INVESTIGATING THE GENETIC PROFILE OF THE E-CADHERIN GENE IN SQUAMOUS CARCINOMA OF THE ESOPHAGUS

by

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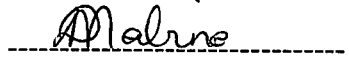
DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my original (except where otherwise indicated), independent and has not in its entirely or part been submitted to any university for a degree.

All the sources I have made use of or quoted have been acknowledged by complete references.



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ABBREVIATIONS

APC – adenomatous polyposis coli

APS – ammoniumperoxodisulphate

arm – armadillo

bp – base pair

CDH1 – E-cadherin gene

cDNA – complementary DNA

CIS – carcinoma in situ

CpG – cytosine followed by guanine on the same strand

CP – cytoplasmic domain

CTNNA1 – alpha catenin gene

CTNNB1 – beta catenin gene

CTNND1 – $p120^{ctn}$ gene

DNA – deoxyribonucleic acid

dNTPs – deoxynucleoside-triphosphates

EC – esophageal cancer

EC_1-EC_5 – extracellular domains

E-cadherin – epithelial cadherin

EDTA – ethylenediamine-tetraacetic acid

EGFR – epidermal growth factor receptor

ESCC – esophageal squamous cell carcinoma

FB_1 –fumonisin

GSK – glycogen synthetase kinase

HAV – histidine alanine valine

HPV – human papilloma virus

H pylori – Helicobacter pylori

JUP – gamma catenin gene

kb- kilo base pair

kDa – kilo Dalton

L-CAM – E-cadherin gene in chicken

LEF/TCF – leucocyte enhancer factor/ T-cell factor

LOH –loss of heterozygosity

MD – moderately differentiated

$MgCl_2$ – magnesium chloride

N-cadherin – neural cadherin

NH_2 – amino terminal

NMR – nuclear magnetic resonance

OD_{260} – optical density at 260nm

PD – poorly differentiated

PCR – polymerase chain reaction

P-cadherin – placental cadherin

PRE – precursor

Rb- retinoblastoma

RIP – regulated intramembrane proteolysis

SCC – squamous cell carcinoma

SSCP – single-stranded conformational polymorphism

Taq – Thermus aquatus

TBE – Tris–Borate–EDTA buffer

TEMED – *N,N,N′,N′*- tetramethylenediamine

TM – transmembrane domain

VEGF – vascular epidermal growth factor

WD – vell differentiated

WNT – wingless

CHAPTER 1

Esophageal Cancer

1.1 Esophageal cancer

Esophageal cancer (EC) in humans ranks as the eighth most common cancer in the world (World Cancer Research Fund and American Institute for Cancer Research, 1997; World Health Organisation, 1997). This malignancy exists in two main forms with distinct etiological and pathological characteristics, namely: squamous cell carcinoma (SCC) and adenocarcinoma. More than 90% of esophageal cancers worldwide are SCC (Stoner *et al.*., 1995; Beer and Stoner, 1998).

The esophagus is divided into three regions, namely: the cervical, the mid and the distal region (Figure1). The cervical and mid esophageal tumours tend to develop SCC which is often multifocal characterized by a rapid increase in the size of the tumour (Haruma *et al.*., 1991). Distal and GE junction lesions are generally adenocarcinomas (Lee *et al.*., 1984).

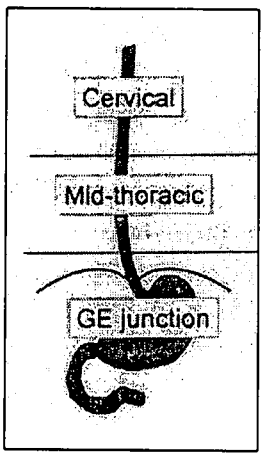

Figure 1.1 Regions of the esophagus. From Harrison, 2000.

Regardless of cell type, esophageal cancer is a disease that rarely occurs in children or young adults (Blot, 1994). The economic climate of many countries is changing which it leads elevated incidence of esophageal cancer (Blot and McLaughlin, 1999; Day and Varghese, 1994; Gao *et al.*., 1994). Rates of occurrence of ESCC vary markedly around the world. In certain parts of Iran and North Central China, annual rates of esophageal cancer mortality exceed 100 per 100,000 population, one of the highest rates for any cancer worldwide (Parkin *et al.*., 1992; Munoz and Day, 1996). Pockets of elevated esophageal cancer mortality have been reported in South Africa and parts of France, but in most countries rates are less than 10 per 100,000 (Brown *et al.*., 1988).

1.2 Esophageal cancer in South Africa

Cancer of the esophagus was an uncommon disease in the South African black population during the 1920s and 1930s. Since then an alarming increase in incidence has occurred (Rose, 1973). Figures from hospitals in Johannesburg which have served the black population show an increase from 2% of all tumours in men in the 1930s, to 11% in the early 1950s and 28% in the early 1960s (Cook, 1971).

High incidences of esophageal squamous carcinoma have been reported in the adult black male population in the Transkei region of South Africa. In 1994, esophageal cancer accounted for 45.8% of all malignancies in this region and the figures keep on increasing (Klimstra, 1994). Transkei has one of the highest rates in the world of carcinoma of the esophagus. Much work has gone into the search for a specific carcinogen, but no single candidate has been found that can explain the local high level of the disease (Sitas *et al.*., 1996). Multifactorial etiology is the most commonly held hypothesis and proposed risk factors include carcinogens, dietary deficiency of vitamins and trace elements and alcohol (Sammon, 1998).

1.3 Diagnosis

Esophageal squamous cell carcinoma (ESCC) is usually diagnosed in its very late stage because the symptoms develop very slowly and are painless. Early tumour growth causes the smooth muscle to dilate readily due to the esophagus lacking a serosal covering. During this period the patient is generally asymptomatic. Evidence from prospective studies suggests that esophageal SCC probably develops through a progressive sequence from mild to severe dysplasia (abnormal development of the skin, bone or other tissue), carcinoma *in situ* and, finally, invasive carcinoma (Anani *et al.*., 1991; Kuwano *et al.*., 1993). When the esophageal circumference is more than half infiltrated with tumour, dysphagia (difficulty swallowing) occurs (Allen *et al.*., 1997). Dysphagia is usually one of the initial signs of the disease.

Dysphagia is the most common presenting symptom and often heralds incurable disease due to local spread (Allen *et al.*., 1997). Most patients who are over 45 years and suffer from dysphagia also have ESCC. Other less likely presenting symptoms include coughing or choking, hoarseness, or, more rarely, shock (Allen *et al.*., 1997). The prognosis of this type of cancer is poor compared with other types of cancer of the gastrointestinal tract, such as stomach and colon cancers (Nishihira *et al.*., 1993).

1.4 Prognosis

The 5-year survival rate of esophageal cancer patients remains bleak and is estimated at 3-10% (Cilley *et al.*., 1989). Seventy percent of patients die of the disease within 1 year of diagnosis (Cilley *et al.*., 1989). This is partially due to the fact that approximately 50% have advanced stage disease, with irresecteable lesions at initial presentation (Allen *et al.*., 1997).

Shimada *et al.*. (1999) researched eleven molecular biological markers together, using immunohistochemical tests. They concluded that one oncogene

(cyclin D1) and one cell-adhesion molecule (E-cadherin) are predictive death factors of ESCC. E-cadherin has already been proposed as a significant prognostic factor not only in ESCC (Miyata *et al.*., 1994; Tamura *et al.*., 1996), but also in various other carcinomas. In ESCC, a correlation between E-cadherin reduction and lymph node metastasis (Miyata *et al.*., 1994) or hematogenous recurrence (Tamura *et al.*., 1996) has been reported. The Research Committee on Malignancy of Esophageal Cancer (Japanese Society for Esophageal Diseases, 2001) found no association between reduced expression of E-cadherin and recurrence. Therefore, the reason why E-cadherin reduction is associated with the prognosis of ESCC remains uncertain.

1.5 Treatment

The treatment of ESCC is based on its stage at presentation (Forastiere, 1992). Despite improvements in surgical techniques, rapid fatal recurrence is common in patients with advanced disease (Isono *et al.*., 1990). Surgical resection alone rarely results in long-term survival; efforts are now focused on combined multi-modality treatments in an attempt to improve local control and eliminate micro-metastasis present at the time of surgery (Shimada *et al.*., 2002). Esophagectomy may be performed for cure or palliation (Allen *et al.*., 1997).

1.6 Diseases associated with ESCC

Among the disease processes that predispose a patient to the development of ESCC are achalasia, dysphagia, lye strictures, Plummer-Vinson Syndrome and tylosis. Interestingly, SCC of the esophagus associated with achalasia, a disease primarily of the body of the esophagus and the lower esophageal sphincter, nearly always occurs in the middle third of the esophagus (Meijssen *et al.*., 1992). Plummer-Vinson Syndrome is associated with carcinoma of the

hypopharynx and the upper third of the esophagus (Larson *et al.*., 1975). Patients with tylosis, a rare genetic syndrome characterized by symmetrical late onset keratosis involving the palms of the hands and the soles of the feet, have a 90-95% probability of dying of ESCC before age 65. Familial esophagael cancer can also occur in patients without tylosis (Ghardirian, 1985; Marger and Marger, 1993).

1.7 Inhibitors of ESCC

Drinking of green tea, which contains flavonoids, isothiocyanates, phenols, and other compounds have been postulated to play a protective role (Yang and Wang, 1993). Green tea has also been associated with a significant decrease in the risk of ESCC in Shanghai (Gao *et al.*., 1994).

In South Africa, a significant association has been shown between EC and consumption of beans as well as with a traditional diet (maize, pumpkin and beans) in the people living in the Transkei region. Their diet is based on maize, pumpkin and beans (Sammon, 1998). Solanum nigrum (a wild vegetable), lima beans and pumpkin have a major feature in common. They all contain protease inhibitors. Luminal proteases, including pepsin and trypsin, are secreted from the esophageal mucosa and they degrade growth factors. Growth factors are involved in the repair and also provide a proliferative drive (Playford *et al.*., 1995). Solanum nigrum inhibits pepsin activity (Akhtar and Munir, 1989), whereas beans and pumpkin contain high amounts of trypsin inhibitor (Aletor *et al.*., 1989; Krishnamourthy *et al.*., 1990).

1.8 Risk factors

Both genetic and environmental factors play a role in the etiology of squamous cell carcinoma of the esophagus (Allen *et al.*., 1997). It could be argued that

either the genetic or environmental factors are more important, but the combination of the two would yield better results. Excessive alcohol and tobacco usage are some of the major risk factors known to play a contributing role to ESCC.

1.8.1 Tobacco and alcohol

Two major contributing factors to ESCC are cigarette smoking and alcohol consumption (International Agency for Research on Cancer, 1986, 1988). Large cohort studies indicate that the risk of esophageal cancer are approximately five times higher among cigarette smokers than nonsmokers, with the excess increasing to nearly 10-fold among heavy smokers. Part of the increase among smokers is due to their increased alcohol consumption, but the risk is also increased among smokers who do not drink.

The consumption of specific alcoholic beverages has been implicated in several clusters of elevated SCC of the esophagus mortality rates around the world (Blot, 1992). In northern France, which leads all western areas in the incidence of esophageal cancer, the drinking of apple brandies appears to be a major contributor, whereas maize beer in the South African Transkei, sugar-distilled beverages in Puerto Rico and South America, and moonshine whiskeys in South Carolina have been linked to excess risks (Blot, 1992). It seems likely that the underlying cause of these associations worldwide is ethanol intake. The variation in the risk of ESCC with regard to the specific alcoholic beverages suggests a contributory factor from other ingredients in the beverages (William *et al.*., 1999).

In South Africa, the risk associated with the consumption of traditional beer may not rest solely in the quantity of alcohol consumed. The increase in risk associated with the use of maize meal as the major ingredient of beer (Segal *et al.*., 1988) accords with the findings by Cook (1971) of an association in Africa with the use of maize for beer making.

1.8.2 *Fusarium verticillioides*

A positive correlation has been reported for the occurrence of ESCC in humans and the presence of *Fusarium*-contaminated maize in the Transkei region of the Eastern Cape Province of South Africa (Marasas *et at.*, 1988) and the Henan Province of the People's Republic of China (Yoshizawa *et al..*, 1994). The fungal metabolite fusarin C was found in healthy and visibly *Fusarium*-infected maize kernels from rural households in South Africa (Gelderblom, *et al..*, 1986). Bever *et al..* (2000) suggested that fusarin C is a possible etiological agent for the high incidence of human ESCC in South Africa. Fumonisin B_1 (FB_1) has also been associated with the etiology of ESCC in South Africa (Rheeder *et al..*, 1992) and this has been supported by immunolocalization of FB_1 in esophageal cancer tissue (Myburg, 1998). There is an increased risk of consumption of fumonisins since maize is the staple diet of the South African rural population (Sydenham *et al..*, 1990).

1.8.3 Diet and nutrition

Diet and nutrition have played an important role in the occurrence of ESCC (van Rensburg, 1981). The intake of fruits and vegetables in adults play a significant role in the inihibition of ESCC (William *et al..*, 1999). Athough specific food nutrients may be involved, only one reported randomized trial investigating the effects of vitamins and minerals on ESCC risk has been that from Linxian, China, where esophageal cancer rates are exceptionally high (Li *et al..*, 1993; Blot *et al..*, 1993). For more than 5 years, the Chinese population was supplemented with food that contained a combination of beta-carotene, vitamin E, and selenium. Death rates decreased by 13% (Blot *et al..*, 1993).

1.8.4 Human Papilloma Virus

Human Papilloma Virus (HPV) is a member of the papovavirus family, a family of closed circular double-stranded DNA (7.9kb) viruses. To date 73 different HPV genotypes have been described (Zur Hausen and de Villiers, 1994). HPV DNA sequences has been detected in 25/48 (52%) esophageal cancers and HPV 16 was present in 84% of the HPV-positive cases of ESCC in South Africa (Cooper *et al.*., 1995; Sur and Cooper., 1998).

1.8.5 Other factors

Pickled vegetables, hot food and drinks and moldy grains have also been implicated as risk factors for ESCC (Weiss, 1995; Norell *et al.*., 1983). Ionizing radiation may also increase the risk of ESCC (Smith, 1984). Other physical irritants of the esophagus may predispose to increased risk of SCC (William *et al.*., 1999).

Although social class has been linked to ESCC in a number of studies (Segal *et al.*., 1988; Yu *et al.*., 1993), the underlying exposures or characteristics responsible for the association remain unclear (Brown *et al.*., 2001). Some of these factors, such as nutritional status, may affect susceptibility to environmental carcinogens, but the mechanisms still need to be clarified (Tollefson, 1985).

1.8.6 Genetic influence

A number of genetic changes in ESCC have been consistently observed regardless of patient origin and the suspected etiological factors. These include (i) mutation of the p53 tumour suppressor gene, giving rise to a variety of

disturbances in growth control, DNA replication, repair and apoptosis; (ii) deregulation of cell cycle control by disturbance of the cyclin-dependent kinase-*RB* pathway of cell cycle control, and (iii) genetic alteration of oncogenes, causing deregulation of signal transduction (Mandard *et al.*., 2000).

These molecular and cytogenetic studies suggest that many oncogenes and tumour suppressor genes are involved in the initiation and development of esophageal cancer. Microsatellite marker loss of heterozygosity (LOH) studies have shown that allelic losses on chromosomes 1pter-21, 3p21, 5q, 9p21, 11q, 13q, 17 and 18q are frequent in ESCC (Mandard *et al.*., 2000). Other genes that are altered in ESCC include, *Rb, cyclin D1, INT-2, p16, APC, MCC, DCC, Ki-67, Bcl12, EGFR, VEGF, Mdm2, AMF* and E-cadherin to mention but a few. Unlike neoplastic development in the colon, *RAS* oncogene activation is seldom seen in ESCC (Mandard *et al.*., 2000).

While it has been stated that there is no single gene directly associated with esophageal cancer so far (Lu, 2000), it is assumed that *C1orf10* gene expression is restricted only to the esophagus (Xu *et al.*., 2000). The expression of this gene has been undetectable in 15 other adult tumours and so it supports the esophageal-specific expression pattern. *C1orf10* is composed of three exons and is expressed at a high level in normal esophageal mucosa, but is undetectable or barely detectable in 94.6% EC tissues. The high frequency of loss of C1orf10 expression in primary ESCC supports the notion that *C1orf10* is important in human ESCC. The precise functions of *C1orf10* are currently unknown. (Xu *et al.*., 2000).

Approximately 90% of human cancers originate from epithelial cells (Takeichi, 1995; Nollet *et al.*., 1999) and immunohistochemical studies have demonstrated that loss of E-cadherin expression is a frequent event in many types of carcinomas (Jiang, 1996; Papadavid and Katsambas, 2001). It could thus be postulated that E-cadherin should have an important role in cancer development (Nollet *et al.*., 1999).

1.8.6.1 E-cadherin

E-cadherin is a member of the cadherin family that is known to play an important role in the regulation of intercellular adhesion in epithelial tissues (Takeichi, 1991). Because one of the first changes in the metastatic process is a decrease in this adhesion (Doki *et al.*., 1993; Shiozaki *et al.*., 1996), it has been postulated that abnormal or reduced E-cadherin expression acts to facilitate tumour invasion and sub-sequent formation of metastases. Indeed, E-cadherin expression has been found to be associated closely with tumour invasiveness, dedifferentiation, the formation of metastases and poor prognosis in various human carcinomas (Becker *et al.*., 1994; Tamura *et al.*., 1996).

CHAPTER 2

E-cadherin

2.1 E-cadherin – the gene

The human E-cadherin gene locus (*CDH1*) is localized on chromosome 16q22.1. This location was detected by the use of human and mouse somatic-cell hybrids (Mansouri *et al.*., 1988) and by analysis of interstitial deletions at 16q (Natt *et al.*., 1989). The human P-cadherin gene is located only 32kb upstream from E-cadherin and the M-cadherin gene is also positioned on chromosome 16 (16q24.1 – qter) (Kauppmann *et al.*., 1992). The localization suggests a cluster of cadherin genes, originating from gene duplication, while possible co-evolution might be explained by gene conversion (Gally and Edelman, 1992).

The E-cadherin gene, in common with all classical cadherin genes, has 16 exons separated by 15 introns. Comparison of the human E-cadherin exon borders to those of the other reported cadherin genes, shows a remarkably high conservation of the splice sites among various species and also among various cadherin types. The exon structure of the E-cadherin gene is also well conserved, since the physical map of the human gene is homologous to that of the chicken (L-CAM) (Berx *et al.*., 1995; Gallin *et al.*., 1987). Comparison of exon size of the mammalian E-cadherins with chicken L-CAM shows 5 small-size differences in exons coding for the mature protein (Berx *et al.*., 1995). Compared to the human gene, most of the introns are 2 to 4-fold increased in size in the mouse E-cadherin gene. Discrepancies in exon size between human and mouse E-cadherin are found only in exons 1 and 2, coding for signal and precursor sequences, and in the 3' untranslated region encoded by exon 16 (Berx *et al.*., 1995). Intron 2 contributes more than 50% of the overall length of the human E-cadherin gene (65kb versus 100kb) (Berx *et al.*, 1995). The size of the E-cadherin gene exons varies between 190 bp and 378 bp.

DNA sequencing of intron 1 (EMBO/GenBank Accession No. L36526) and further analysis according to Gardiner-Garden and Frommer (1987) and Larsen *et al.*. (1992) discovered a sequence of about 1500bp with the features of a high-density CpG island. This putative island covered the region from exon 1 to exon 2 of the human E-cadherin gene. In contrast, the other exons including exon 16 of 2245bp lacked such features (Berx *et al.*, 1995).

2.1.1 Transcriptional regulation of E-cadherin

The promoter of the E-cadherin gene is specifically active in E-cadherin-expressing cells and inactive in E-cadherin-deficient cells, for example in fibroblasts and dedifferentiated carcinoma cells (Behrens *et al.*., 1991). The basis for this specificity seems to reside in a negative regulatory DNA element named the E-box that represses promoter activity in E-cadherin-negative cells (Figure 2.1.4) (Giroldi *et al.*., 1997).

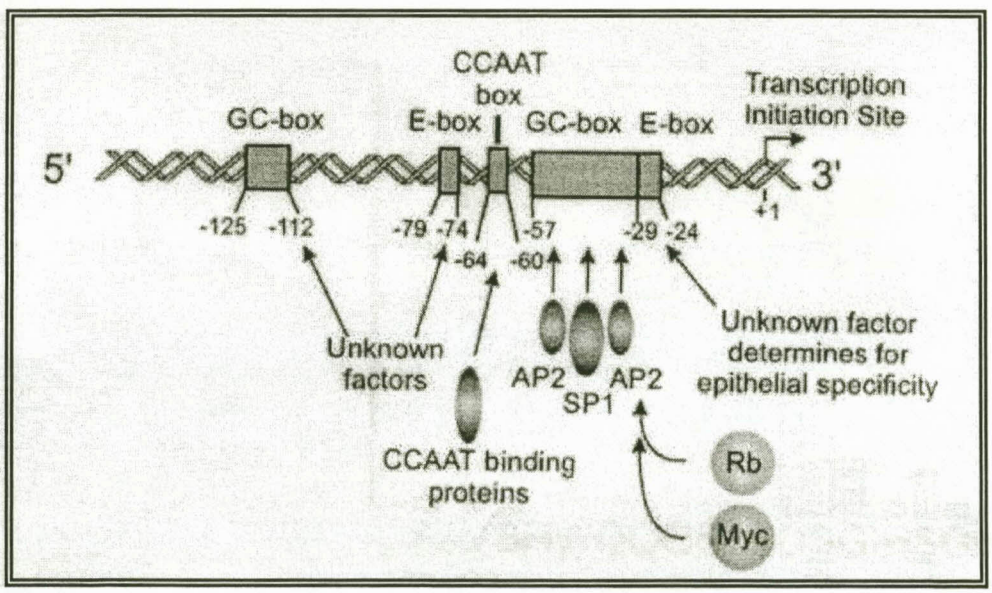

Figure 2.1. Overview of the modular structure of the human E-cadherin promoter. Adapted from Nollet *et al.*., 1999.

The CCAAT box and the GC-box (-29 to –57) exert a positive effect on the E-cadherin promoter activity. The upstream regulatory sequences of the mouse and human E-cadherin genes have been characterized. Both promoters contain two conserved E-boxes which are of major importance for the epithelial-specific expression of the E-cadherin gene (Behrens *et al.*., 1991; Giroldi *et al.*., 1997). It appears that specific repression in non-epithelial cells rather than activation in epithelial cells controls E-cadherin expression and points to a role of transcriptional repressors of the E-cadherin promoter that become activated in carcinomas (Behrens, 1994).

2.2 Cadherins

The cadherins were first identified as a family of single-pass transmembrane glycoproteins mediating calcium-dependent cell-cell adhesion, and it is now well recognised that they play essential roles in development, cell polarity and tissue morphology (Takeichi, 1991 and 1995). They are organised in cell-cell attachment sites called zonulae adherens or adherens junctions, which contain a cytoplasmic 'undercoat' associated with the actin cytoskeleton (Dahl *et al.*., 1996). Cadherins instruct particular cells to remain at one particular site, to associate specifically with their neighbouring cells or to disrupt these associations and migrate directionally. Extensive research has led to the discovery of many cell adhesion molecules, which are classified into four protein families, namely the immunoglobulin-like protein family, the integrin family, the cadherin family and the selectin family. During the last decade, numerous new members of these protein families have been isolated and characterized (Nollet *et al.*., 2000).

The cadherins constitute an ever-growing family of proteins, for over 100 members of this superfamily have been identified to date (Nollet *et al.*., 1999). The number of genes involved has doubled by the recent description of 52 human protocadherin genes by Wu and Maniatis (1999). Nollet *et al.*. (2000)

assumed that the large superfamily of cadherins originated from a need of multicellular organisms for many types of specific intercellular interactions.

The cadherins are traditionally classified according to tissue distribution or to the origin from which they were discovered. For example, the cadherin seen in epithelial cells is named E-cadherin, P-cadherin in placental tissue and N-cadherin in neural tissues. They were the first cadherins to be identified (Takeichi, 1991). P-cadherin and N-cadherin were characterized soon after the identification of E-cadherin (Miyatama *et al.*., 1989; Nose and Takeichi, 1988). These three cadherins, previously termed " classical cadherins" (Munro and Blaschuk, 1996) or "type-I cadherins" (Tanihara *et al.*., 1994), show a high degree of protein sequence similarity (Nollet *et al.*., 2000). Although some of the cadherin subclasses share similar properties, such as a high degree of homology and molecular weight, each subclass is characterized by a unique tissue distribution pattern and discriminating interactions (Takeichi, 1991). E-cadherin is the most extensively studied cadherin family member (Miyatama *et al.*., 1989; Nose and Takeichi, 1988).

2.3 E-cadherin – expression

The human E-cadherin was first identified by Damsky *et al.*. as cell-CAM 120/80 using polyclonal antibody (Damsky *et al.*., 1983). It is also known as uvomorulin in mouse (Schuh *et al.*., 1986) or L-CAM in chicken (Gallin *et al.*., 1983). E-cadherin is well understood so that it can be considered as a prototype molecule for the whole cadherin family. E-cadherin is confined to the epithelial cells originating from ectodermal, mesodermal and endodermal tissue and is the key component of adherens junctions between epithelial cells (Shiozaki *et al.*., 1996).

De novo synthesis of E-cadherin begins at the late two-cell stage of embryonic development. It has also been found that E-cadherin is transiently expressed in parts of the mouse embryonic brain (Roose *et al.*., 1999). Initially, it is uniformly distributed on the cell surface, but it later clusters at sites of cell

adhesion in cells of eight-cell-stage embryos that are destined for epithelial differentiation (Larue *et al.*., 1994).

Two E-cadherin molecules from the same cell form lateral, parallel dimers on the cell surface, using their extracellular domains (Grunwald, 1993) (Figure 2.2). This process of lateral dimerization is required for homophilic adhesion of E-cadherin molecules (Yap *et al.*., 1997). The parallel dimers have been proposed to interdigitate with dimers from adjacent cells to form the points of adhesion (Shapiro *et al.*., 1995). The complementary processes of cell adhesion and cell motility are not only critical for development, but also for the maintenance of normal tissue structure and integrity. However, recent evidence suggests that the parallel dimers might represent intermediate structures that dissociate to allow the formation of adhesive anti-parallel dimers between cells (Chitaev *et al.*., 1998). In epithelial tissues, adherens junctions form a belt around each cell, creating a continuous zipper of cell adhesion (Chitaev *et al.*., 1998). Cells that come in contact with each other will use already present E-cadherin molecules on the cell surface to rapidly form an adhesion structure. Once synthesized, E-cadherin has a short half-life of 5-10 hours (Jiang and Mansel, 2000).

E-cadherin is not only a cell-cell adhesion protein that is involved in the suppression of tumours but it is also involved in growth suppression.

2.4 E-cadherin as a growth suppressor

It has recently become clear that E-cadherin is involved in 'contact inhibition' of cell growth by inducing cell cycle arrest (Croix *et al.*., 1998). 'Contact inhibition' is a phenomenon in normal epithelial cells: When the density of the cells reaches a certain degree, there is a reduction in the rate of growth and proliferation. Sequential activation and inactivation of a family of cyclin-dependent kinases govern the cell cycle. One such cyclin-dependent kinase inhibitor is $p27^{kip1}$, which results in cell cycle arrest. It is now established that E-cadherin has the

ability to inhibit cell proliferation by upregulation of $p27^{kip1}$ (Croix *et al.*., 1998). The degradation of $p27^{kip1}$ is regulated by phosphorylation of the molecule, i.e. increased phosphorylation leading to its degradation and vice versa (Jiang and Mansel, 2000).

Presently, it is not clear how E-cadherin mediates the accumulation of $p27^{kip1}$ in the cells (Jiang and Mansel, 2000). Inhibition of the activity of mitogenic pathways perhaps via EGFR, which in turn regulate the level of $p27^{kip1}$ in cells, has been suggested as a possible mechanism. Therefore, E-cadherin, generally described as an invasion suppressor (Vleminckx *et al.*., 1991), is also a major growth/proliferation suppressor (Wijnhoven *et al.*., 2000).

2.5 E-cadherin – structure and components

E-cadherin is produced from a 135kDa precursor that undergoes cytoplasmic trimming of what will become the extracellular N-terminal end of the mature molecule. Following the trimming process, E-cadherin is routed towards the basolateral surface of the epithelial cell. The mature E-cadherin protein (120kDa) is composed of a highly conserved carboxy-terminal cytoplasmic domain, the transmembrane domain and an extracellular domain. Several of the cadherins feature a conserved, distinctive sequence motif in their extracellular segments and this sequence is known as the Histidine-Alanine-Valine (HAV) sequence (Hatta *et al.*., 1988). The HAV sequence is situated on the first extracellular (EC1) domain (Figure 2.2) (Blaschuk *et al.*., 1990).

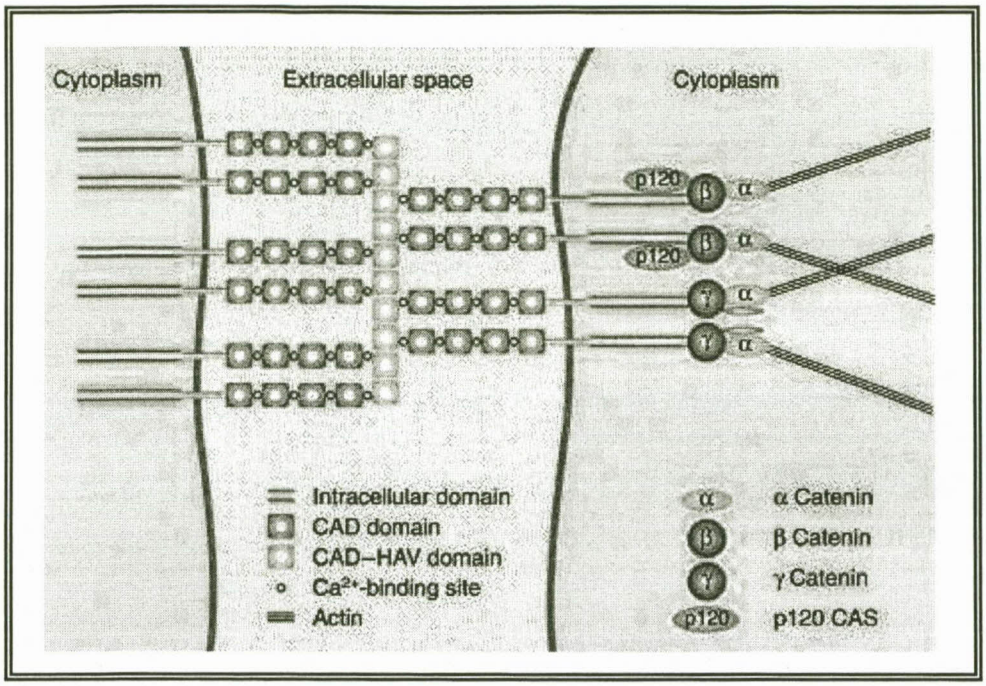

Figure 2.2 E-cadherin-mediated cell-cell adhesion. E-cadherin homodimers expressed on the plasma membranes of adjacent cells interact in a zipper-like fashion. The most N-terminal CAD domain on each E-cadherin molecule contains the HAV motif that is thought to interact with E-cadherin molecules on adjacent cells. The intracellular adhesion complex, which consists of α-,β-, γ-catenin (plakoglobin) and p120cas, links E-cadherin homodimers to the actin cytoskeleton. Adapted from Christofori and Semb, 1999.

2.5.1 The extracellular domain

The extracellular domain is situated on the amino terminus of the E-cadherin protein. The extracellular domain consists of five tandemly repeated cadherin-motif subdomains (EC_1-EC_5), each harbouring two conserved regions representing the putative calcium binding sites which is highly conserved (Overduin *et al.*., 1995; Shapiro *et al.*., 1995). This domain is encoded by exons 7, 8 and 9 (Ringwald *et al.*., 1987; Berx *et al.*., 1995; Soares *et al.*., 1997). These exons have been reported to be frequent mutation sites of the E-cadherin gene (Berx *et al.*., 1995). The extracellular domain, compared to the transmembrane and cytoplasmic domains, exhibits the least homology amongst cadherin proteins

(Pokutta *et al.*., 1994). This domain is responsible for homophilic binding of E-cadherin molecules to each other (Figure 2.2). The EC_1 domain, closest to the N-terminal end, contains an HAV sequence (Pokutta *et al.*., 1994).

2.5.1.1 The HAV sequence

The highly conserved HAV sequence, as determined by nuclear magnetic resonance (NMR), is important for homophilic binding of cadherin molecules to each other. It distinguishes the classical cadherins from other cadherins, for only classical cadherins contain the HAV sequence. The exact mechanism of this interaction is, however, still a matter of debate (Noë *et al.*., 1999).

The HAV sequence is comprised of 113 amino acids and antibodies raised against the EC1 domain inhibit cadherin function and block cell adhesion (Nose *et al.*., 1990). The inhibition of cadherin function by peptides suggest that cadherin fragments containing an HAV sequence may stimulate invasion, a process that is counteracted by the expression of a functional E-cadherin/catenin complex (Vleminckx *et al.*., 1991). The synthetic HAV peptides also inhibit compaction of mouse preimplantation embryos, which normally occurs through E-cadherin-induced cell adhesion (Blaschuk *et al.*., 1990b).

Although the HAV sequence is responsible for homophilic binding of E-cadherin molecules to each other, calcium binding is required for the stability of the E-cadherin molecules (Grunwald, 1993; Chitaev and Troyanvosky, 1998).

2.5.1.2 Calcium binding

The extracellular regions of the E-cadherin protein requires Ca^{2+} binding necessary for the adhesiveness, rigidity and stability of the protein (Grunwald, 1993). The co-operativity of Ca^{2+} binding is enhanced by dimer formation. One of the essential roles of Ca^{2+} ions is to ensure the proper orientation of cadherin

molecules in an aligned extracellular lattice suitable for proper adhesive contacts (Figure2.3).

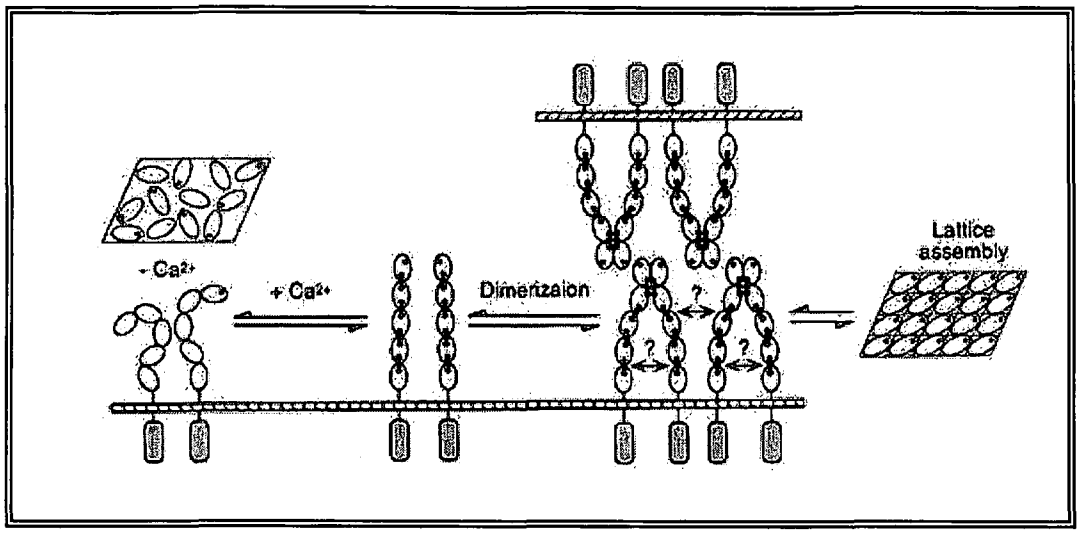

Figure 2.3. Schematic representation of the proposed mechanism by which Ca^{2+} organized cadherin assemblies are formed at the cell surface. Extracellular cadherin repeats are represented by open ovals, Ca^{2+} ions by small shaded spheres, adhesion sites by asterisks and the cytoplasmic domain by a rectangle. Only the two N-terminal repeats are shown to interact in dimers. The cytoplasmic domain and/or the molecules from the opposite cell may also contribute to the cadherin lattice assembly. The disordered ($-Ca^{2+}$) cadherin lattice is represented at the top, where adhesion sites might be unformed or assume random orientations. Adapted from Alattia *et al.*., 1997.

Figure 2.3 illustrates the proposed mechanism by which the Ca^{2+} dependent assembly of cadherin molecules forms a stable cell adhesion surface on the plasma membrane. First, apo-cadherin monomers with flexible linkers bind Ca^{2+} ions, resulting in an overall rigidification of the molecule. Similarly important is that calcium binding restrains the positions of the individual adhesion sites to those suitable for the formation of a uniform cell-cell adhesion lattice (Alattia *et al.*., 1997). Such organized structures at low concentrations would associate loosely, as exemplified by the weakness of the E-cadherin dimer solution.

However, the key to stable adhesion would lie in the collective assembly of a number of properly oriented structures (Alattia *et al.*., 1997).

The crystal structure of the E-cadherin dimer shows that Ca^{2+}ions are co-ordinated by backbone and side chain oxygen atoms of residues that are strategically located at the interface between the first and second cadherin domains. Some of the residues that are involved in calcium co-ordination form hydrogen bonds with the opposite monomer, either directly or via bound water molecules. Thus, an intricate network of interacting Ca^{2+}ions, water molecules and E-cadherin side chains results in a very specific geometry that would correct the orientation between the individual monomers. Increasing calcium concentration from 0 to 1mM results in a shift from disordered cadherin structure to a rigid rod-like structure (*cis* dimer), then a *trans* dimer of multiple *cis* dimers, the *trans* dimers forming 'zipper' structure (Jiang and Mansel, 2000).

Lateral clustering interactions between cadherin monomers on the surface of the plasma membrane are probably not limited to the N-terminal tip of the cadherin molecule. Another extracellular part and/or the cytoplasmic domain could possibly be involved (Yap *et al.*., 1997). Calcium binding is also important for the protection of E-cadherin molecules against trypsin. Trypsin is a proteolytic agent that degrades E-cadherin molecules in the absence of calcium ions.

Calcium binding is important for proper interactions of E-cadherin molecules to each other, while the cytoplasmic domain is important for the proper functioning of the E-cadherin molecule.

2.5.2 The cytoplasmic domain

The cytoplasmic domain is situated at the carboxy-terminus of the E-cadherin protein (Figure 2.2). It has been observed that an antibody reacting with the cytoplasmic tail of E-cadherin may label tumour cell nuclei strongly. This suggests that E-cadherin may be cleaved at the cell membrane through the

process of regulated intramembrane proteolysis (RIP) and translocated to the nucleus. Presently, the function of the cytoplasmic tail in the nucleus is not known (Bremnes *et al.*., 2002).

The cytoplasmic tail of E-cadherin associates with several proteins termed catenins and this is thought to be its main function (Ozawa *et al.*., 1990b).

2.6 Catenins

Catenins are intracellular or cytoplasmic proteins that connect the E-cadherin protein to the microfilament network or the actin filaments (Takeichi, 1991; Kemler, 1992a). The interaction of these molecules is a prerequisite for the proper formation of functional intact adherens junctions (Nagafuchi *et al.*., 1991; Ozawa and Kemler, 1992). The linkage between trans-membranous cadherins and actin filaments of the cytoskeleton is necessary for strong cell-cell adhesion (Figure 2.2) (Nagafuchi and Takeichi, 1988 and 1989).

Alteration in expression or structure of the catenins may lead to the disassembly of the adherens junctions and the generation of more invasive cells (Shimoyama *et al.*., 1992). Deletion of the intracellular catenin-binding domain of E-cadherin or the alteration of the functional active catenins results in the loss of ability of E-cadherin to establish cell-cell adhesion, even if the extracellular binding domain is intact (Nagafuchi and Takeichi, 1988 and 1989).

Each E-cadherin-catenin complex includes α-catenin and either β-catenin or γ-catenin. The α-catenin links the β-catenin or γ-catenin to the actin cytoskeleton (Ozawa, 1998).

2.6.1 α-Catenin

α-Catenin is a 102kDa protein encoded by a gene (*CTNNA1*) on chromosome 5q21-22 (Herrenknecht *et al.*., 1991). It is a multifunctional protein with multiple interaction sites, including amino-terminal β-/γ-catenin-binding and homodimerization sites (Koslov *et al.*., 1997), a central region for α-actinin binding (Nieset *et al.*., 1997), and amino-terminal, as well as carboxyl-terminal actin-binding sites (Rimm *et al.*., 1995). The carboxyl-terminal region of α-catenin (residues 612-906) is sufficient to trigger the adhesive activity of E-cadherin, provided it is covalently linked to E-cadherin or associated with the E-cadherin adhesion complex through its interaction with β-/γ-catenin via its amino-terminal β-/γ-catenin-binding sites (Ozawa, 1998).

Two isoforms of α-catenin have been identified, namely αE-catenin and αN-catenin. αE-catenin is predominantly expressed in all epithelial tissues, whereas the expression of αN-catenin seems to be largely restricted to neuronal cells (Nollet *et al.*., 1999). These isoforms show 82% similarity to each other (Hirano *et al.*., 1992).

Cells lacking α-catenin are unable to form stable contacts despite high expression levels of E-cadherin and β-catenin. The ability of these cells to adhere to one another is increased dramatically by the introduction of αN-catenin. Similarly, cells expressing α-catenin and β-catenin, but lacking cadherins, can be induced to form an epithelioid phenotype by the introduction of N-cadherin or E-cadherin (Hirano *et al.*., 1992). This shows that different cadherin subtypes can interact with different isoforms of α-catenin and vice versa (Näthke *et al.*., 1993).

In some human cancers, such as that of the breast, esophagus and prostate, decreased expression of α-catenin has been noted (Shimoyama *et al.*., 1992). In human esophageal cancer tissue, loss of α-catenin expression correlates with the degree of infiltration and the extent of lymph node metastasis (Kadowaki *et al.*., 1994).

α-Catenin exhibits 30% sequence similarity to vinculin within three conserved domains (Herrenknecht *et al.*., 1991). Vinculin is a highly conserved 117kDa cytoskeletal protein that is found in both cell-cell and cell-extracellular matrix adherens-type junctions (Geiger *et al.*., 1980; Weiss *et al.*., 1998). In such junctions, vinculin is thought to be one of a number of interacting proteins that links the cytoplamic face of adhesion receptors of the cadherin or integrin family to the actin cytoskeleton. Similarities between α-catenin and vinculin are restricted to the amino-terminal, central, and carboxyl-terminal regions and are the highest for the carboxyl-terminal regions. Vinculin, like α-catenin, associates with E-cadherin complexes via β-catenin (Hazan *et al.*., 1997).

2.6.2 β-catenin

The β-catenin gene (*CTNNB1*) maps to chromosome 3p21 and the gene product is a 92kDa protein which appears to be important in the functional activities of both APC and E-cadherin (Nagasawa *et al.*., 1999). β-Catenin was initially discovered as an associated protein in the cadherin complex, but it was soon realised to be a central player in a complex of signalling events (Hinck *et al.*., 1994). This was proved by its interaction with an array of other molecules important in cellular signalling and gene expression, including the cadherin binding site, α-catenin, axin, GSK3β and APC (Hinck *et al.*., 1994; Näthke *et al.*., 1994).

Although the catenin-binding domain of E-cadherin is required for cell adhesion (Miller and Moon, 1996), E-cadherin-mediated cell adhesion is maintained *in vitro* when β-catenin is artificially eliminated from the complex by expression of an E-cadherin-α-catenin fusion protein (Nagafuchi *et al.*., 1994). This suggests that β-catenin has a regulatory, rather than mechanical, role in cell adhesion (Guilford, 1999). β-Catenin is usually sequestered in the E-cadherin adherens junction or in tight-junction complexes. Non-sequestered, free β-

catenin is rapidly phosphorylated by glycogen synthetase kinase 3β (GSK-3β) in the adenomatous polyposis coli (APC)-GSK3β complex and subsequently degraded by the ubiquitin-proteasome pathway (Gumbiner, 1997; Rubinfeld *et al.*., 1997). GSK-3β is a protein kinase that forms a complex with APC protein, axin and free β-catenin within the cytosolic pool (Figure2.4) (Christofori and Semb, 1999).

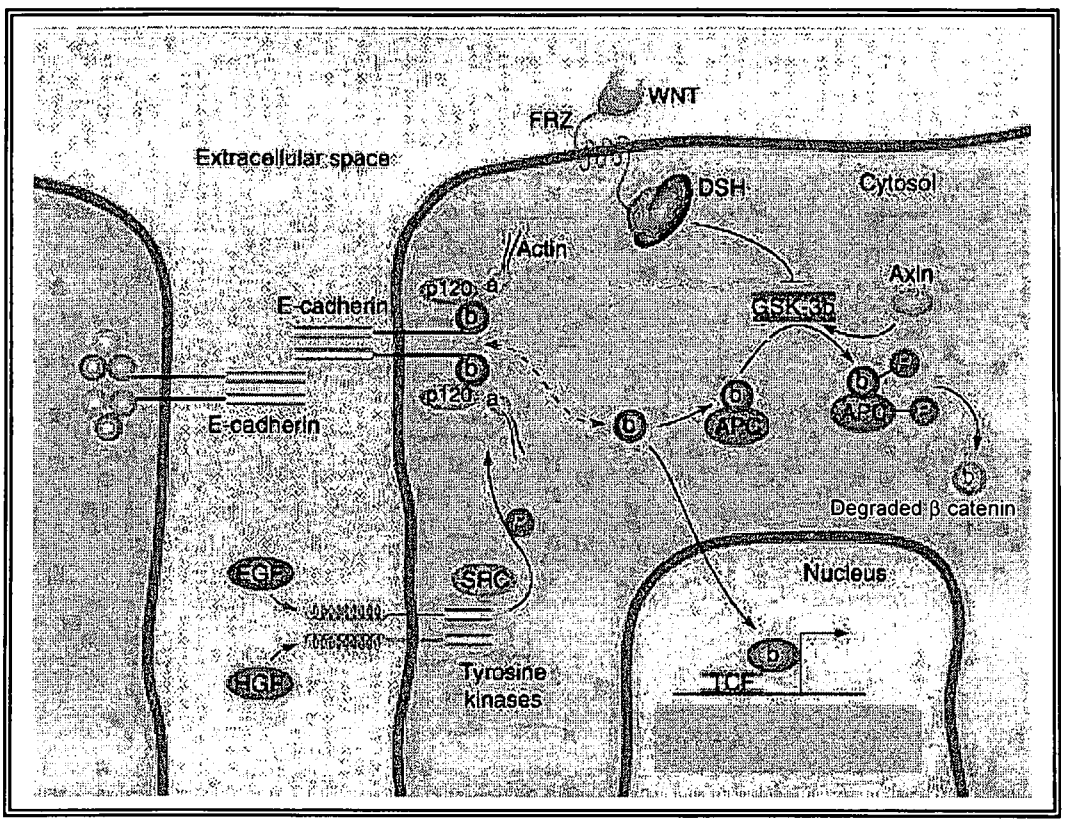

Figure 2.4 The E-cadherin-catenin complex and signalling. Adapted from Christofori and Semb, 1999.

When in complex, GSK-3β acts together with axin to phosphorylate β-catenin and APC (Dierick and Besjovec, 1999). The phosphorylated β-catenin undergoes ubiquitination followed by degradation mediated by proteosome (Aberle *et al.*., 1997). It can thus be seen that inhibition of GSK-3β will have the

effect of reducing β-catenin degradation, therefore increasing the pool of free β-catenin.

Wnt-1 protein also takes part in the increase of the cytoplasmic β-catenin levels in target cells. The *Wnt-1* gene is the human homologue of the *Drosophila Wingless* gene (Jiang and Mansel, 2000). Recently, several groups have reported that β-catenin, besides being a major component of the E-cadherin adhesion complex, is also part of the WNT-mediated signalling pathway (Gumbiner, 1997; Molenaar *et al.*., 1996). If the stimulus that activated the Wnt-1 pathway also resulted in the activation of EGFR, then it is clear that movement back to the cell adhesion complex would not be possible. It follows that free β-catenin may heterodimerise with members of the leucocyte enhancer factor/ T-cell factor (LEF/TCF) family allowing translocation to the nucleus (Figure 2.4). The complex of β-catenin and LEF/TCF induces DNA bending and transcription of Wnt-responsive genes (Behrens *et al.*., 1996). The nature of the target genes has been largely unknown until recently (Beavon, 2000). LEF binding sites have also been found contained within the promoter region of the E-cadherin gene and it has been proposed that binding of the complex of β-catenin and LEF/TCF downregulates the expression of the E-cadherin gene (Huber *et al.*., 1998).

In contrast to Wnt-1 and GSK-3β, APC can complex with, and degrade, β-catenin, which helps to control the level of free cytoplasmic β-catenin. Most of the mutations in APC result in truncated APC protein, which can complex with but not degrade β-catenin. The net result of the *APC* mutation is therefore an increase in cytoplasmic β-catenin, which may then trigger a cascade of events resulting in the initiation of adenomas. When the truncated APC is bound to β-catenin, it may not be available for incorporation into the E-cadherin-catenin family. In this way, as well as deregulated β-catenin signalling, the *APC* mutation may also result in disrupted E-cadherin function. Thus, the very first stages of adenoma development involve loss of control of β-catenin, a protein involved in organization of tissue architecture. The loss of the normal architecture may then

mean some loss of normal control mechanisms (such as signals from the basement membrane), resulting in abnormal tissue growth (Klimstra, 1994).

Ninomiya *et al.*. (2000) suggests that the accumulation of β-catenin protein could be responsible for carcinogenesis in a sub-set of esophageal squamous-cell carcinomas. Unlike in colorectal carcinomas, β-catenin expression in esophageal squamous carcinoma is increased. This expression has been associated with cytoplasmic distribution. There is an increase in expression but also nuclear localization of the β-catenin protein in ESCC, yet no mutation of either the β-catenin or of the *APC* gene have been found (Ninomiya *et al.*., 2000). The mechanism of accumulation of the β-catenin protein might be independent of genetic alteration of either the β-catenin or the *APC* gene as shown in other tumours. γ-Catenin, like β-catenin is normal in colorectal cancer (Ghadimi *et al.*., 1999).

The sequence of β-catenin shows approximately 65% similarity to armadillo (arm), a segment polarity gene in *Drosophila* (McCrea *et al.*., 1991). The arm family includes the adhesion-related proteins β-catenin and plakoglobin. β-Catenin and γ-catenin share approximately 60% sequence similarity, for both bind to amino acid (aa) positions 832-862 in the cytoplasmic domain of the E-cadherin molecule (Ozawa *et al.*., 1989; Ozawa, 1998). The sites in β-catenin and γ-catenin which mediate the interaction with E-cadherin, appear to be less defined and are located in the multiple armadillo repeats (Hülsken *et al.*., 1994).

2.6.3 γ-Catenin

γ-Catenin, also known as plakoglobin, is a 83kDa protein encoded by a gene (*JUP*) located on chromosome 11q11 (Hajra and Fearon, 2002). It binds directly to the cytoplasmic domain of the E-cadherin protein in the absence of β-catenin. The soluble form of plakoglobin associates with α-catenin and/or the tumour suppressor protein APC via the central repeats domain (Hinck *et al.*., 1994). The

central region of the plakoglobin molecule contains imperfect sequence repeats of approximately 40 amino acids known as arm repeats (Peifer *et al.*., 1994). This region is involved in binding to most, if not all, known plakoglobin partners. It has become clear, however, that each interaction requires distinct binding sites (Troyanovsky *et al.*., 1996).

In the adherens junction, plakoglobin is associated with E-cadherin and α-catenin, which provide anchorage for F-actin and α-actinin (Ozawa *et al.*., 1989; Rimm *et al.*., 1995). In the intracellular plaques of adherens junctions, plakoglobin is also linked to the vinculin-related protein α-catenin (Knudsen *et al.*., 1995; Gumbiner, 1996). Plakoglobin's function in the adherens junction is to link the transmembrane cadherin molecule to α-catenin, which then links the entire complex to the microfilaments (Knudsen *et al.*., 1995).

β-catenin and plakoglobin bind directly to α-catenin and also interact with the APC gene product, unlike $p120^{ctn}$ (Jou *et al.*., 1995).

2.6.4 $P120^{ctn}$

$P120^{ctn}$ (also known as $p120^{cas}$) is a 120kDa protein encoded by a gene (*CTNND1*) on chromosome 12p13. This cadherin-associated protein was originally discovered in *c-src* mutational analyses (Reynolds *et al.*., 1989). From its discovery $p120^{ctn}$ was known to be membrane-associated. Evidence linking $p120^{ctn}$ with cell adhesion came when cloning of the *p120*^{ctn} gene identified 10 copies of a characteristic 42 amino acid *armadillo* repeat placing it in the *arm* family of proteins (Peifer and Wiechaus, 1990; Reynolds *et al.*., 1992).

$P120^{ctn}$ is involved in the lateral clustering of cadherin molecules as well as in the negative modulation of E-cadherin (Aono *et al.*., 1999; Ohkubo and Ozawa, 1999). In E-cadherin, $p120^{ctn}$ binds to the juxtamembrane domain, within the last 37 carboxy-terminal residues (Yap *et al.*., 1998). Deletion of these residues abolishes the ability of E-cadherin to co-precipitate $p120^{ctn}$ (Shibamoto *et al.*., 1995), although there is a possibility that the carboxyl-terminal regions of

E-cadherin has another binding site for $p120^{ctn}$, which is dependent on the conformation for binding. The juxtamembrane domain of cadherins has been implicated in the regulation (suppression) of the invasive and motile behaviour of cancer cells (Gold *et al..*, 1998). The exact role of $p120^{ctn}$ in cadherin mediated cell adhesion is yet to be clarified. However, Anastasiadis and Reynolds (2000) proposed that $p120^{ctn}$ may be involved in both 'positive' and 'negative' regulation of cell adhesion, possibly depending on the functional status of the protein. Interestingly, $p120^{ctn}$ is found to be able to mediate nuclear signalling similar to that of β-catenin. This is perhaps achieved by direct interaction with the transcription factor Kaiso (Van Hengel *et al..*, 1999; Mariner *et al..*, 2000).

Co-localization and immunoprecipitation experiments confirmed the hypothesis that $p120^{ctn}$ functions as part of the E-cadherin-catenin complex (Reynolds *et al..*, 1994).

2.7 The E-cadherin-catenin complex

The E-cadherin-catenin complex begins to form during the passage of E-cadherin to the cell membrane. The first catenin to interact with E-cadherin is β-/γ catenin (Ben-Zeév and Geiger, 1998; Hinck *et al..*, 1994; Nagafuchi and Takeichi, 1990). Both β-catenin and plakoglobin associate with cadherins immediately following synthesis, indicating that binding is a constitutive process and does not occur in response to cell contact. Newly synthesized α-catenin, on the other hand, is only found in association with newly synthesised cadherin at 30-60 minutes after synthesis (Ozawa and Kemler, 1992). β-Catenin appears in sites of cell contact with the same kinetics as cadherin after cell contact is initiated, whereas α-catenin is not found at these sites until later times (Näthke *et al..*, 1993). A large portion of α-catenin is not associated with cadherin that makes α-catenin to be more easily removed from cadherin than β-catenin (McCrea and Gumbiner, 1991; Ozawa and Kemler, 1992). These observations suggest that α-catenin may be able to exchange from one cadherin molecule to

another at the cell surface, thus modulating cadherin function (Näthke *et al.*., 1993). The binding of α-catenin to the E-cadherin-β/γ-catenin complex results in the formation of stable bonds between the complex and the actin cytoskeleton (Aberle *et al.*., 1994). The binding domain responsible for the link between α-catenin and the actin cytoskeleton is located at the N-terminus and is also responsible for the linkage of spectrin to the complex (Lombardo *et al.*., 1994).

Linkage with the actin cytoskeleton is not the only interaction that occurs between the E-cadherin-catenin complex and the dynamic structural components of the cell. Linkages are also made with other classes of cytoplasmic structural proteins, such as fodrin and ankyrin, to create an effective continuum between the cytoskeleton of adjacent cells (Shiozaki *et al.*., 1995). Fodrin and ankyrin are components of the membrane-associated cytoskeleton of mammalian cells. These interactions are necesssary for proper epithelial cell functionality and tissue integrity (Guilford, 1999).

It is thought that the formation of cadherin-catenin complexes following the formation of cell-cell attachment, sets off a signalling pathway that results in attraction of E-cadherin. With time, free diffusing E-cadherin becomes trapped by the immobilised cadherin-catenin complexes resulting in an increase of the local concentration of E-cadherin, which forms lateral bonds, strengthening cell-cell adhesion (Adams *et al.*., 1996; Adams and Nelson, 1998).

Given the central role of E-cadherin-mediated cell adhesion in development and homeostasis, disruption of normal E-cadherin function would be expected to result in the onset of diseases that are characterized by abnormal tissue morphology and aggressive cell migration (Guilford, 1999).

2.8 E-cadherin and cancer

Since an intact E-cadherin adhesion complex is required for maintencance of normal intercellular adhesion, several investigators have proposed E-cadherin as an invasion suppressor molecule in carcinoma cells (Bremnes *et al.*., 2002). In

colorectal tissues, Gagliardi and co-workers (1996) found a steady decrease in E-cadherin expression, from normal mucosa through adenoma, primary cancer and metastatic lesions, indicating the critical involvement of the molecule in the progression of cancer. It has, however, been debated whether the loss of the E-cadherin-mediated cell-cell adhesion is a prerequisite for tumour progression or consequence of de-differentiation during tumour progression (Christofori and Semb, 1999).

Immunohistochemical studies of E-cadherin shows that E-cadherin protein is continuously re-generated in healthy individuals. These individuals process small amounts of soluble E-cadherin into the blood flow, resulting in a serum E-cadherin concentration of 2μg ml^{-1}. Elevated levels of soluble E-cadherin are detected more frequently in patients with malignancy but not in patients with diabetes mellitus or acute hepatitis. This implies that degradation, release and shedding of E-cadherin on the tumour cells is related to proteolytic action by those cells, required for penetrating the extracellular matrix (Goldfarb and Liotta, 1986). This leads to the markedly increased soluble E-cadherin (80kDa) in the circulation of cancer patients, and it can reasonably be derived from proteolytic digests of cell-surface E-cadherin (Katayama *et al.*., 1994; Shiozaki *et al.*., 1991). These proteases, for example stromelysin 1, are activated during tumour progression (Lochter *et al.*., 1997). Thus, the proteolytic degradation of E-cadherin in cancers is associated with malignancy, invasiveness or the metastatic ability of tumour cells at the primary sites. Serum E-cadherin can thus be used as a clinical marker specific to detecting epithelial carcinomas by calculating the concentration of soluble E-cadherin (Katayama *et al.*., 1994).

The E-cadherin molecule was originally presented as a paradigm of invasion suppressor (Vleminckx *et al.*., 1991), and this concept was further substantiated by elegant studies on the generation of pancreatic carcinomas in a transgenic mouse model (Perl *et al.*., 1998). Perl and co-worker (1998) used a transgenic mouse model of pancreatic β-cell tumourigenesis and demonstrated that loss of the E-cadherin-mediated intercellular adhesion is causally involved in

the transition from well-differentiated adenoma to invasive carcinoma (Figure 2.4).

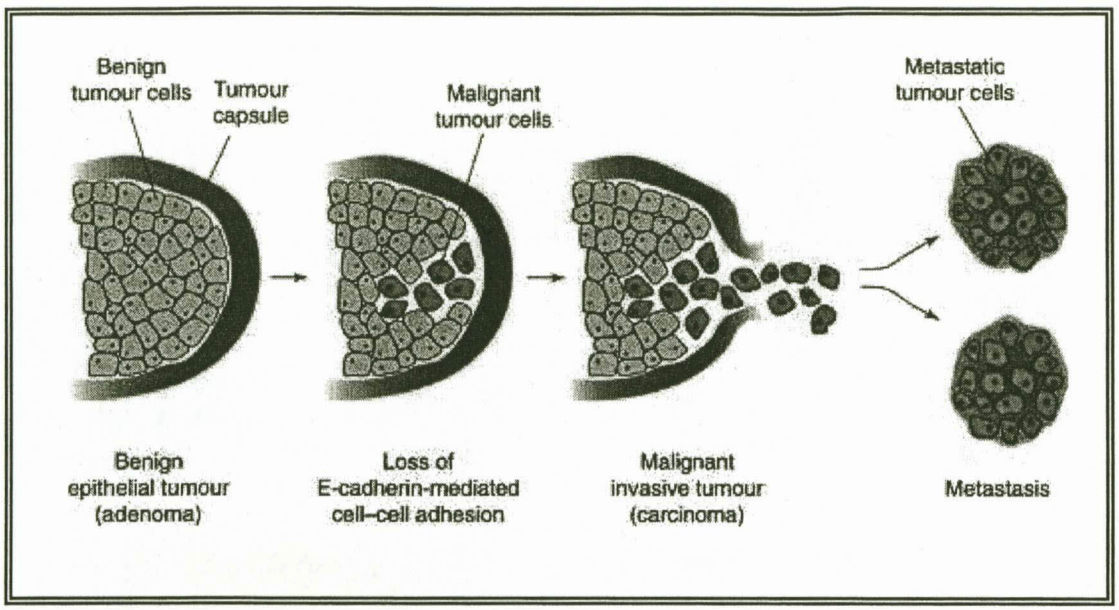

Figure 2.5. Loss of E-cadherin-mediated cell-cell adhesion contributes to the transition from benign, non-invasive tumours (adenoma) to malignant, invasive tumours (carcinoma). Adapted from Christofori and Semb, 1999.

Maintenance of E-cadherin expression during β-cell tumourigenesis resulted in arrest of tumour development at the adenoma stage. By contrast, expression of a dominant negative E-cadherin induces early invasion and metastasis. These results demonstrate that loss of E-cadherin-mediated cell-cell adhesion is a rate-limiting step in the progression from adenoma to carcinoma *in vivo* (Christoforia and Semb, 1999).

The realisation of the role of reduced E-cadherin expression in tumour cell invasiveness originated from work on cultured human carcinoma cells (bladder, breast, lung, pancreas) in which E-cadherin negative variants were invasive. Re-establishing the functional cadherin complex, for example by forced expression

of E-cadherin, resulted in a reversion from an invasive to a benign epithelial tumour-cell phenotype (Birchmeier and Behrens, 1994; Frixen *et al.*., 1991; Vleminckx *et al.*., 1991). In contrast, downregulation is either absent or less pronounced in well differentiated tumour types, such as ductal breast cancers and intestinal-type gastric cancers (Guilford, 1999). Immunohistochemical studies have shown that normal E-cadherin expression in primary tumours is downregulated in a variety of tissues, including the stomach, breast, prostate, esophagus and thyroid (Chitaev *et al.*., 1998).

Immunohistochemical studies on lymph node and distant metastases from a variety of primary tumours have shown that the low expression of E-cadherin in poorly differentiated tumours is sometimes transient (Mareel *et al.*., 1995). This might be owing to the clonal expansion of E-cadherin-positive cells. Regardless of the mechanism, re-expression of E-cadherin is likely to enhance tumour-host adhesion at the metastatic site. Alternatively, it might be owing to reversible E-cadherin expression within cells of the tumour, perhaps related changes in the local tissue environment following metastasis and invasion (Guilford, 1999).

Invasiveness of the transfected cells could be restored by treatment with E-cadherin antibodies or by reducing E-cadherin expression with an E-cadherin antisense RNA (Vleminckx *et al.*., 1998). A direct role for E-cadherin in the suppression of tumour invasion has been demonstrated by the reversion of the invasive phenotype in malignant epithelial tumour cells following transfection with E-cadherin cDNA (Vleminckx *et al.*., 1998). Downregulation of E-cadherin coincides with the transition from well-differentated adenoma (a benign neoplasm of epithelial cell origin that forms glandular patterns) to invasive carcinoma (a malignant neoplasm of epithelial cell origin). Abrogation of E-cadherin-mediated cell adhesion by expression of a dominant-negative E-cadherin transgene, induced tumour invasion and metastasis (Perl *et al.*., 1998). Tumour aggressiveness often correlates with a decreased or less polarized expression of E-cadherin (Berx, 1994).

The correlation between E-cadherin downregulation and metastasis might be related not only to the ability of E-cadherin-negative cells to invade

surrounding tissue. It might also be due to the increased likelihood of weakly adherent cells detaching from the tumour mass in response to low shear forces, such as those found in lymphatic vessels and venules (Byers, 1995). The carcinoma cells lose E-cadherin expression during the process of detaching from the primary sites and infiltrating other sites (Matsuura *et al.*., 1992). Expression of different members of the cadherin family in primary tumours might also play a role in determining the site of metastases (Guilford, 1999).

The loss of cell-cell adhesion alone is not sufficient to induce active tumour invasion and metastasis. Additional genetic and/or epigenetic events seem to be involved (Christofori and Semb, 1999).

Alterations of the E-cadherin-catenin complex may be either reversible (epigenetic) or irreversible (genetic). Phosphorylation of the E-cadherin-catenin complex is the best way to document post-translational regulation. This phosphorylation or downregulation of the E-cadherin protein leads to the progression of cancer (Stappert and Kemler, 1994).

2.9 E-cadherin-catenin complex and cancer

To assure that the entire E-cadherin adhesion complex is intact and functional, the normal expression and function of E-cadherin as well as each catenin is critical (Shimoyama *et al.*., 1992; Vermeulen *et al.*., 1995). Reduced expression of components of the cadherin-catenin complex may also be related to genetic abnormalities, transcription problems, molecular abnormalities, and/or protease cleavage of the peptides (Frixen *et al.*., 1991; Shimoyama *et al.*., 1992). Alterations in E-cadherin, α-catenin, β-catenin, γ-catenin and $p120^{ctn}$ have been implicated in the lack of adhesion and increased invasiveness of many types of cell lines and tumours (Takeichi, 1993; Birchmeier and Behrens, 1994). Expression of E-cadherin, α-catenin, β-catenin and plakoglobin has a prognostic significance in esophageal carcinomas (Nakanishi *et al.*., 1997). A delicate balance of the catenins is essential for normal cell function (Bremnes *et al.*.,

2002). Several other *in vitro* studies have demonstrated that disturbance of the E-cadherin-catenin complex is the cause, rather than the consequence of de-differentiation and tumour invasiveness (Frixen *et al.*., 1991; Vleminckx *et al.*., 1991).

Inactivating mutations in *CTNNA1*, the gene encoding α-catenin, have been demonstrated only in lung, prostate, ovarian and colon cell lines that lack normal cadherin-dependent cell-cell adhesion and not in tumours *in vivo* (Shimoyama *et al.*., 1992; Vermeulen *et al.*., 1999). Additionally, immunohistochemical analysis has demonstrated loss of α-catenin in some primary tumours (Papadavid and Katsambas, 2001). Cultured human cancer cell lines with a genetically altered α-catenin regained their cell-cell adhesiveness when transfected with wild-type α-catenin c-DNA (Breen *et al.*., 1993). Therefore α-catenin meets the criteria of an invasion suppressor gene (Wijnhoven *et al.*., 2000).

Recently, a mutation in γ-catenin has been described in a gastric cancer cell line, but no mutations have been reported in sporadic gastric cancers (Caca *et al.*., 1999). Re-introduction of γ-catenin has been found to suppress tumorigenicity (Simcha *et al.*., 1996).

Loss of β-catenin expression has been shown to correlate with high grade tumours (Takayama *et al.*., 1996). Altered expression of β-catenin (nuclear localization) has been shown in a subset of early lesions (Valizadeh, *et al.*., 1997) and mutations altering adhesion protein interactions have been found in colon cancer cell lines (Ilyas *et al.*., 1997). Truncated β-catenin disrupts the interaction between E-cadherin and α-catenin: a cause of loss of intercellular adhesiveness in human cancer cell lines (Oyama *et al.*., 1989; Morin *et al.*., 1997), but they are uncommon in actual human tumours (Kitaeva *et al.*., 1997). Mutation in or loss of regions in exon 3 of β-catenin is associated with the malignant transformation and growth pattern in cancer cells (Nagasawa, *et al.*., 1999). Interestingly, it has recently been demonstrated that one of the other target genes of the β-catenin-TCF-4 signal pathway is the *TCF-1* gene. *TCF-1*

may act as a feedback repressor of β-catenin-TCF-1 targeted genes, such as *c-myc* and *cyclin D1* (He *et al..,* 1998).

The interaction between E-cadherin and β-catenin at the adherens junction provides one obvious mechanism by which the mutation of *CDH1* could disrupt growth signalling and initiate tumourigenesis. Loss of functional E-cadherin shifts the cellular equilibrium of β-catenin away from the adherens junctions towards the pool of free β-catenin. Increased free β-catenin activates transcriptional targets of the Wnt signalling pathway, which includes the oncogene *c-myc* (He *et al..*, 1998).

In some tumours, including esophageal cancer, the staining pattern of the E-cadherin-catenin complex does not always indicate an absence or reduction in expression but shows a redistribution from the cell membrane to the cytoplasm (El-Hariry *et al..*, 1999). The mechanism responsible for this redistribution in tumour cells remains elusive. These studies have shown that the expression of the proteins does not necessarily imply that they are functioning; binding of the E-cadherin-catenin complex to the cytoskeleton is essential for its role in cell adhesion (Wijnhoven *et al..*, 2000).

The loss of E-cadherin mediated cell adhesion is a rate-limiting step in tumour progression. However, in addition to increasing the rate of cancer progression by promoting tumour invasion and metastasis, reduced E-cadherin expression might also be involved in the initiation of tumourigenesis (Guilford, 1999).

2.9.1 Tumourigenesis mechanisms

There are multiple mechanisms that are found to underlie the loss of E-cadherin function during tumourigenesis:

(i) transcriptional repression of the E-cadherin gene, for example by hypermethylation,

(ii) *Helicobacter pylori* infection,

(iii) Snail and twist,

(iv) tyrosine phoshorylation and

(v) mutations or deletions of the E-cadherin gene itself (Berx *et al.*., 1998).

2.9.1.1. Promoter hypermethylation

Epigenetic inactivation of gene expression by the hypermethylation of promoter sequences provides a further mechanism of the downregulation of gene transcription (Yoshiura *et al.*., 1995). In normal tissue, CpG islands are generally unmethylated or hypomethylated while hypermethylation is often associated with transcriptional silencing in imprinted alleles and genes in the inactive X-chromosome (Baylin *et al.*., 1998). This is probably the reason men are more affected than women because they don't possess the extra X-chromosome.

The role of methylation of the E-cadherin promoter has been clearly demonstrated in a study using serial dilution of cells with different adhesion and invasion properties (Graff *et al.*., 2000). There exists a heterogeneous pattern of E-cadherin promoter region methylation at an early stage of tumour development and it occurs before invasion. The density of E-cadherin promoter methylation increases (concomitant with E-cadherin level reduction) when invasion begins to occur (Melki *et al.*., 2000). However, when invasive tumour cells are cultured in an environment that favours cell-cell adhesion, the methylation reduces dramatically and the E-cadherin level is restored (Jiang and Mansel, 2000).

Hypermethylation of CpG islands in the *CDH1* promoter has been demonstrated in several human carcinomas and cell lines. Approximately 83% of colorectal cancers have promoter methylation (Hirohashi, 1998). The study by Si *et al.*. (2001) provided the first information that decrease or loss of E-cadherin expression in ESCC was associated with CpG island methylation in the promoter region of the E-cadherin gene. Of the six ESCC cell lines they examined, they found evidence of E-cadherin promoter methylation in four. This suggests that E-cadherin promoter methylation contributes significantly in ESCC (Si *et al.*., 2001).

2.9.1.2 H pylori infection

Helicobacter pylori is a bacterium that causes peptic ulcers. It has been postulated that *H pylori* is involved in the development of gastric cancer. Although the causal role of H pylori in gastric carcinogenesis awaits definite confirmation, results from an ever-increasing number of studies strongly suggest that *H pylori* is indeed the single most important etiologic factor for gastric cancer development (Forman and Webb, 1994; Parsonnet *et al.*., 1991; Eurogast Study Group, 1993). It is known that *H pylori* infection is associated with the downregulation of E-cadherin in gastric mucosa and so might play a role in the onset of neoplastic growth (Terres *et al.*., 1998).

2.9.1.3. Snail and twist

A recent surprising discovery demonstrated that the loss of E-cadherin transcriptional expression may involve Snail (Cano *et al.*., 2000). Snail is a family of zinc-finger transcription factors that regulate the transition from epithelial to mesenchymal cells. The family was initially discovered to have two members, Snail and Slug and was known to be involved in embryogenesis and a number of diseases (Twigg and Wilkie, 1999).

Recently, it was reported that Snail, but not Slug, can act as a repressor of transcription of the E-cadherin gene (Cano *et al.*., 2000). Snail directly downregulates E-cadherin expression through binding to the E-box elements of the E-cadherin promoter. An inverse relationship between the level of E-cadherin and Snail exists in cancer cells. Transfection of cells with Snail resulted in loss of expression of E-cadherin and occurrence of migration and invasiveness of cancer cells *in vitro* and metastasis *in vivo* (Jiang and Mansel, 2000).

2.9.1.4. Tyrosine phosphorylation

The ability of the E-cadherin-catenin complex to mediate cell adhesion can also be eliminated by post-translational events, including phosphorylation (Vleminckx *et al.*., 1994). These post-translational mechanisms for modulating cell adhesion might allow the cell to respond rapidly to the local environment. Strong evidence suggests that tyrosine phosphorylation can initiate disassembly of the complex during normal cell migration (Gumbiner, 1996). Tyrosine phosphorylation has previously been implicated in the regulation of cadherin function. Both β-catenin and E-cadherin are phosphorylated. This post-translational modification results in functional changes, such as decreased cell-cell adhesion, increased migration and increased invasiveness, without affecting the overall expression of either of the catenins or the cadherins (Behrens *et al.*., 1993). β-catenin is tyrosine phosphorylated by the non-receptor tyrosine kinase SRC, and this modification might lead to disassembly of the cadherin-catenin complex and subsequent loss of cell adhesion (Behrens *et al.*., 1993). Phosphorylation of the E-cadherin-catenin complex, principally of β- and γ-catenin is associated with the de-localization of membrane-bound E-cadherin from the adherens junctions (Efstathiou and Pignatelli, 1998).

The inhibition of tyrosine phosphorylation restores cadherin function to normal. Other studies show that upregulation of tyrosine phosphorylation of β-catenin and p120 occurs frequently in surgical specimens of colorectal and lung cancer, and that phosphorylation of β-catenin correlates well with poor survival of patients after surgery (Takayama *et al.*., 1998). These results suggest that tyrosine phosphorylation of the catenins might be a significant mechanism that modulates their function and, in turn, that of E-cadherin-catenin; this may have important prognostic value (Wijnhoven *et al.*., 2000).

The molecular mechanism for E-cadherin downregulation during tumour progression in esophageal cancers remains largely unknown (Si *et al.*., 2001).

2.9.1.5. E-cadherin gene mutation

The E-cadherin gene (*CDH1*) plays a major role in the downregulation of E-cadherin protein. The E-cadherin gene is often termed a metastasis suppressor gene because the E-cadherin protein can suppress tumour cell invasion and metastasis. Inactivation of the E-cadherin gene occurs in undifferentiated solid tumours by both genetic and epigenetic mechanisms (Crawford *et al.*., 1999). Furthermore, there is experimental evidence that when a normal E-cadherin gene is transfected into tumour cells, the cells lose their invasiveness (Behrens *et al.*., 1989). Conversely, using anti-E-cadherin antibodies can induce invasive behaviour in normal cells (Vleminckx *et al.*., 1991).

The irreversible mutation of *CDH1* provides a simple mechanism for the inactivation of E-cadherin-mediated cell adhesion (Guilford, 1999). However, the requirement for cyclical regulation of E-cadherin expression during development and homeostasis provides a complex array of transcriptional and post-transcriptional mechanisms for E-cadherin downregulation. Activation of these mechanisms during tumorigenesis will diminish cell adhesion and enhance tumour invasion (Guilford, 1999).

2.9.1.5.1. Germline *CDH1* mutation

Perhaps the strongest evidence in support of a causal role for cadherin alterations in cancer pathogenesis is the observation that germline mutations in *CDH1* strongly predispose affected individuals to diffuse-type gastric cancer (Gayther *et al.*., 1998; Guilford *et al.*., 1998). Heterozygous, inactivating germline *CDH1* mutations have been detected in approximately 50% of families with a history of early-onset, diffuse gastric cancer. In contrast, no germline *CDH1* mutations have been identified in families that are affected by the well-differentiated, intestinal form of gastric cancer (Gayther *et al.*., 1998).

In some kindreds segregating a germline *CDH1* mutation, individuals have been identified with colorectal (Guilford *et al..,* 1998; Richards *et al..*, 1999), breast (Guilford *et al..,* 1999) and prostate (Gayther *et al..*, 1998) cancers. However, the elevated risk of these carcinomas in individuals who carry germline *CDH1* mutations is less certain than the markedly elevated risk for gastric cancer (Hajra and Fearon, 2002). A causal role for E-cadherin inactivation in cancer has been supported by data showing that, in a mouse model of pancreatic β-cell cancer development, E-cadherin inactivation is a rate-limiting step in the progression from adenoma to carcinoma (Li *et al..*, 1998).

However, it is not yet clear why gastric cancer, and not cancer of other epithelial tissues, predominates in families with heterozygous *CDH1* mutations. The carriers of these mutations have no other known medical problems, indicating that the second, non-mutated *CDH1* allele is generally sufficient for normal development and homeostasis. However, the stability of expression from this allele might differ in various epithelia (Guilford, 1999).

2.9.1.5.2 Sporadic *CDH1* mutation

In addition to the germline mutation observed in hereditary diffuse gastric cancer families, somatic mutations in *CDH1* are common in poorly differentiated breast and gastric cancer (Berx, *et al..*, 1998). No mutations have been observed in invasive ductal carcinomas or well-differentiated, intestinal-type gastric carcinoma (Batsché *et al.*, 1998). Intriguingly, the breast cancer mutations are characteristic frameshift or nonsense mutations (Guilford, 1999). Figure 2.6 shows the distribution, type and frequency of the E-cadherin mutations in human tumours reported so far.

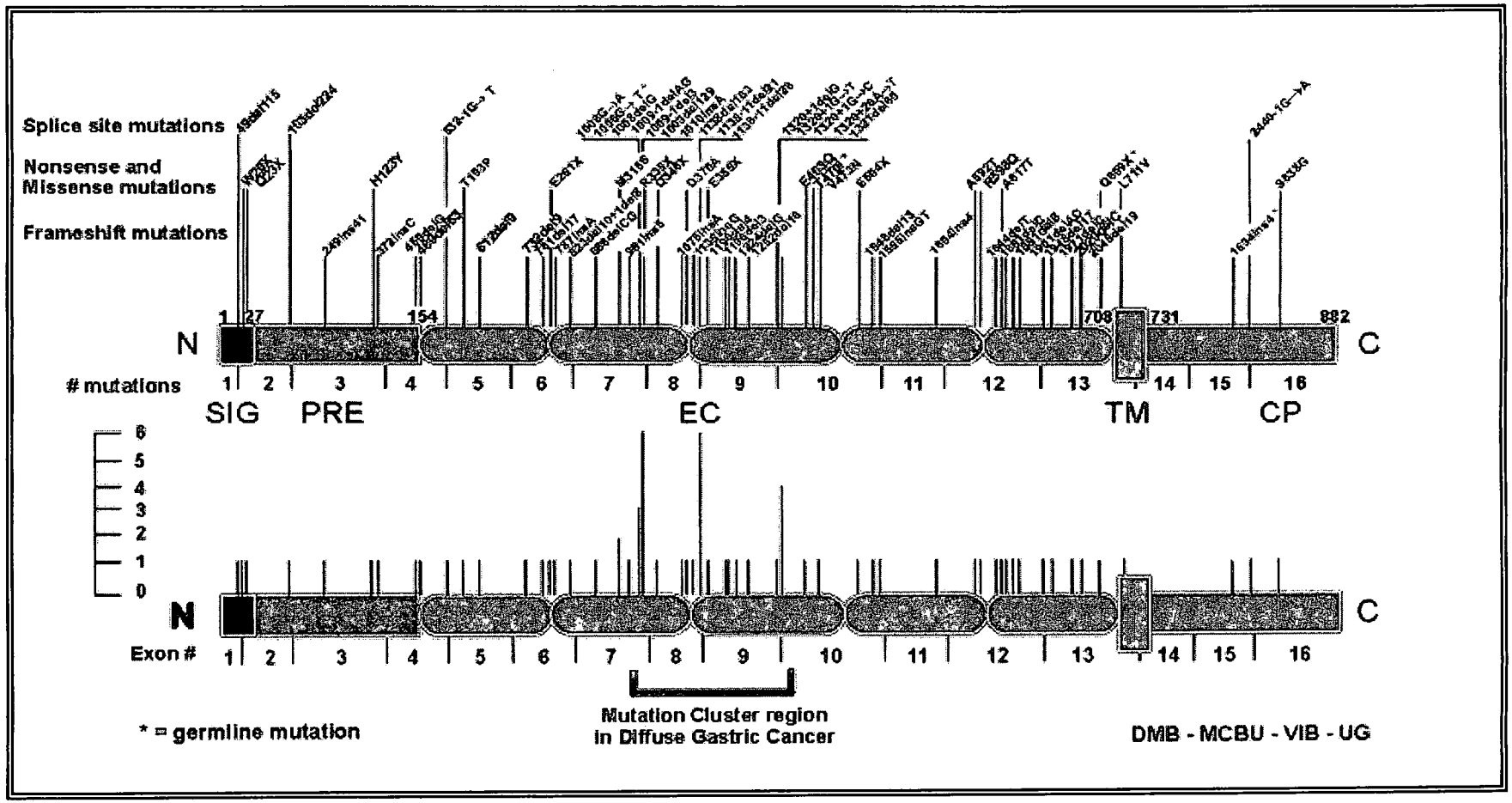

Figure 2.6. Distribution, type and frequency of the so far reported E-cadherin mutations in human tumours and tumour cell lines. Protein domains comprise of: SIG, signal peptide; PRE, precursor sequence; EC, extracellular domains with Ca^{2+}-binding motifs; TM, transmembrane domain; CP, cytoplasmic domain. Numbers directly on top of the protein symbol denote codon numbers at domain borders. Numbers below the protein symbol denote the encoding exons. Adapted from Berx *et al.*., 1998.

Somatic mutation in one *CDH1* allele has been shown to occur in combination with inactivation of the other allele (Berx *et al.*., 1998). Thus, *CDH1* inactivation seems to adhere to the two-hit (biallelic) model for tumour suppressor gene inactivation (Knudson, 1985). The majority (93%) of sporadic breast tumours that carry mutations in *CDH1* show loss of heterozygosity of the second *CDH1* allele (Berx *et al.*., 1993; Berx *et al..,* 1998). The inactivation of both alleles is consistent with the two-hit hypothesis for tumour suppressor genes (Guilford, 1999).

Although there have been several reports on E-cadherin gene mutations in human cancers (Berx *et al.*., 1998), mutations are rare in adenocarcinoma of the esophagus, thyroid and colorectal carcinomas (Wijnhoven *et al.*., 1999). This brings us to our aim, which is to investigate the genetic profile of the E-cadherin gene in esophageal squamous carcinoma.

2.10 Aims of this study

In the light of the above-mentioned involvement of E-cadherin in various cancers and the fact that nothing could be found in the scientific literature on the status of the gene in squamous cell carcinoma of the esophagus, we have embarked on a project to investigate it. The plan was to compare the single stranded conformational polymorphism (SSCPs) profile of DNA isolated from blood and tumour from the same patient in order to detect somatic mutations. In order to do this, the method first had to be optimised in our hands. Furthermore, since the biopsies were very small and the amount of blood that could be drawn from seriously ill, cachectic patients were sometimes less than half a milliliter, a method had to be devised to enable all exons to be amplified from limiting amounts of DNA. Finally, if new polymorphisms were found or if blood and biopsy DNA from the same patient showed different patterns, these were to be sequenced to include in the mutation database.

CHAPTER 3

MATERIALS AND METHODS

3.1 Patients

All the specimens were obtained from newly diagnosed untreated patients undergoing esophageal dilatations. These subjects were referred to the weekly gastroscopy clinic at the Universitas Hospital in Bloemfontein, Free State Province. All participating subjects were asked to sign a consent form. The Ethics Committee of the Faculty of Health Sciences approved this project at the University of Free State with the ETOVS number 145/02. The subjects were mostly from the Free State, but also included patients from the Northern Cape Province and Eastern Cape. Thirty-one subjects were selected for this study on the basis of availability of both blood and tumour biopsies and to approximate the epidemiological profile of the disease. The female to male ratio was 8:23, and the Whites to Blacks ratio was 3:28. The age of the patients ranged from 40 to 95 years, with a median of 61 years.

3.2 Sample collection

Blood and biopsies were taken from each of the patients. Endoscopic biopsies were taken by qualified surgeons from the tumour mass using a flexible Olympus endoscope equipped with a sampling channel. The biopsies were frozen individually at -70°C until they were analysed. Duplicate biopsies were taken and put into buffered formalin for histological analysis. Whenever possible, five millilitres of periferal blood was collected in EDTA tubes.

3.3 DNA isolation

Genomic DNA was extracted from both blood and tumour biopsies. The Promega Wizard genomic DNA Purification kit was used for the isolation of DNA from blood samples. Due to the small size of the biopsies we used a breast cancer tumour that was available in the laboratory to select the best protocol for the isolation of DNA. The tumour was cut into 0.1mg sizes to correspond to the sizes of the biopsies. We observed that out of all the methods used the Amersham Life Sciences Nucleon Bacc3 for blood and cell cultures kit produced the best results. The protocol for hard tissue and paraffin sections was chosen. The DNA was resuspended in only 40μl tris-EDTA buffer due to the size of the biopsies.

3.4 Polymerase Chain Reaction (PCR)

3.4.1 Exon PCR

Amplification of the individual *CDH1* exons was done according to the method of Berx, *et al.*. (1995), with a number of alterations. A Perkin Elmer 2400 PCR machine was used for all the reactions. The primers shown in Table 3.1 were purchased from Integrated DNA Technologies, Inc. Polymerase Chain Reaction (PCR) was carried out in a total reaction volume of 25μl containing up to 250ng genomic DNA, 10pmol of each primer, 250mM of each d-NTP, 2.5μl of reaction buffer, 2mM $MgCl_2$ and 2.5 units of *Taq* polymerase (Roche 1 146 173). The PCR cycles were as follows: An initial denaturation step at 94°C for 2 minutes, 25 cycles at 94°C for 30 sec, appropriate annealing temperature for 1 min and 72°C for 45 sec. The final elongation step consisted of one cycle at 72°C for 7 min. The results of the PCR were observed by mixing 5μl of the PCR product with 1 μl of loading buffer and the total volume was run on a 2% agarose gel.

Table 3.1
Primers used for PCR amplification of E-cadherin exons

Exon number	Sequence of forward primer (5' - > 3')	Sequence of reverse primer (5' -> 3')	Amplicon length (bp)	Ta °C
1b	NEW GCGGAAGTCAGTTCAGACTCCAG	NEW AATGCGTCCCTCGCAAGTCAGG	190	60
2	(-34) TCACCCGGTTCCATCTAC	(+229) CAACCTCCTCTTCTTTAT	378	50
(2b)	as above	NEW CTACTCCGCCCAGGGAC	210	50
3	(-54) GCTCTTGTCTTTAATCTGTC	(+75) GTACCAAGGCTGAGAAACCT	360	60
4	(-25) CTTGTTCCTCATCTTCTTTC	NEW (+33) TCCTTGGTACTTCTCTGCCAAATC	204	55
5	NEW (-69) GATTTGGCAGAGAAGTACCAAGGA	(+151) CCCATCACTTCTCCTTAGCA	376	55
(5B)	NEW GGATCCTTCTTTACTAATTC	NEW GTGTCAACAAGCTTCTAAG	246	55
6	(-18) CTCACTTGGTTCTTTCAG	(+60) AACCTTTGGGCTTGGACA	246	55
7	(-37) AGCTTGTCTAAACCTTCATC	(+116) GCTTAGACCATCACTGTATT	329	60
8	(-24) TTGGTTGTCTCGATCTCTCT	(+70) CAGTGGTACCCTTAGTTCAT	223	55
9	(-33) GTACTTGTAATGACACATCTC	(+36) TGCCAGTTTCTGCATCTTGC	252	55
10	(-24) ACTTCATTGTTTCTGCTCTC	(+41) AACCAGTTGCTGCAAGTCAG	311	60
11	(-47) GTTGTTGCTGGTCCTATTC	(+48) GAACTAGCTAGGAGGTCGAG	253	60
12	(-54) TGGGGATTCATTACTGTTGC	(+27) GCATGGCAGTTGGAGCAAAG	326	60
13	(-44) TTTCCTCCCCTGGTCTCATC	(+25) TGAGTCACTTGCCAGCTGGA	302	60
14	(-36) CTCTCAACACTTGCTCTGTC	(+22) AGAGATCACCACTGAGCTAC	209	60
15	(-67) CATAGCCCTGTGTGTATGAC	(+33) CGGATGCTTTGGCTTTCCAC	248	60
16	(-57) AGATGACAGGTGTGCCCTTC	(+51) ATTTCTGCATTTCCCAGCAC	315	60

[a]Positions of the forward and reverse primers are indicated by the number of nucleotides upstream (-y) or downstream (+y) from the nearest exon (or stop codon for the reverse primer of exon 16).
Bold temperatures – indicate the temperatures that are not the same as in Berx, 1995.

3.4.2 Long PCR

Due to the small size of the tumour biopsies, primers were designed to amplify the whole *CDH1* gene in five fragments to generate enough template for the exon PCRs. The primers have been designed with the aid of the Omiga 1.1 software package (Oxford Molecular) to be 28-32 bp in size and to have optimal annealing temperatures as close to 68°C as possible. They are listed in Table 3.2. The reaction volume was 25μl consisting of 0.5μl genomic DNA, 1μl primer mixture (10pmol of each primer), 500μM dNTPs, 2.5μl Roche Expand Long Template Buffer 3, 0.5μl polymerase mixture (Roche Expand Long Template PCR system, 1 681 842). The cycles were as follows: initial denaturation step at 95°C for 1 min, 35 cycles at 95°C for 30 sec and 68°C for 7 min. The final elongation step consisted of 68°C for 7 min.

Table 3.2

Primer	**Sequence (5'-3')**	**Exon**	**Size (bp)**
1F	CATAACCCACCTAGACCCTAGCAACTCC'	1-2	4244
1R	AGTTCCTCAGATTGAACGTGTGATTTTACCA		
2F	GTTTCCTAGAATGAATGGTGTTATCGACTG	3	2550
2R	TATCAAGAGTCTTCAGCGAGAAAAGAAGATGC		
3F	CATTAAATTCAAACTGTACACTGCCCACAGAA	4-10	8683
3R	TATCTATTTCACTACTGCAAGCTTTCAACCCA '		
4F	CTAAAACGGTGTAAACCGCGTCTCAACTAAC	11-13	6054
4R	CAATGGAAACAGGTTTAAGGACTATGGCAG		
5F	AGCAACATTTAAAACAGAGGCAATGCCA	14-16	7713
5R	CACCAGTGTAGTAATGAGCAGAAGCACACA		

3.5 Single Stranded Conformational Polymorphism (SSCP) analysis.

For standardisation of the technique, a number of anonymous Caucasian DNA samples from the department of Genetics were used. None of these subjects had cancer of the esophagus, although they formed part of families being studied for familial breast cancer. The individual exons were amplified according to 3.4.1 and 20µl of the PCR product was mixed with 8µl of denaturation buffer. The denaturation buffer contained 95% formamide, 0.0025% bromophenol blue and 0.0025% xylene cyanol. The mixture was denatured for 5 min at 95-99°C and immediately placed in an ice block for 5 min. Twelve microlitres of the mixture was loaded on to 0.5 x MDE (FMC) gels. The volumes and chemicals for a single gel of the 0.5 x MDE gels consisted of: 2 x MDE (BioWhittaker Molecular Applications) (11.3ml), dH_20 (25ml), 5 x TBE (9ml), 10% APS (MERCK) (300µl) and TEMED (Gibco BRL Life Technologies) (30µl). The total volume for a single 0.5 x MDE, with 1 mm spacers, was 45ml. The denatured samples were electrophoresed at constant voltage (260v) for ±16 hours at temperatures ranging between 4°C and 16°C in 1 x TBE buffer. The results were visualised by using the BDH Electran 'Instaview' Silver Staining kit (Sugano *et al.*., 1993). The gels were dried onto filter paper using a gel drier (BioRad). For reproduction in this thesis, the dried gels were scanned on a flat bed scanner (Hewlett Packard) and the image converted to a negative for better visualisation.

3.6 Sequencing

Sequencing of selected PCR products was done by Dr Parry Guilford in Dunedin New Zealand and was done by using fluorescent primers and the dideoxy chain termination method.

CHAPTER 4

Results and Discussion

4.1 Exon-specific amplification

The PCR method of Berx *et al.*. (1995) was used for the amplification of the E-cadherin exons and the method worked excellently for most of the exons except for a few annealing temperatures that had to be adjusted. The annealing temperatures of the four exons that differed from that of Berx *et al.*. (1995) are as indicated in Table 4.1.

Table 4.1 Differences between the annealing temperature of Berx *et al.* (1995) and ours

Exons	Berx *et al.*.	This work.
1	70	60
2	55	50
2b	55	50
8	55	50

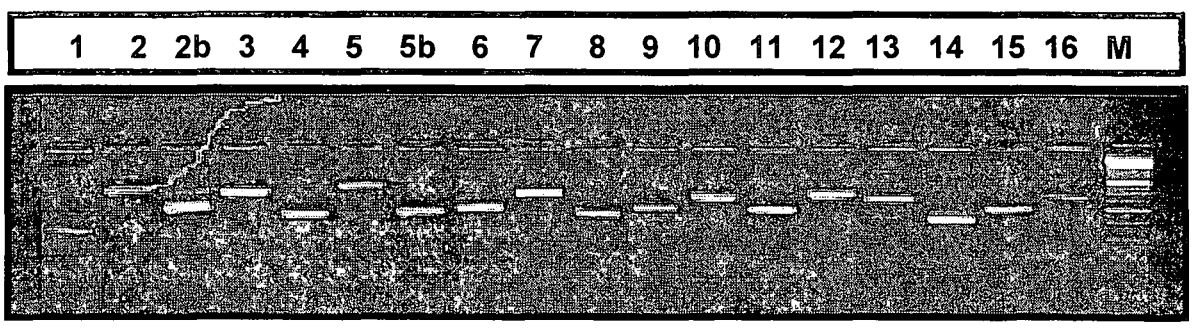

Figure 4.1 PCR products of each exon, loaded on a 2% agarose gel. M-Roche molecular size marker XIII. Exon 1 is the smallest at 190bp while exon 2 is the biggest at 378bp.

We used those temperatures that produced less uncertainity or that produced a clearer band. Difficulty in amplifiying exon 1 was due its high G+C content. Exon 2 and 5 are the two largest exons and two sets of primers were used to amplify each exon into two amplicons, exons 2 and 2b, exon 5 and 5b. Each PCR reaction had a total reaction volume of 25μl and 5μl of the PCR product was loaded on agarose gels to check the results, an example of which is given in Figure 4.1. It is clear from this photograph that most exons produced very clean single bands, while some, eg exon 13, showed a ghost band which could be removed by increasing the annealing temperature, but at the price of reduced yield. In these cases a compromise was struck between yield and purity.

4.2 DNA isolation from biopsies and long PCR

Compared to blood, the quality and quantity of DNA recovered from the tumour biopsies were generally not adequate for doing 17 separate amplification reactions. Yields were variable, with some biopsies showing virtually no DNA when an aliquot was run on an agarose gel. This prompted development of a nested approach where the genomic DNA would first be amplified in large fragments to generate enough template for the second round exon-specific PCR. Ideally, such a preamplification should be a multiplex reaction to include all the fragments. We were worried initially about the difference in size between the smallest (2550 bp) and largest (8683) fragments which could enrich the smaller fragments to the detriment of the larger ones. Indeed, some fragments turned out to be present in higher amounts than others (Figure 4.2), but that did not seem to be important for the second round PCR as long as only 1 microliter of first round product was used as template. The products of the nested PCR could not be distinguished from that originating from good quality genomic DNA isolated from blood (results not shown). The long PCR was not optimised further and used throughout the study.

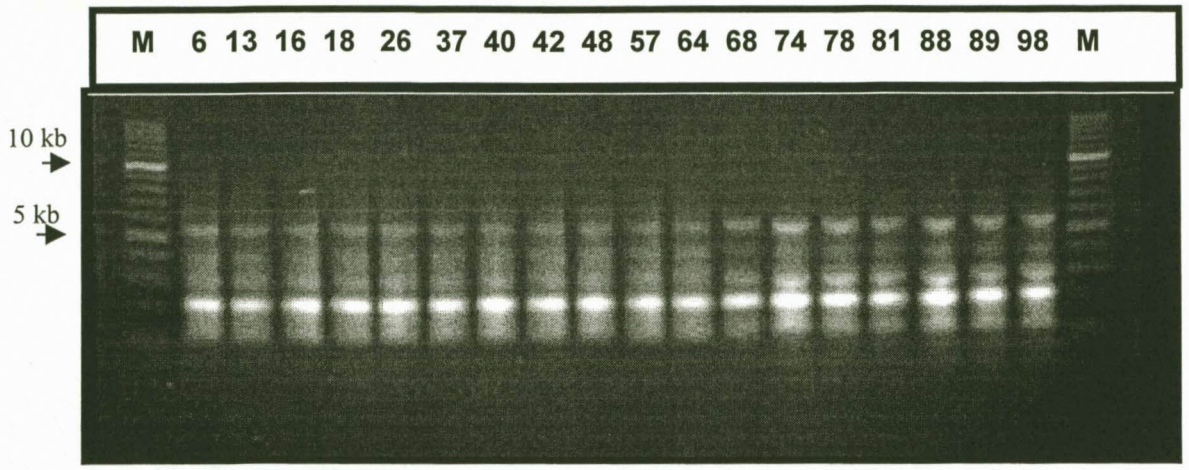

Figure 4.2 Amplification products from the multiplex long PCR separated on a 0.5% agarose gel. M – Promega 1 kb ladder

4.3 SSCP and sequencing analysis

SSCP is a technique that detects uncharacterized sequence variations. Each polymorphism require particular conditions in order to become visible, therefore a range of conditions have to be tried for each exon. In our experience, the optimal conditions for performing a specific SSCP are dependent on many variables, eg the equipment used, the type of separating medium (gel), the loading and electrophoresis buffers and the temperature at which the electrophoresis is done. Of these variables, only the running temperature was varied for the purpose of optimization. The standardization of the different SSCPs was done from 4°C to 20°C in steps of two degrees, running gels for 18 hours. Table 4.2 shows the optimal temperatures of individual exons.

Table 4.2 SSCP temperature for each exon.

Temp.°C	Exons
6	1,2,2b,5b,7,14
8	4,5
10	8
12	3,10,12
14	6,9,13,15
16	11,16

Polymorphisms were detected in exons 4, 5 and 12 and in none of the others. The extra bands were all present in addition to the normal pattern, suggesting that the patients could be heterozygotes for the particular polymorphisms. In the case of exon 4, a fairly common polymorphism was seen in both the control group (38%), which were of Caucasian origin and the patient group (32%), which were Africans (Fig 4.4). Although not clearly visible in the photographs , the extra bands were seen on the original gels before drying, a general observation for all presented photographs. This could be the polymorphism reported by Berx *et al.* (1995), although it could not be confirmed by sequencing as the exon 4 reactions failed. Exon 5 shows no polymorphisms for the control group (Fig 4.5), but a clear band can be seen in 6 (20%) patient samples. The same extra band is seen in blood and tumour, indicating that it did not originate in the tumours. Sequencing revealed a change in the intron at position -4 (**T**CAG/ATCAAA-> **A**CAG/ATCAAA). Two different polymorphisms are visible in exon 12, the more common one identified by sequencing as C1896T (H632H), previously described by Huiping *et al.* (2001).

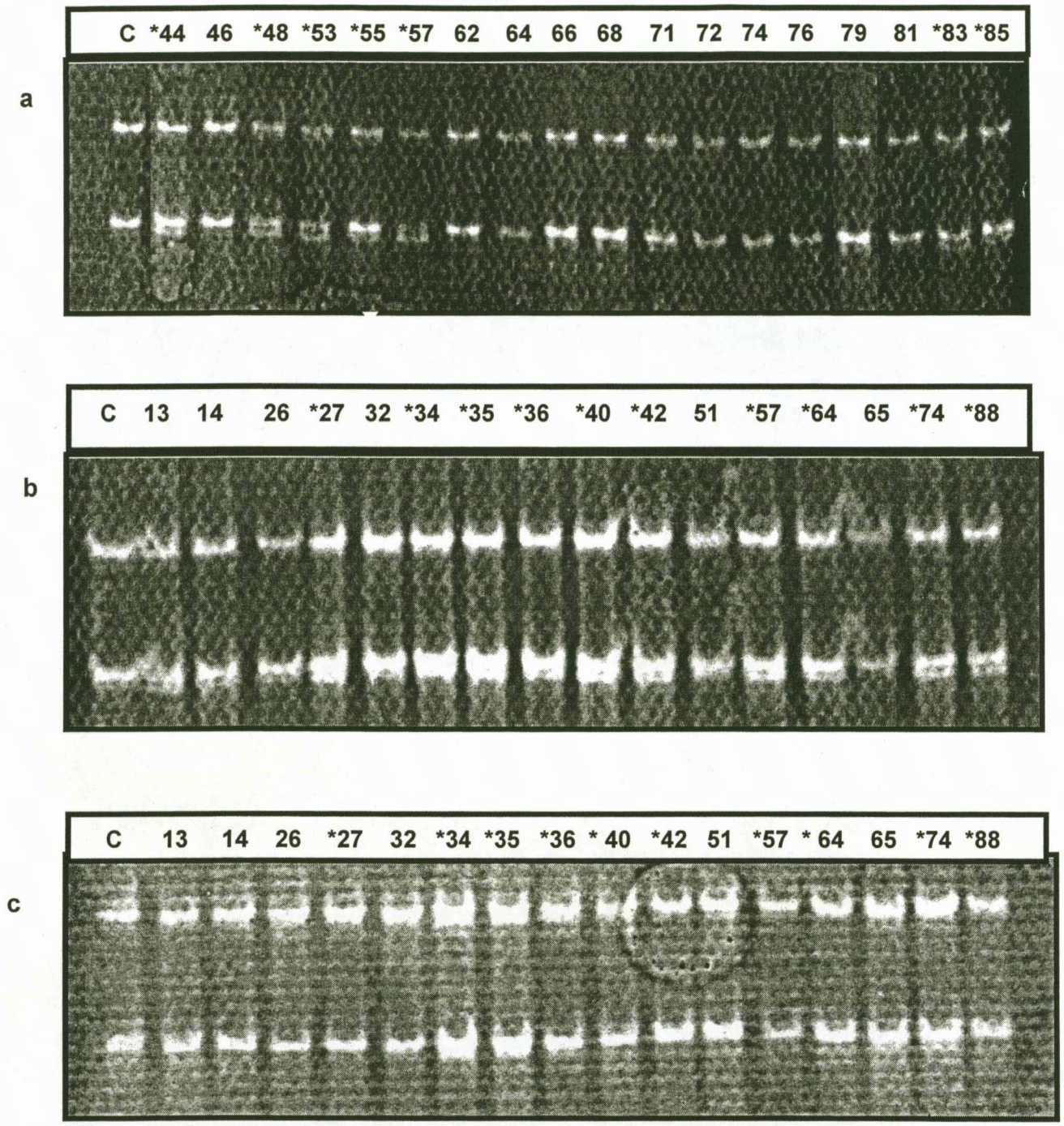

Figure 4.4. Representative SSCP analysis of exon 4. a –normal control, b- patient blood DNA, c- patient tumour DNA. C- control. Arrows indicate the band shift and asterisks indicate subjects with polymorphism.

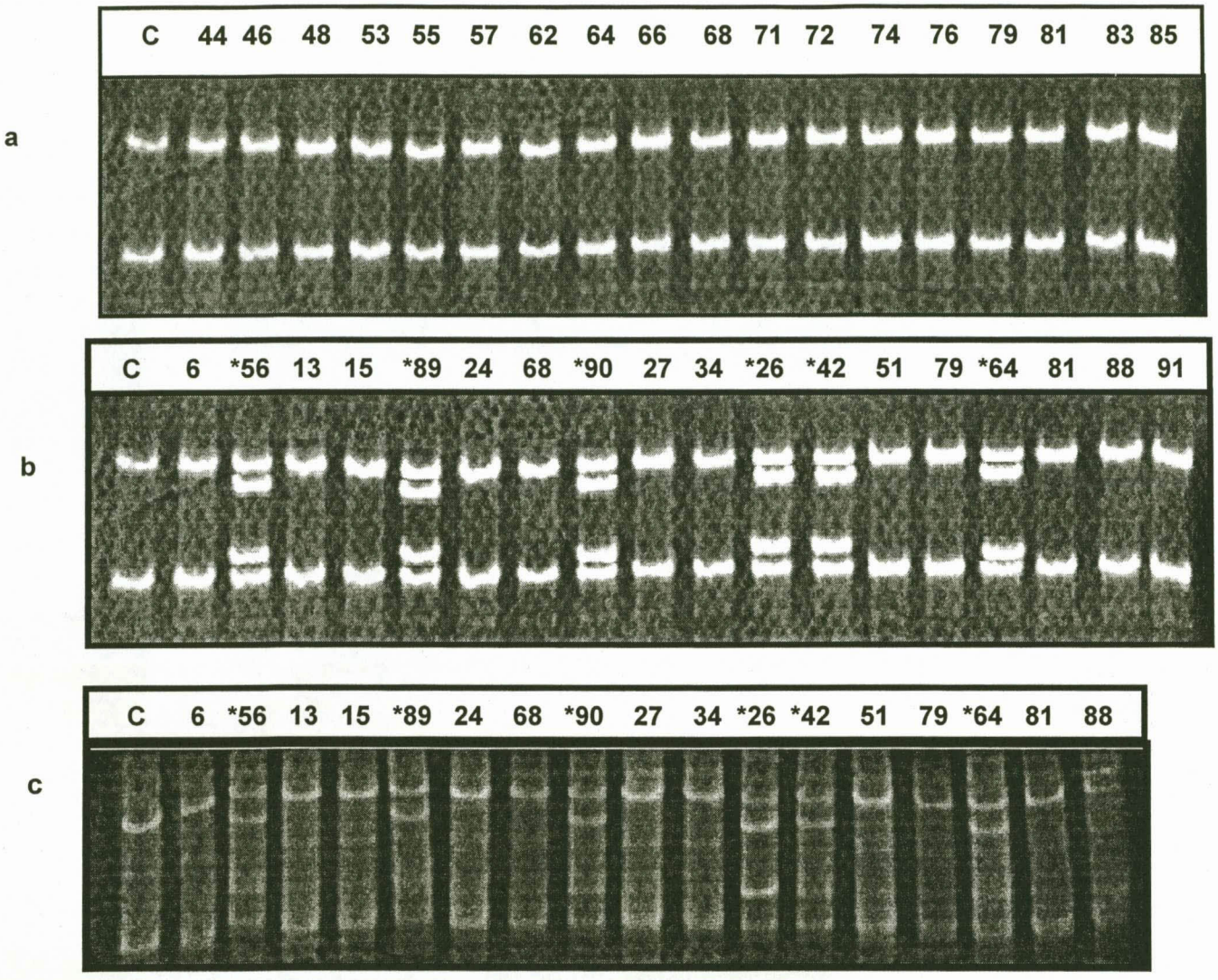

Figure 4.5 Representative SSCP results of exon 5. a– normal control, b – patient blood DNA, c- patient tumour DNA C- control, asterisks indicate subjects with polymorphisms.

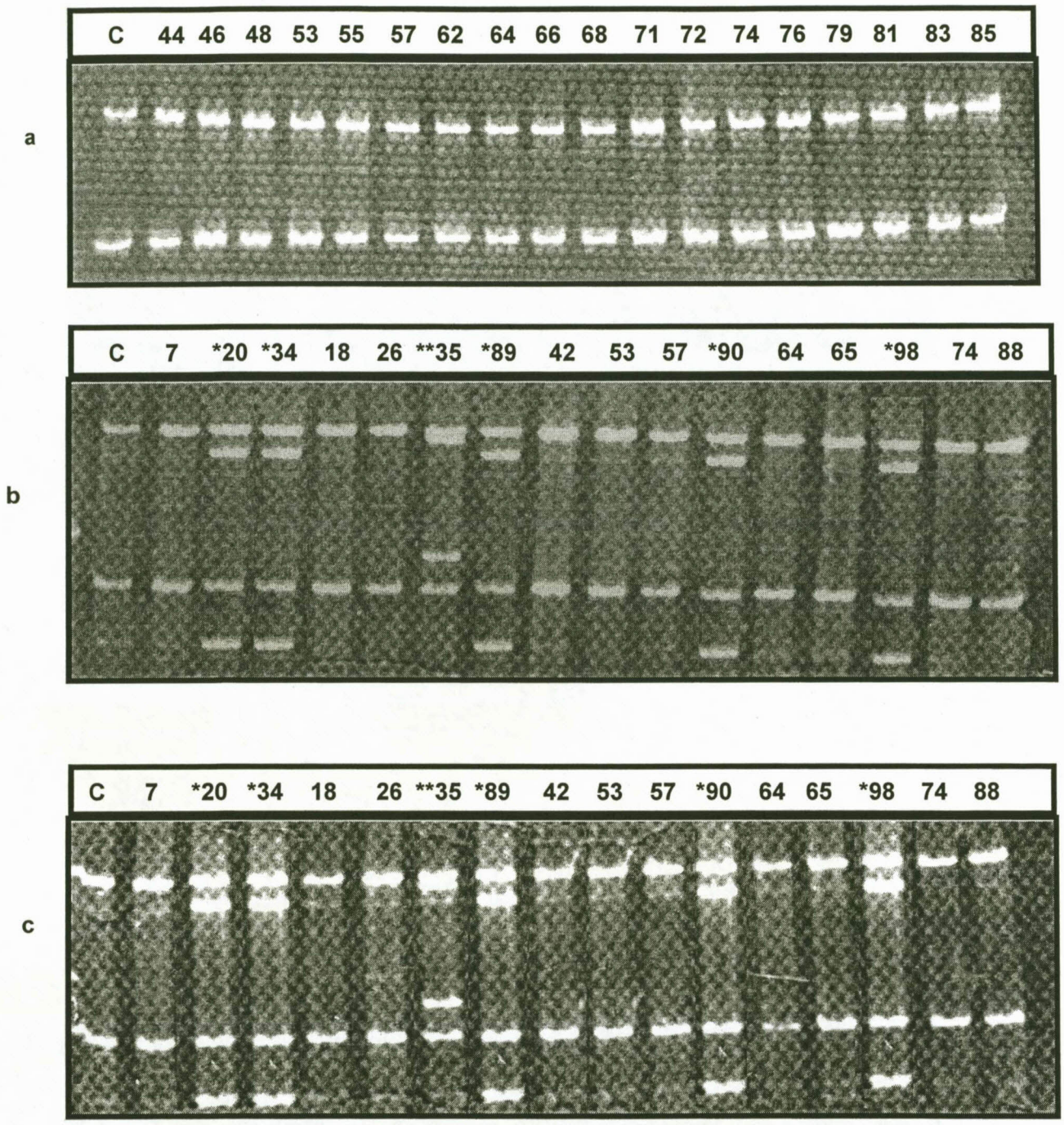

Figure 4.6 Representative SSCP results of exon 12. a – normal controls, b- patient blood DNA, c- patient tumour DNA. Asterisks indicate subjects with polymorphisms.

Table 4.3. Summary of patient data and .SSCP results for variant exons

Patient No	Sex	Age	Histology[1]	SSCP Result of exons[2] 4	5	12	Variant patients
6	M	66	PD	N	N	N	N
7	M	51	PD	N	N	N	N
13	M	47	MD	N	N	N	N
14	M	72	MD	N	N	N	N
15	M	40	PD	N	N	N	N
18	F	51	MD	N	N	N	N
20	M	61	PD	N	N	P1	P
24	M	45	WD	N	N	N	N
26	M	56	WD	N	P	N	P
27	F	78	MD	P	N	N	P
32	M	74	MD	N	N	N	N
34	M	48	NA	P	N	P1	P
35	M	74	MD	P	N	P2	P
36	M	95	MD	P	N	N	P
40	M	62	PD	P	N	N	P
42	M	60	MD	P	P	N	P
51	F	66	PD	N	N	N	N
53	F	48	MD	N	N	N	N
56	M	61	MD	N	P	N	P
57	M	53	CIS	P	N	N	P
64	M	73	PD	P	P	N	P
65	M	64	NA	N	N	N	N
68	F	65	MD	N	N	N	N
74	M	74	MD	P	N	N	P
79	F	59	WD	N	N	N	N
81	F	64	MD	N	N	N	N
88	F	65	WD	P	N	N	P
89	M	76	MD	N	P	P1	P
90	M	64	MD	N	P	P1	P
91	M	65	PD	N	NA	N	N
98	M	56	MD	N	N	P1	P
Variant exons (%)				**10 (32)**	**6 (20)**	**6 (19)**	**16 (52)**

1. CIS: Carcinoma in situ, MD: Moderately differentiated, PD: Poorly differentiated,
   WD: Well differentiated, NA: Not available.
2. N: Normal, P: Polymorphism, NA: Not Available

The other bandshift (P2) seen in patient 35 was identified as G1849A (A617T), first described by Risinger *et al.* (1994) in endometrial carcinoma.

Taken together this data means that somatic mutation of the CDH1 gene is not a high-frequency event in squamous epithelial carcinoma of the esophagus, extending the finding of Wijnhoven *et al.*. (1999) on esophageal adenocarcinoma that mutation of this gene is rare in that cancer.

4.4 Discussion

In this project a start was made at investigating the possible involvement of the E-cadherin gene in squamous epithelial carcinoma of the esophagus. No tumour-specific polymorphisms were found, but the level of polymorphism in this gene in the study population was substantial, with 52% of patients showing one or more polymorphisms in exon 4, 5 or 12. The chosen screening technique is not always informative, as some polymorphisms do not show up as band shifts on SSCP gels (Tsai *et al.*, 1993), meaning that there may be other undetected base changes in the gene. It is impractical at this stage, however, to use complete sequencing as a screening tool.

The exon 4 polymorphism is shared with the Caucasian population in our control group, while the others may be specific to the African population as germline polymorphisms. One of these polymorphisms (A617) in particular, has been found as somatic mutation combined with loss of heterozygosity in endometrial carcinoma (Risinger te al, 1994). It is unclear whether this germline polymorphism may be involved in predisposition to cancer of the esophagus. Epidemiological data does not indicate any familial tendecies for this cancer in South Africa (Sitas *et al.*, 1998; Sammon, 1996).

If it is assumed that E-cadherin function is abrogated in ESCC (Chitaev *et al.*, 1998) and if as this study suggests, mutational inactivation in the coding region or intron-exon borders is not a major contributor to the decrease in activity, other pathways may be involved. Promoter activity may be decreased or blocked by hypermethylation of CpG islands (Si *et al.*, 2001) or polymorphisms at certain sites in the promoter (Nakamura *et al.*, 2002). Moving away from the

gene itself, many other possibilities open up. To illustrate one avenue: β-catenin regulates E-cadherin mediated cell-cell adhesion while itself being involved in the Wnt signal transduction pathway and disruption of this cascade by mutation or dysregulation may lead to decreased E-cadherin expression and hence loss of tissue architecture (Ninomiya *et al.*, 2000; Klimstra *et al.*, 1994). Alteration of the cellular distribution of the cadherin-catenin complexes may also be involved in reduction of function, for instance, El-Hariry *et al.* (1999) found a redistribution of these complexes from membrane to cytoplasm in some tumours. The molecular basis for this redistribution is not known.

It is thus clear that this study has only scratched the surface of E-cadherin involvement in ESCC. Before the gene can be firmly implicated or cleared of involvement, more work needs to be done to investigate the full regulatory framework of E-cadherin function.

References

Aberle H, Bauer A, Stappert J, *et al.*. β-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J* 1997; **16**: 3797-3804.

Adams CL and Nelson WJ. Cytomechyanics of cadherin-mediated cell-cell adhesion. *Curr Opin Cell Biol* 1998; **10**: 572-577.

Adams CL, Nelson WJ and Smith SJ. Quantitative analysis of cadherin-catenin-actin reorganization during development of cell-cell adhesion. *J Cell Biol* 1996; **135**: 1899-1911.

Akhtar MS and Munir M. Evaluation of the gastric antiulcerogenic effects of Solanum nigrum, Brassica oleracea and Ocimum basilicum in rats. *J Ethnopharmacol* 1989; **27**: 163-176.

Alattia J-R, Ames JB, Porumb T. *et al.*. Lateral self-assembly of E-cadherin directed by cooperative calcium binding. *FEBS Lett* 1997; **417**: 405-408.

Allen JW, David Richardson J and Edwards MJ. Squamous cell carcinoma of the esophagus: a review and update. *Surg Oncol* 1997; **6**: 193-200.

Anani PA, Gardiol D, Savary M, *et al.*. An extensive morphological and comparative study of clinically early and obvious squamous cell carcinoma of the esophagus. *Pathol Res Pract* 1991; **187**: 214-219.

Anastasiadis PZ and Reynolds AB. The p120 catenin family: complex roles in adhesion, signalling and cancer. *J Cell Sci* 2000; **113**: 1319-1334.

Aono S, Nakagawa S, Reynolds A B, *et al.*. $p120^{ctn}$ acts as an inhibitory regulator of cadherin function in colon carcinoma cells. *J Cell Biol* 1999; **145**: 551-562.

Batsché E, Muchardt C, Behrens J, *et al.*. RB and *c-Myc* activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2. *Mol Cell Biol* 1998; **18**: 3647-3658.

Baylin SB, Herman JG, Graff JR, *et al.*. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998; **72**: 141-196.

Becker KF, Atkinson MJ, Reich U, *et al.*. Exon skipping in the E-cadherin gene transcript in metastatic human gastric carcinoma. *Hum Mol Genet* 1993; **2**: 803-804.

Beer DG and Stoner GD. Clinical models of chemoprevention for the esophagus. *Hematology Oncol Clin North Am* 1998; **12**: 1055-1077.

Behrens J. Cadherins as determinants of tissue morphology and suppressors of invasion. *Acta Anat* 1994; **149**: 165-169.

Behrens J, Löwrick O, Klein-Hitpass L, *et al.*. The E-cadheirn promoter: Functional analysis of a GC-rich region and an epithelial cell-specific palidromic regulatory element. *Proc Natl Acad Sci USA* 1991; **88**: 11495-11499.

Behrens J, Mareel MM, Van Roy F, *et al.*. Dissecting tumour cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-medated cell-cell adhesion. *J. Cell Biol* 1989; **108**: 2435-2447.

Behrens J, Vakaet L, Friis R, *et al.*. Loss of epithelial morphotype and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive *v-src* gene. *J Cell Biol* 1993; **120**: 757-766.

Behrens J, von Kries JP, Kuhl M, *et al.*. Functional interactions of beta-catenin with the transcription factor, LEF-1. *Nature* 1996; **382**: 638-642.

Ben-Zéev A and Geiger B. Differential molecular interactions of β-catenin and plakoglobin in adhesion, signaling and cancer. *Curr Opin Cell Biol* 1998; **10**: 629-639.

Berx G, Becker K-F and Van Roy F. Mutations of the human E-cadherin (*CDH1*) gene. *Hum Mutat* 1998; **12**: 226-237.

Berx G, Cleton-Jansen A-M, Strumane K. *et al.*. E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. *Oncog* 1996; **13**: 1919-1925.

Berx G, Nollet F and Van Roy F, *et al.*. Dysregulation of the E-cadherin/catenin complex by irreversible mutations in human carcinomas, *Cell Adhesion Commun* 1998; **6**: 171-184.

Berx G, Staes K, van Hengel J, *et al.*. Cloning and characterization of the human invasion suppressor gene E-cadherin (*CDH1*). *Genomics* 1995; **26**: 281-289.

Bever Jr. RJ, Couch L/H, Sutherland JB, *et al.*. DNA adduct formation by Fusarium culture extracts: lack of role of fusarin C. *Chemico- Biological Interactions* 2000; **128**: 141-157.

Birchmeier W and Behrens J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta* 1994; **1198**: 11-26.

Blaschuk OW, Sullivan R, David S, *et al.*. Identification of a cadherin cell adhesion recognition sequence. *Dev Biol* 1990; **139**: 227-229.

Blaschuk OW, Pouliot Y and Holand PC. Identification of a conserved region common to cadherins and influenza strain A hemagglutinin. *Dev Biol* 1990b; **211**: 679-682.

Blot WJ. Alcohol and cancer. *Cancer Res* 1992 (suppl); **5**: 2119s-2123s.

Blot WJ. Esophageal cancer trends and risk factors. *Semin Oncol* 1994; **21**: 403-410.

Blot WJ, Li JY, Taylor PR, *et al.*. Nutrition intervention trials in Linxian, China: Supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *J Natl Cancer Inst* 1993; **85**: 1483-1492.

Blot WJ and McLaughlin JK. The changing epidemiology of esophageal cancer. *Semin in Oncol* 1999; **26**: 2-8.

Breen E, Clarke A, Steele GJr, *et al.*. Poorly differentiated colon carcinomas cell lines deficient in alpha-catenin expression express high levels of surface E-cadherin but lack Ca2+-dependent cell-cell adhesion. *Cell Adhes Commun 1993;* **1**: 239-50.

Bremnes RM, Veve R, Hirsch F, *et al.*. The E-cadherin cell-cell adhesion complex and lung cancer invasion, metastasis, and prognosis. *Lung Cancer* 2002; **36**: 115-124.

Brown LM, Hoover R, Silverman D, *et al.*. Excess incidence of squamous cell esophageal cancer among US black men: role of social class and other risk factors. *Am J Epidemiol* 2001; **153:** 114-122.

Brown LM, Blot WJ, Schuman S, *et al.*. Environmental factors and high risk of esophageal cancer among men in coastal South Carolina. *J Nastl Cancer Inst* 1988; **80**: 1620-1625.

Byers SW. Role of E-cadherin in the response of tumor cell aggregates to lymphatic, venous and arterial flow: measurement of cell: cell adhesion strength. *J Cell Sci* 1995; **108**: 2053-2064.

Byers S, Amaya E, Munro S. *et al.*. Fobroblast growth factor receptors contain a conserved HAV region common to cadherins and influenza strain A hemagglutinins: a role in protein-protein interactions? *Dev Biol* 1992; **152**: 411-414.

Caca K, Kolligs FT, Ji X, *et al.*. Beta-catenin and gamma-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. *Cell Growth Differ* 1999; **10**: 369-76.

Cano A, Perez-Moreno MA, Rodrigo I, *et al.*. The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2000; **2**: 76-83.

Chitaev NA, Averbakh AZ, Troyanovsky RB, *et al.*. Molecular organization of the desmoglein-plakoglobin complex. *J Cell Sci* 1998; **111**: 1941-1949.

Chitaev NA and Troyanvosky SM. Adhesive but not lateral E-cadherin complexes require calcium and catenins for their formation. *J Cell Biol* 1998; **142**: 837-846.

Christofori G and Semb H. The role of the cell-adhesion molecule E-cadherin acts as a tumor suppressor gene. *TIBS* 1999; **24**: 73-76.

Cilley R, Strodel W and Peterson R. Cause of death in carcinoma of the esophagus. *Am J Gastroenter* 1989; **84**: 147-149.

Cook P. Cancer of the esophagus in Africa. *Br J Cancer* 1971; **25**: 853.

Cooper K, Taylor L, Govind S, *et al.*. HPV DNA in esophageal carcinoma in South Africa. *J Pathol* 1995; **175**: 273-277.

Crawford HC, Fingleton BM, Rudolph-Owen LA, *et al.*. The metalloproteinase matrilynsin is a target of β-catenin transactivation in intestinal tumors. *Oncog* 1999; **18**: 2883-2891.

Croix B, Sheehan C, Rak JW, *et al.*. E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27 (KIP1). *J Cell Biol* 1998; **142**: 557-571.

Dahl U, Sjodin A and Semb H. Cadherins regulate aggregation of pancreatic β-cells *in vivo*. *Dev* 1996; **122**: 2895-2902.

Damsky CH, Richa J, Solter D, *et al.*. Identification and purification of a cell surface glycoprotein mediating intercellular adhesion in embryonic and adult tissue. *Cell* 1983; **34**: 455-466.

Day NE and Varghese C. Oesophageal cancer. *Cancer Surv* 1994; **20**: 43-54.

Dierick H and Besjovec A. Cellular mechanisms of wingless/Wnt signal transduction. *Curr Topics Cev Biol* 1999; **43**: 153-190.

Doki Y, Shiozaki H, Tahara H, *et al.*. Correlation between E-cadherin expression and invasiveness *in vitro* in a human esophageal cancer cell line. *Cancer Res* 1993; **53**: 3421-3426.

Du Plessis L, DietzxdhE, Van Gele M, *et al.* Mapping of novel regions of DNA gain and loss by comparative genomic hybridization in esophageal carcinoma in the Black and Colored populations of South Africa. *Cancer Res* 1999; **59**: 1877-1883.

Efstathiou JA and Pignatelli M. Modulation of epithelial cell adhesion in grastrointestinal homeostasis. *Am J Pathol* 1998; **153**: 341-347.

El-Hariry I, Jordinson M, Lemoine N, *et al.*. Characterization of the E-cadherin-catenin complexes in pancreatic carcinoma cell lines. *J Pathol* 1999; **51**: 410-412.

Eurogast Study Group. An international association between *Helicobacter pylori* and gastric cancer. *Lancet* 1993; **341**: 1359-1362.

Forastiere AA. Treatment of locoregional esophageal cancer. *Semin in Oncol* 1992; **4:** 57-63.

Forman D and Webb PM. *H pylori* and gastric cancer: the significance of the problem. In: Hunt RH, Tytgat GNJ, eds. *Helicobacter pylori*: Basic *Mechanisms to Clinical Cure*. Dordrecht, the Netherlands: Kluwer Academic Publishers; 1994.

Frixen UH, Behrens J. Sachs M, *et al.*. E-cadherin mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* 1991; **113**: 173-185.

Gagliardi G, Kandemir O, Liu D, *et al.*. Changes in E-cadherin immunoreactivity in the adenoma-carcinoma sequence of the large bowel. *Virchows Arch* 1995; **426**: 149-154.

Gallin WJ, Sorkin BC, Edelman GM, *et al.*. Sequence analysis of a c-DNA clone encoding the liver cell adhesion molecule, L-CAM. *Proc Natl Acad Sci USA* 1987; **84**: 2808-2812.

Gallin WJ, Edelman GM and Cunningham BA. Characterization of L-CAM, a major cell adhesion molecule from embryonic liver cells. *Proc Natl Acad Sci USA* 1983; **80**: 1038-1042.

Gally JA and Edelman GM. Evidence for gene conversion in genes for cell-adhesion molecules. *Proc Natl Acad Sci USA* 1992; **89**: 307-332.

Gao YT, McLaughlin JF, Blot WJ, *et al.*. Reduced risk of esophageal cancer associated with green tea consumption. *J Natl Cancer Inst* 1994; **86:** 855-858.

Gao YT, McLauglin, JK, Gridley G, *et al.*. Risk factors for esophageal cancer in Shangai, China: II. Role of diet and nutrients. *Int J Cancer* 1994; **58**: 197-202.

Gardiner-Garden and Frommer. CpG islands in vertebrate genomes. *J Mol Biol* 1987; **196**: 261-282.

Gayther SA, Gorringe KL, Ramus SJ, *et al.*. Identification of germ-line E-cadherin mutations in gastric cancer families of European origin. *Cancer Res* 1998; **58**: 4086-4089.

Gelderblom WCA, Thiel PG, Jaskiewiez K, *et al.*. Investigations on the carcinogenicity of fusarin C – a mutagenic metabolite of *Fusarium moniloforme*. *Carcinogenesis* 1986; **7**: 1899-1901.

Ghadimi BM, Behrens J, Hoffman I, *et al.*. Immunohistological analysis of E-cadherin, α-, β-, and γ-catenin expression in colorectal cancer: Implications for cell adhesion and signalling. *Eur J Cancer* 1999; **35**: 60-65.

Ghadirian P. Familial history of esophageal cancer. *Cancer* 1985; **56**: 2112-2116.

Giroldi LA, Bringuier PP, de Weijert M, *et al.*. Role of E-boxes in the repression of E-cadherin expression. *Biochem Biophys Res Commun* 1997; **241**: 453-458.

Gold JS, Reynolds AB and Rimm D. Loss of p120ctn in human colorectal cancer predicts metastasis and poor survival. *Cancer Letters* 1998; **132**: 193-201.

Goldfarb RK and Liotta LA. Proteolytic enzymes in cancer invasion and metastasis. *Semin Thromb Hemost* 1986; **12**: 294-307.

Graff JR, Gabrielson E, Fujii H, *et al.*. Methylation patterns of the E-cadherin 5 CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J Biol Chem* 2000; **275**: 2727-2732.

Grunwald GB. The structural and functional analysis of cadherin calcium-dependent cell adhesion molecules. *Curr Opin Cell Biol* 1993; **5**: 797-805.

Guilford P. E-cadherin downregulation in cancer: fuel on the fire? *Molecular Medicine Today* 1999; **5**: 172-177.

Guilford P, Hopkins J, Harraway J, *et al.*. E-cadherin germline mutations in familial gastric cancer. *Nature* 1998; **392**: 402-405.

Guilford P, Hopkins J, Grady WM, *et al.*. E-cadherin germline mutations define an inherited cancer syndrome dominated by diffuse gastric cancer. *Hum Mut* 1999; **14**: 249-255.

Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 1996, **84**: 345-357.

Gumbiner BM. Carcinogenesis: a balance between β-catenin and APC. *Curr Biol* 1997; **7**: R443-446

Hajra KM and Fearon ER. Cadherin and catenin alterations in human cancer. *Genes, Chromosomes and Cancer* 2002; **34**: 255-268.

Haruma K, Tokutomi T, Yoshihara M, *et al.*. Rapid growth of untreated esophageal squamous cell carcinoma in 10 patients. *Clin Gastroenter* 1991; **13**: 129-134.

Hazan RB, Kang L, Roe S, *et al.*. Vinculin is associated with E-cadherin adhesion complex. *J Cell Chem* 1997; **272**: 32448-32453.

He T-C, Sparks AB, Rago C, *et al.*. Identification of c-MYC as a target of the APC pathway. *Science* 1998; **281:**1509-1512.

Herrenknecht K, Ozawa M, Eckerskorn C, *et al.*. The uvomorulin-anchorage protein α-catenin is a vinculin homologue. *Proc Natl Acad Sci USA* 1991; **88**: 9156-9160.

Hinck L, Näthke IS, Papkoff J, *et al.*. Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J Cell Biol* 1994; **125**: 1327-1340.

Hirano S, Kimoto N, Shimoyama Y, *et al.*. Identifiation of a neural α-catenin as a key regulator of cadherin function and multicellular organization. *Cell* 1992; **70**: 293-301.

Hirano S, Nose A, Hatta K, *et al.*. Calcium-dependent cell-cell adhesion molecules (cadherin): subclass specificities and possible involvement of actin bundles. *J Cell Biol* 1987; **105**: 2501-2510.

Hirohashi S. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* 1998; **153**: 333-339.

Hoschuetzsky H, Aberle H and Kemler R. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J Cell Biol* 1994; **128**: 949-957.

Huber O, Bierkamp C and Kemler R. Cadherins and catenins in development. *Curr Opin Cell Biol* 1998; **8**: 685-691.

Huiping C, Kristjansdottir S, Jonasson JG, Magnusson J, Egilsson V and Ingvarsson S. Alterations of E-cadherin and β-catenin in gastric cancer. *BMC Cancer* 2001, **1**:16-22

Hülsken J, Birchmeier W and Behrens J. E-cadherin and APC compete for the interaction with β-catenin and the cytoskeleton. *J Cell Biol* 1994; **127**: 2061-2069.

Ilyas M and Tomlinson IPM. The interactions of APC, E-cadherin and β-catenin in tumour development and progression. *J Pathol* 1997; **182**: 128-137.

Ilyas M, Tomlison IPM, Rowan A, *et al.*. Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc Natl Acad Sci USA* 1997; **94**: 10330-10334.

International Agency for Research on Cancer: Alcohol drinking, in Evaluation of Carcinogenic Risks to Humans, Vol **44**. Lyon, France, International Agency for Research on Cancer, 1988.

International Agency for Research on Cancer: Tobacco smoking, in Evaluation of Carcinogenic Risks to Humans, Vol **38**. Lyon, France, International Agency for Research on Cancer, 1986.

Jiang WG and Mansel RE. E-cadherin complex and its abnormalities in human breast cancer. *Surg Oncol* 2000; **9**: 151-171.

Jiang WG. E-cadherin and its associated protein catenins, cancer invasion and metastasis. *Br J Surg* 1996; **83**: 437-446.

Jou TS, Stewart DB, Stappert J, *et al.*. Genetic and biochemical dissection of protein linkages in the cadherin-catenin complex. *Proc Natl Acad Sci USA* 1995; **92**: 5067-5071.

Kadowaki T, Shiozaki H, Inoue M, *et al.*. E-cadherin and α-catenin expression in human esophageal cancer. *Cancer Res* 1994; **54**: 291-296.

Katayama M, Hirai S, Kamihagi K, *et al.*. Soluble E-cadherin fragments increased in circulation of cancer patients. *Br J Cancer* 1994; **69**: 580-585.

Kaupmann K, Becker-Follmann J, Scherer, *et al.*. The gene for the cell adhesion molecules M-cadherrin maps to mouse chromosome 8 and human

chromosome 16q24.2 – qter and is near the E-cadherin (uvomorulin) locus in both species. *Genomics* 1992; **14**: 488-490.

Kemler R. Classical cadherins. *Seminars Cell Biol* 1992a; **3**: 149-155.

Kitaeva MN, Grogan L, Williams JP, *et al.*. Mutations in beta-catenin are uncommon in colorectal cancer occurring in occasional replication error-positive tumors. *Cancer Res* 1997; **57**: 4478-4481.

Klimstra DS. Pathologic prognostic factors in esophageal carcinoma. *Semin Oncol* 1994; **21**: 425-430.

Knudson AC. Hereditary cancer, oncogenes, and antioncogenes. *Cancer Res* 1985; **45**: 1437-1443.

Knudsen KA, Soler AP, Johnson KR, *et al.*. Interaction of α-actinin with the cadherin/catenin cell-cell adhesion complex via α-catenin. *J Cell Biol* 1995; **130**: 67-77.

Knudsen AK and Wheelock MJ. Plakoglobin or an 83 kD homologue distinct from beta-catenin, interacts with E-cadherin and N-cadherin. *J Cell Biol* 1992; **118**: 671-679.

Krishnamourthy R, Gong YX, Richardson M, *et al.*. A new protein inhibitor of trypsin and activated Hageman factor from pumpkin (Cucurbita maxima) seeds. *FEBS Lett* 1990; **273**: 163-167.

Kuwano H, Watanabe M, Sadanaga N, *et al.*. Squamous epithelial dysplasia associated with squamous cell carcinoma of the esophagus. *Cancer Lett* 1993; **72**: 141-147.

Larsen F, Gundersen G, Lopez H, *et al.*. CpG islands as gene markers in the human genome. *Genomics* 1992; **13**: 1095-1107.

Larson LG, Sandstorm A, Wesling P. Relationship of Plummer Vinson disease to cancer of upper alimentary tract in Sweden. *Cancer Res* 1975; **35**: 3308-3316.

Larue L, Ohsugi M, Hirchenhain J, *et al.*. E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proc Natl Acad Sci USA* 1994; **91**: 8263-8267.

Lauffenburger DA and Horwitz AF. Cell migration: a physically integrated molecular process. *Cell* 1996; **84**: 359-369.

Lee DJ, Harris A, Gillette A, *et al.*. Carcinoma of the cervical esophagus diagnosis, management, and results. *South Med J* 1984; **77**: 1365-1367.

Li C, Bapat B and Alman BA. Adenomatous polyposis coli gene mutation alters proliferation through its β-catenin-regulatory function in aggressive fibromatosis (desmoid tumour). *Am J Pathol* 1998; **153**: 709-714.

Li JY, Taylor PR, Li B, *et al.*. Nutrition intervention trials in Linxian, China: Multiple vitamin/mineral supplementation, cancer incidence and disease-specific mortality among adults with esophageal dysphasia. *J Natl Cancer Inst* 1993; **85**: 1492-1498.

Liotta LA and Stetler Stevenson WG. Tumor invasion and metastsiss: an imbalance of positive and negative regulation. *Cancer Res* 1991; **51**: 5054s-5059s.

Lochter A, Galosy S, Muschler J, *et al.*. Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J Cell Biol* 1997; **139**: 1861-1872.

Lombardo CR, Rimm DL, Koslov E, *et al.*. Human recombinant alpha-catenin binds to spectrin. *Mol Biol Cell* 1994; **5**: 47a

Lu SH. Alterations of oncogenes and tumor suppressor genes in esophageal cancer in China. *Mutat Res* 2000; **462**: 343-353.

Lu SJ, Ronai ZA, Li MH, *et al.*. *Fusarium moniliforme* metabolites: genotoxicity of culture extracts. *Carcinogen* 1988; **9**: 1523-1527.

Mandard A-M, Hainaut P and Hollstein M. Genetic steps in the development of squamous cell carcinoma of the esophagus. *Mut Res 2000;* **462**: 335-342.

Mansouri A, Spurr N, Goodfellow P, *et al.*. Characterization and chromosomal localization of the gene encoding the human cell adhesion molecule uvomorulin. *Differentiation* 1988; **38**: 67-71.

Marasas WFO, Jaskiewiez K, Venter FS, *et al.*. *Fusarioum moniliforme* contamination of maize in oesophageal cancer areas in Transkei. *S Afr Med J* 1988; **74**: 110-114.

Mareel M, Bracke M and Van Roy F. Cancer metastasis: negative regulation by an invasion-suppressor complex. *Cancer Detect Prev* 1995; **19**: 451-464.

Marger RS and Marger D. Carcinoma of the esophagus and tylosis. A lethal genetic combination. *Cancer* 1993; **72**: 17-19.

Mariner DJ, Wang J and Reynolds AB. ARVCF localizes to the nucleus and adherens junction and is mutually exclusive with p120ctn in E-cadherin complex. *J Cell Sci* 2000; **113**: 1481-1490.

McCrea PD and Gumbiner B. Purification of a 92kDa cytoplasmic protein tightly associated with the cell-cell adhesion molecule E-cadherin (uvomorulin). *J Biol Chem* 1991; **266**: 4514-4520.

McCrea PD, Turck CW and Gumbiner B. A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin. *Science* 1991; **254:**1359-1361.

Meijssen NAC, Tilanus HW and van Blankenskin M. Esophageal carcinoma and achalasia: another call for screening? *Gut* 1992; **33**: 155-159.

Melki JR, Vincent PC, Brown RD, *et al.*. Hypermethylation of E-cadherin in leukemia. *Blood* 2000; **95**: 3208-3212.

Miller JR and Moon RT. Signal transduction through β-catenin and specification of cell fate during embryogenesis. *Genes and Dev* 1996; **10**: 2527-2539.

Miyata S, Koshikawa N, Higashi S, *et al.*. Expression of trypsin in human cancer cell lines and cancer tissues and its tight binding to soluble form of alzheimer amyloid precursor protein in culture. *J Bio-chem* 1999; **125**: 1067-1076.

Miyatama S, Shimamura K, Hatta M, *et al.*. Neural cadherin: role in selective cell-cell adhesion. *Science* 1989; **245**: 631-635.

Molenaar M, van de Wetering M, Oosterwegel M, *et al.*. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* 1996; **86**: 391-399.

Morin PJ, Sparks AB, Korinek V, *et al.*. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or Apc. *Science* 1997; **275**: 1787-1790.

Munoz N and Day NE; Esophageal cancer, in Cancer Epidemiology and Prevention (ed 2). New York, NY, Oxford University Press, 1996, pp 681-706.

Munro SB and Blaschuk OW. The structure, function and regulation of cadherins. In Cell Adhesion and Invasion in Cancer Metastasis, ((Brodt P. Ed.) , pp. 17-34, R.G. Landes Company Austin). 1996

Myburg R. Immunolocalisation of Fumonisin B_1 in cancerous esophageal tissue and the cytotoxic evaluation of this metabolite on cultured cells (Master's Thesis). Durban, South Africa: University of Natal, 1998.

Nagafuchi A, Ishihara S and Tsukita S. The role of catenins in the cadherin-mediated cell adhesion: functional analysis of E-cadherin-α-catenin fusion molecules. *J Cell Biol* 1994; **127**: 235-245.

Nagafuchi A and Takeichi M. Cell binding function of E-cadherin is regulated be the cytoplasmic domain. *EMBO J* 1988; **7**: 3679-3684.

Nagafuchi A and Takeichi M. Transmembrane control of cadherin-mediated cell adhesion: a 94-kDa protein functionally associated with a specific region of the cytoplasmic region of E-cadherin. *Cell Regul* 1990; **1**: 37-44.

Nagasawa Y, Miyoshi Y, Iwao K, *et al.*. Transformation and morphological changes of murine L cells by transfection with a mutated form of β-catenin. *Cancer Res* 1999; **59**: 3539-3542.

Naitoh H, Shibata J, Kawaguchi A, *et al.*. Overexpression and localization of cyclin D1 m-RNA and antigen in esophageal cancer. *Am J Pathol* 1995; **146**: 1161-1169.

Nakanishi Y, Ochiai A, Akimoto S, *et al.*. Expression of E-cadherin, alpha-catenin, beta-catenin and plakoglobin in esophageal carcinomas and its prognostic significance: immunohistochemical analysis of 96 lesions. *Oncol* 1997; **54**: 158-165.

Näthke IS, Hinck LE and Nelson WJ. Epithelial cell adhesion and development of cell surface polarity: possible mechanisms for modulation of cadherin function, organization and distribution. *J Cell Sci* 1993; **17**: 139-145.

Natt E, Magenis RE and Zimmer J. Regional assigment of the human loci for uvomorulin (UVO) and chymotrypsinogen B (CTRB) with the help of two overlapping deletions on the long arm of chromosome 16. *Cytogenet Cell Genet* 1989; **50**: 145-148.

Nieset JE, Redfield AR, Jin F, *et al.*. Characterization of the interactions of alpha-catenin with alpha-mediating the attachment of F-actin to the membrane adhesion complex. *J Cell Sc* 1997; **110**: 1013-1022.

Ninomiya I, Endo Y, Fushida S, *et al.*. Alteration of β-catenin expression in esophageal squamous-cell carcinoma. *Int J Cancer* 2000; **85**: 757-761.

Nishihira T, Hashimoto Y, Katayama M, *et al.*. Molecular and cellular features of esophageal cancer cells. *J Cancer Res Clin Oncol* 1993; **119**: 441-449.

Noë V, Willems J, Vandekerckhove J, *et al.*. Inhibition of adhesion and induction of epithelial cell invasion by HAV-containing E-cadherin-specific peptides. *J Cell Sci* 1999; **112**: 127-135.

Nollet F, Kools P and van Roy F. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides solitary members. *J Mol Biol* 2000; **299**: 551-572.

Nollet F, Berx G and van Roy F. The role of the E-cadherin/catenin adhesion complex in the development and progression of cancer. *Mol Cell Biol Res Commun* 1999; **2**: 77-85.

Norell S, Ahblom A, Lipping H, *et al.*. Esophageal cancer and vulcanization work. *Lancet* 1983; **1**: 462-463.

Nose A and Takeichi M. A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *Cell Biol* 1986; **10**: 2649-2658.

Nose A, Tsuji K and Takeichi M. Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell* 1990; **61**: 14-155.

Novak A, Hsu S-C, Leung-Hagesteijn C, *et al.*. Cell adhesion and the integrin-linked kinase regulate the LEF-1 and β-catenin signaling pathways. *Proc Natl Acad Sci USA* 1998; **95**: 4374-4379.

Ohkubo T and Ozawa M. P120ctn binds to the membrane-proximal region of the E-cadherin cytoplasmic domain and is involved in modulation of adhesion acitivity. *J Biol Chem* 1999; **274**: 21409-21415.

Overduin M, Harvey TS, Bagby S, *et al.*. Solution structure of the epithelial cadherin domain responsible for selective cell adhesion. *Science* 1995; **267**: 386-389.

Oyama T, Kanai Y, Ochiai A, *et al.*. A truncated beta-catenin disrupts the interaction between E-cadherin and alpha-catenin: a cause of loss of intercellular adhesiveness in human cancer cell lines. *Cancer Res* 1994; **54**: 6282-6287.

Ozawa M, Baribault H and Kemler R. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independet proteins structurally related in different species. *EMBO J*1989; **8**: 1711- 1717.

Ozawa M. Identification of the region of α-catenin that plays an essential role in cadherin-mediated cell adhesion. *J Biol Chem* 1998; **273**: 29524-29529.

Ozawa M, Engel J and Kemler R. Single amino acid substitution in one Ca^{2+} binding site of uvomorulin abolish the adhesive function. *Cell* 1990a; **63**: 1033-1038.

Ozawa M and Kemler R. Molecular organization of the uvomorulin-catenin complex. *J Cell Biol* 1992; **116**: 989-996.

Ozawa M and Kemler R. Correct proteolytic cleavage is required for the cell adhesive function of uvomorulin. *J Cell Biol* 1990; **111**: 1645-1650.

Ozawa M, Ringwald M and Kemler R. Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc Natl Acad Sci USA* 1990b; **87**: 4246-4250.

Papadavid E and Katsambas A. The interactions and role of epithelial cahderin and catenins in tumorigenicity. *Int J Dermatol* 2001; **40**: 254-257.

Papkoff J, Rubinfeld B, Schryver B, *et al.*. Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. *Mol Cell Biol* 1996; **16**: 2128-2134.

Parkin M, Muir C, Whelan S, *et al.*. Cancer incidence in five continents. Vol **VI.** Lyon, France, International Agency for Research on Cancer, 1992.

Parsonnet J, Friedman GD, Fullerton F, *et al.*. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127-1131.

Peifer M and Wiechaus E. The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homologue of human plakoglobin. *Cell* 1990; **63**: 1167-1178.

Peifer M, Berg S and Reynolds AB. A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell* 1994; **76**: 789-791.

Peifer M, McCrea PD, Green KJ, *et al.*. The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the Drosophila segment polarity gene armadillo form a multigene family with similar properties. *J Cell Sci* 1992; **104**: 681-691.

Perl AK, Wilgenbus P, Dahl U, *et al.*. A causal role for E-cadherin expression by epithelial in the transition from adenoma to carcinoma. *Nature* 1998; **392**:190-193.

Pokutta S, Herrenknecht K, Kemler R, *et al.*. Conformational changes of the recombinant extracellular domain of E-cadherin upon calcium binding. *Eur J Biochem* 1994; **223**: 1019-1026.

Research Committee on Malignancy of Esophageal Cancer, Japanese Society for Esophageal Diseases. Prognostic significance of Cyclin-D1 and E-cadherin in patients with esophageal squamous cell carcinoma: multiinstitutional retrospective analysis. *J Am College of Surgeons* 2001; **192**: 708-718.

Reynolds AB, Roesel DJ, Kanner SB, *et al.*. Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cell expressing oncogenic variants of the avian cellular src gene. *Mol Cell Biol* 1989; **9**: 629-638.

Reynolds AB, Herbert L, Cleveland JL, *et al.*. P120, a novel substrate of protein tyrosine kinase receptors and of $p60^{v\text{-}src}$, is related to cadherin-binding factors β-catenin, plakoglobin and *armadillo. Oncogene* 1991; **7**: 2439-2445.

Reynolds AB, Daniel J, McCrea PD, *et al.*. Identification of a new catenin: tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Mol Cell Biol* 1994; **14**: 8333-8342.

Rheeder JP, Marasas WFO, Thiel PG, *et al.*. *Fusarium moniliforme* and fumonisins in maize in relation to human esophageal cancer in Transkei. *Phytopathol* 1992; **82**: 353-357.

Richards FM, McKee SA, Rajpar MH, *et al.*. Germline E-cadherin gene (*CDH1*) mutations predispose to familial gastric cancer and colorectal cancer. *Hum Mol Genet* 1999; **8**: 607-610.

Riethmacher D, Brinkman V and Birchmeier C. A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proc Natl Acad Sci USA* 1995; **92**: 855-859.

Rimm DL, Koslov ER, Kebriaei P, *et al.*. α(E)-catenin is an actin-binding and actin–bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc Natl Acad Sci USA* 1995; **92***:* 8813-8817.

Ringwald M, Schuh R, Vestweber D, *et al.*. The structure of the cell adhesion molecule uvomorulin. Insights into the molecular mechanisms of Ca^{2+} - dependent cell adhesion. *EMBO J* 1987; **6**: 3647-3653.

Risinger JI, Berchuck A, Kohler MF and Boyd J. Mutations of the E-cadherin gene in human gynecologic cancers. *Nature Genet.* 1994; **7**: 98-102

Roose J, Huls G, van Beest M, *et al.*. Synergy between tumour suppressor APC and the β-catenin TCF-4 target TCF-1. *Science* 1999; **285**: 1923-1926.

Rose EF. Oesophageal cancer in the Transkei 1935-1969. *J Natl Cancer Inst* 1973; **51**:7.

Rubinfeld B, Robbins R, El-Gamil M, *et al.*. Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 1997; **275**: 1790-1792.

Sammon AM. Protease inhibitors and carcinoma of the esophagus. *Cancer* 1998; **83**: 405-408.

Schuh R, Vestweber D, Riede I, *et al.*. Molecular cloning of the mouse cell adhesion molecule uvomorulin: c-DNA contains a B1-related sequence. *Proc Natl Acad Sci USA* 1986; **83**: 1364-1368.

Segal I, Reinach SG and de Beer M. Factors associated with oesophageal cancer in Soweto, South Africa. *Br J Cancer* 1988; **58**: 681-686.

Shapiro L, Fannon AM, Kwong PD, *et al.*. Structural basis of cell-cell adhesion by cadherins. *Nature* 1995; **374**: 327-337.

Shibamoto S, Hayakawa M, Takeuchi K, *et al.*. Association of p120, a tyrosine kinase substrate, with E-cadherin/catenin complexes. *J Cell Biol* 1995; **128**: 949-957.

Shimada Y, Imamura M, Shibagaki I, *et al.*. Genetic alterations in pateints with esophageal cancer with short- and long-term survival rates after curative esophagectomy. *Ann Surg* 1997; **226**: 162-168.

Shimada H, Hoshino T, Okazumi T, *et al.*. Expression of angiogenic factors predicts response to chemoradiotherapy and prognosis of oesophageal squamous cell carcinoma. *Br J Cancer* 2002; **86**: 552-557.

Shimoyama Y, Nagafuchi A, Fujita S, *et al.*. Cadherin dysfunction in a human cancer cell line – possible involvement of loss of alphal-catenin expression in reduced cell-cell adhesiveness. *Cancer Res* 1992; **52**: 5770-5774.

Shiozaki H, Oka H, Inoue M, *et al.*. E-cadherin mediated adhesion system in cancer cells. *Cancer*1996; **77**: 1605-1613.

Shiozaki H. Effect of epidermal growth factor on cadherin-mediated adhesion in human oesophageal cancer cell line. *Br J Cancer* 1995; **71**: 250-258.

Shiozaki H, Tahara H, Oka H, *et al.*. Expression of immunoreactive E-cadherin adhesion molecules in human cancer. *Am J Pathol* 1991; **193**: 17-23.

Si HX, Tsao SW, Lam KY, *et al.*. E-cadherin expression is commonly downregulated by CpG island hypermethylation in esophageal carcinoma cells. *Cancer Lett* 2001; **173**: 71-78.

Simcha I, Geiger B, Yehuba-Levenberg S, *et al.*. Suppression of tumorigenicity by plakoglobin: an augmenting effect of N-cadherin. *J Cell Biol* 1996; **133**: 199-209.

Sitas F, Terblache M and Madhoo J. Incidence and Geographical Distribution of Histologically Diagnosed Cancer in South Africa, 1990 and 1991. National Cancer Registry of South Africa, South African Institute for Medical Research, Johannesburg, 1996.

Smith PG: Late effects of x-ray treatment of ankylosing spondylitis, in Boice J, Fraumeni J (eds): Radiation Carcinigenesis. New York, NY, Lippincott-Raven, 1984, pp 107-118.

Soares P, Berx G, van Roy F, *et al.*. E-cadherin gene alterations are rare events in thyroid tumors. *Int J Cancer* 1997; **70**: 32-38.

Stappert J and Kemler R. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes Commun* 1994; **2**: 319-327.

Stoner GD and Rustgi AK. Biology of esophageal squamous cell carcinoma. *Gastrointest Cancers Biol Diagn Ther* 1995; **8**: 141-146.

Sur M and Cooper K. The role of the human papilloma virus in esophageal cancer. *Pathol* 1998; **30**: 348-354.

Sydenham EW, Thiel PG, Marasas WFO, *et al.*. Natural occurrence of some *Fusarium* mycotoxins in maize from low and high esophageal cancer prevalence areas of the Transkei, southern Africa. *J Agric Food Chem* 1990; **38**: 1900-1903.

Takayama T, Shiozaki H, Shibanoto S, *et al.*. Beta-catenin expression in human cancers. *Am J Pathol* 1996; **148**: 39-46.

Takayama T, Shiozaki H, Doki Y, *et al.*. Aberrant expression and phosphorylation of beta-catenin in human colorectal cancer. *Br J Cancer* 1998; **77**: 605-613.

Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 1991; **251**: 1451-1455.

Takeichi M. Cadherins in cancer: implications for invasion and metastasis. *Curr Opin Cell Biol* 1993; **5**: 806-811.

Takeichi M. Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol* 1995; **7:** 619-627.

Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion. *Annu Rev Biochem* 1990; **59**: 237-252.

Tamura S, Shiozaki M, Miyata M, *et al.*. Decreased E-cadherin expression is associated with haematogenous recurrence and poor prognosis in patients with squamous cell carcinoma of the oesophagus. *Br J Surg* 1996; **83**: 1608-1614.

Tanihara H, Sano K, Heimark RL, *et al.*. Cloning of five human cadherins clarifies characteristic features of cadherin extracellular domain and provides further evidence for two structurally different types of cadherin. *Cell Adhes Commun* 1994; **2**:15-26.

Terres AM, Parajes JM, O'Toole D, *et al.*. *H pylori* infection is associated with downregulation of E-cadherin, a molecule involved in epithelial cell adhesion and proliferation control. *J Clin Pathol* 1998; **51**: 410-412.

Tollefson L. The use of epidemiology, scientific data, and regulatory authority to determine risk factors in cancer of some organs of the digestive system. 2. Esophageal cancer. *Regul Toxicol Pharmacol* 1985; **5**: 255-275.

Troyanovsky RB, Chitaev NA and Troyanovsky SM. Cadherin binding sites of plakoglobin: localization, specificity and role in targeting to adhering junctions. *J Cell Sci* 1996; **109**: 3069-3078.

Tsai MY, Holzknecht RA and Tuchman A. Single-strand conformational polymorphism and direct sequencing applied to carrier testing in families with ornithine transcarbamylase deficiency. *Hum Genet* 1993, **91**: 321-325.

Twigg SR and Wilkie AOM. Characterization of the human snail (SNAIL) gene and exclusion as a major disease gene in craniosynostosis. *Hum Genet* 1999; **105**: 320-326.

Valizadeh A, Karayiannakis AJ, Elhariry I, *et al.*. Expression of E-cadherin-associated molecules (α-, β-, γ-catenin and $p120^{ctn}$) in colorectal polyps. *Am J Pathol* 1997; **150**: 1977-1984.

Van Hengel J, Vanhoenacker P, Staes K, *et al.*. Nuclear localisation of the p120ctn armadillo-like catenin in counteracted by a nuclear export signal and by E-cadherin expression. *Proc Natl Acad Sci USA*1999; **96**: 7980-7985.

Van Rensburg SJ. Epidemiologic and dietary evidence for specific nutritional predisposition to esophageal cancer. *J Natl Cancer Inst* 1981; **67**: 45.

Vermeulen SJ, Bruyneel EA, Bracke ME, *et al.*. Transition from the non-invasive to the invasive phenotype and loss of α-catenin in human colon cancer cells. *Cancer Res* 1995; **55**: 4722-4728.

Vermeulen SJ, Nollet F, Teugels E, *et al.*. The alpha-catenin gene (*CTNNA1*) acts as an invasion-suppressor gene in human colon cancer cells. *Oncog* 1999; **18**: 905-15.

Vleminckx KL. Enlarged cell-associated proteoglycans abolish E-cadherin functionality in invasive tumour cells. *Cancer Res* 1994; **54**: 873-877.

Vleminckx K, Vakaet LJr, Mareel M, *et al.*. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* 1991; **66**: 107-119.

Weiss N. Cancer in relation to occupation exposure to pershloroethylene. *Cancer Causes Control* 1995; **6**: 257-266.

Weiss EE, Kroemkar M, Rudiger A-H, *et al.*. Vinculin is part of the cadherin-catenin junctional complex; complex formation between α-catenin and vinculin. *J Cell Biol* 1998; **141**: 755-764.

Wijnhoven BP, de Botha NJ, van Dekken H, *et al.*. E-cadherin gene mutations are rare in adenocarcinomas of the esophagus. *Br J Cancer* 1999; **80**: 1652-1657.

Wijnhoven BPL, Dinjens WNM and Pignatelli M. E-cadherin-catenin cell-cell adhesion complex and human cancer. *Br J Surg* 2000; **87**: 992-1005.

World Health Organization. *The World Health Report*, WHO. 1997.

Wu Q and Maniatis T. A striking organization of a large family of human neural cadherin-like cell adhesion genes. *Cell* 1999; **97**: 779-790.

Xu Z, Wang M-R, Xu X, *et al.*. Novel human esophagus-specific gene C1orf10: c-DNA cloning, gene structure and frequent, loss of expression in esophageal cancer. *Genomics* 2000; **69**: 322-330.

Yang CS and Wang ZY: Tea and cancer. *J Natl Cancer Inst* 1993; **85**: 1038-1049.

Yap AS, Niessen CN and Gumbiner BM. The juxtamembrane region of the cadherin cytoplasmic tail support lateral clustering adhesive strengthening and interaction with p120ctn. *J Cell Biol* 1998; **141**: 779-789.

Yap AS, Brieher WM and Gumbiner BM. Molecular and functional analysis of cadherin-based adherens junctions. *Annu Rev Cell Dev Biol* 1997; **13**: 119-146.

Yoshiura K, Kanai Y, Ochiai A, *et al.*. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc Natl Acad Sci USA* 1995; **92**: 7416-7419.

Yoshizawa T, Yamashita A and Luo Y. Fumonisin occurrence in corn from high- and low-risk areas for human esophageal cancer in China. *Appl Environ Microbiol* 1994; **60**: 1626-1629.

Yu Y and Taylor PR. Retrospective cohort study of risk-factors for esophageal cancer in Linxian, People's Republic of China. *Cancer Causes Control* 1995; **6**: 311-320.

Zur Hausen H and de Villiers EM. Human papilloma viruses. *Annu Rev Microbiol* 1994; **48**: 427-447.

APPENDIX A

CONSENT FOR DNA ANALYSIS AND STORAGE

I, _____

request that an attempt be made to assess the probability that I have a mutated gene that may be the possible cause of my esophageal squamous cell carcinoma. I understand that the DNA analysis will be used for academic research study of the E-cadherin gene. I also understand that the DNA will be obtained from blood and tumour.

I also understand that:

1. The research is specific to the genetic study mentioned above and cannot be determine any other research.
2. I understand the steps that will be obtained for the extraction of my blood and tumour.
3. I may withdraw my consent at any time without giving a reason and without this affecting my future medical care.
4. The DNA bank is under an obligation to respect medical confidentiality.

A portion of the DNA obtained may be stored to enable later testing. The following conditions apply to my DNA that is being stored: (circle any and all that you wish).

a. To be used without any restrictions.
b. To be used for research this particular research and no other studies.
c. To be used for any studies without my knowledge at the discretion of the Head of Haematology and Molecular Biology, UFS and that the information form such research will remain confidential.
d. Other, _____.

I understand that I may change my mind and withdraw my consent for DNA banking at any time.

ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND ALL MY QUESTIONS WERE ANSWERED BY:

_____

_____

Patients signature

_____ _____

Witnessed consent Date: