

**P-CADHERIN IN OVARIAN CANCER:
A NOVEL MARKER FOR DISEASE PROGRESSION**

by

ILA SAROJ PATEL

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Department of Obstetrics and Gynecology

The University of British Columbia
Vancouver, Canada

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Abstract

These studies have focused upon role(s) of the calcium-dependent cell adhesion molecules, known as the cadherins, in ovarian cancer. Recent studies have characterized one of the cadherins, E-cadherin, as a tumour suppressor gene. The normal human ovarian surface epithelium, as well as primary ovarian tumours, has been shown to express this cadherin subtype. Although E-cadherin expression levels are high in ovarian surface epithelial cells undergoing neoplastic transformation, there is a marked reduction in the expression levels of this cell adhesion molecule with progression to later stages of the disease state when the tumour cells acquire the ability to detach from the primary tumour. This in turn allows the cells to disseminate into the peritoneal cavity and subsequently interact with the mesothelial cells of the peritoneum. In our studies, we have found that P-cadherin is the predominant cadherin subtype present in ovarian tumor cell aggregates recovered from the ascites of patients. Interestingly, we have also determined that normal human peritoneal cells express P-cadherin which raises the possibility that this cell adhesion molecule plays an important role in the progression to the late stages of the disease state by mediating, at least in part, ovarian surface epithelial tumour-peritoneal cells interactions. We believe that these studies give us novel insight into the role(s) of the cadherins in ovarian cancer.

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List of Abbreviations

APC	Adenomatous polyposis coli
BSA	Bovine serum albumin
°C	Degrees centigrade
CaCl ₂	Calcium chloride
Cad	Cadherin
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine 3',5'-monophosphate
CAR	Cell adhesion recognition
CDNA	Complementary DNA
CP	Cytoplasmic
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
E2	17β-estradiol
EC	Extracellular
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetate
ER	Estrogen receptor
FSH	Follicle-stimulating hormone
Fn	Fibronectin
g	Grams
g	Gravity
h	Hours
HRE	Hormone responsive element
IPTG	Isopropyl-β-D-thiogalactoside
kb	kilobases
kDa	kiloDaltons
LH	Luteinizing hormone
MDCK	Madine Darby canine kidney
min	Minutes
ml	Milliliter
mM	Millimoler
MMP	Matrix metalloproteinase
Mr	Molecular weight
mRNA	Messenger RNA
μl	Microliter
μM	Micro-Molar
Na ⁺ ,K ⁺ -ATPase	Sodium, potassium-adenosine triphosphatase
nM	Nano-Molar
NaCl	Sodium chloride
P4	Progesterone
PBS	Phosphate-buffered saline

PMSF	Phenylmethyl sulfonyl fluoride
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RT-PCR	Reverse-transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SSPE	Standard saline phosphate-EDTA
TGF-β	Transforming growth factor-β
TM	Transmembrane
Tris-HCL	Tris (hydroxymethyl)-aminomethane-Hydrochloric acid

Introduction

Ovarian cancer is the fifth-most common malignancy in North American women and the most frequent cause of death from gynecological neoplasms (Landis, 1999). Whereas germ cell teratomas and granulosa-thecal cell tumors are the most frequent ovarian tumors in the prepubertal female, 80-90% of ovarian cancers in the adult originate from the surface epithelium (Dietl et al., 1993; Rao et al., 1991; Murdoch, 1996).

The early detection of ovarian cancer, when the tumor is confined to the ovary (Stage I) (Hamilton, 1992), has an excellent prognosis and is usually treated by surgery (Davis et al., 1996). Unfortunately, the disease is typically asymptomatic at these early stages, and the condition is therefore most often recognized at later stages (Stage II or later) when the tumor has disseminated within the peritoneal cavity (Friedlander, 1998; Chuaqui et al., 1998). Treatment approaches at these later stages are limited and are usually confined to the reduction of tumor bulk and treatment with cytotoxic agents or radiation (McGuire et al., 1998). The emergence of drug resistance, coupled with the spread of the tumor cells within the peritoneal cavity, leads to the extremely poor prognosis for these later stages of the disease state (Gajewski et al., 1998; McGuire et al., 1998). It has been estimated that less than 15% of patients with advanced disease survive beyond 5 years (Murdoch, 1996). A better understanding of the adhesive mechanism(s) which allows tumor cells to detach from the primary ovarian tumor mass and interact with the mesothelial cells of the peritoneum is likely to result in the identification of novel target genes for the development of cell based therapies.

Structure and Function of the Ovary

The ovary is comprised of 3 distinct cellular compartments: the ovarian surface epithelium, the cortex, and the medulla. The organisation of the ovary allows this dynamic organ to perform its two major functions, steroidogenesis and folliculogenesis.

Surface Epithelium

Ovarian surface cells have a mesodermal origin shared with the epithelia of the urogenital system and the adrenal cortex. During early embryonic development, mesoderm segregates into pluripotent mesenchyme and coelomic epithelium (peritoneal mesothelium) (Murdoch, 1995). In the adult, the ovarian surface epithelium (OSE) arises after invagination of the coelomic mesothelium over the embryonic gonadal ridge (Shoham, 1994). This single layer varies from squamous to cuboidal and low columnar mesothelial cells. With advancing age the cells tend to become flattened. The surface cells are supported by a basement membrane (the basal lamina), and by the tunica albuginea, a thin, fibrous, connective tissue layer. The ovarian surface epithelium is directly continuous with the flat mesothelial cells of the peritoneal cavity, and has a protective function. In addition to the contribution of pregranulosa cells during fetal development and its role in follicular maturation, the OSE in the adult has been shown to be involved in the transport of metabolites. OSE has also been shown to be responsive to changes in levels of gonadal steroids and are believed to mediate cell death (Murdoch, 1996). For example, in studies of rat OSE lysosomal hydrolases were released in response to prostaglandin $F_{2\alpha}$ (Murdoch, 1996), enzymes which may contribute to the breakdown of the stroma surrounding the preovulatory follicle (Kruk et al., 1992). Kruk

and Auersperg (1992) reported that in culture human OSE demonstrated the ability to physically remodel extracellular matrix, by matrix contraction, a process that can serve as a model for wound repair. This is of particular importance in ovulatory wound repair.

Cortex

The tunica albuginea separates the OSE from the ovarian cortex. The cortex makes-up more than 50% of the volume of the ovary in women of reproductive age. It contains the ovarian follicles at different stages of development, surrounded by the closely packed, spindle-shaped cells known as the cortical stroma. These cells give rise to ovarian stromal or interstitial lutein cells. These specialized cells surround growing follicles, and are then referred to as theca-lutein cells. The theca layer of the developing follicles contains a rich vascular plexus (Bychkov V., 1996), and is separated from the underlying granulosa cells by a basement membrane.

The cortex is also referred to as the outer, cellular zone. The ovary also contains an inner zone, called the medulla or the zona vasculosa. The medulla is composed of connective tissue and thick walled blood vessels. Surrounding the blood vessels are fibroblasts, mast cells and strands of smooth muscle cells. (Russell et al., 1989). Hilus cells are also located predominantly in medulla. They are morphologically identical to the testicular Leydig cells, and are found in close proximity to nerve fibres. Hilus cell clusters are more prominent during pregnancy and at menopause (Bychkov V., 1996; Russell et al., 1989).

Steroidogenesis

The major functions of the female gonad are: (1) oogenesis – the differentiation and release of the mature oocyte for fertilization and production of offspring (McGee et al., 2000); and (2) steroidogenesis. The ovary is a dynamic organ that undergoes a highly regulated series of cellular events that is initiated during fetal development and continues through adult life until the cessation of ovulation at menopause. The ovary produces steroidal sex hormones, estrogen, progesterone and the peptide hormone inhibin, which participate in the development of female secondary sexual characteristics, as well as the maintenance of pregnancy. Of the approximately 6 million oogonia initially formed during gestation, only 2 million are incorporated into primordial follicles – when a single layer of pregranulosa cells surround each oocyte (McGee and Hsueh, 2000). Depletion of the primordial follicles occurs throughout childhood, and at puberty approximately 400,000 remain. The number of primordial follicles continues to decrease progressively with time, with follicle depletion occurring at a rate of approximately 1000/month during the female reproductive years (Macklon and Fauser, 1998). Only 400 follicles are destined to ovulate. The majority of primordial follicles are found in the superficial cortex, immediately beneath the tunica albuginea (Russell and Bannatyne, 1989).

Complete follicle maturation takes at least 85 days (Gougeon, 1986; Macklon, 1998). Initiation of the growth of the primordial cycle occurs continuously, while the progression of some primordial follicles to the pre-antral and early antral stage occurs throughout the menstrual cycle. During recruitment there is a stimulation of a group of primordial follicles in response to gonadotrophins, primarily follicle-stimulating hormone

(FSH). This leads to initial oocyte enlargement (Russell and Bannatyne, 1989; Macklon et al., 1998; McGee and Hsueh, 2000). Approximately one week into the cycle a dominant follicle is selected, and begins to enlarge prior to ovulation, while the other previously recruited follicles undergo atresia. The dominant follicle continues to grow despite decreasing FSH levels, in part due to an increased sensitivity to FSH (Macklon and Fauser, 1998).

As the secondary follicle is being formed, the surrounding granulosa cells express receptors for FSH, estrogen and androgens. As the follicle grows, the connective-tissue cells, which surround the granulosa cells, differentiate into the outer, or theca, layer. The theca cells begin to express luteinizing-hormone (LH) receptors. The granulosa cells are the source for progesterone and estradiol; however, it is only with the cooperation of the theca cells, which produce androgens, that estrogen is produced. The acquisition of aromatase by the granulosa cells enables the conversion of the thecally produced androgens into estrogen (Falck, 1959; Zeleznik, 1993). The interactions between the theca cells and the granulosa cells have led to the 2 cell, two gonadotrophin theory (Armstrong and Dorrington, 1977; Macklon and Fauser, 1998). The secondary follicle also develops a fluid-filled cavity, the antrum. The antral fluid contains steroids, plasma and secreted proteins. In the selected dominant follicle, the follicular enlargement is mainly due to the expanding antrum. Estrogen secretion rises rapidly during the late follicular stage, which triggers the LH surge through a positive feedback loop between the ovary and the hypothalamus. The LH surge stimulates maturation of the oocyte, resumption of meiosis, ovulation, and the formation of the corpus luteum.

In the luteal phase the corpus luteum secretes estrogen, progesterone and inhibin, although progesterone dominates. In the absence of fertilisation, the corpus luteum lasts for approximately 14 days. It then undergoes involution atresia (Russell and Bannatyne, 1989).

The ovarian surface epithelial cells are believed to play a pivotal role in the process of ovulation. OSE cells excrete lysosomal bodies from their basal surfaces before ovulation. The enzymes contained in the lysosomal bodies are believed to participate in the breakdown of the tunica albuginea, and therefore in follicular rupture (Godwin et al., 1992). The OSE has also been shown to participate in the repair of the resulting wound, which occurs in the ovarian surface after ovulation (Auersperg et al., 1991). It is postulated that the OSE also participates in the production of a new tunica albuginea (Godwin et al., 1992). There is much speculation on the many different regulatory molecules that may influence OSE cells by autocrine, endocrine or paracrine mechanisms. For example, a paracrine mechanism exists in that the ovarian surface is bathed in follicular fluid at the time of follicular rupture (Godwin et al., 1992). Estrogen receptors have been detected in the normal rat ovarian surface epithelium (Adams et al., 1983), while follicular fluid itself contains estrogen, suggesting a role for this hormone to influence the growth and/or function of these cells. Estrogen receptors have also been detected in malignant ovarian tumors originating in the OSE (Hamilton et al., 1981;1983). However, there is little direct laboratory evidence that examines the role of

different hormones and growth factors that influence the OSE (Bast et al., 1992; Godwin et al., 1992).

Epithelial Ovarian Cancer

Epidemiology and Etiology

Epithelial ovarian cancer is the fifth leading cause of new cancer cases and accounts for 4% of all cancer in women. It is estimated that 1 in 55 women will develop ovarian cancer during her lifetime (Meisler, JG, 2000). Today there are considered to be two major groups at higher risk: nulliparous women and women with a familial history of gynaecological cancer (Meisler, JG, 2000).

The occurrence of familial ovarian cancer has been increasingly recognized (Perez et al., 1991; Shoham, 1994), and three distinct hereditary conditions are identified which predispose women to this disease (Lynch et al., 1981;1986; Shoham, 1994; Murdoch, 1996): 1) site-specific familial ovarian cancer syndrome, where women are at risk for developing ovarian cancer only; 2) breast/ovarian cancer syndrome (Lynch et al., 1978), where a woman has an increased risk of developing ovarian cancer if a first-degree relative (mother or sister) has a personal history of breast or ovarian cancer; and 3) Lynch type-II cancer family syndrome, characterized by ovarian cancer inherited together with non-polyposis colorectal and endometrial cancers (Lynch et al., 1985). For families affected with breast and ovarian cancer syndrome or site-specific ovarian cancer, mutation of the gene BRCA₁, which encodes a predicted protein which inhibits tumour

growth, has been linked to early-onset and a predisposition to these cancers (Godwin et al., 1994; Miki et al., 1994; Murdoch, 1996). Recent research suggests that women of Eastern European ancestry may also be at increased risk (Murdoch, 1996; Meisler, 2000). Age is one of the significant risk factors predisposing women to ovarian cancer. With age the incidence curve rises exponentially around the time of menopause (about age 45) and continues to climb until the late 70s, when the incidence begins to taper off (Meisler, 2000). In 1972 Fathalla, (Byers et al., 1992; Peluso et al., 1996) proposed the “incessant ovulation hypothesis” based on the observation that nulliparous women have a high incidence of ovarian cancer. The process of ovulation is associated with the development of non-genetically mediated ovarian cancer (Waleh et al., 1995; Meisler, 2000). More ovulatory cycles mean a greater risk (Tiedemann, 2000), and consequently not just nulliparity, but early menarche and late menopause are considered increased risk factors for ovarian cancer. Reducing the number of ovulatory cycles, through use of oral contraceptives, multiple pregnancies, or prolonged lactation appears to provide protection (Waleh et al., 1995; Tiedemann, 2000). For example, the longer a woman takes the oral contraceptive pill, the greater and more profound is the protection against ovarian cancer – anywhere from a 40% to 60% decrease in the risk of ovarian cancer (Meisler, 2000).

Neoplastic Transformation of OSE Cells

It has been postulated that ovarian cancers originate most frequently from OSE that have invaded the ovarian cortex and formed epithelial inclusion cysts (Hamilton, 1992; Auersperg et al., 1998). Alternatively, the development of these cysts could arise when surface epithelium is trapped as the wound, formed during follicular rupture, is repaired

(Hamilton, 1992; Auersperg et al., 1998). The cysts are lined with surface epithelial cells and are presumed to form as sequelae of ovulation (Cramer et al., 1983a; 1983b). These cellular structures are normally eliminated by apoptosis (Cramer et al., 1983b; Murdoch, 1996). Cysts could also be formed if the ovarian surface became remodelled over the area of a deep crypt (Hamilton, 1992; Auersperg et al., 1998). The persistence of inclusion cysts has been observed in patients with de novo ovarian cancer (Bell and Scully, 1994). Several epidemiological studies have implicated the ovulatory process as a key event in tumorigenesis, with a subsequent reparative proliferation of OSE in the vicinity of the ruptured follicle as the target site for gene distortions (Sundfeldt et al., 1997). Benign epithelium adjacent to borderline or malignant epithelium in a stage of transition, expressing specific histologic changes in ovarian tumors has been detected (Puls et al., 1992). These observations suggest that a number of aberrant steps are required in the formation of the malignant tumor (Murdoch, 1996; Sundfeldt et al., 1997). This proposal is supported by the high incidence of benign tumors observed early in life as compared with the malignant tumors that occur most frequently after the age of 50 years (Sundfeldt et al., 1997).

The formation of epithelial inclusion cysts clearly demonstrates a balance between OSE cell-cell adhesion and exposure of the cyst-encapsulated cells to a new tissue environment and potential stimuli. These stimuli may include elevated levels of gonadal steroids as well as growth factors such as the transforming growth factors TGF α , TGF β and basic fibroblast growth factor (bFGF) that are synthesized by ovarian stromal cells (Heintz et al., 1985; Whittemore et al., 1992;1993). We have shown that estrogens increase the expression of the

cell adhesion molecule, known as E-cadherin (E-cad), in mouse OSE (MacCalman et al., 1994). Epidermal growth factor and TGF α have been shown to decrease E-cad mediated adhesion through phosphorylation of one of the cadherin linker proteins, β -catenin (Bendell and Dorrington, 1990). bFGF signaling in rat ovarian granulosa cells has been shown to be modulated through N-cadherin (Sporn and Roberts, 1992). Hence the OSE in inclusion cysts would be exposed to a number of cadherin-modulating or adhesion-modifying agents that could result in their transformation to the invasive phenotype.

Disease Progression

There are four basic stages of disease advancement in ovarian cancer according to the system adopted by the International Federation of Gynaecologists and Obstetricians. The most important determinant of prognosis is tumor stage, but other factors affecting survival include the tumor's substage and cell type (Tiedemann, 2000). Stage I is characterized by the tumor being limited to one or both ovaries (IA and IB), with IC characterized by malignant cells extruded into the peritoneal cavity when an inclusion cyst ruptures. Peritoneal exposure to cancerous cells is what marks Stage II, with subclassifications depending on the degree of pelvic extension. Stage IIC marks the development of ascites fluid. Stage III is characterized by the development of tumors involving one or both ovaries with peritoneal implants outside the pelvis (Murdoch, 1996). By Stage IV there is a tumor of one or both ovaries with distant metastases. The cancer has spread to organs outside of the peritoneal cavity, such as the liver or lungs.

The main route of metastatic dissemination of epithelial ovarian cancer is believed to be by the exfoliation of the tumor cells (Davies, 1998). The cells then migrate, implant and invade throughout the peritoneal cavity. The molecular mechanisms that underlie this process are as yet poorly characterised. However, it has been suggested that specific cell adhesion molecules must mediate the interaction between the ovarian cancer cells and the peritoneal mesothelium (Davies, 1997; Zhang et al., 1999). Recent research has been focused on the role of the cell adhesion molecules cadherins and their role in the neoplastic transformation of epithelial cells. The loss of cadherin-mediated adhesion is believed to be one of the main factors leading to the reduced cell-cell adhesion characteristics of tumor cells, and may play a pivotal role in the acquisition of the invasive and metastatic potential of these cells (Darai et al., 1997).

The Cadherins

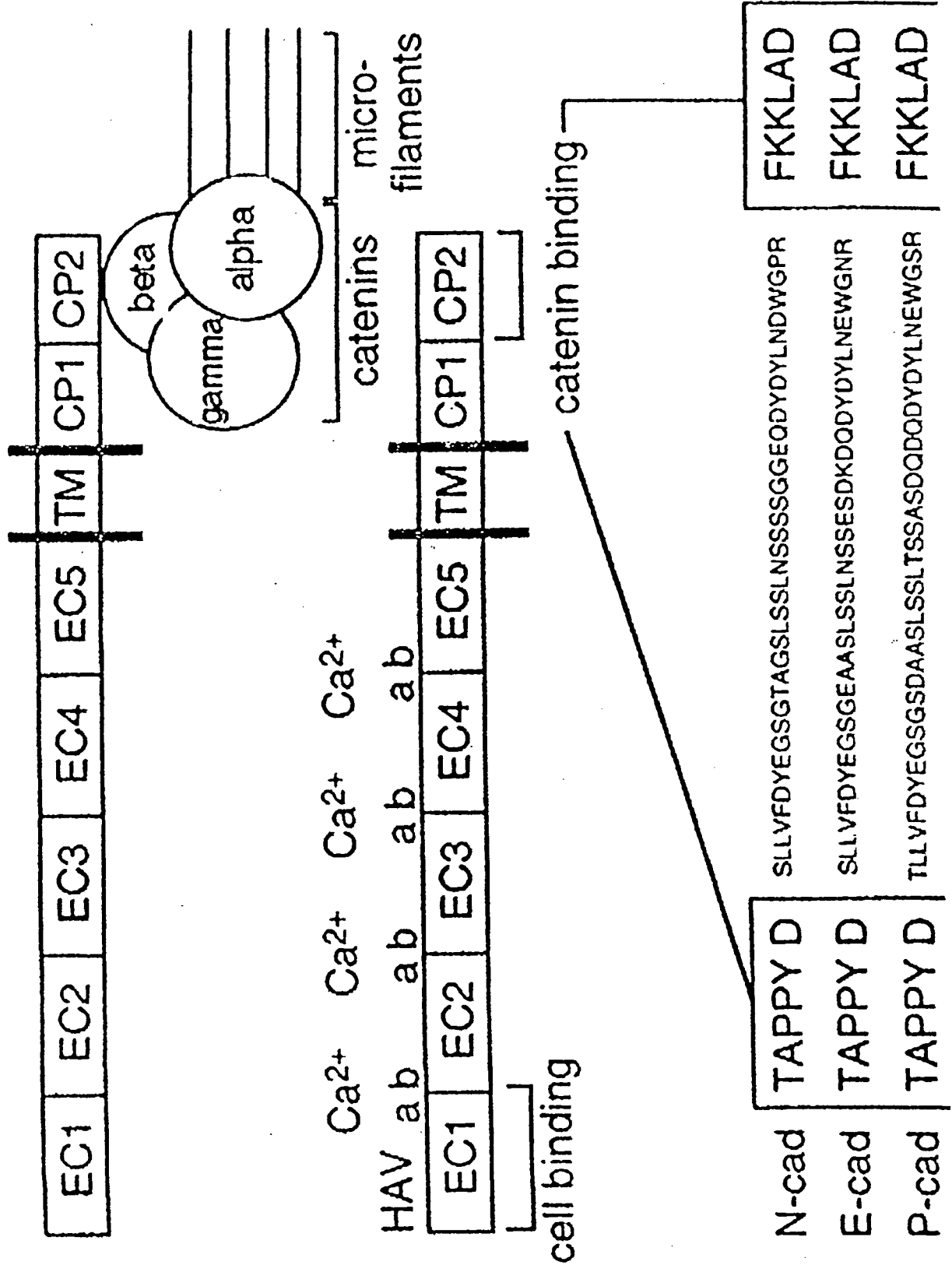
The cadherins constitute an expanding family of integral transmembrane glycoproteins that mediate calcium-dependent cell adhesion in a homophilic manner (Knudsen et al., 1995; Takeichi, 1991; 1995). The cadherin family is comprised of several distinct groups based on amino acid sequence comparisons and structural features. These include the classical cadherins (type 1 and type 2); desmosomal cadherins, (cadherins associated with cell-cell junctions such as desmocollins, desmogleins); protocadherins; truncated cadherins and other unclassified sub-family members (Suzuki, 1996; Peluso, 1997; Potter et al., 1999).

Type 1 Classical Cadherins

The type 1 classical cadherins contain the 3 initially identified cadherins E-cadherin (E-cad), P-cadherin (P-cad), and N-cadherin (N-cad), as well as R-cadherin or cad-4. They are named after the tissues where they were found prominently expressed (Hatta et al., 1988; Suzuki et al., 1991; Potter et al., 1999). The classical cadherins are composed of an amino-terminal extracellular region including five repeated subdomains, a single transmembrane domain, and two cytoplasmic domains (Geiger, 1992; Takeichi, 1995; Potter et al., 1999;) (Fig 1). The first four extracellular domains contain the calcium binding regions, while the first extracellular domain contains the cell adhesion recognition (CAR) amino acid sequence, His Ala Val (HAV) (Blaschuk, 1990). This first extracellular domain has been implicated in selective cadherin binding. The nonconserved amino acid residues immediately adjacent to the CAR sequence modulate the ability of the cadherins to interact with one another in a homotypic manner (Nose et al., 1990; MacCalman et al., 1997). The intracellular cytoplasmic domain of the classical cadherins is highly conserved, and associates with at least four intracellular proteins known as the catenins.

Figure 1. Schematic representation of the basic cadherin structure. The cadherins are comprised of five extracellular subdomains (EC1-EC5), a single transmembrane domain (TM) and 2 cytoplasmic domains (CP1 and CP2). The first four extracellular domains contain the acidic motifs, DXNDN and DXD (a and b), which have been shown to bind calcium. The CAR sequence, HAV, is present in the most distal extracellular domain (EC1) of classical cadherins.

In order to identify the cadherin subtypes present in the peritoneum, ovarian tumor masses, or ascites tumor cells, degenerate oligonucleotides encoding the amino acids present in two conserved regions of the cytoplasmic domain were prepared and used as primers in RT-PCR reactions. The amino acid sequences of the two conserved regions (boxed areas) and the intervening region are represented by the single letter amino acid code.



Interactions Between the Classical Cadherins and the Catenins

The catenins, α -, β -, γ -, and p120^{ctn}, were initially identified by coimmunoprecipitation with E- and N-cadherin (Knudsen et al., 1995). The adhesive function of adherens junctions is dependent on the interactions between members of the cadherin superfamily and these cytoplasmic proteins (Miller and Moon, 1996). The catenins are regarded as important intracellular regulators of cadherin-mediated cell adhesion. These cell adhesion molecules cannot promote cell adhesion unless they are complexed with the catenins (Nagafuchi et al., 1991; Shimoyama et al., 1991; Blaschuk et al., 1994).

β -, γ -, and p120 catenin are classified as members of the family of proteins containing the armadillo repeat domains. Catenins share a conserved binding domain that allows for direct interaction with the cadherins. β -catenin binds directly with the cytoplasmic domain of the cadherins, and forms a complex with α -catenin (Nagafuchi et al., 1991; Kemler, 1993; Miller and Moon, 1996). γ -catenin, or plakoglobin, plays a similar role to β -catenin in regulating cadherin adhesive activity in adherens junctions, and also modulates the activity of desmosomal cadherins (Gumbiner, 1996; Miller and Moon, 1996). P120 catenin, originally identified as a substrate of Src and several receptor tyrosine kinases, also interacts with the cadherin- β -catenin complex and may play a part in regulating the adhesive functions of the cadherins (Miller and Moon, 1996). Recent studies indicate that the formation and localization of the two types of complexes are differentially regulated during cellular differentiation and development. Complexes containing β -catenin are more abundant in poorly differentiated cells while the number of complexes containing γ -catenin increase with cellular differentiation (Ranscht, 1994;

Butz and Larue, 1995). Furthermore γ -catenin could not be substituted for β -catenin during the early embryogenic development of β -catenin null mice (Haegal et al., 1996). Taken together, these studies indicate that these two cadherin-catenin complexes mediate the formation of two structurally and functionally distinct membrane domains.

α -catenin is an actin-binding protein, similar to vinculin, which interacts with β - and γ -cadherin-catenin complexes and links them to the actin-based cytoskeleton (MacCalman et al., 1997; Ozawa and Kemler, 1992). $p120^{ctn}$ is not present in α -catenin immunoprecipitates and is therefore not believed to link the cadherins to the cytoskeleton, indicating a fundamentally different role from that of other catenins (Daniel and Reynolds, 1995; Thoreson et al., 2000). It binds to the juxtamembrane domain of the cadherin cytoplasmic tail, distinct from the classical cadherin-catenin binding site (Gumbiner, 2000). $p120^{ctn}$ has acted as an inhibitor of cadherin-mediated adhesion in some cell types, possibly due to a deletion of the cadherin juxtamembrane domain (Gumbiner, 2000). The phosphorylation of β -catenin and γ -catenin reduces their association with the cadherin-catenin complex, resulting in the disassembly of intercellular junctions and a decrease in cell-cell adhesion. For example, the disruption of β -catenin genes in mouse embryos results in detachment of ectodermal cells in the embryo (Haegal et al., 1995). The expression of a truncated β -catenin leads to a loss of cell adhesion in human cancer cell lines (Oyama et al., 1994). The catenins are not limited to their roles in cell-cell adhesion. β -catenin has been shown to be an integral part of the Wnt signaling pathway, which determines cell fate during development (Miller and Moon, 1996). Activation of the wnt signaling pathway leads to increased

levels of β -catenin or γ -catenin/plakoglobin. This has been found to promote the formation of the cadherin-catenin complex at the plasma membrane, and therefore to enhance cadherin-mediated cell adhesion in some cell lines (Gumbiner, 2000). Furthermore, adenomatous polyposis coli protein (APC), a tumor suppressor protein found in discrete regions of the cell cortex, interacts with β - and γ -catenin/plakoglobin, but not the cadherins or p120^{cas} (Daniel and Reynolds, 1995). These binding interactions may modulate catenin stability and possibly reduce the availability of catenins for interactions with the cadherins, affecting cell adhesion and migration (Nathke et al., 1994; Miller and Moon, 1996).

Cell biology of Type I Cadherins

Type I cadherins have been implicated in key morphoregulatory processes, from tissue morphogenesis, cell migration, differentiation and the subsequent formation of tissues (Gumbiner, 1996; Takeichi, 1991; 1995; Steinberg and McNutt, 1999). A number of examples exist which demonstrate a relationship between specific differentiated cell types and the expression of specific subtypes. Cadherin subtype expression is correlated with the terminal differentiation of many different cell types, including bone, nerve, skeletal muscle, and epithelia (Steinberg and McNutt, 1999). The spatiotemporal expression of the cadherin subtypes is highly regulated during development. One of the earliest observations relating to cadherins demonstrated that when populations of cells expressing different cadherins were mixed, cells progressively sorted out into distinct subpopulations that had the same cadherin (Nose et al., 1986; Yap, 1998). Embryonic cells displaying different classical cadherins segregate from one another and it is believed

that these CAMs provide the molecular basis for the sorting of discrete populations of cells and the subsequent formation of tissues (Takeichi, 1995). For example, progenitor cells in the embryonic ectoderm that are destined to differentiate into the neural tube switch gradually cease to express E-cadherin and begin to express N-cadherin (Thiery et al., 1984; Nose et al., 1986; Takeichi, 1988). The switch in cadherin subtype expression allows the cells that are expressing N-cad to separate from the ectodermal cell layer and subsequently form these neural structures. Differential cadherin expression promotes cell sorting during folliculogenesis and spermatogenesis (Blaschuk et al., repro paper). For example, N-cadherin mediates Sertoli cell-germ cell adhesion and its expression decreases as the germ cells differentiate (Byers et al., 1993;1994; MacCalman et al., 1998).

The type 1 cadherins have been localized to different tissues in the adult mouse (Takeichi, 1988). In general, E-cad is expressed by epithelial cells, but not most muscle or neural cells. In contrast, N-cad has been localized primarily to cells of muscle and neural origin. P-cad is expressed in both the mouse placenta and decidua (Nose and Takeichi, 1986; Kadokawa et al., 1989), first appearing in developing mouse embryos in the extraembryonic ectoderm and the visceral endoderm at the egg cylinder stage (Nose and Takeichi, 1986), as well as in several epithelial cell types (Nose and Takeichi, 1986; Radice et al., 1997b). R-cad is expressed at later stages of organogenesis in several tissues including the eye, brain, skeletal muscle, kidney, pancreas and the gastrointestinal tract (Inoue et al., 1997). N-cadherin is predominantly expressed in neural tissues, but can also play an essential role in the formation of strong cell-cell contacts, which allow

the cells in cardiac muscle to beat co-ordinately (Knudsen and Wheelock, 1992; Islam et al., 1996). Mesothelial cells, lens epithelial cells and fibroblasts also express N-cadherin. E-cad is expressed in virtually all epithelial cells. P-cadherin is found in epithelial tissues, including lung epithelia, basal cells of the skin, and myoepithelial cells of the mammary glands (Peralta Soler et al., 1997a). These tissue distributions are maintained in the adult human with the major exception being that P-cad is not expressed in the human placenta (Shimoyama et al., 1989; 1991; MacCalman et al., 1997).

Classical cadherins mediate the formation of the intercellular junctional complex in epithelial cells, which includes tight, adherens, desmosomal, and gap junctions, before themselves being localized to the membrane domain of adherens junctions (Gumbiner et al., 1988; Marrs et al., 1995). Adherens junctions are prominent in the epithelia of cells and are critical to the function of the normal epithelium (Knudsen et al., 1995). In particular, E-cadherin-mediated adhesion appears to be essential for normal epithelial cell polarization and the formation of junctions (Birchmeier and Behrens, 1994). Antibodies generated against E-cad disrupt the formation of tight junctions, desmosomes and gap junctions in Madine Darby canine kidney (MDCK) cells (Gumbiner et al., 1988) and keratinocytes (Wheelock and Jensen, 1992), respectively.

The cadherins are responsible for the establishment of epithelial cell polarity (McNeill et al., 1990). In particular, E-cad has been shown to regulate the distribution of membrane-associated proteins within the plane of the epithelial cell plasma membrane. For example, E-cad restricts the distribution of the sodium, potassium-adenosine

triphosphatase (Na^+ , K^+ - ATPase) to the basolateral domain of polarised epithelial cells, including the cells of the mural trophectoderm in the mammalian blastocyst (McNeill et al., 1990; Watson et al., 1990). The interaction between E-cad and the Na^+ , K^+ - ATPase may, at least in part, explain why E-cad deficient embryos fail to form a normal blastocoel in utero (Larue et al., 1994). Collectively, these observations suggest that E-cad mediates an early adhesive event, which serves as a prerequisite for the recruitment of components of intercellular junctions, and membrane complexes that are involved in maintaining the differentiated state of epithelial cells. Further evidence to support the hypothesis that E-cad can act as an inducer of cell surface polarity was obtained by transfecting fibroblasts with a full-length E-cad cDNA. In the transfected cells, Na^+ K^+ -ATPase and cytoskeletal component, known as fodrin, become localised to areas of cell-cell contacts, which resembled the expression pattern in the polarised epithelial cells. In contrast, Na^+ K^+ -ATPase was not redistributed in fibroblasts transfected with truncated E-cad lacking the catenin-binding region (McNeill et al. 1990). In view of these observations, it has been proposed that the homophilic binding of E-cad on adjacent fibroblasts initiated a cascade of molecular events that result in the assembly of a membrane complex composed of E-cad and Na^+ K^+ -ATPase, which in turn was linked to the cytoskeleton by fodrin (McNeill et al., 1990).

The coordinated expression of different classical cadherin subtypes is essential for the maintenance of tissue integrity. For example, a monolayer of MDCK cells cultured in the presence of function-perturbing antibodies specific for E-cad, disassemble their intercellular junctional complexes and acquire a motile and invasive fibroblast-like

phenotype (Behrens et al., 1989). Furthermore, the transfection of full-length E-cad cDNA into invasive human carcinoma cells not only decreases the invasive potential of these cancer cells but also induces a reversion to the polarized epithelial cell state (Frixen et al., 1991). The importance of cadherin expression in the maintenance of tissue integrity becomes apparent in the study of tumor development when the expression levels of these CAMs are frequently observed to be reduced or absent.

Type 2 Classical Cadherins

The type 2 classical cadherin subfamily includes human cadherin-5, -6, -8, -11, -12 and -14 (Suzuki et al., 1990; Tanihara et al., 1994; Shibata et al., 1997) as well as other cadherin subtypes identified in the rodent (Korematsu and Redies, 1997), chicken (Nakagawa and Takeichi, 1995) and *Xenopus* (Espeseth et al., 1995). Type 2 cadherins are structurally similar to the classical cadherins, however there is low overall amino acid homology between the two cadherin subfamilies (Suzuki et al., 1990; Tanihara et al., 1994; Takeichi, 1995). In particular, type II cadherins do not contain the cell adhesion recognition (CAR) sequence, HAV, highly conserved among the classical cadherins. Although the CAR sequence for type 2 cadherins has not been determined, these CAMs can mediate calcium dependent cell-cell adhesion (Kimura et al., 1995; Nakagawa et al., 1997). Type 2 classical cadherins contain the highly conserved cytoplasmic domains specific for most cadherins, domains that contain the amino acid sequences which comprise the catenin binding regions. Shibata et al (1996) have shown that type 2 cadherin-11 interacts with β -catenin *in vitro*. Pishvaian et al (1999) demonstrated that

cadherin-11 forms complexes containing both α - and β -catenin *in vivo* in their studies involving breast cancer cell lines.

Cell Biology of Type 2 Cadherins

Although the function and patterns of expression of type 1 cadherins has been studied extensively, the cell biology of the type 2 classical cadherins continues to be poorly understood. However, there is increasing evidence to suggest that these CAMs play a central role in tissue morphogenesis. For example, cad-11 expression has been associated with bone formation in the mouse and human (Okazaki et al. 1994; Cheng et al. 1998) and the formation and organization of the human placenta (MacCalman et al., 1996;1997). Cadherin-11 is also expressed in cells differentiating into a variety of mesenchymal tissue in the mouse embryo (Kimura et al., 1995; Takeichi, 1995). In addition, cadherin-6, (cad-6) is believed to play a central role in the formation of the human and rodent kidney. The spatiotemporal expression of three type 2 classical cadherins, cad-6, cad-11, and cadherin-8 in the embryonic mouse brain provides further evidence to suggest that this cadherin subfamily may play specific role(s) in the formation and organization of the central nervous system during development (Redies and Takeichi 1996; Inoue et al. 1997; Korematsu and Redies 1997;).

Similar to the type 1 classical cadherins, the expression of type 2 cadherins appears to be complementary during cellular differentiation. For example, cad-6 mRNA levels decrease and the levels of the cad-11 mRNA transcript increase in human granulosa cells

undergoing spontaneous luteinisation in culture (MacCalman et al. 1997b). Similarly, cad-6 is present in undifferentiated human myoblasts but not in myotubes (Symonds et al. 1996). It has also been suggested that reciprocal expression of E-cadherin and cadherin-11 may play a role in trophoblast-endometrial cell interactions during implantation of the mammalian embryo (MacCalman et al., 1996). There is a marked increase in cad-11 mRNA levels following the formation of the terminally differentiated myotube. To date, the roles of type 2 cadherins in these developmental processes have not been elucidated. However, recent studies indicate that type 2 cadherins are capable of mediating cell sorting in the *Xenopus* embryo (Hadeball et al. 1998).

Cadherins, Catenins, and Cancer

Cell adhesion is believed to be critical in the dynamic process involving tissue morphogenesis in embryos and in the maintenance of complex differentiated tissues in adult organisms (Pignatelli, 1998). Cancer is characterized by disturbances in the orderly patterning of cells within tissues, and it has therefore been postulated that aberrant morphogenetic processes may play important roles in tumor biology, leading to an increasing number of studies on cadherin cell adhesion molecules and their contribution to tumor progression (Yap, 1998).

E-cadherin is likely the best characterized cadherin and the subject of a number of research studies in an attempt to understand its role in epithelial cancer. It has been

postulated that in terms of epithelial oncogenesis, dysfunction of cadherin-mediated adhesion is a significant factor leading to the reduced cell-cell adhesion characteristic of tumor cells and the acquisition of invasive and metastatic properties by neoplastic epithelial cells (Darai et al., 1997). Loss of E-cad expression and/or function has been associated with the neoplastic transformation of epithelial cells in vivo (Birchmeier et al., 1994; Blaschuk et al., 1994). A reduction in cadherin expression correlates strongly with invasiveness and metastasis. For example, in a study on colon carcinoma, during the process of malignant transformation epithelial cells became apolar and highly invasive when E-cadherin function was lost (Munroe, 1995). In general, poorly differentiated, metastatic human carcinomas frequently contain either reduced or undetectable levels of E-cadherin (Mareel et al., 1994; Blaschuk et al., 1995). Taken together, these studies demonstrate that E-cadherin serves as a regulator of the differentiated, non-invasive epithelial cell state (Blaschuk et al., 1994;1995). These observations have led to the hypothesis that E-cad is the product of a tumor suppressor gene (Birchmeier et al., 1991). The mechanisms by which E-cadherin negative tumor cells escape apoptosis remains to be determined.

Recently the consequence of inappropriate expression of N-cad in an E-cad expressing squamous epithelial cell line has been examined (Blaschuk et al., 1994; Islam et al., 1996). Transfection of these cells with N-cad resulted in a phenotypic transformation, from an epithelial to a loosely-aggregated fibroblastic morphology. In addition, E-cad expression was significantly reduced. The screening of a number of squamous epithelial cell carcinomas of the head and neck revealed that those tumors that expressed N-cad were

invasive (Islam et al., 1996). P-cadherin has been found to be expressed in a subset of high grade breast carcinomas, which were also found to have reduced E-cadherin expression (Palacios et al., 1995). The presence of P-cadherin in breast cancer tissue has been correlated with poor patient survival (Peralta Soler et al., 1999). P-cadherin deficient mice are subject to increased hyperplasia and dysplasia of the mammary epithelium with age (Radice et al., 1997b). These findings suggest that inappropriate cadherin expression in epithelia can affect native cadherin expression, allow for phenotypic alteration, and promote the acquisition and progression to the neoplastic state.

E-cad gene mutations, which result in the expression of non-functional isoforms, have been identified in human carcinomas (Birchmeier et al., 1991; Islam et al., 1996). In addition, the loss of or mutation in the genes for the catenin proteins also compromise cadherin-mediated adhesion. Alterations in the expression of α -, β -, γ -catenin, and p120ctn have also been described in several human carcinoma cells, (Sommers et al., 1994; Rimm et al., 1995b; Hiscox and Jiang, 1997; Dillon et al., 1998). In particular, studies in melanoma, colon and prostate cancer have found mutations in the regulatory region of β -catenin (Morin, 1999). This suggests a common target in the down-regulation of E-cad containing complexes as a characteristic of cellular invasion. Studies in gastric cancers, (Jawhari et al., 1997), and prostate cancers (Richmond et al., 1997) have shown that E-cadherin and β -catenin appear to be prognostic markers.

Type 2 cadherins also appear to play a role in tumorigenesis and metastasis. For example, cad-11 is expressed in gastric and renal cancer cell lines (Shibata et al., 1996; Shimazui et

al., 1996), and it has been proposed that this type 2 cadherin mediates interactions between signet cell carcinoma and the underlying stroma during invasion (Shimazui et al., 1996). Cad-11 is also expressed in the most invasive breast cancer cell lines, but not in the noninvasive cell lines, suggesting it may play a significant role in facilitating tumor cell invasion and the formation of metastatic tumors (Pishvaian et al., 1999). Cad-6 has been found in a number of human carcinoma cell lines, and in particular in the kidney (Shimazui et al., 1996). It has been suggested that this CAM may play a role in the neoplastic transformation of renal cells, and could serve as a cellular marker for progression of these tumors (Shimazui et al., 1996).

Cadherins, Folliculogenesis, and Ovarian Cancer.

The hormone-mediated transformations that occur in the ovary present challenges for the maintenance of tissue integrity. The OSE that surrounds a mature follicle approaching ovulation will experience the disassembly of intercellular junctions. Cadherin expression patterns have been shown to be tightly regulated during folliculogenesis. In particular, E-, N-, P-cad, cad-11 and cad-6 are spatiotemporally expressed in the oocyte, theca and granulosa cells of the rat ovary (Machell et al., 2000; summarized in Fig 2), suggesting that these CAMs play an integral role in the formation and organization of the developing ovarian follicle (MacCalman et al., 1994; 1995; 1998). Granulosa cells in healthy follicles have been shown to express N-cad and cad-6 (Peluso, 1996; MacCalman et al., 1997a). As the follicle undergoes atresia the number of junctions decrease and the

granulosa cells dissociate. In an associated developmental process N-cad expression increases in granulosa cells undergoing luteinisation (MacCalman et al., 1997a).

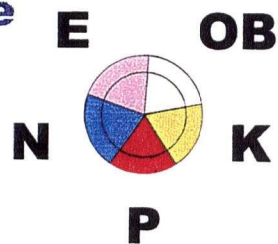
Figure 2: Localisation of the cadherins in the developing ovarian follicle.

Key: E: E-cad; N: N-cad; P: P-cad; K: cad-6; OB: cad-11.

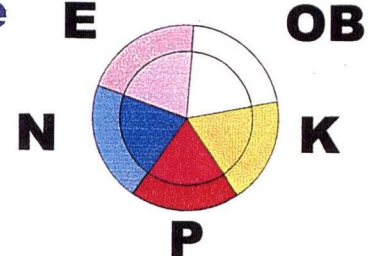
The central circle corresponds to the oocyte plasma membrane (oolemma) and the outer circle to the basement membrane of the follicle (encircling the follicular granulosa cells). The intensity of the color shading corresponds to the intensity of the immunostaining observed. Note that E-cadherin is expressed initially in primordial follicles (oocyte and granulosa) but diminishes with follicular growth and is absent from the follicle at the antral stage. N-cadherin is maintained in the follicular cells and oocyte throughout follicular development as is cad-6. Cad-6, however, diminishes in intensity in the preantral-antral transition in the granulosa cells, which is consistent with the absence of cell proliferation and antrum formation at this stage. P-cad is expressed in follicles and oocytes at the early stages but is excluded from the oocyte at later stages of follicle development. Note the complete absence of cad-11 staining in the follicular compartment (it is expressed in the theca interstitium).

Summary of Follicular Cadherin Expression

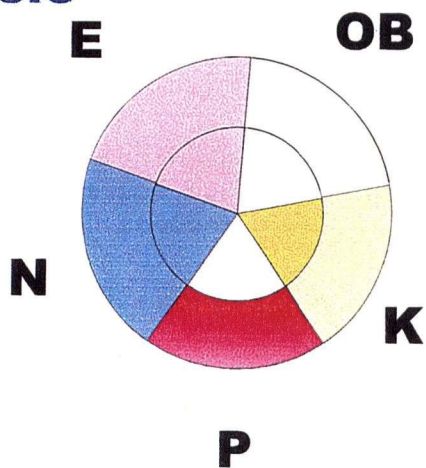
Primordial Follicle



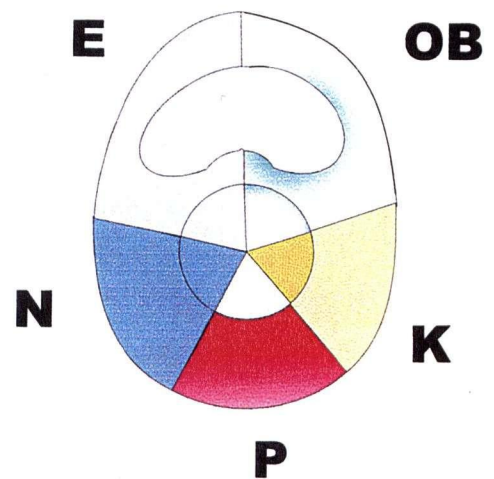
Small Primary Follicle



Large Primary Follicle



Antral Follicle



In contrast, low levels of E-cadherin have been detected in normal ovarian surface epithelial cells (Maines-Bandiera and Auersperg., 1997; Inoue et al., 1992; Veatch et al., 1994). In the normal ovary, Sundfelt et al., (1997), found that E-cad expression was restricted to the epithelial cells lining inclusion cysts and in epithelial invaginations present in the ovarian stroma. This is of interest as several studies have implicated the ovulatory process and subsequent proliferation of OSE involved in wound repair as key events in tumorigenesis. N-cad has also been detected in epithelial ovarian cells (Peralta Soler et al., 1995; Darai et al., 1997). The expression of both E- and N-cad is restricted to the more cuboidal and columnar shaped OSE cells. It has been suggested that N-cadherin appears to maintain the epithelial integrity of the OSE, while E-cadherin regulates cell shape and inter-relationships (Davies et al., 1997; Maines-Bandiera and Auersperg, 1997; Wong et al., 1999). α - and β -catenins have also been detected around the lateral membranes of normal OSE, as well as in epithelial inclusion cysts (Davies et al., 1997). Collectively these studies suggest that cadherins and catenins play important roles in the development and functioning of the normal ovary.

In contrast to studies that suggest E-cad as a general tumor suppressor, E-cad expression appears to increase with the neoplastic transformation of ovarian surface epithelium (Veatch et al, 1994; Darai et al., 1997; Peralta Soler et al., 1997b; Wong et al., 1999). E-cad expression has been observed in ovarian epithelial cells in tumours ranging from benign adenomas to undifferentiated adenocarcinomas *in vivo* (Sundfeldt et al., 1997). Analysis of cell lines derived from human OSE demonstrates an indirect correlation between E-cad expression and the invasive capacity of these cells *in vitro* (Auersperg et

al., 1998). The expression of E-cad and N-cad in ovarian carcinomas has not been correlated. However, a change in E-cad expression most often occurs in the early stages of disease progression, from benign to borderline lesions, while alterations in N-cad expression appear to be detectable in the transition from borderline to malignant tumours (Darai et al., 1997). Similarly, the metastatic lesions of advanced ovarian cancers contain reduced levels of E-cad as well as α - and β -catenin mRNAs, although mRNA transcripts encoding these molecules are present in the primary tumor (Mareel et al., 1992; Fujimoto et al., 1997). A reduction or complete loss of α - and β -catenin proteins in some ovarian adenocarcinomas has also been observed (Davies et al., 1997).

As has been previously discussed there are two types of tumor cell populations present in ovarian cancer: free-floating tumor cells in the ascites and solid tumor masses (Veatch et al., 1994). The ascites tumor cells are morphologically distinct and are considered highly malignant. Behrens et al., (1992) noted that in order for tumor cells to migrate and invade, they must detach from the primary tumor site, severing intercellular adhesion forces. In correlation with other results concerning the inverse expression of E-cadherin and cell invasiveness, a reduced E-cadherin expression has been detected in the ascites tumor cells compared to the cells present in the solid tumor. In general, it has been postulated that the loss of cadherin-mediated adhesion, resulting from either the loss of the cadherin or catenin, may be the underlying cause for the acquisition of invasiveness and metastatic potential.

Rationale

Taken together, these observations suggest that E-cad plays a key role in the neoplastic transformation of human epithelial cells, including human OSE, and that a reduction in the expression of this CAM promotes the metastatic potential of these cells. Epithelial ovarian cancers increase in size, begin to invade, and metastasize mainly in the peritoneal cavity. Although Veatch et al., in 1994, speculated that the loss of E-cadherin could be associated with metastatic and invasive capacity of the tumour cells, the role(s) of the cadherins in the progression to the late stages of the disease state, when the tumor cells have spread within the peritoneal cavity have not been determined. To address these outstanding issues, we have undertaken a comprehensive examination of the cadherin subtypes present in normal peritoneum and primary ovarian mass, ascites tumor cells obtained from women diagnosed with stage I or stage II ovarian carcinoma. In addition, we hypothesized that the expression of the predominant cadherin subtype(s) present in primary tumor masses and/or ascitic tumor cell aggregates can serve as cellular marker(s) for tumor progression in ovarian carcinoma. We further propose that this cadherin mediates, at least in part, interactions between tumor cells and peritoneal cells in a homophilic manner. In the event of the latter, a novel therapeutic approach to late stage ovarian cancer may be uncovered.

Research Accomplished

Tissues

Specimens of ovarian tumor mass, ascites fluid, and normal peritoneum were collected from women diagnosed with stage I or stage II primary ovarian carcinoma. The tissues used in these experiments were obtained with the approval of the Committees for Ethical Review of Research involving Human Subjects, University of British Columbia, and McGill University. The stages of clinical disease were established according to the criteria of the International Federation of Gynecologists and Obstetricians (FIGO).

Materials and Methods

RT-PCR

Total RNA was prepared from specimens of ovarian tumor mass, ascites fluid, and normal peritoneum obtained from women diagnosed with stage I (n=2) or stage II primary ovarian carcinoma (n=2) by the phenol-chloroform method of Chomczynski and Sacchi (1987).

The reverse transcription-polymerase chain reaction (RT-PCR) was performed using the method described by Suzuki et al. (1990). Degenerate oligonucleotides corresponding to two conserved regions in the cytoplasmic using degenerate oligonucleotides encoding

amino acid sequences that are conserved among all of the known cadherins were used as primers (see Fig. 1). The sequences of these oligonucleotides are as follows: Forward primer 5'-GAATTCACNGCNCCNCCNTAYGA-3', Reverse Primer 5'-GAATTCCTCNGCNARYTTYTTAAR-3'; where R is either A or G, Y is either C or T and N is either A, C, G, or T. Template cDNAs were synthesized from the total RNA extracted from the peritoneum, ovarian tumor masses and ascites tumor cells. Briefly, the RT-PCR reaction mixture contained 24.5 μ l RNase-free water, 4 μ l dNTP mix (10 mM, Gibco BRL, Life Technologies, Rockville, MD), 2.5 μ l DTT solution (0.1 M, Gibco BRL), 1 μ l Rnasin[®] RNase inhibitor (Gibco BRL), 1 μ l each of downstream and upstream primers, and 5 μ l template RNA, 10 μ l 5x RT-PCR buffer, 1 μ l (200 units) of Moloney Murine Leukemia Virus – Reverse Transcriptase (MMLV-RT, Gibco BRL), and 1 μ l of Taq DNA polymerase (Gibco BRL). These components were added to a 0.2 ml PCR tube on ice, mixed, and centrifuged briefly prior to incubation for 30 minutes at 50°C. The cycling program used consisted of denaturation at 95°C for 1 minute, annealing at 55°C for 2 minutes, and polymerization at 68°C for 3 minutes. This program was repeated for 35 cycles.

Isolation and Amplification of Cadherin cDNAs Generated by RT-PCR

The resultant PCR products (160 bp) were subcloned into the pCR[®]2.1 plasmid (Invitrogen, Carlsbad, CA) by a blunt-end ligation using the Invitrogen[®] Original TA Cloning[®] Kit. The ligation reaction was performed by mixing 2 μ l of the PCR product with 1 μ l of 10X ligation buffer, 2 μ l of the pCR[®] 2.1 vector (25 ng/ μ l), 4 μ l of sterile

water and 1 μ l of T4 DNA ligase. The reaction was incubated for 4 hours at 14°C. The plasmid reaction mixture was then used to transfect competent *E.coli* (TOP10F').

The bacteria were incubated with 2 μ l each of β -mercaptoethanol (0.5M) and the ligation product for 30 minutes on ice. The bacteria were then heat shocked for 30 seconds at 42°C and placed on ice for 2 minutes. An aliquot (250 μ l) of SOC medium (2.0% tryptone, 0.5% yeast extract, 10.0 mM NaCl, 2.5 mM KCl, 10.0 mM MgCl₂ 6H₂O, and 20.0 mM glucose) was added to the bacteria and the resultant mixture incubated for a further 1 hour at 37°C in an environmental shaker (225 rpm). An aliquot (50 μ l) was plated on a Luria-Bertani (LB) plate for amplification and selection.

LB solution contains 1.0% bacto-tryptone, 0.5% bacto-yeast extract and 1.0% NaCl in deionised water, adjusted to pH 7. For liquid LB medium, this solution was autoclaved and allowed to cool to 55°C prior to the addition of ampicillin (50 ug/ml). Ampicillin allows for selection against bacterial clones not containing the pCR[®]2.1 plasmid. LB plates were prepared by the addition of 1.5% agar to the LB solution before autoclaving. Plates were poured after the solution had cooled to 55°C and the antibiotic had been added to the mixture. The plates were allowed to cool prior to being stored at 4°C. Prior to use, the plates were warmed by incubation at 37°C for 10 minutes. Isopropyl- β -D-thiogalactoside (IPTG; 40 μ l, 1M) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal; 40 μ l, 40 mg/ml in dimethylformamide) were spread over each plate. IPTG and X-gal allows for the screening of bacterial colonies containing a plasmid with the cDNA insert (white) as compared to those containing an intact plasmid (blue). The plates were

then incubated for a further 30 minutes at 37°C to allow diffusion of the solution into the plate and evaporation of the dimethylformamide. Approximately 20 white clones/tissue sample were selected, streaked on separate LB agar plates, and incubated at 37°C. Clones from the streaked plates were used to inoculate 6 ml of liquid LB. The bacterial cultures were incubated at 37°C overnight in an environmental shaker.

Plasmid Purification

The plasmids were purified from the bacterial cultures using the Wizard™ *Plus* Minipreps DNA Purification System (Promega, Madison, WI). All reagents used were provided in this kit. The bacteria were removed from solution by centrifugation of the liquid culture at 1,400 x g for 10 minutes. The pellet was resuspended in 300 µl of Cell Resuspension Solution (50 mM Tris HCL, pH 7.5, 10 mM EDTA, 100 ug/ml RNase A) and lysed by the addition of 300 µl Neutralisation Solution (1.32 M sodium acetate, pH 4.8). Cellular debris and chromosomal DNA were removed by centrifugation of the bacterial lysate at 10,000 x g for 5 minutes.

Clear lysate was pushed through 1 ml of resuspended DNA Purification Resin in a Wizard™ Minicolumn attached to a 3 ml Luer-Lok® syringe, and washed by 2 ml of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris HCl, pH7.5, 40 uM EDTA) containing ethanol. The contents of the column were centrifuged at 10,000 x g for 2 minutes to dry the resin. Plasmid DNA was eluted from the resin by adding 50 µl of deionised water followed by centrifugation at 10,000 x g for 20 seconds.

To confirm the presence of the PCR product in the selected clones, a restriction enzyme digestion of the plasmid was performed. Six μ l of plasmid DNA, 10 μ l of deionized water, 2 μ l of 10X reaction buffer, and 2 μ l of EcoRI were incubated in an Eppendorf tube for 30 minutes at 37°C. The digestion products were analysed by DNA gel electrophoresis. The digestion products were mixed with 4 μ l DNA loading buffer and loaded into an 1.8% agarose gel. Gel was prepared using 0.5X TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA), agarose, and ethidium bromide (EtBr). A 100 base-pair ladder (Gibco BRL) was loaded onto the gel to assess the molecular weight of the digestion products.

DNA sequence analysis of the isolated plasmids was performed using an automated DNA sequencer (Applied Biosystems) employing the Taq DyeDeoxy sequencing reagents. The resultant DNA sequences were compared to the nucleotide databases of GenBank using the BLAST computer program.

Generation of Human P-cadherin cDNA

Total RNA was prepared from human foreskin using the phenol-chloroform method of Chomczynski and Sacchi (1987). RT-PCR was performed using this total RNA extract and primers specific for P-cadherin (P-cadF: 5'-AAAGAGCAGCTGACGGTGAT-3', P-cadR 5'-GAACACCAAGAGGGTGTCGT-3'). This pair of P-cad primers, which correspond to bp 2043 and 2532 of the human P-cad cDNA, yield a PCR product of 490 bp in length. The PCR conditions that were used are as follows: denaturation at 94°C for

1 min, annealing at 58 C for 1 min and extension at 72 C for 1 min. This cycle was repeated 22 times.

The resultant RT- PCR product was subcloned into the PCR II vector by blunt end ligation for subsequent DNA sequence analysis. A total of 5 plasmids were selected. The BLAST computer program was used to confirm that these cDNA clones encoded human P-cad (Fig 3).

Figure 3: DNA sequence analysis of a P-cad cDNA generated from human foreskin by RT-PCR. The BLAST computer program confirmed 100% homology between this clone and the nucleotide sequence for human P-cad cDNA deposited in Genbank.

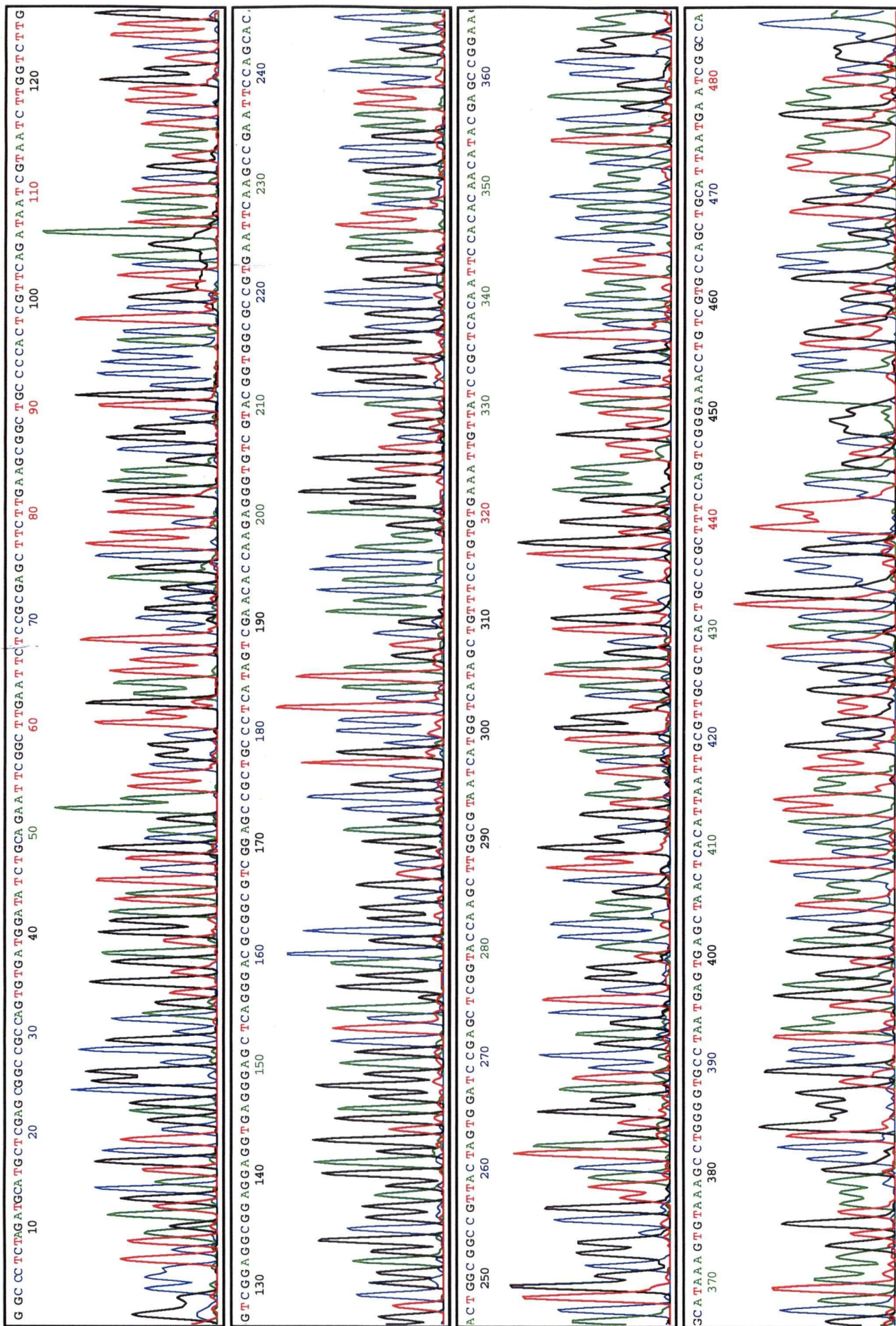


Model 377
Version 3.3
ABI100
Version 3.2

13/T7
Ila Patel
13/T7
Lane 38

Signal G:81 A:63 T:48 C:67
DT {BD Set Any-Primer}
dRhod Matrix
Points 1046 to 8260 Pk 1 Loc: 1046

Page 1 of 2
Wed, Sep 8, 1999 8:07 AM
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Spacing: 10.98{10.98}



Northern Blot Analysis

For Northern blot analysis, the RNA species present in total RNA extracts prepared from the ovarian tissues and the cells present in the ascites fluid was resolved by electrophoresis in 1% agarose gels containing 3.7% formaldehyde. Approximately 20 µg of total RNA was loaded per lane. The fractionated RNA species were then transferred onto charged nylon membranes.

Northern blots were probed with the P-cad cDNA probe described above, according to the methods of MacCalman et al. (1992). The blots were then washed twice with 2X SSPE at room temperature, twice with 2X SSPE containing 1% SDS at 55 C and twice with 0.2 X SSPE at room temperature. The blots were subjected to autoradiography in order to detect the hybridisation of the radiolabeled probe to the P-cad mRNA species. To standardize the amounts of total RNA in each lane, the blots were then probed with a radiolabeled synthetic oligonucleotide specific for 18S rRNA according to the protocols described by MacCalman et al. (1992). The blots were again subjected to radioautography to detect the hybridisation of the radiolabeled probe to the 18S rRNA. The autoradiograms were then scanned using an LKB laser densitometer. The absorbance values obtained for the P-cad mRNA transcript were normalized relative to the corresponding 18S rRNA absorbance value.

The Northern blots containing total RNA extracted from normal peritoneum, ovarian tumor masses and ascites tumor cells obtained from women diagnosed with stage I or stage II ovarian cancer were probed with a radiolabeled human β -catenin cDNA

according to the protocol described above. This cDNA has been described in detail elsewhere (Chen et al., 1998b).

Detergent Extraction and Western Blot Analysis

Samples of the ovarian and peritoneal tissue samples were homogenised in the presence of 100 µl of cell lysis buffer (Tris HCl, pH 7.5 containing 0.5% NP-40, 0.5 mM CaCl₂ and 1.0 mM PSF). Ascites cells were also incubated in this lysis buffer at 4 °C for 30 min on a rocking platform. The lysates were then centrifuged at 10,000 x g for 20 min and the supernatants used for Western blot analysis or immunoprecipitation studies. The concentration of protein in the cell lysates was determined using the BCA kit (Pierce Chemicals, Rockford, IL).

For Western blot analysis, aliquots (20 µg) of the cell lysates were taken from the samples and subjected to SDS polyacrylamide gel electrophoresis under reducing conditions, as described by Laemmli (1970). The stacking gels contained 5% acrylamide and the separating gels were composed of 7.5% acrylamide. The proteins were electrophoretically transferred from the gels onto nitrocellulose paper according to the procedure of Towbin et al. (1979). The nitrocellulose blots were probed with the mouse monoclonal antibodies directed against P-cad. The Amersham ECL system was used to detect antibody bound to antigen.

Western blots containing total protein extracts obtained from normal peritoneum, ovarian tumor masses and ascites tumor cells obtained from women diagnosed with stage I or

stage II ovarian cancer were probed with a mouse monoclonal antibody directed against human β -catenin (Transduction labs, Lexington, KY) according to the methods described above.

Immunoprecipitation

Immunoprecipitations were performed at 4 °C with constant mixing on a rocking platform according to the methods described by Pishviain *et al.* (1999). Briefly, aliquots (250 μ g) of cell lysates prepared from the ovarian tumors or asitic cells were precleared with 10 μ l of a protein A-agarose conjugate (2mg/ml; Transduction Labs) for 30 min. The agarose was removed from the cell lysates by centrifugation at 10, 000 x g for 4 min. The resultant supernatants were incubated with 5 μ g of a mouse monoclonal antibody directed against human β -catenin for 1 h. The immune complexes were then incubated with 5 μ g of a rabbit anti-mouse IgG antibody (Jackson ImmunoResearch Labs, West Grove, PA) for 30 min after which protein A-agarose was added to the lysates and the reaction mixture incubated for a further 30 min. The agarose-bound immune complexes were centrifuged at 10, 000 x g for 4 min, and washed five times with cell lysis buffer before being resuspended in Laemmli sample lysis buffer and resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions. The proteins were then electrophoretically transferred from the gels onto nitrocellulose paper. The immunoblots were probed with a mouse monoclonal antibodies directed against P-cad. The Amersham ECL system was used to detect antibody bound to antigen.

Results

Identification

The cadherin subtypes identified in the peritoneum, ovarian tumor masses and ascites tumor cells obtained from patients diagnosed with stage I ovarian cancer are summarized in Tables 1 and 2.

As expected, the majority of cDNA clones isolated from the ovarian mass encoded E- or N-cad. Interestingly, although the peritoneum is considered to be an epithelial tissue, the number of E-cad cDNA clones generated from this tissue was surprisingly low. Instead, the greatest number of cDNA clones generated from the peritoneum as well as the ascites tumor cells encoded P-cad.

Tables 1 and 2: Repertoire of Cadherins present in the Peritoneum, Ovarian Masses and Ascites fluid Obtained from Women with Stage I Ovarian Cancer

Patient 1

Cadherin Subtype	P-cad	E-cad	N-cad	R-cad	cad-6	Cad-11
Peritoneum Total=22 clones	11	4	0	4	3	0
Ovarian Mass Total =20 clones	1	8	6	2	2	1
Ascites Fluid Total = 20 clones	10	1	2	4	3	0

Patient 2

Cadherin Subtype	P-cad	E-cad	N-cad	R-cad	cad-6	Cad-11
Peritoneum Total=20 clones	12	4	0	2	2	0
Ovarian Mass Total =20 clones	2	7	7	2	2	0
Ascites Fluid Total = 20 clones	9	0	3	5	3	0

The cadherin subtypes identified in the peritoneum, ovarian tumor masses and ascites tumor cells obtained from patients diagnosed with stage II ovarian cancer are summarized in Tables 3 and 4.

Tables 3 and 4: Repertoire of Cadherins present in the Peritoneum, Ovarian Masses and Ascites fluid Obtained from Women with Stage II Ovarian Cancer

Patient 1

Cadherin Subtype	P-cad	E-cad	N-cad	R-cad	cad-6	Cad-11
Peritoneum Total=20 clones	10	3	0	4	3	0
Ovarian Mass Total =20 clones	6	4	5	2	3	0
Ascites Fluid Total = 20 clones	12	0	2	4	2	0

Patient 2

Cadherin Subtype	P-cad	E-cad	N-cad	R-cad	cad-6	Cad-11
Peritoneum Total=21 clones	13	3	0	2	3	0
Ovarian Mass Total=20 clones	8	4	4	2	2	0
Ascites Fluid Total = 21 clones	12	1	3	2	3	0

A similar repertoire of cadherins was observed in these tissues with the notable exception that a greater number of P-cad cDNA clones were generated from the stage II ovarian mass.

Correlation of P-Cadherin

To determine whether P-cad steady state mRNA and protein expression levels are increased in poorly differentiated ovarian carcinomas, we performed Northern and

Western Blot analysis using total RNA or protein extracts prepared from stage I or stage II ovarian tumor masses, ascites tumor cells and normal peritoneum.

A single P-cad mRNA transcript (4kb) was detected in total RNA extracts prepared from the ascites and peritoneum of women diagnosed with stage I (n=3) or stage II (n=3) ovarian cancer (Figs 4 and 5, respectively). We failed to detect significant P-cad mRNA levels in the extracts prepared from stage I ovarian tumor masses. However, the mRNA transcript encoding P-cad was readily detectable in total RNA extracts prepared from ovarian tumor masses obtained from women diagnosed with stage II ovarian cancer.

In agreement with the Northern blot analysis, a single P-cad protein species (120 kDa) was detected in the total protein extracts prepared from the peritoneum and ascites tumor cells obtained from women diagnosed with either stage I (n=3) or stage II (n=3) ovarian cancer. Similarly, P-cad expression was readily detectable in the extracts prepared from type II but not type I ovarian masses.

Figure 4: Representative radioautograms of Northern Blot probed with radiolabeled P-cad (upper panel) and then reprobbed with a radiolabeled synthetic oligonucleotide specific for 18S ribosomal RNA (lower panel). The blot contains total RNA extracted from ovarian tumor, ascites and peritoneum of a woman diagnosed with stage I ovarian cancer (lanes A-C, respectively). The positions of the 28S and 18S rRNA species are shown on the left-hand side of the upper panel.

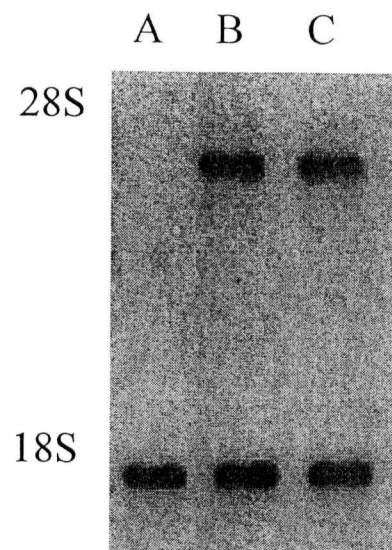


Figure 5: Representative radioautograms of Northern Blot probed with radiolabeled P-cad (upper panel) and then reprobbed with a radiolabeled synthetic oligonucleotide specific for 18S ribosomal RNA (lower panel). The blot contains total RNA extracted from ovarian tumor, ascites and peritoneum of a woman diagnosed with stage II ovarian cancer (lanes A-C, respectively). The positions of the 28S and 18S rRNA species are shown on the left-hand side of the upper panel.

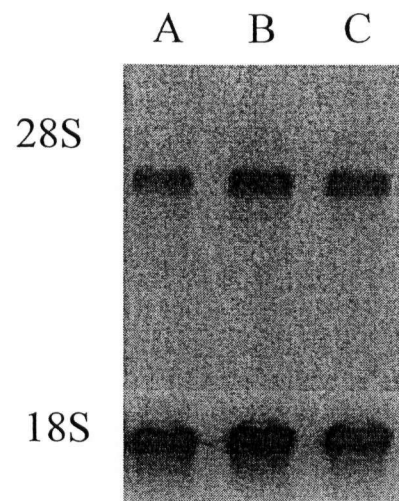


Fig. 6: Western blot analysis of P-cad expression levels in total protein extracts prepared from ovarian tumor, ascites and peritoneum of a woman diagnosed with stage I ovarian cancer (lanes A-C, respectively). Twenty μ g of protein were loaded in each lane. Western blot analysis was performed using mouse monoclonal antibodies directed against human P-cad. The Amersham ECL system was used to detect antibody bound to antigen.

A B C

**P-cadherin
(220 kDa)**

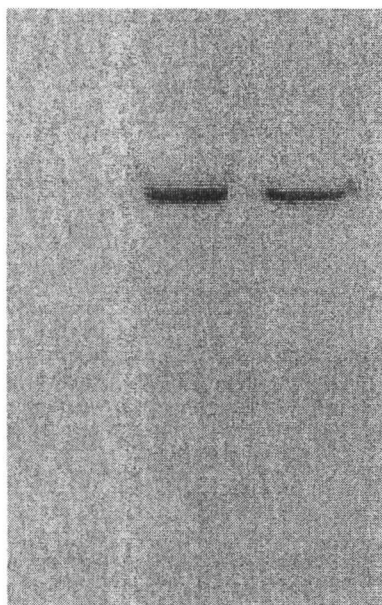
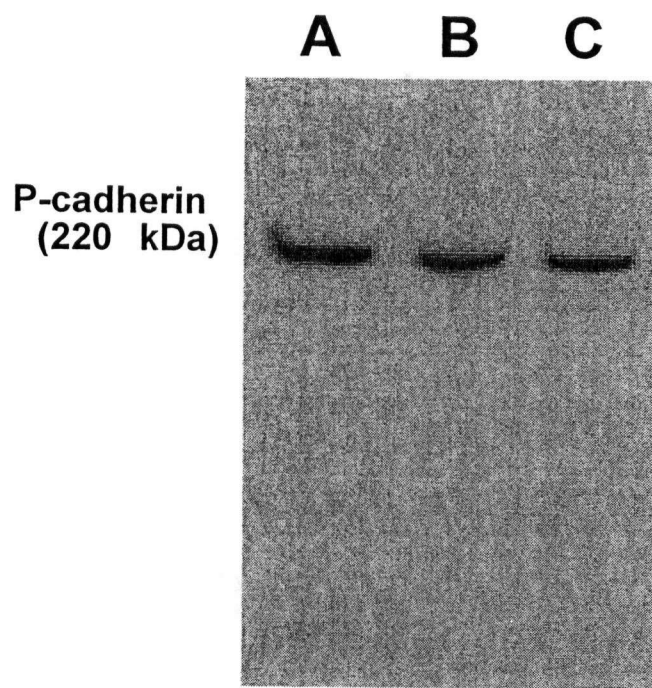


Fig. 7: Western blot analysis of P-cad expression levels in total protein extracts prepared from ovarian tumor, ascites and peritoneum of a woman diagnosed with stage II ovarian cancer (lanes A-C, respectively). Twenty μg of protein were loaded in each lane. Western blot analysis was performed using mouse monoclonal antibodies directed against human P-cad. The Amersham ECL system was used to detect antibody bound to antigen.



Correlation of β -Catenin

As a first step in identifying the cellular mechanisms underlying the role(s) of P-cad in the progression of ovarian cancer, we have examined β -catenin mRNA and protein expression levels in normal peritoneum, ovarian tumor masses and ascites tumor cells obtained from women diagnosed with stage I or stage II ovarian cancer using Northern and Western blot analysis. The ability of P-cad to interact with this cytoplasmic protein was determined by immunoprecipitation.

A single β -catenin mRNA transcript (3.3 kb) was detected in all of the total RNA extracts prepared from the peritoneum, ovarian tumor mass or ascites tumor cells obtained from women with stage I or stage II ovarian cancer (Figs 8 and 9, respectively). Similarly, a single β -catenin protein species (92 kDa) was detected in all of the total protein extracts prepared from these tissues (Figs 10 and 11, respectively).

Immunoprecipitation studies demonstrated that β -catenin was capable of interacting with P-cad in the peritoneum and ascites tumor cells obtained from women diagnosed with stage I or stage II ovarian cancer and stage II ovarian tumor masses (Figs 12 and 13).

Figure 8: Representative radioautograms of Northern Blot probed with radiolabeled β -catenin (upper panel) and then reprobbed with a radiolabeled synthetic oligonucleotide specific for 18S ribosomal RNA (lower panel). The blot contains total RNA extracted from ovarian tumor, ascites and peritoneum of a women diagnosed with stage I ovarian cancer (lanes A-C, respectively). The positions of the 28S and 18S rRNA species are shown on the left-hand side of the upper panel.

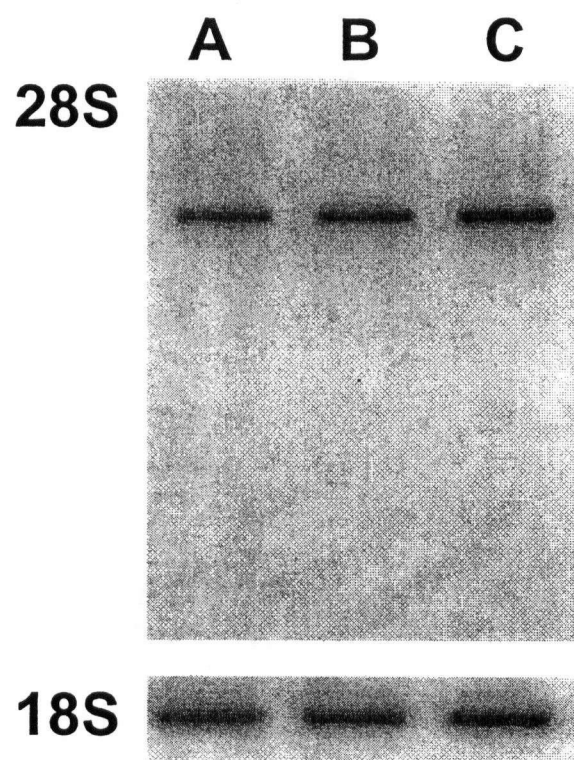


Figure 9: Representative radioautograms of Northern Blot probed with radiolabeled β -catenin (upper panel) and then reprobbed with a radiolabeled synthetic oligonucleotide specific for 18S ribosomal RNA (lower panel). The blot contains total RNA extracted from ovarian tumor, ascites and peritoneum of a woman diagnosed with stage II ovarian cancer (lanes A-C, respectively). The positions of the 28S and 18S rRNA species are shown on the left-hand side of the upper panel.

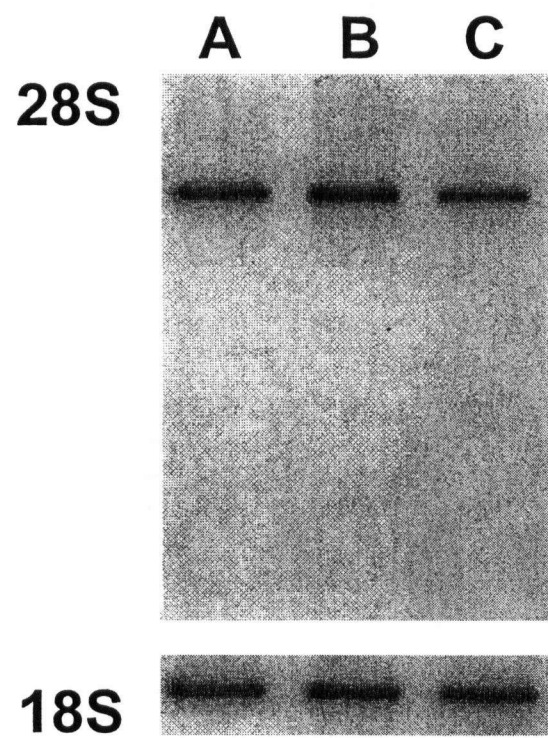


Fig. 10: Western blot analysis of β -catenin expression levels in total protein extracts prepared from ovarian tumor, ascites and peritoneum of a woman diagnosed with stage I ovarian cancer (lanes A-C, respectively). Twenty μ g of protein were loaded in each lane. Western blot analysis was performed using mouse monoclonal antibodies directed against human β -catenin. The Amersham ECL system was used to detect antibody bound to antigen.

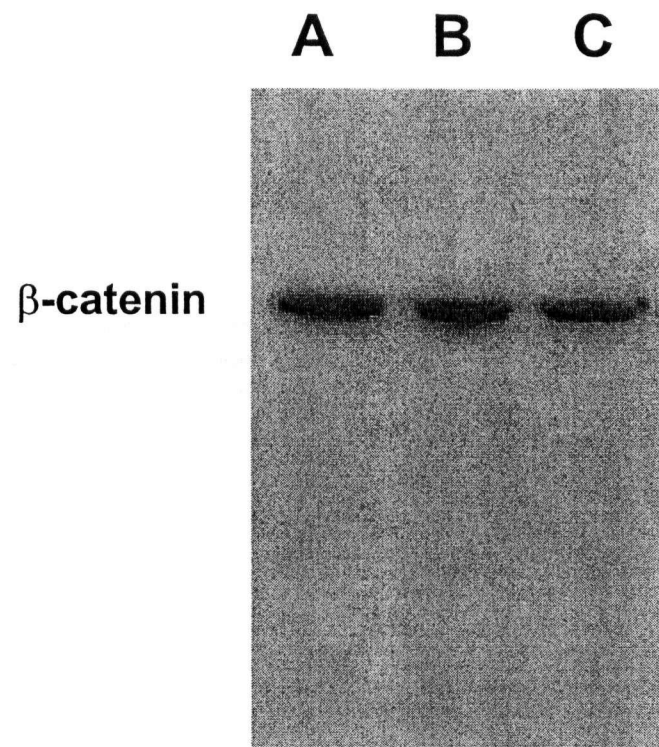


Fig. 11: Western blot analysis of β -catenin expression levels in total protein extracts prepared from ovarian tumor, ascites and peritoneum of a woman diagnosed with stage II ovarian cancer (lanes A-C, respectively). Twenty μ g of protein were loaded in each lane. Western blot analysis was performed using mouse monoclonal antibodies directed against human β -catenin. The Amersham ECL system was used to detect antibody bound to antigen.

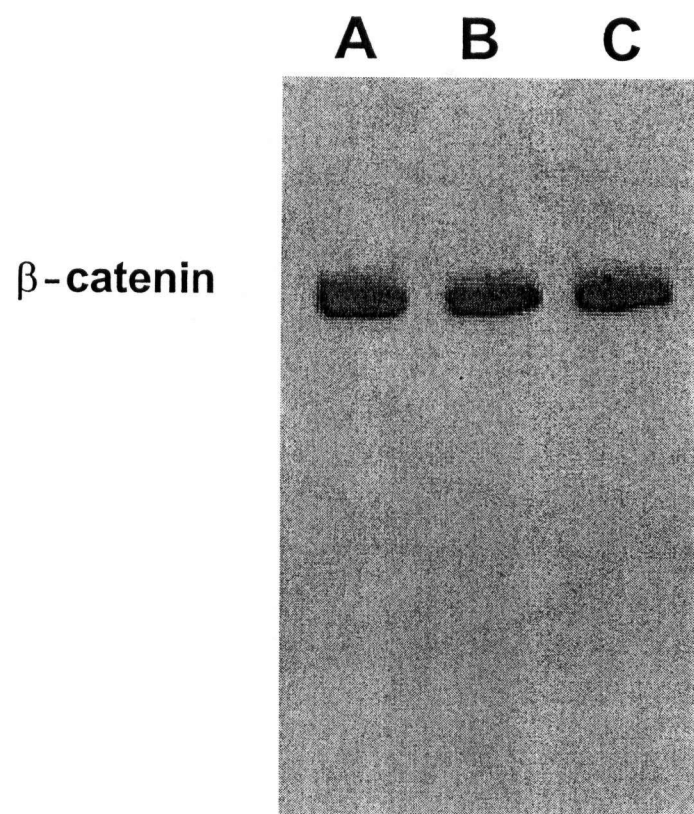


Fig 12. Autoradiogram of an immunoblot containing lysates prepared from ovarian tumor mass, ascites tumor cells or peritoneum obtained from woman with stage I ovarian cancer which were immunoprecipitated with a mouse monoclonal antibody directed against human β -catenin. The immunoblot was probed with a mouse monoclonal antibody directed against P-cad. The Amersham ECL system was used to detect antibody bound to antigen.

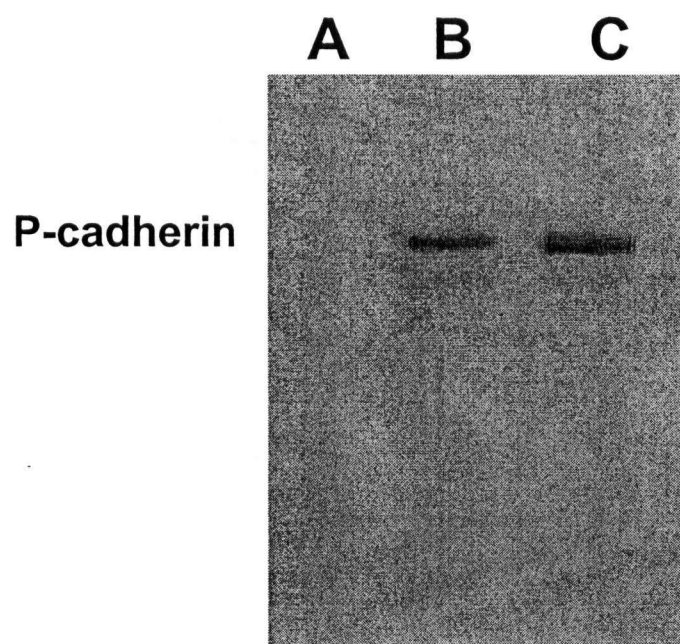
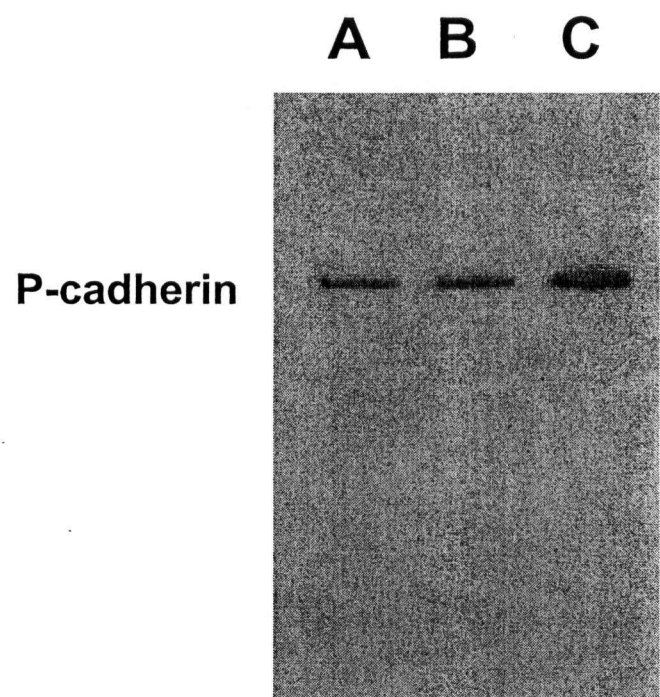


Fig 13. Autoradiogram of an immunoblot containing lysates prepared ovarian tumor mass, ascites tumor cells or peritoneum obtained from woman with stage II ovarian cancer which were immunoprecipitated with a mouse monoclonal antibody directed against human β -catenin. The immunoblot was probed with a mouse monoclonal antibody directed against P-cad. The Amersham ECL system was used to detect antibody bound to antigen.



Discussion

Ovarian epithelial tumours account for approximately 90% of all ovarian tumours and have the highest mortality rate among gynecological cancers in the industrialised world (Sundfeldt et al., 1997; 2001). Cadherins and their associated proteins, the catenins, have important roles in embryonic morphogenesis, epithelial morphogenesis and carcinogenesis (Davies et al., 1997). Correlation between cadherin expression and the metastatic potential of cells has been reported for gastric (Guilford et al., 1998), prostate (Umbas et al., 1994), and breast carcinomas (Oka et al., 1992; Takeichi, 1993). Epithelial cells express the cell-cell adhesion molecule E-cadherin, and as such, epithelial ovarian cancer has been the target of a number of studies involving the expression of E- and other cadherins. In tumors other than ovarian, loss of E-cadherin expression has been associated with the acquisition of invasive and metastatic properties. Paradoxically, a number of studies have demonstrated retained expression of E-cadherin in ovarian tumours, and some increased expression as the tumours become dedifferentiated and malignant (Sundfeldt et al., 1997; 2001; Wong et al., 1999). However, metastatic lesions of advanced ovarian cancers demonstrated a suppression of E-cadherin (Fujimoto et al., 1997). Darai et al., (1997), found that the expression of N-cadherin was retained by ovarian tumor cells, while Wong et al., (1999), found N-cad to be the constitutively expressed cadherin of normal and metaplastic OSE. A distinguishing factor in mucinous ovarian tumours is the differential expression of N-cadherin. While serous and endometrioid tumours express both E-cad and N-cadherin, mucinous tumours have a conspicuous absence of N-cadherin, raising an interesting debate around mucinous

tumours having a different origin from the mesoderm-derived OSE serous and endometrioid tumours (Peralta Soler et al., 1997b; Potter et al., 1999).

P-cad is the least studied of the classical cadherins, although there are a number of studies that underline its role in cancer. E- and P-cadherin were down-regulated in node-positive tumours in breast cancer (Madhavan et al., 2001). P-cadherin and β -catenin are expressed in dysplastic, well and moderately differentiated carcinomas in ulcerative colitis associated dysplasia and colorectal cancer, while being undetectable in the poorly differentiated carcinomas (Haq et al., 2001). These studies suggest a role for P-cad related to early neoplasia. P-cad also appears to be involved in the progression of two other cancers: P-cad becomes the predominant cadherin in high grade uterine squamous intraepithelial lesions (SILs), whereas E-cad levels decrease during the transformation of normal squamous epithelium to SILs (De Boer et al., 1999); similarly, there appears to be an increase in P-cad expression and a concomitant decrease in E-cad levels during the progression of Barrett's esophagus to adenocarcinoma (Bailey et al., 1998).

A recent study examined the expression of E-cadherin and β -catenin in primary as well as peritoneal metastatic ovarian carcinoma (Fujioka et al., 2001). Sixty percent of peritoneal metastatic lesions demonstrated reduced expression of E-cadherin compared with the primary lesion. Interestingly, only 20% of the metastatic lesions demonstrated a reduced expression of β -catenin. This study suggests that β -catenin could be associating with another cadherin. Our present results have found that P-cadherin is the predominant cadherin subtype present in ovarian tumor cell aggregates recovered from the ascites of

ovarian cancer patients. Normal human peritoneal cells also express P-cadherin. Together with the studies that implicate P-cadherin in the progression of two cancers, these observations support our hypothesis that P-cad can play an important role in the late stages of tumorigenesis in the ovary and that this CAM may serve as a useful cellular marker of disease progression.

Previous studies have failed to identify the cadherin subtype(s) expressed in the human peritoneum. In particular, Hashimoto et al. (1989) who performed a comprehensive immunohistochemical survey of E-cad expression in normal and benign reproductive tissues reported that this CAM was not expressed in the human peritoneum. Here we demonstrate that P-cad is the predominant cadherin subtype expressed in this tissue. This finding is not altogether surprising, as P-cad expression has been detected in other epithelial tissues. For example, P-cad is expressed in discrete subpopulation(s) of epithelial cells in the in adult mouse mammary gland and disruption of this CAM results in spatially specific disruptions within this tissue (Daniel and Reynolds, 1995; Radice et al., 1997b). In mouse lung morphogenesis, both E- and P-cad play an active role, but the E-cad-mediated effects appear to predominate (Hirai et al., 1989a). Finally, Hirai et al. (1989b) have found that in fetal mouse lip skin organ culture, P-cad rather than E-cad plays a major role in both the organization of the basal keratinocytes and in the development of hair follicles.

We also found P-cadherin to be the predominant cadherin subtype expressed by ovarian tumor ascites cells. Tumor cells present in the ascites are considered to be

morphologically distinct and highly malignant. Veatch et al. (1994), in one of the few studies that looked at cadherins present in the tumor ascites cells, found a reduced expression of the E-cad gene in ascites tumor cells as compared to the solid tumor cells. We speculate that P-cadherin may mediate ovarian tumor cell – peritoneal mesothelial interactions. There are several examples of coregulation of cadherin levels during, for example, development. Embryonic cells expressing different classical cadherins segregate from one another. The reciprocal expression of E-cadherin and cad-11 plays a role in trophoblast-endometrial cell interactions during implantation of the mammalian embryo (MacCalman et al., 1996). It has been suggested for a number of years that in cancer, paradoxically, cell-cell adhesion molecules may be involved in the processes of detachment, metastasis and reattachment (Takeichi, 1993). Islam et al., (1996) speculated that the aberrant expression of N-cadherin by squamous cell carcinomas facilitates the cells detaching from the tumor and invading the underlying stroma, which also expresses N-cadherin. The expression of P-cad in breast carcinomas derived from epithelium that normally does not express P-cad may indicate the proliferative ability of these tumors. The growth pattern exhibited by breast carcinomas showed reduced E-cad expression, while P-cadherin was expressed in a subset of high-grade breast carcinomas (Palacios et al., 1995).

P-cad mRNA and protein expression levels in the ovarian tumor masses appear to be coordinately regulated during progression to the later stages of the disease state. We have previously demonstrated that there is a marked increase in both the mRNA transcripts and protein species of E-cad, N-cad, and cad-11 in mouse OSE, granulosa

cells and human endometrial stromal cells respectively in response to gonadal steroids (MacCalman et al., 1994; MacCalman et al., 1995; Chen et al., 1998a). To date, there have been no reports in the literature regarding the regulation of P-cad expression levels in mammalian tissues or cells. The nucleic acid sequence of the P-cad gene promoter has been determined. It contains estrogen response elements (ERE) but no other steroid response elements (Jarrard et al., 1997). These observations indicate that P-cad gene expression, like E-cad, is directly regulated by estrogens. Gonadal steroids have been shown to be regulators of cadherin expression in human reproductive tissue, such as breast carcinoma cell lines (Jednak et al., 1993), and the endometrium (MacCalman et al., 1998). The antiprogesterone RU486 decreased the levels of cad-11 mRNA and protein expression in human endometrial stromal cells undergoing decidualisation in vitro (Chen et al., 1998a). In view of the presence of EREs in the P-cadherin gene promoter, it is tempting to speculate on the ability of estrogens and anti-estrogens in regulating the P-cadherin protein in ovarian carcinoma, with a view to altering the metastatic and invasive capacity of the carcinoma cells.

A single β -catenin mRNA transcript of 3.3 kb was detected in all of the total RNA extracts prepared from tissue samples of normal peritoneum, ovarian tumor mass and ascites tumor cells obtained from women diagnosed with stage I or stage II ovarian cancer. This β -catenin mRNA transcript has been previously detected in human endometrial stromal cells (Chen et al., 1998b) and breast, gastric, and colon carcinoma cell lines (Oyama et al., 1994; Munemitsu et al., 1995; Byers et al., 1996). Similarly, a 92 kDa protein species corresponding to β -catenin has been detected in a wide variety of

tissues and cells, including *Xenopus* embryos, human liver, epidermoid cells and murine neuroblastoma cell lines (McCrea and Gumbiner, 1991; Aberle et al., 1997; Miyoshi et al., 1998). As the loss of catenin expression also compromises cadherin-mediated adhesion, it has been proposed that this may be a mechanism by which tumor cells acquire an invasive phenotype. Our studies indicate that the progression of ovarian cancer to the later stages of the disease, when the tumor cells are capable of detaching from the primary tumor, is not the result of a reduction or loss of β -catenin expression in these cells. These findings are in direct contrast to Fujimoto et al., (1997) who reported a marked reduction in the levels of the β -catenin mRNA transcripts present in the metastatic lesions of advanced ovarian cancers using semiquantitative RT-PCR. However, this discrepancy may be attributed to the differences in the stages of ovarian cancer examined and/or the sensitivity of the techniques used in these two studies. Davies et al., (1997), showed variable results, with 68% of the ovarian carcinomas studies showing some expression of β -catenin while 34% showed reduced, inappropriate or no expression of the protein. In contrast a recent study by Fujioka et al., (2001) demonstrated that only 2 out of the 10 metastatic lesions from ovarian carcinoma showed reduced expression of β -catenin. To our knowledge, our studies are the first to demonstrate that β -catenin is expressed in the primary ovarian mass and corresponding ascites tumor cells.

P-cad cannot mediate cell adhesion unless it forms intracellular complexes with the catenins (Takeichi, 1988; 1995). Our studies suggest that the regulated expression of P-cad and its ability to interact with β -catenin in the ovarian tumor mass represents a

critical step in the progression of this disease. In particular, the expression of P-cad by poorly differentiated tumor cells would allow these cells to detach from the more differentiated E-cad expressing cells within the tumor. A similar switch in cadherin expression is believed to mediate cell sorting and migration during embryogenesis (Takeichi, 1988; 1995). In addition, the presence of a functional P-cad/ β -catenin in ascitic tumor cells suggest that this CAM may also allow these cells to interact with the mesothelial cells of the peritoneum in a homophilic manner.

There have been several studies that have looked at serum levels of P-cadherin or E-cadherin in cancer of the reproductive tissues. Although soluble fragments of P-cadherin were found in serum from patients with breast carcinomas, the level of P-cadherin was not reflective of the presence of a P-cad positive tumor. There has also been no correlation found between E-cadherins serum levels and the presence of breast cancer (Knudsen et al., 2000). Conversely, concentration of soluble E-cadherin was significantly higher in cystic fluid taken from patients with borderline or malignant ovarian tumors as compared to those with benign tumors (Darai et al., 1998; Sundfeldt et al., 2001). These results suggest a role for soluble E-cad levels as a future diagnostic tool to determine benign versus malignant ovarian tumors. Our preliminary findings suggest that P-cadherin may have an important role in establishing interactions between peritoneal cells and primary ovarian cancer cells. If P-cadherin levels in primary ovarian carcinomas and/or ascites do serve as markers of disease progression, then future studies which assess if proteolytic fragments of P-cadherin can be detected in fluids of patients

with ovarian cancer, such as ascites, could also potentially serve as useful diagnostic tools, particularly in the later stages of the disease.

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