugation (1,000rpm, 20 min, 4°C). The pellet was gently re-suspended in a small volume (3-5x PCV) of 50mM $CaCL_2$-containing 50% (v/v) glycerol. 100 and 200µl aliquots were stored at –70°C until required.

5.5.2 Ligation of fragments

DNA fragments gel purified and quantitated spectrophotometrically $A_{260/280}$ were ligated into pGEM-T Easy cloning vector (Promega). The following reaction set up was used.

2X Rapid Ligation Buffer -	5µl
pGem-T Easy vector -	1µl (50ng)
Fragment -	100ng
T4 DNA ligase (3 weiss units/µl) -	1 µl
Distilled water to a final volume of	10µl.

The reaction was mixed by gentle pipetting (x3) and incubated overnight at 4°C.

5.5.3 Transformation of competent cells

A 100µl aliquot of the competent cells was mixed with 5 or 10µl of the ligation mixture and incubated on ice for 1h. The cells were heat-shocked for 1min at 42°C followed by addition of 500µl of LB. The mixture was incubated for 1h at 37°C. 100µl of the transformation mixture was plated on LB-agar plates (section 5.11) containing 50µg/ml Ampicillin. Plates were incubated overnight at 37°C. Transformation efficiency was confirmed by transformation with 1ng of the empty pGEM-T easy vector; competent cells should give 10^7-10^8 colonies per µg plasmid DNA.

5.5.4 Screening plasmids for inserts

5ml of LB containing 50µg/ml Ampicillin was inoculated with a single bacterial colony and incubated for 16h at 37°C with shaking (200rpm). 300µl of the overnight culture was mixed with 500µl of sterile glycerol. After thorough mixing by shaking, the mixture was left to stand for 1h in a sterile lamina flow hood before it was stored at –70°C as glycerol stock. 2µl of the overnight culture was heated for 5min at 95°C and used directly as a template in a PCR reaction. The lysate was incubated in 1x PCR buffer in the presence of the 4 dNTP's at a final concentration of 0.2mM, 2U of *Taq* DNA polymerase (Takara) and 30 pmol of the following primers (USB Sequenase PCR Product Sequencing Kit):

Forward Primer: 5' - GTTTTCCCAGTCACGACGTTGTA - 3'

Reverse Primer: 5' - TTGTGAGCGGATAACAATTTC - 3'

PCR reactions were carried out in a final volume of 50µl with an initial denaturation step of 2min at 95°C, followed by 35 cycles of denaturation at 95°C (1min); annealing at 60°C (1min) and extension at 72°C (1min). 10µl of the PCR reaction was mixed with 2µl of DNA stop buffer and electrophoresed on a 1.5% agarose gel in 1x TBE. The gel was stained with ethidium bromide (0.25µg/ml) and the bands visualised under a UV light.

5.5.5 Rapid plasmid DNA preparation

Plasmid DNA was extracted using a modified alkaline lysis method of Birnboim and Doly, (1979). Overnight colonies were inoculated into 3ml of LB supplemented with 50 µg/ml Ampicillin and allowed to grow overnight 12-18 h at 37°C in a shaking incubator (200 rpm). 2ml of the overnight culture was transferred into 1.5ml microfuge tubes, centrifuged (13,000 *g*, 4°C, 2min). The cell pellet was resuspended in 100µl of solution 1 (25mM Tris-HCl, pH 8.0, 10mM EDTA, 100µg/ml RNase and50mM glucose) and incubated at room temperature for 5min. Lysis of the bacterial cells was achieved by addition of 200µl of freshly prepared solution 2 (0.25M NaOH and 1.0% (w/v) SDS) and gentle mixing by inverting the tubes (4-6 times) and incubation on ice for 5min. To this suspension, which at this stage was very viscous, 200µl of solution 3 (3M KAc, pH 4.8) was added, the tube inverted 4-6 times and incubated on ice for 10min. The plasmid DNA was obtained by centrifugation (13 000 *g*, 4°C, 5min) to pellet the membranes and cellular debris. 400-500µl of the supernatant was aspirated of after centrifugation and mixed with an equal volume of 100% (v/v) isopropanol. The mixture was incubated for 30min at -20°C. This was followed by centrifuged (13 000 *g*, 4°C, 10min) to collect the precipitate. The pellet was washed with 70% (v/v) ethanol and air-dried. The dried pellet was re-suspended in 40-50µl of TE and subjected to digestion with restriction endonucleases as required.

5.5.6 Purification of plasmid DNA using Qiagen kits

Purification of plasmid DNA was performed using the Qiagen plasmid purification kits for mini or maxi preparations. 50µl of glycerol stocks was used to inoculate 5ml or 200ml of LB supplemented with 50µg/ml Ampicillin. Cells were grown for 12 to 18h at 37°C in a shaking incubator at 200rpm and harvested by centrifugation as described above. The cell pellet was used for plasmid DNA isolation according to the protocol by the manufacturers of the kits. Generally 0.2 to 0.5mg of plasmid was obtained from a 200ml overnight culture. This DNA was sufficiently pure to be used for plasmid sequencing reactions.

5.5.7 Restriction enzyme digestion

50μg of plasmid was digested with restriction enzyme using a ratio of 1μg of plasmid to 1U of restriction enzyme. The digestion was carried out at 37°C after which the digestion mixture was heated for 20min at 65°C to inactivate the enzyme. The success of the digestion was ascertained by agarose gel electrophoresis.

5.6 Sequencing

Cloned PCR products were sequenced using the dideoxy chain termination method (Sanger *et al.,* 1977) with the *T7* Sequenase quick-denature plasmid sequencing kit (Amersham Life Sciences) according to the instructions of the manufacturer. The sequencing reactions were separated on a 6% polyacrylamide gel containing 7M urea as described in section 5.3

5.7 Radiolabelling

5.7.1 Radiolabelling probes for Northern blot analysis

The probes were labelled with [α^{32}P] dCTP using the megaprime DNA labelling system (Amersham, UK). Briefly, ~30ng DNA was mixed with 5μl of primer and heated (95°C, 5 min) then quickly chilled on ice for 1min. The contents were collected by centrifugation (12,000 *g*, 1min, 4°C). 10μl labelling buffer, 50μCi [α^{32}P] dCTP (specific activity ~3000μCi/mmol), 2μl enzyme ("Klenow" DNA polymerase) were added. The volume was made up to 50μl using distilled water. After gentle pipetting (x5), the mixture was incubated for 1h at 37°C. The contents were collected by brief centrifugation (12,000 *g*, 1 min, room temperature).

5.7.2 Purification of labelled probe

Labelled probe was purified using the Nucleotrap system (Macherey-Nagel). Briefly, 200μl of NT2 buffer was added to the labelled probe, followed by 10μl of the nucleotrap suspension. After a brief vortex, the mixture was incubated at room temperature with brief vortexing every 2min. The sample was centrifuged (10,000 *g*, 30sec, room temperature). The supernatant was pipetted off and the pellet resuspended in 500μl of NT3 buffer. After gentle vortexing, the sample was centrifuged (10,000 *g*, 30sec, room temperature). The supernatant was discarded and the washing step repeated. The pellet was centrifuged again briefly to remove residual buffer. The pellet was dried for 10min at 37°C, followed by an addition of 50μl of 5mM Tris-HCl (pH 8.5) to elute the labelled DNA fragment.

After thorough vortexing to resuspend the pellet, the suspension was incubated for 5min at 50°C with vortexing after every 1½min. The suspension was centrifuged (10,000 *g*, 30 sec, room temperature) and the supernatant transferred into a clean tube. The elution procedure was repeated again. The supernatants were pooled and centrifuged (10,000 *g*, 1min, room temperature). The supernatant was carefully pipetted avoiding the loose pellet into a clean tube.

5.7.3 Determination of labelling efficiency

A 5μl aliquot of the labelled DNA was used to determine the efficiency of the labelling procedure. Briefly the aliquot was added to 4mls of Aquasafe 5 scintaillant (Zinsser Analytic, UK). The counts per minute (cpm) were determined using a TRI-CARB 1900CA liquid scintillation analyser (Parkard).

5.7.4 Hybridisation to radiolabelled probes

A cross-linked membrane was prehybridized in 10ml of UltraHyb hybridization buffer (Ambion) in a Hybaid Dual hybridization oven for at least 4h at 42°C. The [α-^{32}P]dCTP labelled DNA probe was denatured at 99°C for 10min, snap-cooled on ice for 5min and added to the pre-hybridization solution at 1-2 X 10^6 cpm/ml for a probe with a specific activity of between 10^8-10^9 cpm/μg DNA. Hybridization was continued for 18h at 42°C in a rotating hybridization oven. The hybridization solution was decanted and the membrane washed twice in 2x SSC, 0.1% (w/v) SDS at room temperature for 15min each, followed by 2 high stringency washes in 0.1x SSC, 0.1% (w/v) SDS at for 15min at 65°C (depending on the probe). The membrane was sealed in a plastic bag and exposed to x-ray film for at least 16 h at -70°C using intensifying screens (if required).

5.8 PROPAGATION OF CELL LINES

5.8.1 Cracking of Cells

Cells (~1ml) archived in liquid nitrogen were thawed on ice and gently re-suspended by pipetting (x3). They were transferred into a 50ml blue capped tube (Greiner Labortechnik) with 49ml of Dulbecco's Modified Eagle's Medium (DMEM) previously at 37°C. The tube was inverted gently (x4) to suspend the cells and centrifuged in a Beckman benchtop

(800rpm, 5min, 4°C) to extract as much DMSO as possible. The culture medium was aspirated off and the pellet re-suspended in 1ml of fresh culture medium.

5.8.2 Culturing of WHCO series of cell lines

A T-75 (Greiner Labotechnik) culture flask with 10 ml of DMEM containing 1% penicillin plus streptomycin, 10% heat inactivated foetal bovine serum was inoculated with 1ml of the thawed cells. The cells were incubated at 37°C with 5% CO_2 humidity. When at least 50% of the cells had attached (6-12h) onto the surface of the flask, the medium was replaced. The cells were grown to 70% confluence before they were trypsinized.

5.8.3 Culturing of KYSE series of cell lines

The KYSE cells were cultured as WHCO cell line except the medium used was DMEM with 1% penicillin plus streptomycin and 5% foetal bovine serum.

5.8.4 Culturing of MDA-MB-231-5 breast cancer cell lines

This cell line was cultured using identical medium as for KYSE cell lines

5.8.5 Passage of cells

The cells in a T-75 flask were washed with 2ml of trypsinization solution (section 5.11) for 30sec and the solution aspirated off. A fresh 3ml of trypsinization solution was added to the cells and the trypsinization process followed by observation under the microscope (Zeiss). The trypsinization process was stopped by addition of 3ml of fresh DMEM medium when 80% of the cells were trypsinized. The cell suspension was aspirated up and down (x5) to disrupt the cell clumps and transferred into a 10ml Falcon tube. The cell suspension was centrifuged in a Beckman bench top (800 rpm, 5min, 4°C), the culture medium aspirated off and cell pellet re-suspended in 1ml of culture medium (37°C). 200μl (1:5 split ration) of the re-suspended cells were used to inoculate 10mls of fresh culture medium in a T-75 flask.

5.8.6 Freezing down of cells

The cell pellet obtained after cell trypsinization in section 5.8.5 was re-suspended in freezing down solution (section 5.11) by gently pipetting (x2) on ice. Thereafter the cells were stored for 24h at -80°C before being archived in liquid nitrogen.

5.9 MICROARRAY ANALYSIS

5.9.1 Making of microarray probe

The following reverse transcription labelling reaction was set up for the normal and tumour sample RNA, respectively.

1. 5x first strand buffer (Life Technologies)	8μl
2. oligo dT 20mer (Life Technologies)	2μl
3. 20x low dNTP mix (Life Technologies)	2μl
4. Cy3 or Cy5 dVTP (Amersham Life Sciences)	4μl
5. 0.1M DTT(Life Technologies)	4μl
6. RNAsin (Life Technologies)	1μl
7. Total RNA (25μg)	7.45μl
8. DEPC-treated water	11.55μl
TOTAL	40μl

The reaction mixture was vortexed and centrifuged briefly. This was followed by 5min incubation at 65°C and a further 2.5min incubation at room temperature. 2μl of Superscript II reverse transcriptase (Life Technologies) was added mixed by pipetting and incubated for 25min at 42°C. A further 2μl of Superscript enzyme was added and the reaction returned to 42°C for a further 35min. The reaction was stopped by addition of 5μl of EDTA (500mM) and quick vortexing. Hydrolysis of residual RNA was performed by addition of 10μl of 1M NaOH, brief vortexing, centrifugation and incubation for 1h at 65°C. The mixture was cooled to room temperature and neutralized using 25μl of 1M Tris-HCl (pH7.5).

5.9.2 Cleaning up of microarray probe

Microcon YM-30 columns (Amicon) were initially washed by addition of 500μl of TE (pH 7.4) and centrifugation (13,000 *g*, 8min, room temperature). 400μl of TE was added to each separately Cy-labelled cDNA and the mixture transferred into a microcon column. This was followed by centrifugation (13,000 *g*, ~8 min, room temperature) and thereafter another 1-2 min until a volume of 20-40μl was left.

The column with Cy3 labelled cDNA probe was inverted into a fresh tube and centrifuged (13,000 x *g*, 1min, room temperature) to recover the probe. The recovered probe was transferred into the column with Cy5-labelled cDNA, 400μl of TE added and the combined probes centrifuged (13,000 *g*) until the volume was 20-30μl as measured by a pipetman.

The unincorporated fluorescent dyes were removed by washing the combined labelled probes with TE. Briefly, 450μl TE was added to the combined probes and centrifuged (13,000 *g*) severally until the volume of the probe was 6-8μl as measured by a pipetman.

The column with the combined washed probe was inverted into a fresh tube and centrifuged (13,000 *g*, 1min) to recover the probe. The volume of the recovered probe was made up to 11μl with TE. A final volume of 17.6μl of the Probe was achieved by adding the following 1μl (10μg) COT-1-DNA (Roche), 1μl (8-10μg) polyA (Pharmacia) 1μl yeast tRNA (4μg) (Sigma), 3.1μl 20x SSC and 0.5μl 10% SDS. The probes were heated for 2min at 100°C, centrifuged (14,000 *g*, 10min, room temperature) and the supernatant carefully pipetted into a clean microfuge tube.

5.9.3 Hybridization of probe to microarray

The probe was re-suspended by gentle pipetting (x5) avoiding formation of bubbles. 17.6μl of the probe was carefully pipetted onto the centre of the 6.5Kb glass array. A cover slip was gently lowered onto the array using forceps. The glass array was transferred into a humid (20μl of water added at each end well) hybridization chamber and the chamber clamped. The chamber was carefully placed at the bottom of a water bath and incubated for 18h at 65°C.

5.9.4 Washing of the hybridized array

The hybridization chamber was removed from the incubator and the margin clamps removed. The array slide was removed and immersed in the first wash (2x SSC, 0.1% SDS) for 1min (the coverslip separated from the array in this step). This was followed by plunging gently for 1 min in 1x SCC, 0.2x SSC, and for 20sec in 0.2x SSC. The slide was dried by centrifugation (100 *g*) until the slide was completely dry. The array was stored in the dark until ready to scan.

5.10 IMMUNOHISTOCHEMISTRY (IHC)

5.10.1 Processing of tissues for IHC

Tissues were fixed in 10% formalsaline for 24h and thereafter processed in a Tissue-Tek vaccum impregnation processing unit (Miles Scientific) set at 37°C as follows; 3 changes in 96% ethanol, 100% ethanol and xylol, respectively. The tissues were then impregnated in 4 molten wax changes at 60°C. Finally the tissues were embedded using a Histocenter II embedding machine.

5.10.2 Coating of slides for Immunohistochemistry

Slides were packed into metal racks and immersed into 1% aqueous ordinary liquid detergent. The slides were washed for 1h by rocking and rinsed in running tap water for 1 h. They were thereafter rinsed in 3 changes (each 20min) of distilled water. The slides were dried in an oven at 37°C overnight. They were coated by dipping the racks into the following; 3-aminopropyltriethoxysilane (APES) (10 dips), Acetone (10 dips), Acetone (10 dips), distilled water (10 dips). After draining off excess liquid by gently blotting the racks on blotting paper the slides were dried overnight at 37°C and subsequently packed into boxes until used

5.10.3 Cutting of tissue sections

Paraffin embedded tissue sections were cut to 2μM thickness using a sliding microtome (Jung). The sections were mounted onto APES coated slides and heat fixed in an oven overnight at 60°C.

5.10.4 Immunohistochemistry using Fibulin-1 antibody

The tissue sections were dewaxed in 4 changes (5mins each) of xylol followed by 4 changes (5min each) of 96% ethanol. The fibulin-1 antigen was retrieved by digesting the sections in 1% trypsin {50mg of trypsin in 50mls 0.05M Tris-HCL buffer (pH 7.6)} for 5min at 37°C. The trypsin was inactivated by rinsing (x2, 2 min) the sections in PBS (without Tween). Endogenous hydrogen peroxide in the tissues was inactivated by immersing the tissue sections in 1% hydrogen peroxide (section 5.11) for 20min at room temperature. This was followed by a 2min rinse in distilled water to remove the hydrogen peroxide.

The sections were blocked for non-specific binding by overlaying the slides with 5% normal rabbit serum (DAKO) in PBS for 10min. Incubation with the primary antibody, detection with secondary antibodies and colour development was carried out using the automated DAKO universal stainer (DAKO) using the IHC software (DAKO). Briefly, the sections were incubated with the primary antibody, Fibulin-1 anti-goat serum {1:30 dilution in antibody diluent (DAKO)} for 30min. This was followed by 2 washes in PBS (with 0.05% Tween 20) and incubation with the secondary antibody, Rabbit anti-goat (DAKO) {1:400 dilution using PBS without Tween 20} for 30min. The antibody was washed off with 2 rinses in PBS (without Tween 20) and overlaid with Avidin horseradish peroxidase (DAKO) {1:500 dilution using PBS (without Tween 20)} for 30min. The chromogenic substrate, diaminobenze (DAB) (DAKO) diluted according to the instructions of the manufacturer was added and the colour development allowed for 5min and thereafter the slides were rinsed in PBS (with Tween 20).

The slides were removed from the Universal stainer and the fibulin stain enhanced by immersing the sections in 1% copper sulphate for 5min. This was followed by a rinse of the sections in running tap water for 1min and counter staining in hematoxylin for 1-3min. After the hematoxylin stain, the slides were rinsed in running tap water for 1min and the colour of the stain enhanced by immersion in Scots solution (section 5.11) for 1min. Thereafter the sections were rinsed in running water for 1 min, dehydrated in 4 changes (1min each) of 96% ethanol followed by 4 changes (1min each) in Xylol (cleaning agent). The sections were mounted in Entellen (Merck) left to air dry for 20min and stored in IHC slide folders until ready for analysis.

5.10.5 Immunohistochemistry using NDRG1 antibody

The tissue sections were initially processed as described in section 5.10.4. The NDRG-1 antigen was retrieved by pressure-cooking the sections in 10mM citrate buffer (pH 6). Briefly, the citrate buffer was brought to boil in an open pressure-cooking pot. The sections were immersed into the boiling buffer, the pot sealed and allowed to come to full pressure. This pressure was maintained for 2min. After the release of the pressure, the slides were transferred into distilled water for 5min to cool.

Endogenous peroxide within the tissues was blocked by incubating the sections in 1% hydrogen peroxide for 20min. This was followed by a 2min rinse in distilled water. The sections were blocked for non-specific binding by using normal goat serum (DAKO) for 20min, which was thereafter drained off. Incubation with the primary antibody, NDRG1 anti-rabbit serum, a kind donation by Teresa Commes {1:500 dilution in antibody diluent (DAKO)}, detection using secondary antibody - Envision^{TM} rabbit peroxidase (DAKO) and development of colour using diaminobenzene (DAB) were carried out using an automated DAKO universal using the IHC software stainer (DAKO). Briefly the sections were mounted onto the staining racks of the DAKO stainer and therafter incubated for 30min with the primary antibody at room temperature. The antibody was rinsed (x2) off using PBS (with 0.05% Tween 20).

The primary antibody was detected using Envision^{TM} rabbit peroxidase for 30min followed by 2 rinses with PBS (with 0.05% Tween 20). Diaminobenzene was used as the chromogenic substrate. The sections were incubated with DAB for 5min and thereafter rinsed in PBS (with 0.05% Tween 20). The stained slides were removed from the automated stainer and processed as described above in section 5.10.4.

5.11 Buffers and solutions

40% ACRYLAMIDE STOCK SOLUTION (38:2)
38% Acrylamide
2% Bisacrylamide
2% ion exchanger V
add water to a final volume of 100ml
The solution was stirred for 16h at 4°C, filtered and then stored at 4°C.

AMINOPROPYLTRIETHOXYSALINE (APES) SOLUTION
3.3% APES in acetone

AMPICILLIN SOLUTION
10mg/ml amplicillin trihydrate salt in sterile water.
Filter sterilize using 0.22μM syringe filter
Store in the dark for 1 month at 4°C

CHLOROPHORM:ISOAMYL ALCOHOL (CIAA) (49:1)
49ml chloroform
1ml isoamyl alcohol

CITRATE BUFFER (pH 6.0) (IHC)
0.01M citric acid
pH to 6 using 1M NaOH

COPPER SULPHATE SOLUTION
1% copper sulphate in distilled water

DEPC-TREATED H_2O
0.05% DEPC in distilled water stir overnight at room temperature and
inactivate DEPC by autoclaving

DEVELOPING SOLUTION
20% developer (Agfa) made up to a litre in distilled water

ETHIDIUM BROMIDE
10mg ethidium bromide
100ml distilled H_2O

FIXING SOLUTION
20% fixer (Agfa) made up to a litre in distilled water

10 X FORMALDEHYDE LOADING BUFFER

1mM EDTA
0.25% Bromophenol blue
0.25% Xylene cyanol
50% Glycerol

FOETAL BOVINE SERUM (FBS)

Foetal bovine serum heat inactivated at 56°C for 30min and stored at –20°C until ready for use.

FORMAMIDE LOADING BUFFER

95% Formamide
10mM EDTA
0.05% Bromophenol blue
0.05% Xylene cyanol

FREEZING DOWN SOLUTION

70% foetal bovine serum
10% Dimethylsulphoxide (DMSO)
20% DMEM

HEMATOXYLIN SOLUTION

0.3mM Hematoxylin monohydrate
105mM Aluminium potassium sulphate dodecahydrate
0.09mM Sodium Iodate
300mM Chloral hydrate
0.4mM Citric acid

Dissolve Hematoxylin monohydrate in water first, heat to 60°C (sunset colour). Add individually the above in order, mix well, let cool, filter before use using Whatmans paper

HYDROGEN PEROXIDE

1% hydrogen peroxide in methanol

LURIA AGAR

10g/l Tryptone
5g/l Yeast extract
5g/l NaCl
15g/l Agar

LURIA BROTH

10g/l Tryptone
5g/l Yeast extract
5g/l NaCl

PENICILLIN/STREPTOMYCIN SOLUTION

Add 5 million units sodium benzylpenicillin to 5ml PBS. Separately add 5g streptomycin sulphate to 15ml PBS. Pool the two antibiotics and make up volume to 500ml with PBS.

Use 5 ml of the stock solution in 500ml of DMEM.

PHOSPHATE BUFFERED SALINE

137mM NaCl
2.7mM KCl
4.3mM $Na_2HPO_4.7H_2O$ (pH 7.4)
1.4mM KH_2PO_4

RNA LOADING BUFFER

1 X RNA Running Buffer
45% deionised formamide
6% formaldehyde
1 X Formaldehyde loading buffer

10 X RNA RUNNING BUFFER

41.8g 3-[N-morpholino] propanesulfonic acid (MOPS) to 800ml DEPC-treated H_2O
Adjust to pH 7.0 with NaOH
16.6ml 3M Na Acetate, pH 5.5
20ml 0.5M EDTA, pH 8.0
Make up to 1 litre with DEPC-treated H_2O

SCOTS SOLUTION

40mM $NaHCO_3$
80mM $MgSO_4.7H_2O$
in tap water

SOLUTION D

4.2M Guanidine thiocyanate salt (GITC)
300mM Sodium Citrate, pH 7.0
0.5% Sarcosyl
Add 360μl 2-mercaptoethanol to 50ml solution D prior to use.

20 X SSC

3M NaCl
0.3M Na_3 citrate

10 X TAE BUFFER

0.4M Tris
0.01M EDTA
pH to 7.8 with acetic acid

10 X TBE BUFFER

890mM Tris
890mM Boric Acid
25mM EDTA
pH to 8.3

1 X TE (TRIS/EDTA) BUFFER

10mM Tris-Cl, pH 8.0
1mM EDTA, pH 8.0

Tris-HCl pH 7.6

50mM Tris salt

pH using 1M HCl

TRYPSINIZATION SOLUTION

0.5g trypsin
8g NaCL
1.45g $Na_2HPO_4.2H_2O$
0.2g KCL
0.2g KH_2PO_4
10mM EDTA, pH 8.0
in a litre of PBS

WASH SOLUTION 1 (NORTHERN ANALYSIS)
2 X SSC
0.1% SDS
in DEPC-treated water

WASH SOLUTION 2 (NORTHERN ANALYSIS)
0.1 X SSC
0.1% SDS
in DEPC-treated water

CHAPTER 6

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