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"Identification of Signalling Pathways influenced by E-cadherin"

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Abstract

E-cadherin is a calcium-dependent glycoprotein that mediates cell-cell adhesion and is important in differentiation, cell growth and maintenance of cell polarity.

The involvement of E-cadherin in tumour development has been extensively demonstrated, with many human carcinomas exhibiting reduced E-cadherin expression. In gastric cancer, the protein is abnormally expressed in more than half of the cases of the sporadic diffuse subset. Furthermore, germline loss-of-function mutations in the CDH1 gene were shown to represent the genetic cause of approximately 1/3 of HDGC cases.

During tumourigenesis, loss of E-cadherin expression and/or function can lead to increased cell motility, cell-cell detachment and, ultimately, to invasion. Moreover, recent evidences point for the possible involvement of the protein in modulating intracellular signalling and thus have a contribution during initial stages of tumour development.

To investigate the hypothesis that E-cadherin modulates intracellular signalling and to understand to what extent two germline mutations localized in different domains of E-cadherin and identified in HDGC cases maintain normal function, we transduced Wild-type, extracellular-mutated and intracellular-mutated forms of E-cadherin to a cell that does not express the protein. We analyzed expression and putative activity changes to key proteins of four signalling pathways implicated in cell survival, proliferation, cell-matrix adhesion and invasion.

We demonstrate that expression of Wild-type E-cadherin inhibits PI3K and RTKs signalling, a capacity most likely dependent on the extracellular domain. We show that MMPs activity and FAK-dependent intracellular signalling is decreased with the expression of normal E-cadherin. Finally, and despite similar outcomes in terms of invasion, motility and overall malignancy, missense mutations localized in different domains of E-cadherin render opposite effects in intracellular signalling. Results obtained for the extracellular mutation were comparable to the lack of protein situation. In contrast, cells with the intracellular mutation behaved more similarly to Wild-type cells.

Taken together, our results show that E-cadherin modulates intracellular signalling. These novel properties may contribute to tumour development by influencing cell

proliferation and survival, cell adhesion, motility and invasion. Although these are preliminary findings, the possibility that causes for malignancy associated with mutations localized at the extracellular domain of E-cadherin may have been identified is very promising, in particular for gastric neoplasias treatment.

Resumo

A caderina-E é uma glicoproteína calcio-dependente que medeia a adesão célula-célula, e é fundamental na manutenção da polaridade celular, no crescimento celular e diferenciação.

O envolvimento da caderina-E no desenvolvimento tumoral foi amplamente demonstrado, com vários carcinomas humanos a exibir redução da sua expressão proteica. Em carcinomas gástricos, a expressão de caderina-E é diminuída ou mesmo perdida em cerca de metade dos casos do tipo difuso. Mutações germinativas no gene CDH1, que causam perda de função da proteína, foram identificadas como a causa genética em um terço dos casos do síndrome HDGC.

Durante a progressão tumoral, a perda da expressão ou função da caderina-E pode provocar o aumento da motilidade celular, a diminuição da adesão célula-célula, e como culminar em invasão tecidular. Recentes linhas de investigação sugerem um ainda maior envolvimento da caderina-E no processo tumorigénico, graças à uma hipotética modulação das vias de sinalização intracelular.

Para investigar a possível capacidade da caderina-E modular vias de sinalização intracelular e para compreender se essa capacidade é alterada ou perdida pela presença de mutações do tipo substituição em diferentes domínios da proteína, infectamos uma linha celular humana que não expressa caderina-E com diferentes formas da proteína – a forma nativa, uma forma com uma mutação na região extracelular e uma forma com uma mutação no domínio intracelular. Os putativos efeitos foram avaliados pela análise da expressão de proteínas, e suas formas activas, centrais na sinalização de quatro vias envolvidas na proliferação e sobrevida celular, na adesão célula-matriz e na invasão.

Nós demonstramos que a expressão de caderina-E nativa inibe a sinalização de PI3K e dos RTKs, muito provavelmente com o contributo do domínio extracelular. Os nossos resultados revelam que a actividade proteolíticas das MMPs e a sinalização intracelular dependente das FAK diminuiu na presença da forma normal da caderina-E.

Quanto às mutações, apesar de provocarem os mesmos efeitos em termos de invasão, motilidade e malignidade, no nosso estudo produziram desfechos opostos. Os resultados obtidos para a mutação extracelular assemelharam-se aos obtidos nas

células sem caderina-E, enquanto as observações retiradas das células com a mutação intracelular se aproximaram mais das obtidas nas células com a forma nativa.

Como conclusão, os nossos resultados demonstram que a caderina-E tem um papel mais alargado na célula, nomeadamente na modulação de vias de sinalização. Tal capacidade pode contribuir ainda mais para o desenvolvimento tumoral, através de efeitos na proliferação, sobrevida, adesão e motilidade celular bem como invasão tecidular. Apesar de estes serem dados muito preliminares, revela-se promissora a possibilidade de estarmos perante a identificação das causas de malignidade associadas às formas mutadas na região extracelular de caderina-E. A confirmar-se tal hipótese, seria de particular importância para o tratamento de neoplasias gástricas.

Abbreviations

AKT	Protein Kinase B
BAD	BCL-2 Antagonist of Cell Death
BSA	Bovine Serum Albumine
CAMs	Calcium-dependent Cell Adhesion Molecules
CDH1	E-cadherin Gene
c-Met	Hepatocyte Growth Factor Receptor
CO_2	Carbone Dioxide
DAPI	4'-6-Diamidino-2-Phenylindole
E-cadherin	Human Epithelial Cadherin
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
ErbB2	V-ERB2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
HDGC	Hereditary Diffuse Gastric Cancer
IP	Immunoprecipitation
kDA	Kilo-Dalton
MMPs	Matrix Metalloproteinases
pAKT	Phosphorylated form of AKT
PBS	Phosphate Buffered Saline
pc-Met	Phosphorylated form of c-Met
PDK1	Phosphatidylinositol-Dependent Protein Kinase
pEGFR	Phosphorylated form of EGFR
pErbB2	Phosphorylated form of ErbB2
pFAK	Phosphorylated form of FAK

PI3K	Phosphoinositide 3'-kinase
pPI3K	Phosphorylated form of PI3K
PY-20	Phosphotyrosine
pβ1-integrin	Phosphorylated form of β1-integrin
Rpm	Rotations per minute
RTKs	Receptor Tyrosine Kinases
SDS-PAGE	SDS-Protein Acrylamide Gel Electrophoresis
TSGs	Tumour-Suppressor Genes

Introduction

Cancer

Cancer is a genetic disease [1]. It is a disease of growth, with accumulation of cells, of differentiation, with changes in cell function, and of tissue organization [2]. Causes of cancer are many and include genetic predisposition, environmental influences, infectious agents and ageing [3].

Carcinogenesis in humans is a multistep process. Each step reflects the acquisition of new genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives [4].

With few exceptions, cancers are derived from single mutated somatic cells and their progeny. Cells in the emerging neoplastic clone accumulate a series of genetic or epigenetic events that lead to changes in gene activity, and so to altered phenotypes which are subject to selection. Ultimately, a cell population evolves and may be able to avoid the normal controls of proliferation and territory, invading adjacent tissues [5].

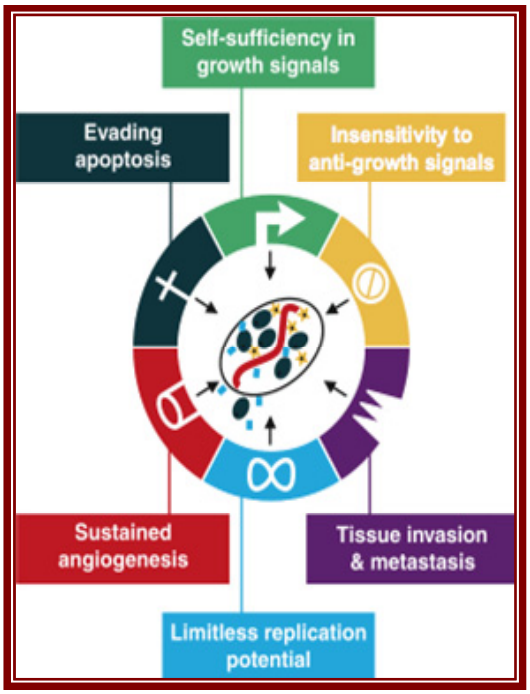

Figure 1| Six essencial alterations in cell that collectively dictate malignant growth. (Hanahan and Weinberg [2])

According to Hanahan and Weinberg [2], cancer cells present six hallmark features that collectively dictate malignant growth (Figure1): self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. Each of these physiologic changes acquired during tumour development, represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues. Hanahan and Weinberg [2] proposed that these six capabilities are shared in common by most and perhaps all types of human tumours.

Although environmental and other non-genetic factors have a role in many stages of carcinogenesis, it is widely accepted that cancer arises because of mutations in cancer-susceptibility genes [4]. Susceptibility genes belong to one of three classes: gatekeepers, caretakers and landscapers [1,6].

Gatekeepers directly regulate growth and differentiation pathways of the cell and comprise oncogenes and tumour-suppressor genes (TSGs). Oncogenes are altered forms of normal cellular genes, called proto-oncogenes [7]. These are growth promoting genes which stimulate in general, when expressed, cell proliferation. Oncogenes can contribute to tumourigenesis if one allele is mutated or inappropriately expressed. TSGs regulate cell proliferation in a negative way. In this case, cancer-causing mutations are generally recessive at the cellular level, and both alleles have to be mutated to cause a phenotypic change. The equilibrium between these two types of genes allows the homeostasis of cells [4].

Caretakers promote tumourigenesis indirectly [8,9]. They function to maintain the genomic integrity of the cell. Mutations on caretakers can lead to genetic instability by increasing the DNA mutation rate and favouring mutations in other genes, including gatekeepers.

Landscapers regulate the cell's interaction with the surrounding microenvironment. Landscaper defects do not directly affect cellular growth, but generate an abnormal stromal environment that contributes to the neoplastic transformation of cells [4].

Gastric Cancer

Gastric cancer is a multifactorial disease. The marked geographic variation, time trends, and the migratory effect on gastric cancer incidence suggest that environmental and/or lifestyle factors are major contributors to the etiology of this disease [10].

It is the fourth most common malignancy worldwide, and ranks second in cancer-related deaths [11].

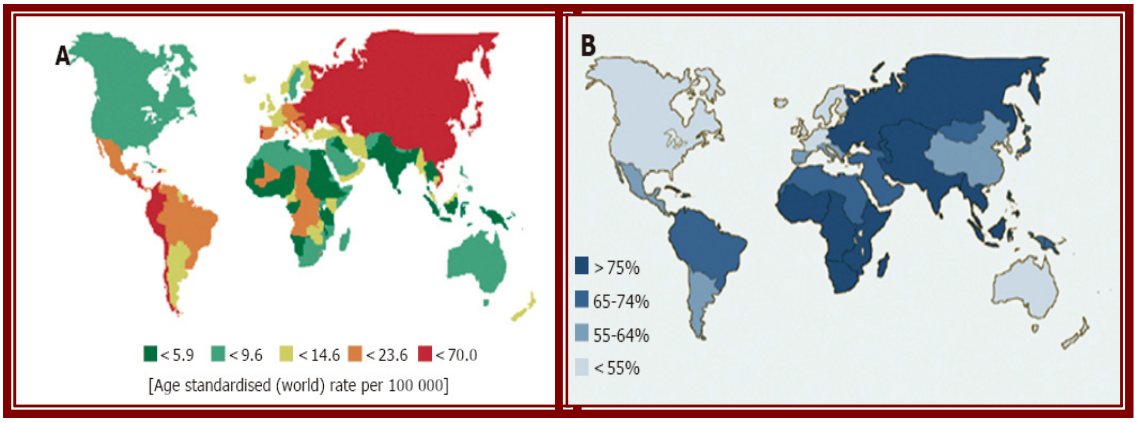

Figure 2| (A) Incidence of stomach cancer in males. **(B)** Prevalence of *H. pylori* infection in asymptomatic adults. (Parkin [16])

There is substantial variation in gastric cancer incidence around the world. Nearly two-thirds of stomach cancers occur in developing countries [12]. Japan and Korea have the highest gastric cancer rates in the world [13,14]. Other high incidence areas include Eastern Europe and parts of Latin America [15], while low incidence rates are found in South Asia, North and East Africa, North America, Australia, and New Zealand (Figure 2A). In Portugal, gastric cancer is the second most incident cancer and the major cause of cancer mortality [15].

One of the most significant advances in the fight against gastric cancer came with the recognition of *Helicobacter pylori* (*H. pylori*) as class I carcinogen by the World Health Organization in 1994 [17]. *H. pylori* is a gram-negative bacillus that colonizes the stomach and may be the most common *chronic* bacterial infection worldwide [18]. Countries with high gastric cancer rates typically have a high prevalence of *H. pylori* infection, and the decline in *H. pylori* prevalence in developed countries parallels the decreasing incidence of gastric cancer [19,20] (Figure 2B).

Despite the worldwide decline in incidence and the major improvements in diagnosis and treatment over the past decades, still less than 20% of patients survive to 5 years, underlining the need for improved early diagnosis of gastric cancer [21].

Pathology of Gastric Cancer

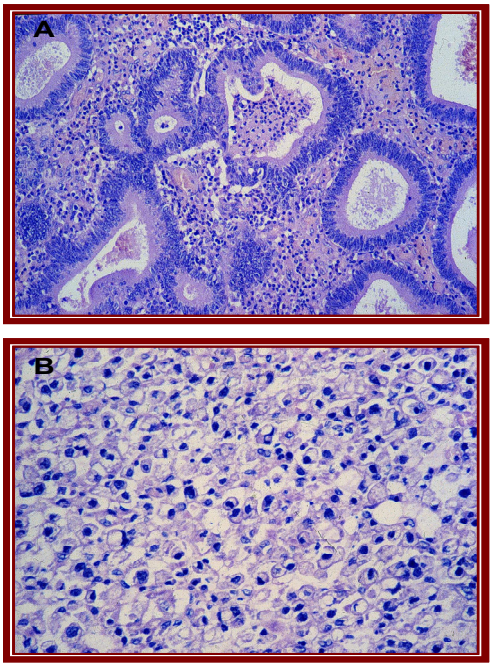

Figure 3| Two major types of gastric carcinomas. A) Intestinal type or glandular carcinoma; B) Diffuse type or isolated-cell type carcinoma.

Over 95% of malignancies of the stomach are adenocarcinomas. Lymphoma, sarcomas and carcinoid tumours constitute the remaining less common neoplasms. Adenocarcinoma of the stomach comprises a spectrum of different conditions classified according to the site of tumour origin and the pathological appearance of the lesion [21].

Morphologically, there are two major types of gastric carcinomas: intestinal type (Lauren, 1965) [22] or glandular carcinoma (Carneiro, 1997) [23]; and the diffuse type (Lauren, 1965) [22] or isolated-cell type carcinoma (Carneiro, 1997) [23]. They differ in their epidemiology, pathogenesis, genetic profile, and clinical outcome [11].

A third category, mixed type gastric carcinoma is used for tumours presenting both major histological types.

The intestinal or glandular type is comprised of malignant cells that are united to form structures resembling functional glands of the gastrointestinal tract. The diffuse or isolated-cell type carcinoma is characterized by isolated cells dispersed in the stroma, no longer capable of gastric function [21].

Intestinal carcinomas occur more commonly in the distal stomach and tend to affect elder patients, mainly men, in geographic areas with high risk of gastric cancer. Diffuse carcinomas have a more uniform geographic distribution, affect younger patients, mainly women, develop throughout the stomach and are associated with worse prognosis [22,23,24].

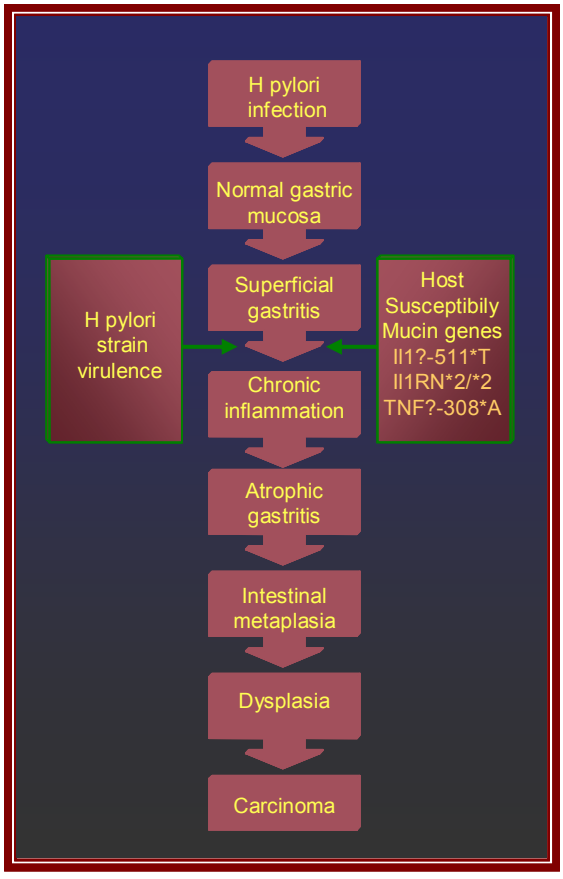

Figure 4| Correa pathological events in gastric adenocarcinoma. (Fox and Wang [25])

The pathogenesis and genetic alterations leading to the two major types of adenocarcinoma are generally considered different. Intestinal tumours follow a progressive evolution through a number of sequential steps, usually initiated by *H.pylori* infection. These steps begin with gastritis which progresses to mucosal atrophy (atrophic gastritis) followed by intestinal metaplasia, dysplasia and carcinoma with subsequent metastatic dissemination [24].

A wide range of genetic and epigenetic abnormalities including point mutation, loss of heterozygosity, microsatellite instability, and hypermethylation are described in intestinal type gastric cancer and its precursor lesions.

By contrast, mutations or epigenetic silencing of the E-cadherin gene appear to be the key carcinogenic event in diffuse gastric cancer development [11]. Futhermore, other than the obvious chronic gastritis that is the hallmark of *H. pylori* pathogenesis, no preceding lesions have been identified [21]. However, in 2004 Carneiro *et al.* [26] proposed a model for the development of diffuse gastric cancer for E-cadherin mutation carriers. An initial in situ signet ring carcinoma with pagetoid spread of signet ring cells

progresses to an invasive carcinoma that may occur without a morphologically detectable in situ carcinoma.

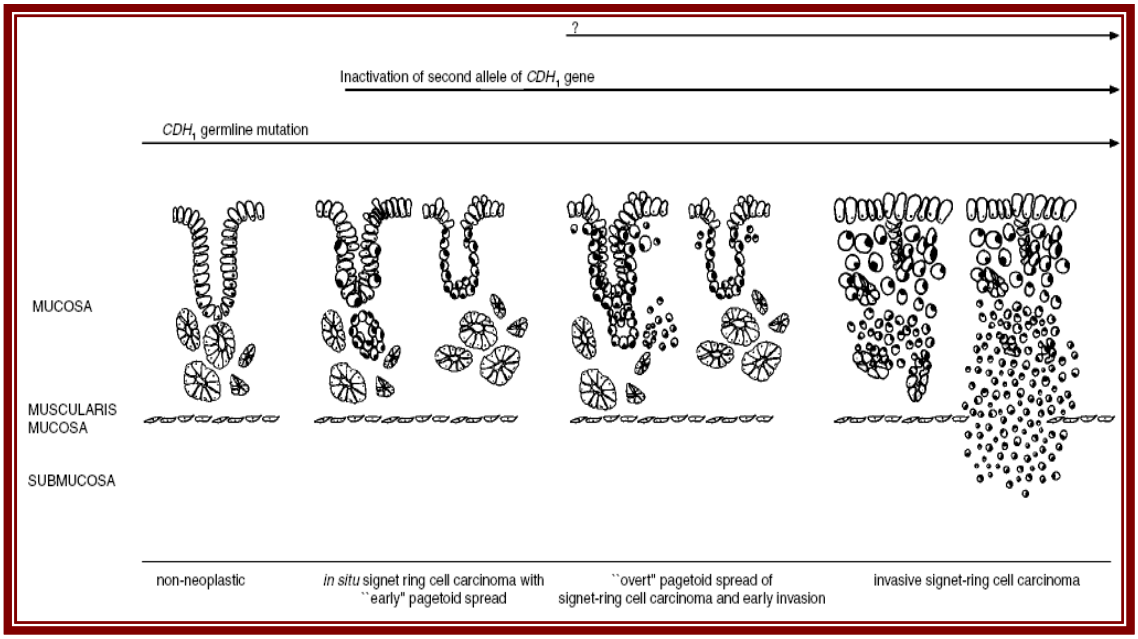

Figure 5| Proposed model for the early development of diffuse gastric cancer in E-cadherin mutation carriers. (Carneiro *et al* [26])

Hereditary Diffuse Gastric Cancer (HDGC)

Hereditary Diffuse Gastric Cancer (HDGC) is an autosomal dominant cancer syndrome. Tumours arising from this syndrome are predominantly histologically diffuse and poorly differentiated. The molecular basis for HDGC was determined by Guilford *et al.* [27] when they found germline truncating mutations in the E-cadherin gene (*CDH1*) in three Maori kindred with early-onset diffuse gastric cancer. Mutations of this gene have since then been reported in several other studies that include HDGC families of European, Japanese, Korean and African American origin. Over 30 mutations of the *CDH1* gene have now been reported in HDGC families, most of which have been identified in families of European origin [28,29]. It has been estimated that mutations of the E-cadherin gene are causal in at least 30% of HDGC cases, and the penetrance of these mutations is in the range 75-85% [29].

Although relatively rare, HDGC families are of extreme importance in the characterization of diffuse gastric cancer, currently not well understood and with poor prognosis.

E-Cadherin

E-cadherin is a cell-cell adhesion molecule with a very important role in epithelial tissues. It is localized on the basolateral surfaces of epithelial cells in regions of cell contact known as adherens junctions [30]. It is essential for multicellular organisms [31,32] and participate in multiple processes, including development, tissue integrity, cell migration, morphology, and polarity [33]. It is also essential for the formation and maintenance of epithelia [34].

As members of a large family of genes coding for calcium-dependent cell adhesion molecules (CAMs) [35,36], the cadherin glycoproteins are expressed by a variety of tissues. Classical cadherins E- and N-cadherins being the best characterized – play important roles in the formation of tissues during gastrulation, neurulation and organogenesis [37].

Expression of E-cadherin in embryonic development occurs very early, at the two-cell stage [38,39]. Epithelial differentiation and polarization occur early in ontogeny in the morula stage, when the embryo compacts and each cell polarizes along its apicobasal axis to generate an epithelial-like phenotype [40]. E-cadherin plays an important role in the adhesion of the blastomeres, and early embryo's ability to compact. It is expressed in the membrane even before compaction of the morula occurs, distributed in a non-polar manner, and does not exhibit adhesive function [41,42].

E-Cadherin Protein

CDH1 encodes a 120kDa glycoprotein with a single transmembrane domain protein whose N-terminus is extracellular and C-terminus is intracellular [43,32]. The C-terminal intracellular domain of ~150 residues is highly conserved in sequence and establishes the interaction with the cytoskeleton. The juxtamembrane region of the cadherin intracellular domain has been identified as a functionally active region supporting cadherin clustering and adhesive strength [44]. The structure of the extracellular domain of classical E-cadherin contains five tandem repeats of a 100-residue amino-acid-motif that include the sites with adhesive activity.

Calcium is required for E-cadherin to mediate its adhesive function, and the extracellular portion of E-cadherin contains several calcium-binding sites [45]. E-cadherin molecules form homodimers on the cell surface (lateral dimerization) and carry out their adhesive function by binding, in an antiparallel fashion, to E-cadherin molecules on adjacent cells (homotypic adhesion), forming points of adhesion (zipper-like structures), which may progress to extensive multimers of E-cadherin [45,46].

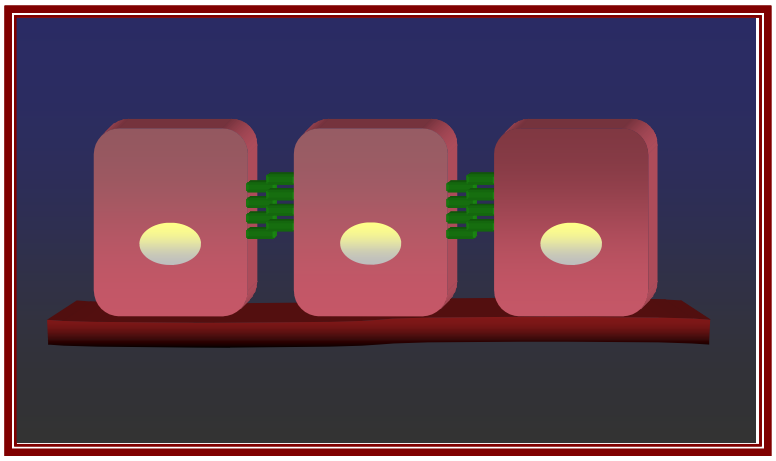

Figure 6| E-cadherin lateral dimerization and homotypic adhesion.

E-cadherin is connected indirectly to the cytoskeleton, via non-covalent linkage of its intracellular C-terminal portion to β-catenin, which in turn is non-covalently linked to α-catenin, which binds to the actin cytoskeleton [47]. The cytoskeletal link is required for maximum homotypic activity of E-cadherin. Additionally, the juxtamembrane region of the intracellular portion binds p120-catenin, which may further contribute to adhesion [48,49].

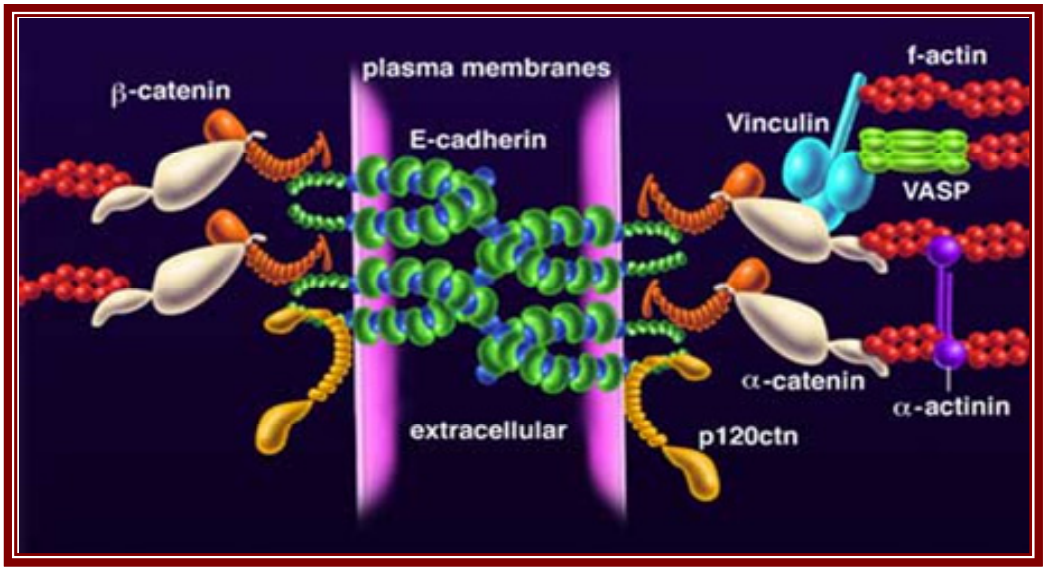

Figure 7| Intercellular adhesion, signalling and the cytoskeleton. (Jamora and Fuchs [50])

In this process, E-cadherin is also a ligand for two integrins, αEβ7 and a α2β1 (heterotypic adhesion). The first interaction might serve to retain intraepithelial lymphocytes in mucosal tissue, while the second may contribute to the organization of epithelial multilayers [37].

E-Cadherin's Role in Malignant Cells

Besides its role in normal cells, this highly conserved protein can play a major role in malignant cell transformation, and especially in tumour development and progression.
Loss of cellular adhesion and increased motility are crucial mechanisms responsible for tumour initiation and progression [37].
Downregulation or a complete shutdown of E-cadherin expression, mutation of the E-cadherin gene, or other molecular mechanism that interfere with the integrity of the adherens junctions, have been observed in carcinoma cells [38]. Currently, the suppression of E-cadherin expression is regarded as one of the main molecular events responsible for dysfunction in cell-cell adhesion and correlates with the loss of the epithelial morphology and acquisition of metastic potential by carcinoma cells.

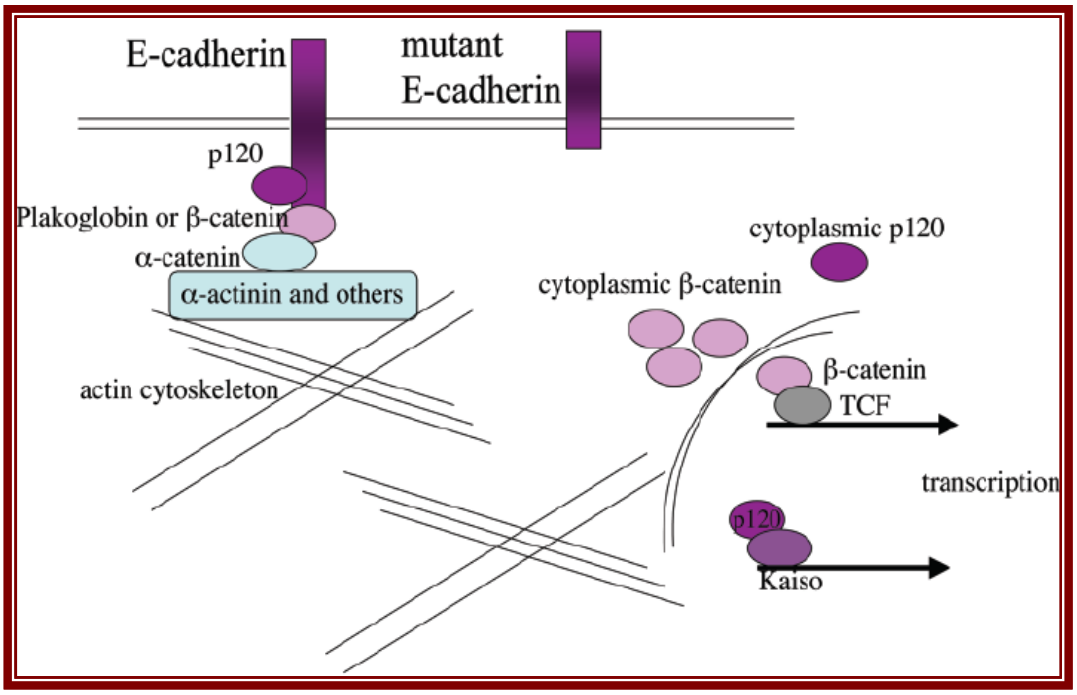

Figure 8| E-cadherin mediated cell adhesion and consequences of E-cadherin loss. (Andl and Rustgi [55])

Most tumours have abnormal cellular architecture, and loss of tissue integrity can lead to local invasion. Loss of function of E-cadherin invasion suppressor protein correlates with increased invasiveness and metastasis of tumours [51], being therefore referred as a tumour suppressor gene [52].

E-cadherin's adhesive functions depend upon interactions with regulatory proteins of the catenin family, namely β-catenin and plakoglobin, which establish a link to the cytoskeleton [36,53]. Mutations and deletions of the intracellular tail of E-cadherin, which contains the β-catenin binding site, result in abolishment of the cadherin/catenin complex formation, leading to the loss of cellular adhesive properties [54].

Another way of regulating E-cadherin mediated cell adhesion is through mutations in the region of E-cadherin binding p120-catenin. This leads to the uncoupling of the E-cadherin–p120 complex, which disrupts cell adhesion [48]. However, the interaction of E-cadherin with β-catenin and plakoglobin remains unaffected in this context. Loss of E-cadherin is not limited to effects upon assembly of adherens junctions. E-cadherin's role in sequestering β-catenin and p120 also fosters transcriptional activity [56]. A shift from membrane-bound catenins to an enlarged cytoplasmic pool is observed after E-cadherin loss, and translocation of β-catenin into the nucleus permits direct or indirect regulation of transcriptional activity. Unbound p120-catenin binds Kaiso, a zinc finger transcription factor [57] which acts as a DNA-methylation dependent repressor [58] while β-catenin participates in the Wnt signalling pathway [59,60].

E-Cadherin in Gastric Cancer

In gastric cancer, E-cadherin expression is frequently lost due to mutation or deletion of the E-cadherin gene *CDH1* [34]. In many sporadic cancers, E-cadherin is also commonly downregulated by hypermethylation of the promoter of *CDH1* [61]. Detection of promoter hypermethylation as a mechanism of *CDH1* gene silencing resulted in the development of a two hit model for E-cadherin silencing in gastric carcinomas: the inactivation of the first *CDH1* allele occurs through mutation or deletion, while the second allele is silenced by promoter methylation [62,63].

In intestinal and diffuse type gastric carcinomas, E-cadherin inactivation does not occur in the same way. It appears that the molecular basis underling the morphological and behaviour differences between intestinal and diffuse gastric cancer could be attributed at least in part to differences in E-cadherin function [28].

Blok *et al*. [34] found that downregulation of E-cadherin is more frequent in diffuse type than intestinal type early gastric carcinoma [34]. Moreover, tumours with *CDH1* promoter hypermethylation are predominantly of the diffuse histotype [64].

An additional mechanism of E-cadherin repression in gastric tumours might be mediated by overexpression of the transcription factors SIP1 or Snail [34]. Becker *et al*. [65] reported E-cadherin inactivation in gastric cancer induced by exon skipping. Biological function of E-cadherin can also be attenuated post-translationally [65].

CDH1 Mutational Status

Mutations in the *CDH1* gene have been described in a number of human cancers including breast, stomach, endometrium, ovary and thyroid [37].

Germline mutations in *CDH1*, accompanied by somatic hypermethylation, mutation or allelic loss cause 30% of HDGC. *CDH1* somatic mutations accompanied by somatic hypermethylation, mutation or allelic loss cause 50-60% of sporadic diffuse and mixed types of gastric cancer, as well as invasive lobular breast cancers, but are not observed in sporadic intestinal gastric cancer [28].

The majority of *CDH1* mutations found in sporadic diffuse gastric carcinomas are missense or in-frame deletions. In contrast to gastric tissues, mutations found in infiltrating lobular breast cancers are out-of-frame, causing premature stop codons. In both models, somatic mutations cluster in exons 7 to 9, corresponding to the extracellular domain of the protein [28].

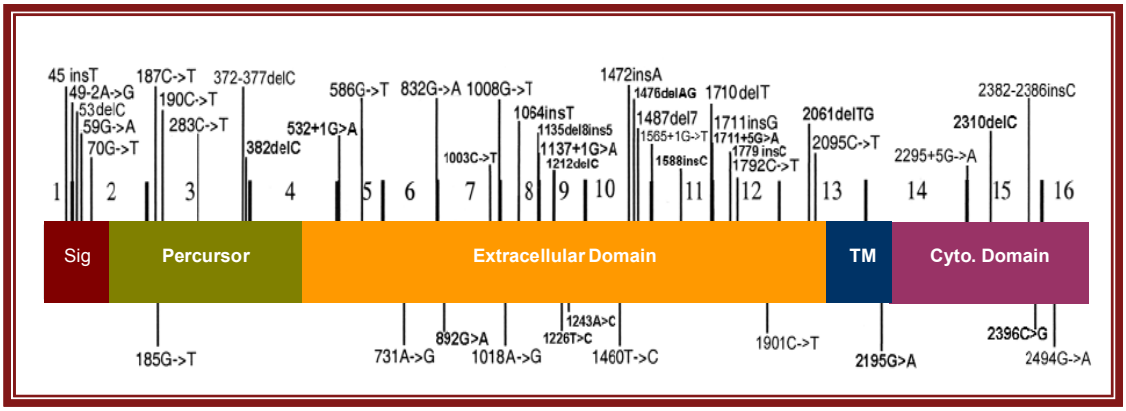

Figure 9| Scheme of *CDH1* gene with germline mutations found in HDGC. Missense mutations are shown below the and truncating mutations above. (Oliveira and Suriano [66])

CDH1 germline truncating and missense mutations resulting in E-cadherin inactivation and/or segregating with the disease have been identified in HDGC. Unlike in sporadic cases, these mutations cover the entire coding region of *CDH1*, without a preferential hot-spot, and 33% are of the missense type.

E-cadherin and Cellular Signalling

The mechanisms of cellular signalling and adhesion are thought to be closely connected, where components have double (or more) functions and interconnect in a signalling structural network [67].

E-cadherin was initially considered as a structural protein [68] but there is evidence that cadherins are not only targets for signalling pathways that regulate adhesion, but may themselves send signals that regulate basic cellular processes, such as migration, proliferation, apoptosis and cell differentiation [69,70,71].

E-cadherins do not exhibit any enzymatic activity; therefore their ability to function as signal transducing receptors depends on their physical interactions with other signal transduction effectors. It was shown that phosphoinositide 3'-kinase (PI3K) was recruited to cell-cell contacts activated by E-cadherin [72] and that E-cadherin interacted with receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) [55].

Clinical intervention to obtain tumour regression is often achieved through inhibition of a specific(s) molecule(s) overexpressed/activated in cancer by a synthetic compound. Efforts are being made to apply this approach on diffuse gastric cancer, characterized by poor prognosis and 5 year survival. Identification of novel E-cadherin functions and possible targets/partners and of pre-neoplasic markers could be decisive in overcoming this invasive carcinoma of the stomach.

Epithelial-Mesenchymal Transition (EMT)

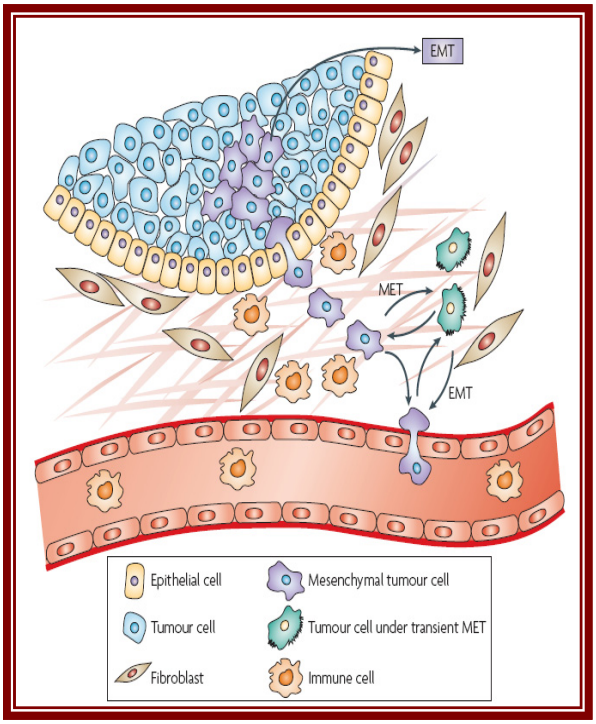

Figure 10| Epithelial Mesenchymal transition process. (Peinado *et al* [75])

Metastasis formation, which starts with dissemination of tumour cells from the primary tumour, is a complex process and is the primary cause of death in cancer patients. It involves the coordination of several signal-transduction pathways that allow cancer cells to proliferate, remodel their surrounding environment, invade and migrate through new tissues, and differentiate [73]. Tumour cells detach from the primary tumour, invade surrounding tissue and basement membranes, intravasate into the lymphatic or blood circulation, and, finally, adhere and extravasate into distant organs (Figure10). Degradation and remodeling of the extracellular matrix (ECM) and basement membranes by proteolytic enzymes is an essential step in this process [74].

Malignant carcinoma cells are characterized in general by poor intercellular adhesion, loss of the differentiated epithelial morphology and increased cellular motility [37]. Such epithelial-mesenchymal conversion is observed in malignant tumours of epithelial origin. This process is similar to developmental events but with the important difference that it is uncontrolled.

The phenotypic change is initially mediated by alterations in the expression of cell-surface molecules known as integrins, release of proteases that remodel the ECM and the deposition of new ECM molecules. These activate signalling cascades that regulate gene expression, cytoskeletal organization, cell adhesion and cell survival. As a result, cancer cells become more invasive, migratory and better able to survive in different microenvironments. Cellular invasion and migration are governed at both the extracellular and intracellular levels by several factors, and depend on the cell's carefully balanced dynamic interaction with the ECM.

Cell adhesion complexes play a major role in the invasive process. It is well known that there is a frequent loss and/or a redistribution of cell adhesion molecules such as the

E-cadherin/catenins in numerous carcinomas [44]. These modifications have two major consequences: a loss of cohesion between tumour cells facilitating their dissemination and migration, and a possible localisation of β-catenin in the nucleus playing a role as a co-factor of transcription regulating various genes implicated in tumour progression (c-myc, cyclin D1, CD44, fibronectin, urokinase-type plasminogen activator, MMP-7, vimentin) [44]. *In vitro* there is a direct correlation between lack of E-cadherin production and loss of epithelial phenotype. The acquisition of the mesenchymal phenotype is associated with invasive behaviour. Therefore, E-cadherin is considered as one of the caretakers of the epithelial phenotype [76].

E-cadherin and Matrix Metalloproteinases (MMPs)

Tumour invasion is greatly dependent on the permissive action of the microenvironment. One critical factor is the production of proteolytic enzymes involved in the degradation and remodelling of the extracellular matrix. Among these enzymes, matrix metalloproteinases (MMPs) represent a large family playing key roles in cell proliferation, angiogenesis, tumour invasion and metastasis. These enzymes principally degrade the ECM components, but have also other substrates such as cytokines, growth factor receptors, cell–cell and cell–matrix adhesion molecules.

MMP activity is regulated at least at three levels: transcription, proteolytic activation of the zymogen form and inhibition of the active enzyme [44]. Most MMPs are secreted from cells in latent forms (proMMPs). Conversion of proMMPs to functionally active forms requires a specific multistep activation process that frequently envolves cleavage others MMPs [77].

MMPs substrate specificity is not yet fully characterized. Known substrates include most of the ECM components (fibronectin, vitronectin, laminin, entactin, tenascin, aggrecan, myelin basic protein, etc). The collagens (types I, II, III, IV, V, VI, VII, VIII, IX, X, XIV) have all been shown to be substrates of different MMPs, with vastly different efficacies. The most common substrates used to study MMP activity are casein and gelatin. While gelatin (heat denatured collagen) might be considered a valid substrate for the gelatinases (MMP-2 and MMP-9), casein is not likely to be a physiologically relevant substrate. Casein is used as a generic proteinase substrate because it is digested by a wide range of proteinases. Other MMPs, such as MMP-3 and MMP-7, cleave a broad range of substrates.

MMPs are physiologically expressed in tissues at various stages of development and in physiological or pathological processes requiring cell migration and tissue remodelling

including wound healing, placentation or tumour progression [78,79,80]. The classic concept of increased MMP activity resulting in greater degradation of the ECM, thus facilitating tumour cell invasion and metastasis formation is currently supplemented by much wider biological function of MMPs in tumour development and progression [78]. There is increasing evidence for important roles of MMPs in interactions with non-matrix proteins including growth factors and their receptors, mediators of apoptosis, and cell adhesion molecules, such as members of the integrin family and CD44 [74,77].

Individual MMPs have been identified to be an increased expression and enhanced activity in tumours. However, although the principal source of MMPs are the stromal cells, in some circumstances, MMPs may be produced *in vivo* by tumour cells. MMPs production by tumour cells is often associated with the expression of other mesenchymal markers and loss of many epithelial characteristics, resulting in epithelial-mesenchymal transition [44].

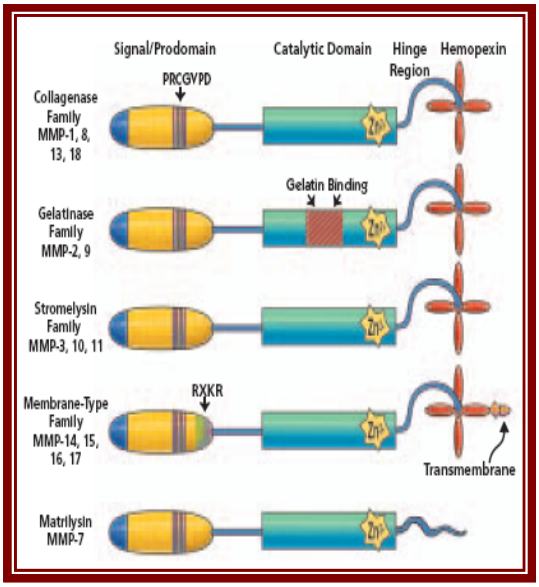

Figure 11| Structural domains of MMPs. (Calbiochem and Oncogene Research[81])

MMPs are classified into distinct categories according to their structure, substrate specificity and cellular localization, into collagenases, stromelysins, gelatinases and MT-MMPs [74,44]. They contain the motif HEXXHXXGXXH as the zinc-binding active site. The MMP family currently consists of at least 26 members, all of which share a common catalytic core with a zinc molecule in the active site.

A functional link between E-cadherin/β-catenin complex and MMPs expression has been demonstrated by several studies [44], as these cell adhesion molecules may also be substrates for MMPs. For example, MMP-7 and MMP-3 was shown to cleave E-cadherin at the cell surface and to release soluble E-cadherin into the medium. The soluble E-cadherin released in turn inhibited E-cadherin functions in a paracrine way by inhibition of the E-cadherin-dependent cell aggregation [82]. Moreover, an up-regulation of MMP-2, MMP-9 and MMP-14 by soluble E-cadherin fragments was demonstrated in lung tumour cells [44].

In gastric cancer, changes in the expression of MMPs are commonly observed. MMP-7 (matrilysin) expression is frequently strongly increased [36]. MMP-7 expression might be a consequence of dysregulated β-catenin signalling, since it is a target of Tcf and was

shown to be over-expressed predominantly in intestinal tumours with high β-catenin signalling [83]. In gastric cancer cell lines, increased gastrin levels can lead to increased MMP-9 expression [34]. Other matrix metalloproteinases such as MMP-1, MMP-2, MMP-3, MMP-11, MMP-12, MMP-19 and ECM remodelling enzymes such as cathepsin K and arylsulfatase E have also commonly been found to be up-regulated [34].

E-cadherin and Integrins

Cadherins and integrins are the major cell-cell and cell-extracellular matrix adhesion receptors [84], respectively the activity of these two receptors must be temporally and spatially coordinated for the proper development and maintenance of tissue architecture. During epithelial-mesenchymal transition of most malignant tumours coordinated disruption of cadherin-dependent cell motility and induction of integrin-dependent cell motility take place, suggesting that a fine-tuned molecular crosstalk must exist between integrins and cadherins [84].

Integrins mediate the binding of cells to ECM proteins such as collagen, fibronectin, laminin, and vitronectin [73,85]. They are heterodimeric glycoproteins consisting of an α- and a β-subunit [86,84] that span the cell membrane, working as transmembrane receptors. All mammalian cells express integrins, but different subtypes are represented on different tissues. In addition to anchoring the cell to the ECM, the binding of ECM proteins to integrins triggers outside–in signal transduction cascades conceptually analogous to those stimulated by ligand-dependent activation of growth factor receptor tyrosine kinases (RTKs). Functional interactions between RTKs and integrins have also been documented downstream of the receptors themselves [85].

Integrins lack intrinsic kinase activity characteristic of RTKs but activate one or more intra-cellular signalling pathways [85]. These pathways typically involve phosphorylation of focal adhesion kinase (FAK), recruitment of adaptor proteins, activation of small GTPases and subsequent activation of downstream effector molecules [73]. These signals, in concert with signals derived from growth factors, regulate cell behaviour in a complex tissue microenvironment [73]. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that provides signalling and scaffolding functions at sites of integrin adhesion. It is involved in the regulation and turnover of these adhesion sites, a process crucial in the control of cell migration [87]. The formation of focal adhesion contacts is a prerequisite for adhesion, spreading, and migration through structures of the ECM [74].

Integrins are also involved in regulating the activities of proteolytic enzymes that degrade the basement membrane, such as MMPs [73].

During tumourigenesis and with increasing dedifferentiation some integrins are lost from the cell surface, whereas others are upregulated or synthesised *de novo*. *In vitro* Integrin subunit β1 is responsible for adhesion to several proteins depending on the substrate (for example collagen and laminin) as well as cell-cell adhesion. Loss of β1 integrin attachment to laminin or collagen has been associated with increased stimulation of MMP-9 [86].

E-cadherin and RTKs (Receptor Tyrosine Kinases)

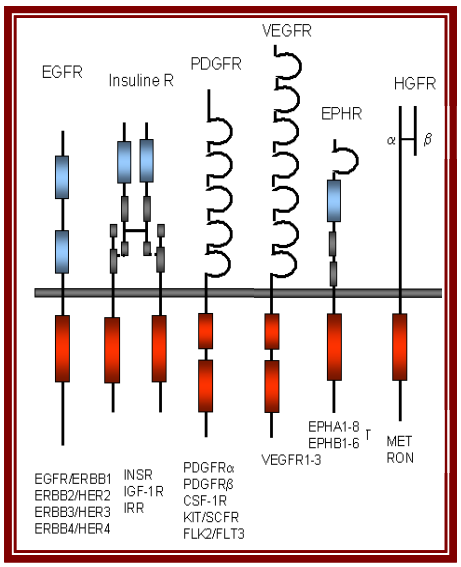

Figure 12| Six subfamilies of human tyrosine kinase. EGFR – epidermal growth factor receptor; InsR – insulin receptor; PDGFR – plateled derived growth factor receptor; VEGF – vascular endothelial growth factor; EPHR – ephrin receptor; HGFR – hepatocyte growth receptor. (Blume-Jensen *et al* [90])

Receptor tyrosine kinases (RTKs) are the main mediators of the signalling network that transmit extracellular signals into the cell. Most growth factors control cell growth, proliferation, differentiation, survival and migration by activating receptors of the RTKs family on specific target cells [88]. Their activity is normally tightly controlled and regulated [89]. Upon ligand binding, cytoplasmic tyrosine residues of the RTKs become autophosphorylated and thus provide docking sites for a variety of phosphotyrosine-binding proteins. The specific recruitment of these proteins, which harbour various catalytic and/or scaffolding domains, determines the signalling output. Since dysregulation of more than 30 RTKs has been associated with cancer [88], it is essential to understand how RTKs are activated and deactivated.

Overexpression of RTKs, by gene amplification or altered transcription/translation, functional alterations caused by mutations that promote ligand independent autophosphorylation, or abnormal stimulation by autocrine growth factor loops contribute to constitutive RTKs signalling, and result in dysregulated cell growth and cancer [89]. However, recent data also showed that failure of RTKs to be appropriately deactivated may be a cause of neoplastic growth [88]. A major deactivation pathway for

RTKs, termed receptor downregulation, involves their ligand-induced internalization by means of endocytosis, followed by degradation in lysosomes [91].

E-cadherin is known to interact with RTKs, like EGFR (epidermal growth factor receptor), ErbB2 (V-ERB2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2) and c-Met (hepatocyte growth factor receptor). These receptors colocalize with E-cadherin to basolateral areas of polarized epithelial cells and form multicomponent complexes that include E-cadherin [68].

The interaction of E-cadherin and EGFR regulates cell adhesion. EGFR activation results in phosphorylation of the cytoplasmic linker molecules and leads to disruption of adherens junctions and increased cell migration.

Low cell surface expression of E-cadherin permits EGFR activity and high surface expression of E-cadherin on adherens junctions leads to immobilization of EGFR through interaction with E-cadherin and alters the affinity of the ligand-binding site within EGFR, resulting in increased cell adhesion [55].

Qian *et al*. [68] demonstrated that there is bi-directional regulation of RTKs and E-cadherin. E-cadherin was found to interact through its extracellular domain with EGFR and other receptor tyrosine kinases, thereby decreasing receptor mobility and ligand affinity. This mechanism by which E-cadherin inhibits RTKs suggests that downregulation of E-cadherin may contribute to the frequently observed activation of RTKs in tumours [55].

The interaction of the extracellular domain of E-cadherin with EGFR is necessary to mediate and regulate EGFR inhibition. E-cadherin mutants that retain their adhesive properties, even if they lack the catenin binding site, are unable to inhibit EGFR. So, not only do RTKs inhibit E-cadherin, which leads to EMT, but E-cadherin may also inhibit signalling through RTKs [55].

On the other hand, Epidermal growth factor (EGF) has been shown to induce tyrosine phosphorylation of β-catenin and plakoglobin after association of EGFR with the cadherin-catenin complex. This has been suggested to induce disassembly of the cadherin-catenin complex with the actin filament network [34]. Furthermore, phosphorylation of plakoglobin through EGFR not only disrupts adherens junctions, but also desmosomes, a structure similar to adherens junctions in which plakoglobin and other cytoplasmic linker molecules mediate anchorage of desmogleins and desmocollins (transmembrane glycoproteins) to the keratin network. Thus, on epithelial cells EGFR may counteract the adhesive function of E-cadherin [72].

Increased cell motility and invasiveness observed in E-cadherin negative tumours and cell lines have been attributed to the loss of cell-cell adhesion and increased β-catenin

nuclear localization with enhanced transcriptional activation of target genes. However, the loss of adhesion-dependent RTK inhibition could also be involved in this phenotype.

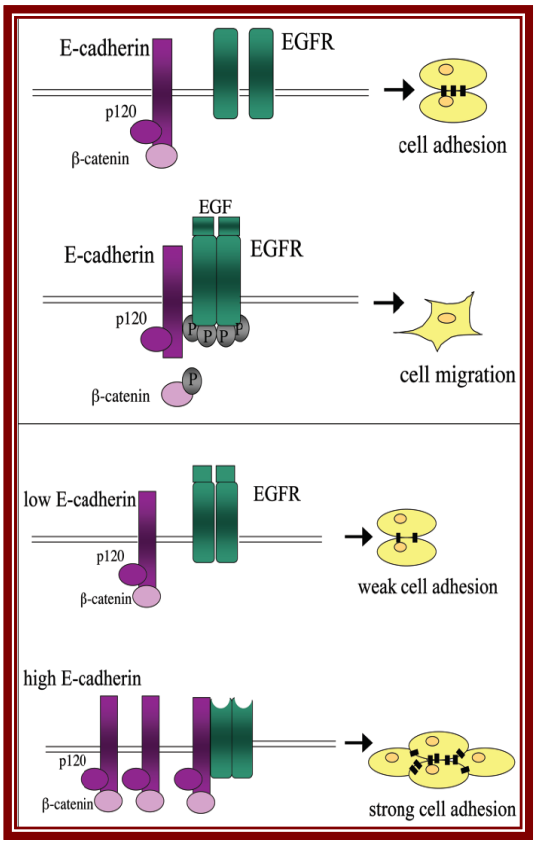

Figure 13| Interaction of E-cadherin and EGFR. (Andl and Rustgi [55])

The relation between EGFR and mutant E-cadherin is dependent on the type of mutation. Truncating mutations of the E-cadherin extracellular domain rendered E-cadherin nonfunctional and led to an upregulation of RTK activity [55]. Thus, mutants lacking the extracellular domain not only lose its adhesive function, but also the inhibitory effect on EGFR signalling. In contrast, mutations located in calcium binding sites abrogated the adhesive functions of E-cadherin but not its signalling functions [92]. However, according to Andl [55], the EGFR and E-cadherin interaction through the extracellular domain still permits E-cadherin dimerization and adhesive function, suggesting a novel functional binding site in the E-cadherin extracellular domain [55].

EGFR overexpression in primary human esophageal keratinocytes leads to enhanced cell migration, but at the same time, induces a shift in the distribution of p120-catenin from the cytoplasm to the membrane, thereby resulting in increased cell adhesion [93]. Thus, there may be different pathways of communication between E-cadherin and EGFR in modulating cell adhesion. E-cadherin mediated inhibition of EGFR activity and the application to downregulation of other receptor tyrosine kinases are important for tumourigenesis. While EGFR overexpression is thought to be an early event in cancer development, the downregulation of E-cadherin through promoter methylation, transcriptional repression or mutation, may occur at later stages of cancer progression, thereby leading to tumour invasion and metastasis [75].

Although not well characterized, E-cadherin seems to establish similar physical interactions with ErbB2 and c-Met. It is likely that deregulation of E-cadherin could also increase the activation status of these receptors.

RTKs in gastric cancer

In gastric cancer, EGF and its receptor (EGFR) have been reported to be frequently upregulated. EGF expression has been detected in 50–60% of advanced gastric carcinomas [94]. EGFR overexpression has been observed in about 34% of advanced gastric cancers [95]. This seems to be only rarely caused by amplification of the EGFR gene [96,97].

Activation of the growth factor receptor ErbB2 is also observed. ErbB2 or HER-2/neu is transmembrane tyrosine kinase receptor. Overexpression of ErbB2 occurs in approximately 10–15% of all cases observed [98]. However, there are differences among subtypes of gastric cancer. ErbB2 overexpression is more common in the intestinal type than in the diffuse type [97]. This overexpression and subsequent increased activation is associated with advanced gastric carcinoma and indicative of a poor prognosis [99].

Another growth factor signalling pathway commonly affected in gastric cancer is the HGF/c-Met pathway. c-Met encodes for a receptor of the hepatocyte growth factor. Both c-Met and HGF are overexpressed in gastric cancer (34%-74%), and in gastric cancer cell lines, c-Met is amplified [100-102]. Furthermore, c-met overexpression is associated with diffuse type histology, advanced stage, and a pattern of infiltrative growth [97].

E-cadherin and PI3K/AKT

One of the major intracellular signalling pathways leading to cell growth involves phosphatidylinositol 3′-kinase(PI3K) [103]. This pathway is activated by many types of cellular stimuli or toxic insults and regulates fundamental cellular functions such as regulation of cell proliferation, adhesion, motility, differentiation, and survival [104]. It has been shown that this pathway can also interfere with cytoskeleton rearrangements, and intracellular trafficking.

Phosphatidylinositol (PI) is unique among membrane lipids because it can undergo reversible phosphorylation at multiples sites to generate a variety of distinct inositol phospholipids. PI3K principally phosphorylates inositol phospholipids rather than other proteins. It can be activated by receptors tyrosine kinases, as well as by other types of cell-surface receptors, including some that are G-protein-linked.

When activated, PI3K catalyses the phosphorylation of inositol phospholipids at the 3 position of the inositol ring to generate lipids called PI(3,4)P_2 PI(3,4,5)P_3. This two lipids then provide docking sites for intracellular proteins, bringing these protein together into

signalling complexes, which transmit the signal into the cell from the cytosolic face of the plasma membrane.

One way in which PI3K signals cells to survive is through indirect activation of protein kinase B (PKB or Akt). This kinase contains a PH domain, which directs it to the plasma membrane when PI3K is activated there by an extracellular survival signal. After binding to $PI(3,4,5)P_3$ on the cytosolic face of the membrane, Akt alters its conformation so that it can now be activated in a process that requires phosphorylation by a phosphatidylinositol-dependent protein kinase called PDK1, which is recruited to the membrane in the same way. Once activated, Akt returns to cytoplasm and phosphorylates a variety of target proteins. One of these, is the protein called BAD, that normally encourages cells to undergo programmed cell death or apoptosis. Akt regulates cellular survival through phosphorylation of downstream substrates that directly or indirectly control the apoptotic machinery or by inhibiting other cell death activators, in some cases by inhibiting the transcription of the genes that encodes them.

An important function of activated PI3K in cells is the inhibition of apoptosis, and Akt is a good candidate for mediating these PI3K-dependent cell survival responses [104]. Overexpression of Akt has an anti-apoptotic effect in many cell types, resulting in a resistance to or delay of cell death [105].

A disturbance in the balance between cell proliferation and survival (apoptosis) causing disturbed activation of the PI3K/Akt pathway has been associated with the development and progression of cancer and of diseases such as diabetes mellitus, and autoimmunity [106-109]. Notably, it has been shown that PI3K/Akt signalling is frequently disrupted in human cancers [104].

Besides PI3K-Akt signalling is associated with cell proliferation and survival (apoptosis) it also plays a major role not only in tumour growth but also in the potential response of a tumour to cancer treatment.

Since cell surface receptors are commonly overexpressed or constitutively activated in a large number of human cancers, downstream signal pathways are often activated as a result [90]. One of the most extensively studied examples is the ErbB2 tyrosine kinase receptor, which is overexpressed as a result of gene amplification in breast and other cancers [90].

Aim

The general aim of this work was to study the influence of mutated E-cadherin on signalling pathways that may contribute to gastric carcinogenesis and be potential targets for clinical intervention.

To this end, the following specific aims were addressed:

1. Determine the effect of mutated E-cadherin in PI3K/AKT pathway;

2. Verify the effect of altered E-cadherin on expression and activation of several RTKs (EGFR, c-Met, ErbB2);

3. Study the influence of E-cadherin mutations in a cell-matrix adhesion signalling pathway (FAK/β1-Integrin);

4. Evaluate the activity of MMP-2 and MMP-9 in the context of mutant forms of E-cadherin.

Material and Methods

Cell Culture and Reagents

MDA-MB 435 cells, derived from human melanoma cells, are E-cadherin negative, due to *CDH1* promoter hipermethylation, fail to aggregate homotypically and to invade into collagen; thus representing a good *in vitro* model for the functional studies of E-cadherin mutants.

MDA-MB 435 were transduced with virus containing Wild-type E-cadherin and missense mutations T340A, localized on the extracellular domain and the V832M localized on the intracellular domain of the protein. As control, E-cadherin negative MDA-MB 435 cells were infected with the empty vector (Mock).

Viruses were created by infecting 293FT cells using lipofectamine 2000 (Invitrogen) reagent with pLenti plasmid containing the specific cDNA together with the packaging mix plasmid (Invitrogen). Virus were produced and released to the medium and recovered after 48 and 72 hours for further infection of desired cells.

Cell lines were maintained in D-MEM medium (GIBCO-BRL), supplemented with 10% fetal bovine serum, 200g/ml streptomycin, 200 international units/ml penicillin (Invitrogen) and 5ug/mL of blasticidin at 37ºC under 5% CO_2 humidified atmosphere.

Experiments were performed on collagen type I-coated flasks in order to assess cells' behaviours in relation to basement matrixes.

Preparation of Cell Lysates

Cells were lysed using cold Catenin Lysis Buffer [1% Triton X-100, 1% Nonidet P-40] with 1:7 proteases inhibitors cocktail (Roche) and 1:100 phosphatases inhibitor cocktail (Sigma). Protein concentration was determined using the Lowry Assay (BioRad).

Previously to SDS-PAGE, proteins were dissolved in sample buffer [90% of Laemmli, 5% of 2-β-mercaptoethanol and 5% of Bromophenol Blue] and boiled for 5 min at 95ºC.

Immunoprecipitation

For immunoprecipitation assays, 450µg of proteins were incubated for 3h or overnight at 4°C with an antibody specific for the protein of interest. Immunocomplexes were incubated for 60min with protein G-Sepharose beads (Amersham Biosciences, Buckinghamshire, UK), washed and eluted in sample buffer. Proteins were separated

by SDS-PAGE and visualized by immunoblot analysis, using antibodies directed to the molecules of interest.

Immunoprecipitation (IP) is a method that uses the antigen-antibody reaction principle to identify a protein from a mixture of proteins so that its quantity or physical characteristics can be examined.

A typical immunoprecipitation experiment contains the following steps (Figure 14): 1) The proteins from the cell or tissue homogenate are precipitated in an appropriate lysis buffer containing an immune complex which includes the antigen of interest (protein), primary antibody and Protein A-, G-, or L-agarose conjugate (Protein A or G binds to the antibody, which is bound to its antigen) or a secondary antibody-agarose conjugate. 2) The immune complex is then captured by centrifugation. Proteins that bound to the antibody are precipitated and proteins that do not are washed away. 3) Components of the bound immune complex (both antigen and antibody) are eluted. 4) Immunoprecipitated proteins are further analyzed by SDS-PAGE and immunoblotted to determine their molecular weights, study their interactions with other proteins, determine specific enzymatic activity, monitor protein post-translational modifications and determine the presence and quantity of proteins.

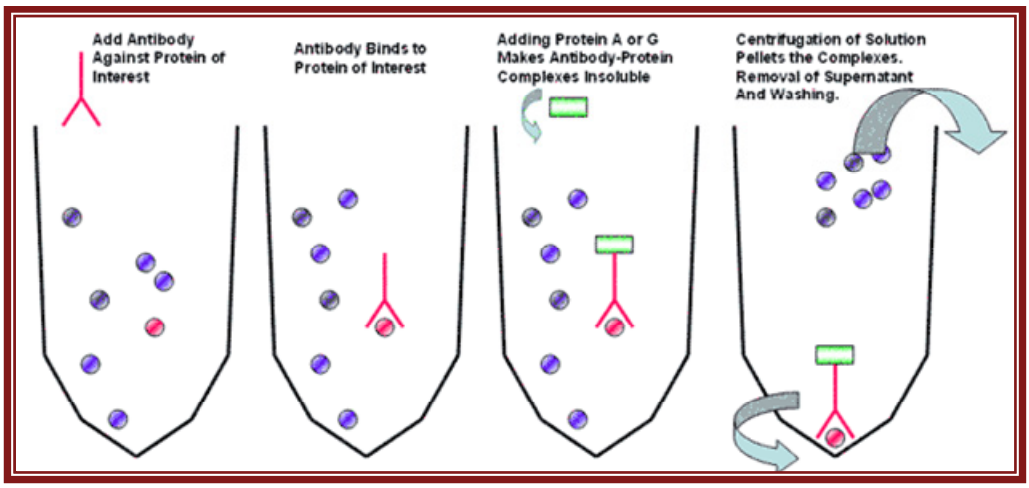

Figure 14| Eschematic figure of Immunoprecipitation process.

Immunoblot Analysis

After electrophoresis, proteins were transferred onto Hybond nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 4% bovine serum albumin (Sigma) in PBS + 0.5% Tween-20 (for detection of phosphorylated proteins) or with 5% nonfat milk in PBS+0.5% Tween-20 (for overall protein detection) and incubated for 60 minutes with the primary antibody specific for the protein of interest. A goat anti-rabbit (Santa Cruz Biotechnology) or a rabbit anti-mouse (Amersham Biosciences) horseradish peroxidase-conjugated secondary antibodies were used, followed by ECL detection (Amersham Biosciences).

Immunocytochemistry

Cells layers established on coverslips were fixed in cold methanol for 10 minutes and subsequently incubated with anti-E-cadherin antibody. After three washes with PBS – Tween 0.5%, cells were incubated for 1 hour with secondary antibody labelled with FITC (Dako, dilution 1/200). Detection of protein was performed under a fluorescence microscope.

Antibodies

The following primary antibodies were used: rabbit anti-c-Met antibody (1:1000, Santa Cruz Biotechnology); mouse anti-E-cadherin (1:3000, BD Transduction Laboratories); mouse anti-EGFR (1:1000, BD Transduction Laboratories); mouse anti-c-erbB2 (1:500, NovoCastra); mouse anti-PI3K (1:1000, BD Transduction Laboratories); rabbit anti-AKT (1:1000, Cell Signaling); mouse anti-Phospho AKT (1:1000, Cell Signaling); mouse anti-FAK (1:1000, BD Transduction Laboratories); mouse anti-Integrin β1 (1:1000, BD Transduction Laboratories) mouse anti-α-Tubulin (1:15000, Sigma); mouse anti-Phosphotyrosine-PY-20 (1:1000, BD Transduction Laboratories); mouse anti-Phosphoserine (1:1000, Sigma).

Zymography

Zymogram gels are used for detecting and characterizing metalloproteinases and various other proteases that can utilize casein and gelatine as a substrate.

To detect MMP enzymatic activity, cells were cultured for 48h on top of collagen type I gels. Conditioned media were collected after 48h, centrifuged at 3220 x *g* for 2 min and passed through 0.2μm pore-size filters (Schleicher & Schu¨ell, Dassel, Germany). 12

µg of protein from conditioned medium of such cultures were loaded on 10% SDS-PAGE containing 1 mg/ml gelatine as substrate. Zymograms were run in Tris/glycine SDS running buffer under nondenaturing conditions. After electrophoresis, gels were washed twice in 2% Triton X-100, to remove SDS. Zymograms were subsequently incubated for 20h at 37°C in the appropriate MMP substrate buffer (10 mM CaCl2 in 50mM Tris-HCl, pH 7.5). Gels were stained with Coomassie blue for 30min and destained with 30% methanol–10% acetic acid. Proteolytic activity (gelatinease) was visualized as the presence of clear bands against the blue background of stained gelatine gels, indicative of proteolysis of the substrate.

Matrigel Invasion Assay

Matrigel Matrix is a solubilized basement membrane preparation extracted from EHS mouse sarcoma. It contains laminin, collagen type IV, heparan sulfate proteoglycans, and entactin (components of the basement membrane) to produce biologically active matrix material resembling the mammalian cellular basement membrane. It provides a physiologically relevant environment for studies of cell morphology, biochemical function, migration or invasion, and gene expression.

Prior to each experiment, 24-well Matrigel-coated invasion inserts of 8µm pore size filters (Becton and Dickinson) were introduced into 24-well plates. For re-hydration, the inner and outer compartments of the system were filled with D-MEM medium, supplemented with 10% fetal bovine serum, 200g/ml streptomycin, 200 international units/ml penicillin and 5ug/mL of blasticidin, and incubated for 60min at 37°C. After rehydration, $5x10^4$ cells were incubated for 24h at 37°C under 5% CO2 humidified atmosphere. Filters were washed in PBS, fixed in 4% paraformaldehyde, removed from the insert, and mounted in Vectashield with 4', 6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Invasive cells were scored in at least 25 microscopic fields (x20 objective), when DAPI-counterstained nuclei passed through the pores of the filter.

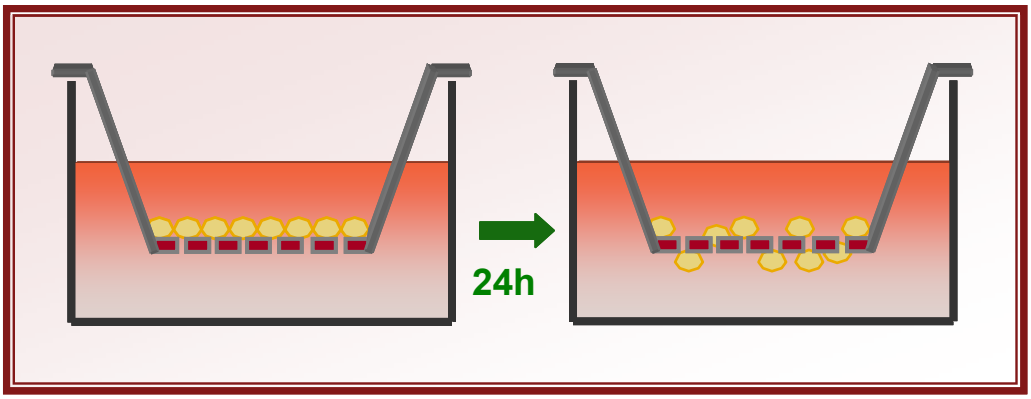

Figure 15| Schematic figure of Matrigel invasion assay process.

Results

MDA-MB 435 cells are E-cadherin negative, representing a good *in vitro* model for the functional studies of E-cadherin mutants.

Cells were transduced with different forms of E-cadherin, namely Wild-type E-cadherin (Wt), an extracellular missense mutated form (hereafter referred by the altered nucleotide, E-cadherin 1018), leading to an Alanine (instead of a Threonine) inserted at aminoacid 340, and an intracellular missense mutated form (hereafter referred as E-cadherin 2494) that results in a Valine to Methionine change at aminoacid 832. As control, E-cadherin negative MDA-MB 435 cells were transduced with the empty vector (Mock).

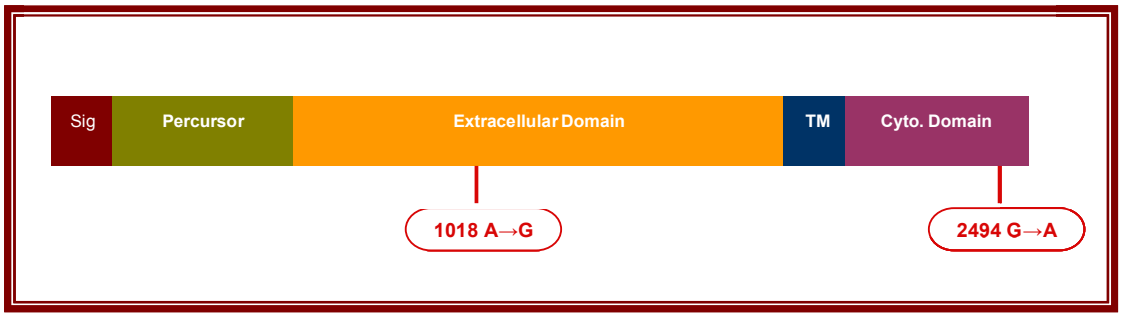

Figure 16| Scheme of CDH1 gene with the germline mutations used in this study. Represented sections correspond to protein domains of E-cadherin. (Adapted from Oliveira and Suriano [66])

In order to identify components of signalling pathways modified by the biological activity of normal and mutant forms of E-cadherin we performed western blot analysis and immunoprecipitation studies. In all experiments performed in this study, at least two independent stable clones were used, in order to exclude clonal dependence of the results observed.

E-cadherin expression analysis

Before addressing each of the aims proposed on this thesis, confirmation of the E-cadherin status on the four cell lines studied was performed by Western blot analysis.

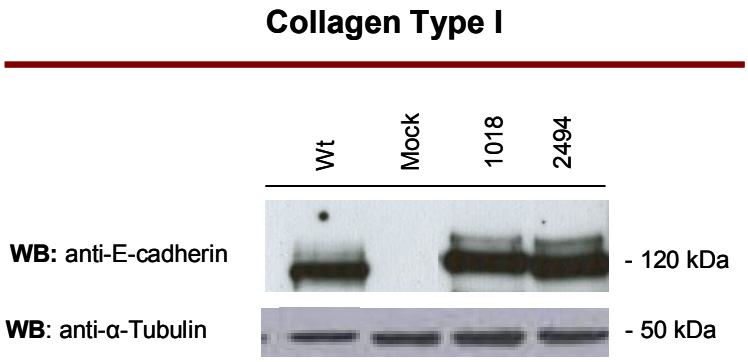

Figure 17| Total cell lysates were immunoblotted with an anti-E-cadherin and reblotted with an anti-α Tubulin antibody.

Total cell lysates were immunoblotted with an anti-E-cadherin and reblotted with an anti-α-tubulin antibody. Equal amounts of protein were loaded in each lane.

Results revealed that the E-cadherin protein was absent from the Mock transduced cell line, and was present in the remaining cell lines, through the detection of the 120kDa E-cadherin band, as expected (Figure 17). All cell lines except Mock cells expressed comparable amounts of transduced E-cadherin.

E-cadherin immunocytochemistry

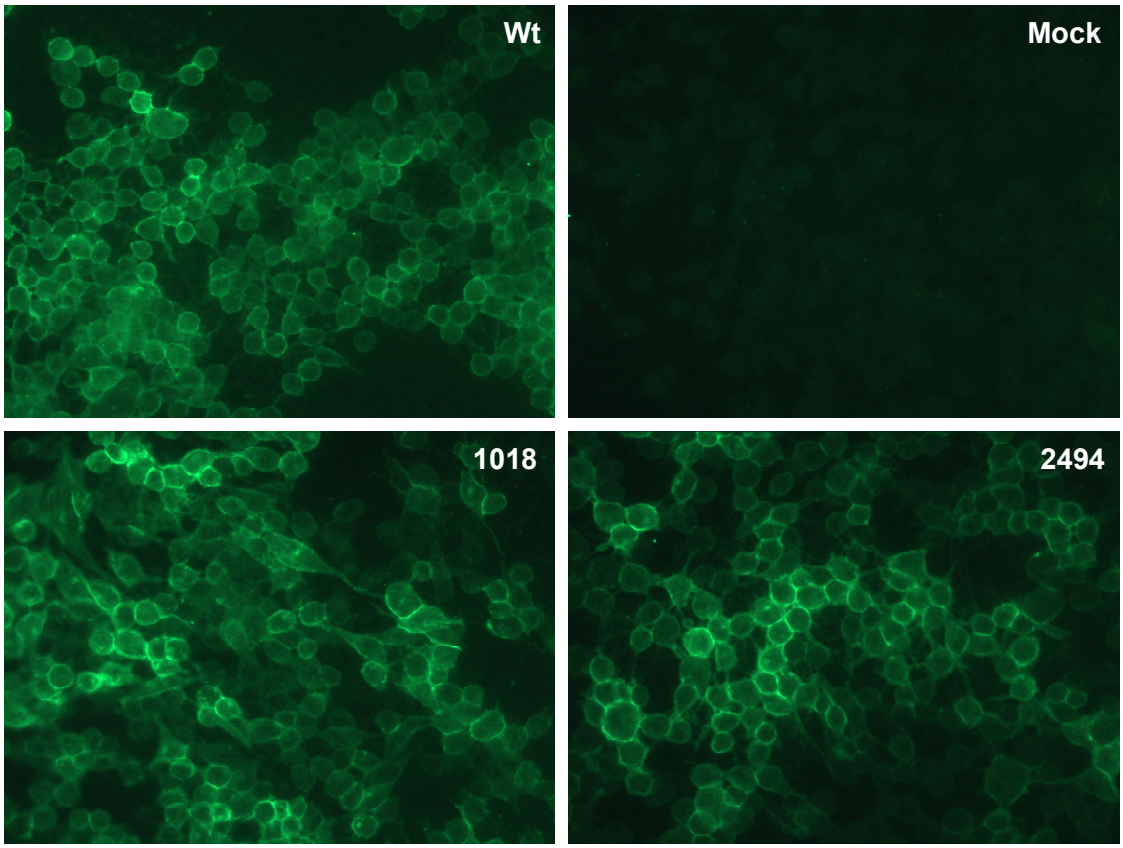

Figure 18| Detection and cellular localization of the different forms of E-cadherin by immunocytochemistry.

E-cadherin expression and correct membrane localization was assessed and confirmed by immunocytochemistry (Figure 18). As expected, Mock cells were negative for the protein. In all cell lines E-cadherin was expressed at the cell membrane forming a typical honey-comb like pattern.

Effect of mutated E-cadherin in PI3K/AKT pathway

To assess if normal E-cadherin and mutant forms modify the biological activity of the survival pathway, the expression and activation of two of its principal components, PI3K and one of its downstream effectors, AKT, was analysed using western blot and immunoprecipitation analysis.

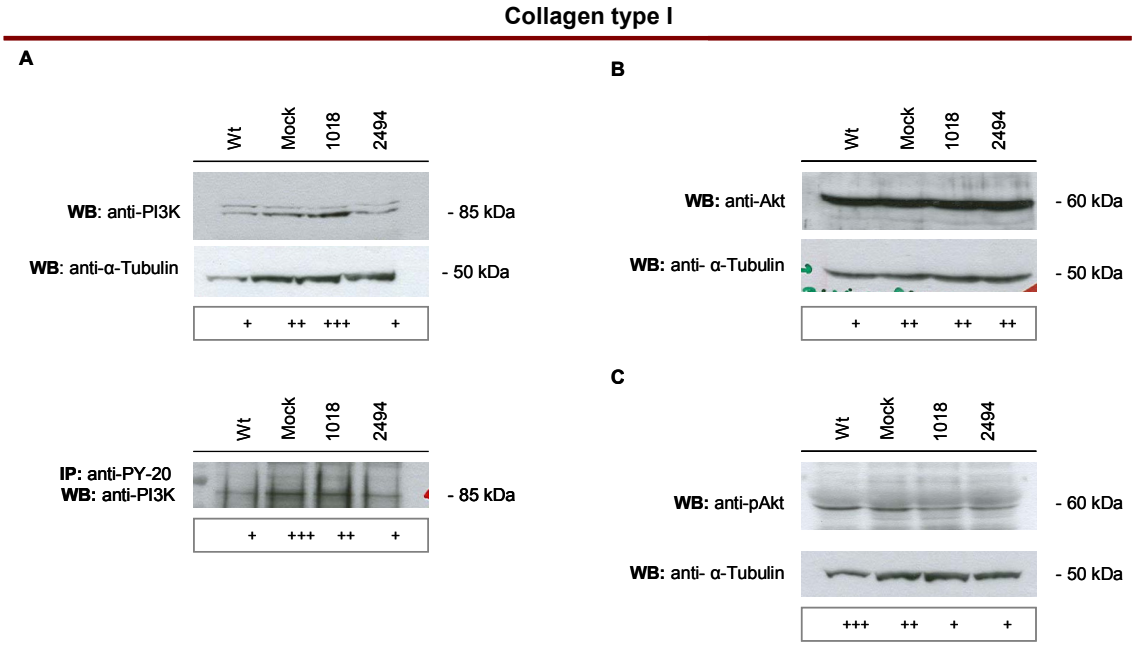

Figure 19| Cells were cultured on collagen type I substrate.**(A)** Cells were lysed, protein lysates obtained were immunoprecipitated with an antibody against tyrosine-phosphorylated residues (PY-20), and immunoblotted with an anti-PI3K antibody. In parallel, total cell lysates were immunoblotted with an anti-PI3K and reblotted with an anti-α-Tubulin antibody. **(B)** Total cell lysates were immunoblotted with an anti-AKT antibody and reblotted with an anti-α-Tubulin antibody. **(C)** Total cell lysates were immunoblotted with an anti-pAKT antibody and reblotted with an anti-α-Tubulin antibody.

PI3K protein was detected on all cell lines studied, although with different expression and phosphorylation levels (Figure 19-A). Expression of total PI3K was higher on the 1018 cell line. Mock cells presented intermediate amounts of PI3K, while Wild-type E-cadherin cells resembled the ones with the intracellular mutated form (2494) by showing lower values. As PI3K has tyrosine kinase activity, we decided to study its tyrosine phosphorylation and consequently its activation status. In this regard, again the mutated 2494 form of E-cadherin behaved similarly to Wild-type by presenting the lowest amounts. Mock cells showed the highest levels and 1018 cells the intermediate amount of phosphorylated PI3K.

As for AKT, a slight decrease in total protein was observed in cells with Wild-type E-cadherin (Figure 19-B). The remaining cells lines presented similar levels of AKT. However, the phosphorylated form of AKT was more abundant in Wild-type than Mock

cells. In addition, cells expressing the mutated forms of E-cadherin expressed lower amounts of pAKT than Mock cells (Figure 19-C). Altogether, these results evidence that cells expressing the extracellular mutated form 1018 present higher expression and tyrosine phosphorylation of PI3K, but less activation of AKT.

Effect of altered E-cadherin on expression and activation of several RTKs (EGFR, c-Met, ErbB2)

In order to investigate the involvement of E-cadherin on the expression and activation of tyrosine kinase receptors (RTKs), three of the most common RTKs associated with cancer, c-Met, EGFR and ErbB2 were studied.

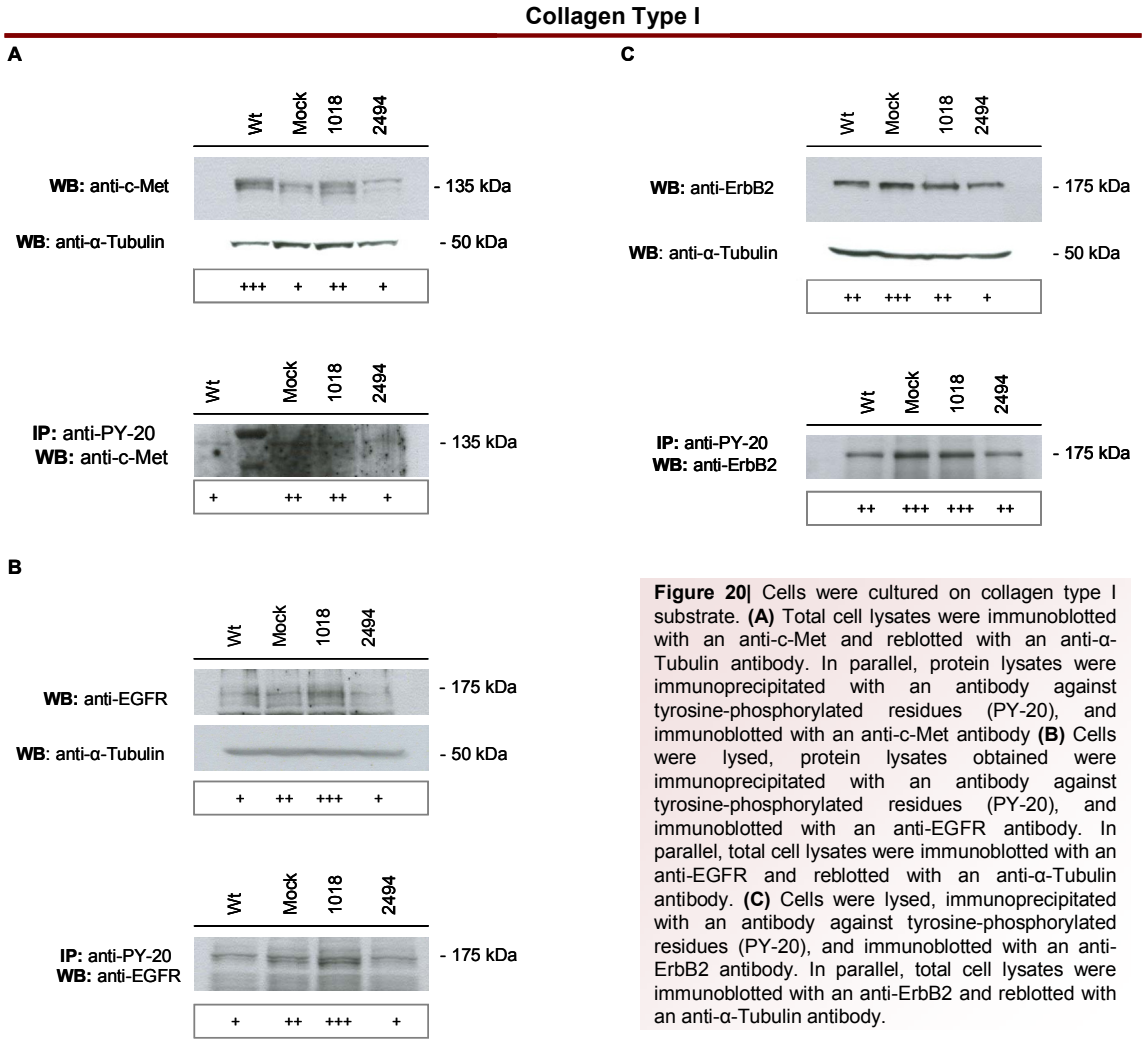

Figure 20| Cells were cultured on collagen type I substrate. **(A)** Total cell lysates were immunoblotted with an anti-c-Met and reblotted with an anti-α-Tubulin antibody. In parallel, protein lysates were immunoprecipitated with an antibody against tyrosine-phosphorylated residues (PY-20), and immunoblotted with an anti-c-Met antibody **(B)** Cells were lysed, protein lysates obtained were immunoprecipitated with an antibody against tyrosine-phosphorylated residues (PY-20), and immunoblotted with an anti-EGFR antibody. In parallel, total cell lysates were immunoblotted with an anti-EGFR and reblotted with an anti-α-Tubulin antibody. **(C)** Cells were lysed, immunoprecipitated with an antibody against tyrosine-phosphorylated residues (PY-20), and immunoblotted with an anti-ErbB2 antibody. In parallel, total cell lysates were immunoblotted with an anti-ErbB2 and reblotted with an anti-α-Tubulin antibody.

Western blot analyses showed decreased c-Met expression in cell lines with the intracellular-altered form (2494) of E-cadherin, or cells without the protein (Figure 20-A). By contrast, Wild-type E-cadherin cells showed more expression than the other cell lines.

The activation of c-Met was more prominent in Mock and 1018 cell lines. Wild-type and 2494 cells behaved in the same way by showing less activation (Figure 20-A).

Total EGFR expression was increased in cells with the extracellular-altered form of E-cadherin (Figure 20-B). Furthermore, Wild-type and 2494 cells showed decreased expression levels when compared to Mock cells. In regard to its activation status, pEGFR levels matched the total expression amounts of the protein observed in the four cell lines (Figure 20-B).

Finally, analysis of ErbB2 showed that cells lacking E-cadherin (Mock) had higher expression (Figure 20-C). Cells with the extracellular mutated form expressed similar levels as cells with the Wild-type protein, while cells with the intracellular mutated form expressed lowest levels of ErbB2. Increased phosphorylation of ErbB2 was observed in Mock and 1018 cell lines. Wild-type E-cadherin cells resembled the ones with the intracellular mutated form (2494) by showing slightly less pErbB2 (Figure 20C). Altogether, these results demonstrate that cells lacking E-cadherin expression (Mock) or expressing the extracellular mutated form (1018) are the ones presenting increased c-Met, EGFR and ErbB2 activity.

Influence of E-cadherin mutations in a cell-matrix adhesion signalling pathway (FAK/β1-Integrin)

The influence of E-cadherin on cell-matrix interactions was investigated through the study of FAK and β1-integrin proteins.

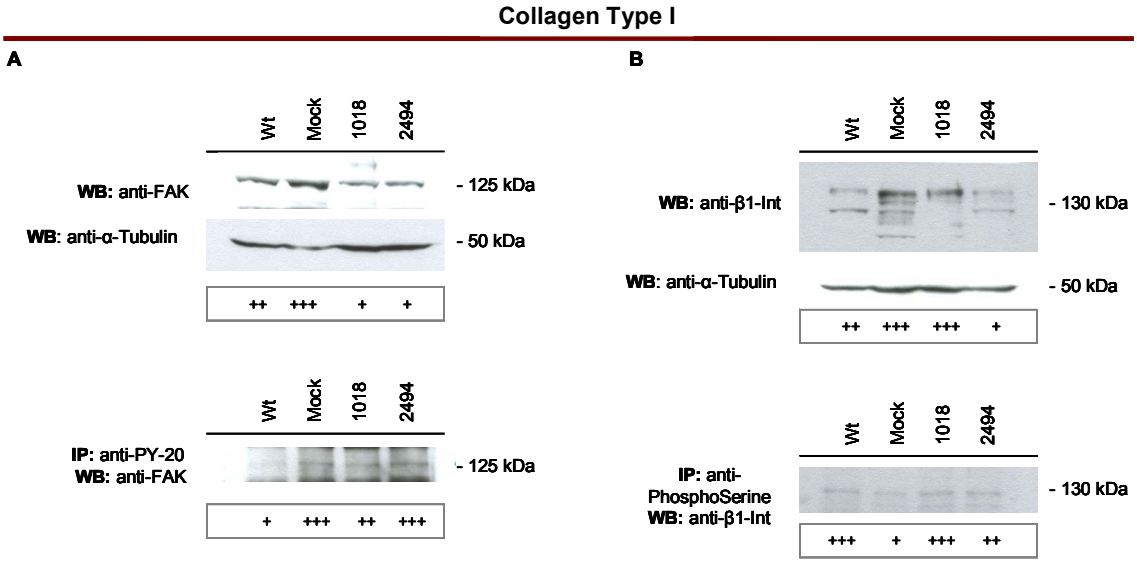

Figure 21| Cells were cultured on collagen type I substrate.**(A)** Cells were lysed, protein lysates obtained were immunoprecipitated with an antibody against tyrosine-phosphorylated residues (PY-20), and immunoblotted with an anti-FAK antibody. In parallel, total cell lysates were immunoblotted with an anti-FAK and reblotted with an anti-α-Tubulin antibody. **(B)** Total cell lysates were immunoprecipitated with an antibody against Phosphoserine, and immunoblotted with an anti-β1-Int antibody. In parallel, total cell lysates were immunoblotted with an anti- β1-Int and reblotted with an anti-α-Tubulin antibody.

A decrease of FAK expression was observed for cells expressing any form of E-cadherin in comparison to cells without the protein (Figure 21-A). This decrease was more pronounced in cells with mutated E-cadherin. As for FAK phosphorylation, it was diminished in Wild-type and 1018 cells, but not on 2494 that showed equal values to Mock cells (Figure 21-A).

β1-integrin was less abundant in cell lines expressing the E-cadherin 2494 mutation and Wild-type E-cadherin (Figure 21-B). Mock and 1018 cells had the same protein amount. In contrast, the activation of β1-integrin, measured as previous proteins by its phosphorylation status, was more frequent in cells with E-cadherin, and particularly pronounced in Wild-type and 1018 E-cadherin cells (Figure 21-B).

Activity of MMP-2 and MMP-9 in the context of mutant forms of E-cadherin

To investigate the role of E-cadherin in cell invasion through MMPs signalling pathway, we studied the proteolytic activity of MMP-9 and MMP-2 on a gelatine substrate. If expressed and in the active form, these MMPs degrade the gelatine gel at a region corresponding to their molecular weight (protein electrophoresis performed is similar to a regular western blot, the only exception being the inclusion of the substrate).

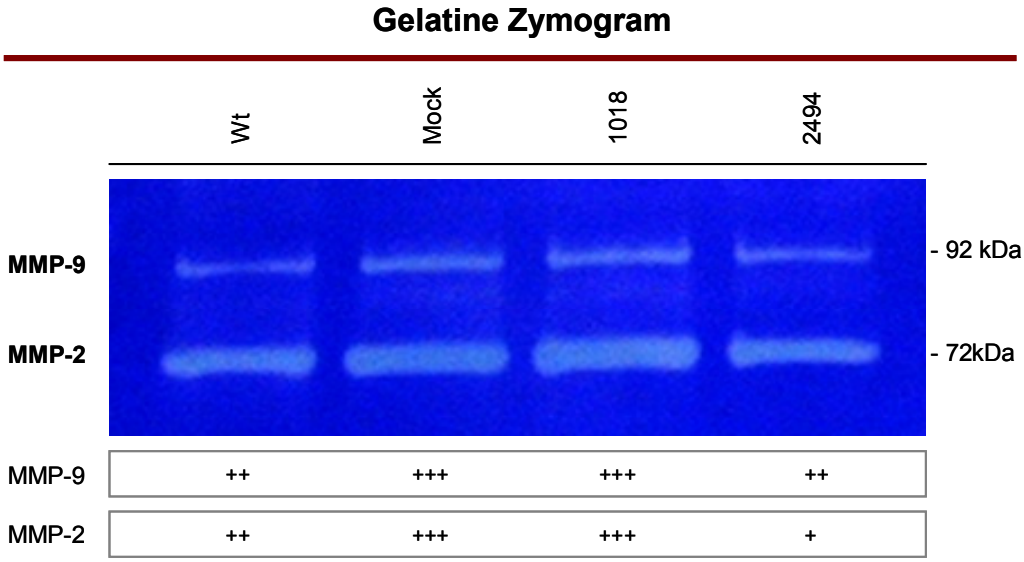

Figure 22| MDA-MB 435 cells were cultured on collagen type I substrate. Filtered culture supernatants were run on a gelatin zymogram to detect MMPs activity. Proteolytic bands were revealed in white on a Coomassie Blue-stained background.

MMP-2 and MMP-9 active forms were detected in all cell lines by zymography experiments (Figure 22). MMP-9 activity, shown by gelatine degradation at the 92kDa region, was increased in Mock and 1018 cells. The intracellular E-cadherin mutation used on this study behaved like Wild-type E-cadherin. Very similar results were observed for MMP-2 activity, detected at the 72kDa. The only variation was that 2494 cells showed less MMP-2 activity than Wild-type cells. Again Mock and 1018 cells had increased activity.

Matrigel Invasion Assay

Despite several attempts, the matrigel invasion assay could not be optimized for the cell line used. For quality control purposes, the same matrigel invasion assay was performed on CHO cells transduced with the same E-cadherin forms. Results obtained were similar to the ones already published [110,111].

We hypothesize two explanations for the difficulties encountered. On one hand, the unusually large size of MDA-MB 435 cells could make it very difficult for them to cross matrigel pores, independently of their invasive properties. On the other hand, the aforementioned large size of and the pre-defined number of cells suggested for the assay may make it easier for cells to adhere to each other and consequently not invade. The latter hypothesis could be tested by reducing significantly the starting number of cells used the assay, but this experiment could not be performed within the time frame of this Thesis.

As such, E-cadherin-dependent invasive properties given by matrigel invasion assay discussed further on will be based on the results obtained for CHO cells published elsewhere [110,111].

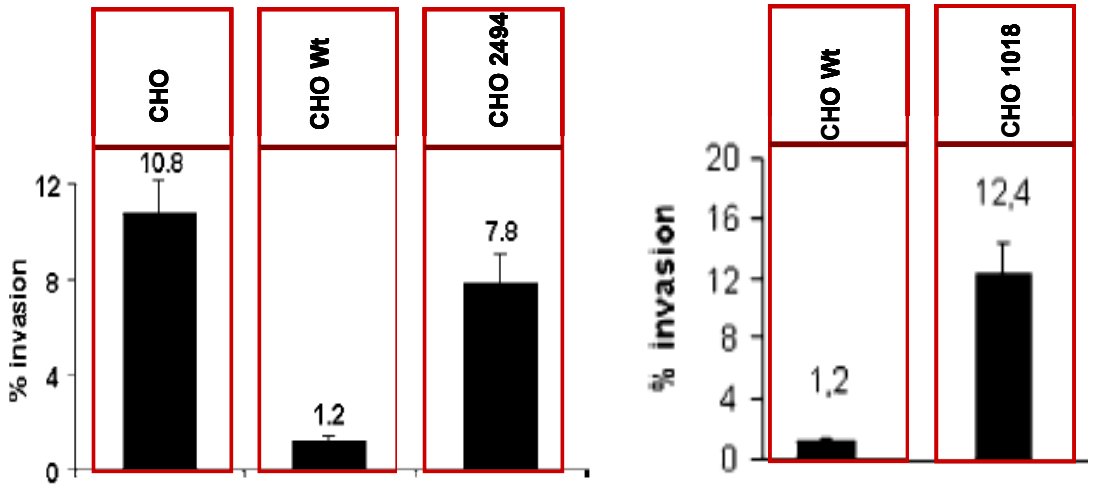

Figure 23| Invasion assay of parental CHO cells, cells expressing Wild-type E-cadherin and cells expressing the mutated forms 1018 and 2494 [110-111].

Discussion

Cancer malignancy and mortality stems from the tumours' ability to invade and destroy nearby tissues, and to spread to other parts of the body. Metastasis is a complex process involving the coordination of several signal-transduction pathways that allow cancer cells to proliferate, remodel their surrounding environment, invade and migrate into surrounding tissues and differentiate [73,74]. During this process, the involvement of the cell-cell adhesion molecule E-cadherin is unequivocal.

In their physiological environment, cells are in contact with surrounding extracellular matrix (ECM) and with neighbouring cells. Cell adhesion receptors are key sensors of cues that stem from the environment, thereby contributing to both the adhesive process and to transmission of signals that result in dependent cellular responses. Indeed, adhesion receptors can modulate or activate intracellular signal transduction pathways in a number of ways, for example, by clustering in the membrane at adhesion sites, by recruiting and interacting with adaptor proteins or proteins that link adhesive structures to cellular cytoskeletal networks, or by directly activating enzymatic proteins at the inner surface of the plasma membrane.

The demonstration of the triggering of tumourigenesis by E-cadherin has come from the identification of germline mutations of its gene (*CDH1*) in hereditary diffuse gastric cancer (HDGC)[28]. These *CDH1* mutations harbour significant functional consequences *in vitro*, supporting their effective pathogenicity in diffuse gastric cancer [110]. In this type of tumour, isolated cancer cells permeate the basal membrane and survive in the gastric wall in the absence of contact with neighbour epithelial cells or with the extracellular matrix.

The role of E-cadherin in suppressing invasion, metastasis, and proliferation has been extensively demonstrated and it is well established that its loss of expression and/or function can lead to increased cell motility, cell-cell detachment and, ultimately, to invasion [110,111]. Recent works have proposed that E-cadherin is also relevant during initial stages of tumour development and that it modulates intracellular signalling [55,112,113]. Thus far, attempts to recognize signalling pathways affected by E-cadherin have not been successful, the major focus being on changes mediated by other components of the adhesion complex (β-catenin, p120, α-catenin). It seems likely that the oncogenic potential of loss-of-function of E-cadherin is exerted through yet to be described interactions with other molecules and pathways.

The goal of this thesis was to study the influence of mutated E-cadherin in activating signalling pathways that are associated with some cancer-related phenotypes.

In our model, we used two distinct missense mutations with the purpose of understanding if a different location of the E-cadherin alteration could activate target molecules that influence distinct cellular phenotypes [110,111]. The 1018 A→G mutation affects the extracellular region of E-cadherin protein, and the 2494 G→A mutation is localized in the intracellular region, more precisely in the cytoplasmic tail (Figure 16). Both mutations were previously shown to confer *in vitro* protein loss of activity, both in terms of cell-to-cell adhesion and suppression of cell invasion [110,111]. For these cell lines, no differences were observed in cell proliferation [112].

To mimic the *in vivo* environment encountered by tumour cells and so providing a surrogate when they are invading, cells were cultured in a reconstituted cell growth matrix of collagen type I.

After confirmation that E-cadherin was expressed and localized correctly to the cell membrane (Figures 17, 18), comparisons were performed between the E-cadherin-expressing cell lines and the Mock-transduced counterpart.

Following, we will discuss each objective separately. In every one, we will start by comparing Mock and Wild-type E-cadherin cells. Afterwards, we will discuss if the missense mutations behave more like the normal protein or if they resemble the lack of protein (or if they show a different result altogether).

Effect of mutated E-cadherin in PI3K/AKT pathway

Several relevant findings were obtained in regard to the effect of E-cadherin on PI3K signalling (Figure 19). Absence of E-cadherin was associated with increased levels of PI3K and AKT and with significantly more abundant pPI3K. In contrast, pAKT levels did not resemble pPI3K, as Wild-type E-cadherin cells were the ones to present more pAKT, even though total AKT levels were lower.

Opposite results were obtained for the two missense mutations studied and PI3K (Figure 19). While the 2494 mutation rendered the same PI3K and pPI3K levels observed in Wild-type cells, the 1018 cells produced intermediate pPI3K amounts and similar PI3K levels to Mock cells. As for AKT, total levels of the protein were equal in cells expressing the two mutations and Mock cells. However, for the two mutations, pAKT was less abundant than in Mock and a lot less abundant than in Wild-type cells.

Our results showed that expression of Wild-type E-cadherin was sufficient to decrease both total PI3K and pPI3K levels. These findings prove that E-cadherin can affect PI3K signalling and in an inhibitory manner. Such influence is likely exerted with the contribution of the extracellular part of E-cadherin, as a single aminoacid change in this domain produced an intermediate signalling inhibition, whereas the intracellular mutation had the same effect as the Wild-type protein. These are novel findings, as no description on the relation of E-cadherin and PI3K was found in the literature.

AKT protein was included in our work for being a possible downstream target of PI3K. However, distinct results were obtained for AKT and pPI3K. In this context, we conclude that increased PI3K activation due to E-cadherin loss does not signal through AKT but other molecular mediators, in accordance with its signalling redundancy. Furthermore, the observed effects on pAKT may be achieved due to the influence of E-cadherin on other protein(s) and independently of PI3K. In this case, such influence has a stimulatory effect on AKT and requires fully functional E-cadherin. Interestingly, both missense mutations produced less AKT activation than the absence of the protein.

In conclusion, and although further experiments are needed, we hypothesize that E-cadherin impairment may ultimately lead to increased cell survival, an outcome favourable for tumourigenic progression, due to its inhibitory effect on PI3K signalling.

Effect of altered E-cadherin on expression and activation of several RTKs (EGFR, c-Met, ErbB2)

Interestingly, none of the membrane receptors and their active forms showed equal protein amounts in cells that only differ on the expression of E-cadherin (Mock vs. Wild-type cells), suggesting that E-cadherin has a clear influence on signalling modulation (Figure 20). The presence of Wild-type E-cadherin was sufficient to alter the expression but, most significantly, the activation status of the three RTKs studied. Mock cells expressed higher levels of ErbB2 and EGFR, and the increased expression of these proteins was accompanied by their increased activity. Furthermore, receptor activity of c-Met was also higher in Mock cells, despite them presenting significantly lower amounts of total protein.

Similarly to PI3K pathway results, clear differences were observed between the two mutated E-cadherin forms (Figure 20). The phosphorylation status of the three RTKs was equal in Wild-type and 2494 cells. By contrast, cells expressing the extracellular mutation either showed the same levels to those on Mock cells, or even higher (as was the case of pEGFR).

From our observations, we conclude that E-cadherin influences the RTKs studied in a negative way. In the same way as with the PI3K pathway, absence of E-cadherin was associated with increased RTKs signalling. These findings are in accordance with previous reports [55].

The mechanism by which such interactions are performed is not yet described. Although we obtained alterations in the amounts of both the total protein and the phosphorylated form for the several RTKs, we speculate that the primary effect of E-cadherin may be exerted on activation status of the receptor, in particular through direct interactions at the cell membrane. Currently, it is accepted that a physical binding between E-cadherin and these other membrane proteins may lead to receptor engagement. This would decrease the total amount of receptor free to bind to its respective ligand, and consequently decrease receptor activation. Most likely, such mechanism is dependent on the extracellular domain of E-cadherin. This conclusion arises from the fact that presence of an E-cadherin protein altered in a single aminoacid at the extracellular domain produced similar receptor activity to the one obtained in the absence of the E-cadherin, while no differences were observed between Wild-type and the intracellular mutated form.

Influence of E-cadherin mutations in a cell-matrix adhesion signalling pathway (FAK/β1-Integrin).

During carcinogenesis, for cells to invade, simultaneous disruption of cadherin-dependent cell junctions and induction of integrin-dependent cell motility should occur. Thus, a molecular crosstalk may take place between integrins and cadherins [84].

We investigated the possible effects of E-cadherin on β1-integrin and β1-integrin-dependent activation of intracellular signalling pathways [85], by studying the downstream target FAK.

Our results showed that expression of both β1-integrin and FAK was upregulated in the absence of E-cadherin (Figure 21). However, when we analyzed β1-integrin activation, we found that cells expressing Wild-type E-cadherin showed more abundant levels than Mock cells. By contrast to what was observed for phosphorylated β1-integrin, phosphorylated FAK amounts were higher in Mock cells.

As for the two missense mutations, some intriguing results were obtained (Figure 21). While in the 1018 cells, total expression of β1-integrin was more similar to Mock cells, β1-integrin activation was equal to Wild-type cells. In addition, 2494 cells presented even lower pβ1-integrin amounts than cells with the normal E-cadherin protein. As for FAK activation, it was intermediate in both mutated cell lines. Even though total FAK levels were lower, these cells presented lower pFAK than Mock cells but higher than Wild-type cells.

From these observations, we speculate that the increased expression of total β1-integrin on Mock cells may be a compensatory mechanism of the cell to overcome any functional consequence on β1-integrin and β1-integrin-mediated cell-matrix adhesion resulting from E-cadherin absence. Despite there being more β1-integrin, the protein may be performing less adequately.

This is supported by the opposite results obtained for activation of β1-integrin and FAK. We included FAK protein in our study for being one of the downstream targets of β1-integrin. In particular, FAK phosphorylation is reported to occur after β1-integrin binding. So, it was puzzling to obtain contradictory results for pFAK in relation to pβ1-integrin.

From our results and previous reports, we conclude that FAK activation is the central event. Cells that assume a more invasive and migratory phenotype might modulate their adhesive capacity and intracellular signalling activities by altering their integrin expression and affinity profiles. In terms of motility and invasion, Mock and E-cadherin mutated cells can be grouped together [110-112]. Regarding our results, the common

observation between these cell lines, in comparison to Wild-type cells, was their increased pFAK levels.

Thus, we speculate that in the presence of normal E-cadherin, β1-integrin may signal preferentially through other molecules rather than FAK or be more involved in cell-matrix adhesion and less in intracellular signalling. Once E-cadherin expression is lost (or with a less severe outcome, mutated E-cadherin is expressed), there is a reversal and intracellular signalling (through FAK phosphorylation) is increased [85], with consequent increase in cell motility.

However, at the moment, influence of another non-studied factor (also altered by E-cadherin) on FAK status and independently of β1-integrin can not be excluded.

Activity of MMP-2 and MMP-9 in the context of mutant forms of E-cadherin

To complete the invasive process, malignant cells need to perforate the surrounding extracellular basement membrane. Thus, the contribution of MMPs in degrading the surrounding matrix components during this process seems to be necessary [78].

Our results demonstrated that on cells expressing Wild-type E-cadherin, a decrease in substrate degradation by MMP-2 and MMP-9 was observed when compared to Mock cells (Figure 22). This result suggests that loss of E-cadherin can also be implicated in increased MMPs activation and consequent matrix degradation.

Again, different outcomes were observed for each E-cadherin mutation (Figure 22). MMPs activity in cells expressing the 1018 alteration was unchanged in relation to Mock cells. As for the intracellular mutation, divergent results were observed. MMP-2 activity was equal to Wild-type cells but MMP-9 was even less active in 2494 cells.

Similarly to our conclusions in the previous objectives, our observations suggest that any influence that E-cadherin exerts on MMPs is dependent on a normal extracellular portion. Thus, results obtained for 1018 cells are expectable. However, the same can not be said for 2494 cells. Although 2494 cells are slightly less invasive than Mock or 1018 cells [110,111], by no means are they comparable to Wild-type cells (Figure 23). One possible explanation is that other MMPs that degrade different matrix components (such as collagen type IV, laminin, and fibronectin) are upregulated in cells with the intracellular E-cadherin mutation.

Interestingly, integrins are also involved in regulating the activities of proteolytic enzymes that degrade the basement membrane, such as MMPs [73], and loss of β1-integrin attachment to laminin or collagen in the cellular matrix has been associated with increased stimulation of MMP-9 [86]. These reports further evidence the interconnection of signalling pathways and cellular outcomes possibly affected by E-cadherin.

From the comparison of Mock MDA-MB 435 cells used in this study as a model, and cells that differ only in the fact that they express Wild-type E-cadherin, several differences were observed. In this work, we show that the expression of normal E-cadherin was able to decrease RTKs signalling as well as signalling through PI3K pathway. In addition, the adhesion protein decreased MMPs activity, and changed the balance of downstream intracellular signalling dependent on β1-integrin and FAK.

Although the magnitude of the differences observed was relatively low, all biological processes studied were affected by E-cadherin. Thus, and despite such relatively effect, their potential contribution to tumourigenesis should not be undervalued.

Despite similar outcomes in terms of invasion, motility and overall malignancy, missense mutations localized in different domains of E-cadherin rendered divergent results in the signalling pathways analyzed. Results obtained for the extracellular mutation were comparable to the ones from Mock cells. In contrast, cells with the intracellular mutation behaved more similarly to Wild-type cells. We conclude that most of the E-cadherin's effects on the signalling pathways achieved through the involvement of the extracellular domain. This suggests that we may have found possible causes for malignancy associated with mutations localized at the extracellular domain of E-cadherin and these findings may be applied for clinical purposes. However, malignancy associated with intracellular mutations remains to be understood. It would be interesting to understand if these findings are applied to other missense mutations and their localization.

Conclusions

Overall, our findings demonstrate that E-cadherin has a broader role in the cell signalling rather than being a simple cell-cell adhesion molecule. Its function as a tumour suppressor may be more relevant than first attributed.

- E-cadherin inhibited PI3K signalling, most likely through its extracellular domain;

- Wild-type E-cadherin expression affected RTKs activation in a negative manner and the same result was observed for the intracellular E-cadherin mutation;

- The extracellular E-cadherin mutation was associated to an increase of c-Met, EGFR and ErbB2 phosphorylation and activity and to PI3K tyrosine phosphorilation;

- FAK-dependent intracellular signalling was increased in cells that do not express E-cadherin or that express E-cadherin mutated forms;

- MMP-2 and MMP-9 activity was increased in the absence of E-cadherin and in cells expressing the extracellular mutation;

- Despite similar outcomes in terms of invasion, and overall malignancy, missense mutations localized in different domains of E-cadherin rendered divergent results in the signalling pathways analyzed.

In conclusion, E-cadherin seems to influence cell attachment, survival and invasion, through modulation of intracellular signalling, and by affecting MMPs activity and cell-matrix interactions.

As future work, it is necessary to demonstrate to what extent these findings can occur at gastric carcinomas and in particular to gastric tumours where E-cadherin mutations and expression changes have been deeply implicated.

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