Cadherins, Catenins And Associated Proteins In Normal Epidermis, Basal Cell Carcinoma And Other Cutaneous Tumours: An Immunohistochemical Study

by

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Dedicated to my parents, my wife
and our three lovely children.

Summary

Background 1: In carcinoma, the detachment of cancer cells from a primary tumour site as a result of disorganised cell-cell association is a critical step leading to tumour invasion and metastasis. Adherens junctions (AJ) are multiprotein complexes localised at intercellular and cell-matrix contacts, mediating the adhesion of cells to each other or to matrix structures. The association of actin filaments with the actin-binding proteins enables them to serve a variety of functions including the stability of cell shape, mechanical support, cell-cell adhesion and cell motility. The latter is thought to be driven largely by the polymerisation of actin monomers into filaments near the plasma membrane. The maintenance of epithelial differentiation requires proper formation of cell-cell junctions where destabilisation of such junctions allows the initiation of the invasive process and the progression of carcinoma. Because there is evidence that down-regulation or complete loss of the major AJ component, E-cadherin, occurs in association with human epithelial cancers, it has been suggested that E-cadherin could play a role as an invasion-suppressor molecule.

Aim 1:

- To investigate the cellular distribution of AJ components (E- and P-cadherin, α- and β-catenin and associated proteins) in the normal human epidermis and in a variety of skin tumours including basal cell carcinoma (BCC), pilomatrixoma (PMX), squamous cell carcinoma (SCC) and precursor lesions and melanocytic lesions. Also to assess whether the decrease in membranous distribution of the AJ major components, particularly cadherins and β-catenin, in human skin cancer progressed further in de-differentiation tumours.
- To investigate the possible co-localisation of E- and P-cadherin in the basal and the first layer of suprabasal cells of the human normal epidermis and BCC with respect to the hypothetical stepwise loss of cadherins by tumour cells.
- To characterise the overall structure of AJ in BCC versus the normal epidermis and to investigate the association of AJ components with the actin-based cytoskeleton. Also to examine the patterns of distribution of filamentous actin in the normal

epidermis and BCC and how actin bundles are arranged in the tumour cells with respect to their shape, migratory behaviour and thus invasiveness.

- To compare the changes in the abundance and distribution of actin and the expression of E-, P-cadherin and β-catenin in nodular and infiltrative BCC with those in migrating keratinocytes in vitro.

Methods 1: Immunoperoxidase and double immunofluorescent staining. Cell culture.

Findings 1:

1. In routinely processed paraffin-embedded BCC, E-cadherin staining was reduced in tumour cell membranes in 30/32 cases, β-catenin in 29/32 cases and P-cadherin in 11/32 cases in comparison with the normal epidermis.
2. E-Cadherin, α- and β-catenin were reduced in tumour cell membrane in all the frozen samples of BCC (12/12). P-Cadherin was reduced in 10/12.
3. In some cases of BCC, tumour lobules expressed reduced E-cadherin membrane staining centrally, while the palisading cells on the peripheries showed positive membrane staining similar to that of normal epidermis
4. In well differentiated SCC, E-cadherin and β-catenin staining was reduced in tumour cell membranes in 8/10 cases and P-cadherin in 6/10 cases. All were reduced in moderately differentiated SCC and keratoacanthoma.
5. In benign hyperplasia, Bowen's disease and actinic keratosis, P-cadherin was expressed in the full thickness of the lesions.
6. E-, P-cadherin and β-catenin membrane staining was reduced in all cases of primary and metastatic melanomas and was variable in benign melanocytic lesions.
7. Double immunolabelling of E- and P-cadherin revealed co-localisation of both cadherins in the basal and first suprabasal layer of the normal epidermis and in a substantial number of BCC tumour cells. However, the majority of tumour cells demonstrated preservation of membranous P-cadherin only.
8. There was a dramatic increase in actin filaments polymerisation (stress fibers) in infiltrative BCC when compared with the nodular subtype and normal epidermis.
9. The double immunolabelling results of normal skin and most tumour cells in nodular and infiltrative BCC have shown separate localisation of E- and P-cadherin, α- and

β-catenin from actin filaments. However, a portion of E- and P-cadherin, α-catenin and α-actinin was co-localised with actin filaments at the plane of the plasma membrane.

10. Keratinocytes cultured in low calcium grew individually in a loose clustered morphology, but they closed the wound partially. They also showed weak E-, P-cadherin and β-catenin membrane immunofluorescent staining and diffuse polymerisation of actin filaments. Keratinocytes, in high calcium conditions, showed stronger staining of the cell adhesion molecules, increased actin filaments polymerisation (fine stress fibers) and associated more closely to close the wound completely.
11. Double immunolabelling of E-, P-cadherin and β-catenin with actin in both low and high calcium keratinocyte cultures revealed no co-localisation.

Main conclusions 1:

1. The reduction in tumour cell membrane E-cadherin, α- and β-catenin in comparison with the normal epidermis is a characteristic in BCC and SCC.
2. The decrease in membranous distribution of E- and P-cadherin, α- and β-catenin in superficial, nodular BCC, well-differentiated SCC and malignant melanoma progressed further in dedifferentiated and metastatic tumours in a direct proportion relationship.
3. The downregulation or loss of E-cadherin but not of P-cadherin, particularly in BCC and SCC tumours with less aggressive behaviour in addition to the double immunolabelling results in BCC may point to a stepwise loss of cadherins during tumour progression.
4. The preserved E-cadherin membrane staining in the palisading cells of the BCC tumour lobules may indicate that E-cadherin is one of multiple biological factors involved in the prevention of free stromal invasion and metastasis.
5. Suprabasal P-cadherin expression in benign hyperplasia and pre-malignant lesions may indicate that this molecule is proliferation-associated.
6. Co-localisation of E- and P-cadherin in normal epidermis suggests that E- and P-cadherin could be in the same junction plaque.

7. The dramatic increase in actin filaments polymerisation (stress fibers) in infiltrative BCC when compared with the nodular subtype and normal epidermis indicates a relation to the infiltrative growth pattern of BCC rather than rapid growth. This was accompanied by decreased membrane staining of the cell adhesion molecules E-cadherin, α- and β-catenin and α-actinin emphasising enhanced tumour cell motility and thus invasion.
8. Co-localisation of α-catenin and α-actinin with the actin filaments could be explained by the interactive role of α-catenin where it connects the membrane associated complex to the actin-based cytoskeleton.
9. Results of keratinocyte culture indicate that the cells grown in low calcium were less motile (similar to nodular BCC) as they showed weak actin staining and migrated into the wound but failed to close it completely, while the cells grown in high calcium were more motile as they showed increased actin filament polymerisation (similar to infiltrative BCC) and migrated in a sheet-like colony and closed the wound completely.

Background 2: During development there is interaction between the Sonic hedgehog (Shh)/Patched (Ptc) and the Wnt/Wingless (Wg) signalling pathways. It has been reported that the Shh/Ptc signalling pathway is dysregulated in BCC. Recently, mutations in β-catenin were found in the hair follicle tumour PMX. In addition to β-catenin's role in supporting cell adhesion at the cell-cell junctions by association with E-cadherin, another role for β-catenin in signal transduction has been proposed where it acts as a key component of the Wnt signalling pathway possessing a nuclear function as it binds transcription factors, Lymphoid Enhancer Factor (LEF-1) and T Cell Factor (TCF). The activation of the Hedgehog/patched signalling pathway in drosophila is associated with transcriptional activation of specific target genes including Wg which may lead to the secondary activation of the Wnt/β-catenin signalling pathway contributing to the development of BCC. The transcription factor Gli-1 is required during the activation of the Shh/Ptc signalling pathway which is associated with transcriptional activation of specific target genes including Wg and Ptc.

Aim 2:

- To investigate whether any decrease in membrane β-catenin expression in epidermal tumours is accompanied by redistribution to the cell nucleus secondary to the activation of the Wnt pathway.
- To investigate the expression of the transcription factor Gli-1 in normal skin and BCC as there is evidence that the activation of the Shh/Ptc-Smo signalling pathway plays a key role in BCC development.

Methods 2: Immunoperoxidase staining.

Findings 2:

1. Routinely processed, paraffin-embedded BCC showed nuclear localisation of β-catenin in 13/32 cases (N-terminus) and 8/32 cases (C-terminus), while frozen BCC showed nuclear staining of both termini in 1/12 cases. This was accompanied by reduced β-catenin membrane staining.
2. Tumour cell membrane staining of N and C termini of β-catenin was evenly reduced in 4/10 PMX but there was strong nuclear staining with both the N and C terminal antibodies in 7/10 tumours.
3. Bowen's disease showed nuclear localisation of C-terminus β-catenin in 2/10 cases accompanied by reduced membrane staining.
4. In routinely processed, paraffin-embedded BCC, tumour cell nuclear staining of Gli-1 was seen in 16/32 cases (N-terminus) and 21/32 cases (C-terminus). Normal epidermis showed variable and weak nuclear staining in the basal cells in a minority (5/32 N-terminus, 6/32 C-terminus).

Main conclusions 2:

1. The detection of nuclear β-catenin staining in Bowen's disease and BCC in addition to PMX is novel as it suggests that dysregulation of β-catenin cellular distribution may be common to several pathways leading to epidermal carcinogenesis.

2. While activation of β-catenin by mutation may be a primary event in PMX, it seems more likely that in BCC and Bowen's disease nuclear localisation of β-catenin would be secondary to activation of the Wnt pathway or possibly due to redistribution of β-catenin as a result of disruption in AJ.
3. Gli-1 nuclear localisation in BCC strongly supports the evidence which indicates that Gli-1 may be a transcriptional activator in which it provides evidence for the activation of the Shh/Ptc signalling pathways in this tumour.

Background 3: BCC is the commonest cutaneous tumour in humans. It is locally invasive but rarely metastasises. Biochemical and molecular studies of BCC were limited by the lack of a suitable and reproducible tissue-culture model system. Unlike other cutaneous tumours, attempts to grow BCC in primary cultures have not been found to be successful.

Aim 3: To grow different subtypes of BCC tumours in vitro.

Methods 3: Cell culture by seeding fresh single cell suspension of BCC derived by collagenase/dispase treatment onto fibroblast-embedded collagen gel model.

Findings 3:

1. Dermal equivalents seeded with tumour single cell suspensions derived from an infiltrative BCC showed massive degradation after 48 hours of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air. Results were confirmed by using cells from a second infiltrative BCC.
2. BCC-derived cells from nodular and superficial BCC did not degrade the dermal equivalents following incubation at 37°C in a humidified atmosphere of 5% CO_2 in air for 5 days as submerged cultures.
3. Dermal equivalent seeded with 1 x 10^5 tumour-derived cells from nodular BCC showed viable basaloid cells reminiscent of BCC cells after 11 days of culture.
4. BCC-derived cell cultures using tissue culture flasks coated with foetal calf serum and/or with Type I collagen failed to yield cell growth. In addition, culture of tumour

cells mixed with or without normal keratinocytes onto fibroblast-free and standard dermal equivalents gave similar negative results.

Main conclusions 3:

1. The massive degradation of dermal equivalents could be due to several reasons: firstly, due to the production of collagenase by infiltrative BCC cells, secondly due to a fibroblast-derived collagenase activity released either from BCC stromal cells or dermal equivalent fibroblasts upon interaction with BCC cells.
2. The absence of dermal equivalent degradation using superficial and nodular BCC in contrast to the massive degradation using infiltrative BCC strongly suggest that the various subtypes of BCC differ in their degrading properties in vitro and may explain the more aggressive behaviour of infiltrating BCC clinically.
3. In general, the results of BCC culture were not satisfactory, however, modification of the technique used in my work may allow future studies using dermal equivalents as a potential model for BCC culture.

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Abbreviations Used

AJ,	Adherens junction
APC,	Adenomatous polyposis coli
BCC,	Basal cell carcinoma
C,	Carboxyl terminus
CB,	Citrate buffer
Ci,	Cubitus interruptus
DPBS,	Diluted phosphate buffered saline
E-Cadherin,	Epithelial cadherin
EDTA,	Ethylenediaminetetraacetic acid
FCS,	Foetal calf serum
Fz,	Frizzled
Gli-1,	Glioma-1
GSK-3β,	Glycogen synthase 3β
Hh,	Hedgehog
IF,	Immunofluorescent
MEM,	Minimal essential medium
N,	Amino terminus
Neg,	Negative
O/N,	Overnight
PC,	Pressure cooker
P-Cadherin,	Placental cadherin
PT,	Pre-treatment
Ptc,	Patched
SCC,	Squamous cell carcinoma
Shh,	Sonic hedgehog
Smo,	Smoothened
TB,	Tris buffer
TBS,	Tris buffer saline
Wg,	Wingless

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Chapter 1: Introduction

1.1. Basal cell carcinoma (BCC)

BCC is the commonest cutaneous tumour in humans, particularly in the white population (MacKie, 1996a). Although accurate data are not available, about 1.2 million BCC cases were managed in the USA in 1995 alone and the incidence of this tumour in the western countries is increasing by 3-6% each year (Bastiaens et al., 1998). Overexposure of fair-skinned individuals to ultraviolet radiation is generally accepted as the main causal factor of BCC; however, this correlation is not complete, since about 20% of tumours still occur in unexposed body areas (MacKie, 1996a; McKee, 1996a). Sun exposure during childhood and adolescence may be the critical factor for establishing adult risk for BCC (Gallagher et al., 1995). Other causes of this tumour include ionising radiation (Lever and Schaumburg-Lever, 1990a) and, less commonly, chronic forms of physical injury e.g. burn scars, tattoos and smallpox vaccination sites (MacKie, 1996a; McKee 1996a; Preston and Stern, 1992). Prolonged intake of inorganic arsenic and exposure to coal tar derivatives may also lead to the development of BCC (McKee, 1996a).

Although there is no formal grading system for BCC, they could be classified into five main clinical subtypes (McKee, 1996a):

1. Nodulocystic/ulcerative BCC (45-60%) starts as a small translucent papule which subsequently becomes nodular with few telangiectatic vessels and ulcerates centrally. The margin of the ulcer is well-defined, slightly raised and rolled (du Vivier and McKee, 1993a). Histologically the tumour is composed of large basaloid lobules varying in size and shape with a loose surrounding stroma usually rich in mucin. This tumour is characterised by separation of the lobules from their associated stroma and retraction which could be related to basement membrane abnormalities of adhesion and fixation resulting in mucin shrinkage (McKee, 1996a).
2. Diffuse (infiltrating and morphoeic) BCC (4-17%) is most likely to be misdiagnosed as a scar. It may present as a slightly elevated, smooth, firm plaque (du Vivier and McKee, 1993a). The morphoeic pattern of this tumour is due to excessive connective tissue proliferation with narrow tongues of epithelium that extend deeply into the adjacent fibrous stroma (MacKie, 1996a; du Vivier and McKee, 1993a).

3. Superficial (multifocal) BCC (15-35%). The lesions are usually multiple and appear as erythematous, slightly scaly plaques often with a fine, rolled margin (Fitzpatrick et al., 1992). This tumour is typified by many discrete downgrowths of small and dark basaloid cells from the dermoepidermal junction (MacKie, 1996a).
4. Pigmented BCC (1-7%) differs from the nodulocystic/ulcerative subtype only by the heavily pigmented margins of the tumour (Lever and Schaumburg-Lever, 1990a). The pigmentation may be evident in both dendritic melanocytes and stromal macrophages. Dense deposits of haemosiderin may also sometimes be present (McKee, 1996a).
5. The fibroepithelioma of Pinkus is frequently found adjacent to a basal cell carcinoma (MacKie, 1996a). It consists of usually only single but occasionally multiple papules or nodules, covered by smooth, slightly reddened skin (du Vivier and McKee, 1993a; Fitzpatrick et al., 1992). The characteristic feature of this tumour is multiple strands of basaloid epithelial cells arising from the epidermis that have anastomosed to create a complicated net-like pattern (McKee, 1996a).

Other variants of BCC include the basisquamous or metatypical type, where squamous differentiation is a feature (MacKie, 1996a). Occasionally some tumours may show focal clear cell change due to glycogen accumulation, hence the clear cell variant, others may be called keratotic where horn cysts are evident or granular due to membrane-bound lysosomal-like granules (McKee, 1996a).

In addition, there are clinical syndromes in which BCC play an important part. They are Gorlin's syndrome (basal cell naevus syndrome) (Howell and Caro, 1959; Gorlin, 1987), linear unilateral basal cell naevus (Anderson and Best, 1962) and Bazex syndrome (Plosila et al., 1981; Goeteyn et al., 1994).

1.1.1. Pathogenesis and biology of BCC

In BCC there is a dual population of a fibrous stroma surrounding lobules of dependent cells which have a large, oval or elongated nucleus and relatively poorly defined cytoplasm that resemble those of basal cells of the epidermis and outer root sheath (ORS) of hair follicle (Lever and Schaumburg-Lever, 1990a). A peripheral palisade is usually present around the edge of the lobule (McKee, 1996a). BCC cells differ from basal cells and ORS cells by having a larger ratio of nucleus to cytoplasm and by the absence of intercellular bridges (Lever and Schaumburg-Lever, 1990a). Recently, (Kore-eda et al., 1998) have reported evidence that BCC cells are more closely related to follicular matrix cells than they are to ORS cells. However, the analysis of the immunophenotype of BCC in comparison with the human vellus hair follicular keratinocytes revealed a close relationship between BCC and ORS cells and the bulge region of the vellus hair follicle (Kruger et al., 1999).
The mechanism of pathogenesis in BCC could be due to flawed rehearsal of embryological differentiation because of stromal dependence and the capacity of the tumour for limited differentiation towards any skin appendage (McKee, 1996a).

Probably, due to the difficulty in ascertaining that BCC excision is complete, basisquamous and infiltrating tumours are more likely to recur (MacKie, 1996a; Sloane, 1977). However, the form of therapy chosen, as well as size, location, and histology play an important role in defining the risk of recurrence. Although BCC is typically a slowly growing tumour that may recur, metastatic cases are a rare entity (Miller, 1991).

Treatment of BCC is with excision with primary closure, skin flaps, or grafts. The use of cryosurgery, curettage and electrosurgery are other options. For lesions in the nasolabial area or around the eyes and in morphoeic BCC, Mohs surgery is the best approach (Telfer et al., 1999).

1.2. Squamous cell carcinoma (SCC) and precursor lesions

Squamous cell carcinoma (SCC) of the skin is a malignant lesion which may occur anywhere on skin and on mucous membranes with squamous epithelium (Lever and Schaumburg-Lever, 1990b). SCC of the skin is the second commonest malignancy following BCC in fair-skinned people (Preston and Stern, 1992; Marks et al., 1988a). SCC is derived from epidermal keratinocytes in sun-damaged skin commonly from solar keratosis or Bowen's disease, and composed of cytologically malignant cells with the capacity for metastatic spread (MacKie 1996b). Bowen's disease and solar keratoses are in situ dysplastic lesions of particular importance. They indicate previous exposure to ultraviolet radiation and most importantly predict the strong possibility of developing SCC of the skin (Marks et al., 1988b).

Keratoacanthoma is histologically similar to SCC and regarded as a squamous cell carcinoma variant rather than as a benign or pseudomalignant tumour (McKee, 1996b).

The true incidence of SCC is variable and difficult to determine with accuracy, but is progressively rising in developed countries (Ko et al., 1994). The most common cause of SCC in Caucasians is ultraviolet irradiation, but exposure to polycyclic hydrocarbons and ingestion of arsenic may induce the tumour. SCC may be a late consequence of the scarring process and skin disease affecting the mucosae, for example, lichen planus and lichen sclerosus et atrophicus, or can be a consequence of certain genetic diseases such as albinism and xeroderma pigmentosum. The tumour may arise as a complication of treatment with photochemotherapy (PUVA), extensive application of topical nitrogen mustard and prolonged use of immunosuppressive agents in renal transplant patients (du Vivier and McKee, 1993a). Human papilloma virus has been implicated in the development of SCC. Typically, the lesion will start as a thickening of the skin and becomes an indurated plaque which grows laterally and vertically. Eventually, the lesion becomes nodular and may ulcerate (du Vivier and McKee, 1993a; MacKie, 1996b).

Histologically, SCC are subdivided into 3 major variants depending upon the degree of differentiation. Well-differentiated tumours are characterised by invading islands of

squamous epithelium that shows obvious and abundant keratinisation with well-formed intercellular bridges readily apparent. Moderately differentiated tumours are typified by more mitotic activity and nuclear pleomorphism with architectural disorganisation. In poorly differentiated variants, there is marked nuclear and cytoplasmic pleomorphism with hyperchromatic and sometimes bizarre nuclei and conspicuous mitotic figures. Rarely, poorly differentiated variants will show intercellular bridges and foci of keratinisation (du Vivier and McKee, 1993a; McKee 1996b). In comparison with BCC, cutaneous and mucosal SCC have a higher metastatic potential. High recurrence and metastasis rates are associated more with poorly differentiated than well-differentiated variants particularly with the presence of perineural, lymphatic and vascular involvement. The frequency of metastasis in SCC is variable and the rate is higher particularly with recurrent SCC (25-45%) (Rowe et al., 1992). SCC metastatic frequency seems to be dependent upon many factors. These include the site of the primary tumour in the skin, histologic subtypes, tumour diameter, aetiology, host immunosupression and prior treatment (Kwa et al., 1992).

1.3. Benign melanocytic lesions and malignant melanoma

In the earliest stage of development, the melanocyte migrates from the neural crest to the dermoepidermal junction (MacKie, 1996c). Melanocytes at the basal layer of the epidermis may be polygonal and epithelioid with eosinophilic cytoplasm and contain uniform round-oval small nuclei with prominent nucleoli (McKee, 1996c).

Benign melanocytic tumours composed of naevus cells derived from the melanocytes in the basal layer are hamartomatous conditions that usually present in childhood and adolescence (McKee, 1996c). They can be divided into 3 main categories. Junctional naevi which are solely intraepidermal, form well-circumscribed nests of many naevus cells with no involvement of the upper epidermis (MacKie, 1996c). Intradermal naevi show no junctional activity and are entirely dermal lesions (du Vivier and McKee, 1993b). In these, naevus cells are small and compact with a relatively epithelioid appearance particularly in the more superficial aspects of the lesion (MacKie, 1996c). With the increasing depth of the lesion, the naevus cells become progressively smaller with minimal cytoplasm and hyperchromatic nuclei and occasionally may adopt a

spindle cell morphology (du Vivier and McKee, 1993b). Compound naevi possess features of both junctional and intraepidermal naevi (Lever and Schaumburg-Lever, 1990c). Other totally benign melanocytic lesions are Spitz and blue naevi. The Spitz naevus in most instances has the basic architecture common to all melanocytic naevi (Hoss and Grant-Kels, 1986). It is composed of large melanocytes nests with spindle and epithelioid features at the junctional and/or dermal regions (Paniago-Pereira et al., 1978). Although it may be histologically misdiagnosed as a malignant melanoma (Okun, 1979), it shows marked retraction artefacts in its junctional components, and striking lateral symmetry in contrast to the disorganised morphology and irregular lateral border of melanoma (McKee, 1996c). In blue naevus, the overlying epidermis is usually normal and the lesion is typically confined to the deeper aspect of the dermis and may extend to the hypodermal region (Lever and Schaumburg-Lever, 1990c). Its pathology is that of a spindle cell population of naevus cells which tend to be large and bipolar interwoven between collagen bundles (MacKie, 1996c).

Atypical naevi whether familial or sporadic show melanocytic architectural and cytologic dysplasia and are associated with an increased incidence of malignant melanoma particularly in the familial variants (Cook and Robertson, 1985; Barnhill, 1991). They may be junctional or compound, displaying the characteristic lentiginous melanocytic hyperplasia along the sides as well as the tips of the epidermal ridges. The dysplastic cells which may be present singly or in clusters usually show increased nuclear size, cytoplasmic pleomorphism and variable hyperchromatism (McKee, 1996c). These features in addition to the occasional mitotic figures seen in atypical melanocytes must be differentiated from the in situ superficial spreading melanoma with radial growth phase.

Malignant melanoma is a very important neoplasm because of the current increase in its incidence rate leading to a rise in morbidity and mortality (MacKie et al., 1991). In Australia, for instance, about 30 males and 24 females/100,000 of the population are affected each year (Jelfs et al., 1994).

The aetiology of malignant melanoma is multifactorial including genetic and racial factors, however, in the majority of tumours, excessive exposure to ultraviolet light is the most important predisposing factor (McKee, 1996c).

Malignant melanoma could be divided on clinical pathological grounds into 4 subsets, these are:

1. Lentigo maligna melanoma is an intraepidermal proliferation of usually spindle-shaped malignant melanocytes replacing the epidermal basal layer particularly in the lateral growth phase. The epidermis is usually atrophic and the dermis shows solar elastosis (MacKie, 1996d)
2. Superficial spreading malignant melanoma is not usually associated with features of severe actinic damage. Malignant cells characteristically spread from one epidermal ridge to the adjacent one and are irregularly distributed, singly or in nests, throughout the epidermis involving the upper layers. These cells are large with abundant cytoplasm and pleomorphic vesicular nuclei. Invasive tumours are usually of epithelioid cell type (du Vivier and McKee, 1993a).
3. Nodular malignant melanoma is characterised by a very sharp transition from normal epidermis to an area of invasive tumour with downgrowths of malignant cells into the dermis (MacKie, 1996d). Melanoma cells in this subset are typically divided into two type. Epithelioid cell type in which melanoma cells are large and rounded with abundant cytoplasm and prominent pleomorphic vesicular nuclei showing abnormal mitotic figures occasionally. The spindle cell type is characterised by elongated cells reminiscent of mesenchymal cells (McKee, 1996c)
4. Acral lentiginous melanoma and its variant subungual malignant melanoma (MacKie, 1996d) have a similar pathology to that of lentigo maligna melanoma except that the epidermis is hyperplastic (du Vivier and McKee, 1993a).

The survival of patients with malignant melanoma can be related to depth of the infiltration of the dermis by using the Clark subclassification.

Level I: where tumour confined to the epidermis.

Level II: infiltration of the papillary dermis by single cells.

Level III: infiltration to the junction of the papillary with the reticular dermis.

Level IV: infiltration of the reticular dermis.

Level V: infiltration of the subcutaneous fat.

Prognosis is good for levels I and II but increasingly poor for III to V (du Vivier and McKee, 1993a).

Overview

One of the prominent phenotypic alterations exhibited by tumour cells is the change in cellular morphology and histological differentiation compared to normal cells. Development of malignant tumours, including malignant cutaneous tumours, is characterised by the ability of tumour cells to overcome the cell-cell adhesion, served by desmosomes and AJ, and to invade surrounding tissue. Many different cell adhesion molecules, including major AJ components, are implicated in human carcinogenesis in which changes in these molecules accompany the tumour de-differentiation.
In the following sections I will give an introduction to the structures, properties and functions of the major AJ components (cadherins, catenins and other associated proteins). Additionally, the cellular distribution of these proteins in normal human tissues and tumours will be reviewed.

1.4. Adherens junctions (AJ)

AJ are multiprotein complexes localised at intercellular and cell-matrix contacts, mediating adhesion of cells to each other or to matrix structures (Knust and Leptin, 1996; Alberts et al., 1998). The initiation of AJ formation is maintained by focal accumulation and homophilic binding between the cell-cell adhesion molecules, cadherins, which require Ca^{2+} ions be present in the extracellular medium (Lodish et al., 1995; Alberts et al., 1998; Hitt and Luna, 1994).

There are 3 different types of AJ localised at cell-cell and cell-matrix contacts which have been demonstrated in tissues and cultured cells: In vitro focal contact, which is formed at sites of attachment of cells to the culture surface; the classical zonula adherens which form a continuous adhesion belt (belt desmosome) around polarised epithelial cells in the apical region of the cell, just below the tight junctions; and a third type, localised at cell-cell contacts which appear structurally similar to desmosomes by conventional electron microscopy (Kaiser et al., 1993). It has been shown that AJ are present in human normal epidermis and oral mucosa (Kaiser et al., 1993).

E-Cadherin is considered to be a key element of the AJ where its cytoplasmic portion is associated with a variety of cytoplasmic proteins, some of which, such as β-catenin, bind directly to E-cadherin, while others, like α-catenin, vinculin, α-actinin or radixin, link the cadherin/β-catenin complex to actin filaments, thereby coupling cell adhesion to the modulation of cell shape (reviewed by Hitt and Luna, 1994; Kemler, 1993; Ranscht, 1994). The regulatory role of E-cadherin in Ca^{2+}-induced adherens junction organisation in cultured keratinocytes may be mediated through activation of protein kinase C (Lewis et al., 1995).

1.5. Cadherin superfamily classification

There are 6 gene families comprising the cadherin superfamily depending on the number of repeats and on sequence homologies (reviewed by Aberle et al., 1996). These gene families are as follows:

1. Classical cadherins (type I) which are mainly localised in AJ and include epithelial (E-); neural (N-); placental (P-); retinal (R-); xenopus blastomere (XB-); ubiquitously expressed during early development (U); related to E- and P-cadherin (EP); brain (B-); chicken liver cadherin (L-CAM); kidney (K-) and osteoblast (OB) cadherin (Xiang et al., 1994; Okazaki et al., 1994), (reviewed by Kemler, 1993; Ranscht, 1994).
2. Classical cadherins (type II) which include cadherin-6 to -12. Apart from cadherin-11 which was localised in the human placenta (Getsios et al., 1998), their localisation and function have not been identified fully. Other classical cadherins which were not classified under the above 2 types include muscle (M-) cadherin and cadherin-5 (Alattia et al., 1999).
3. Desmosomal cadherins which include desmocollins 1-3, desmogleins 1-2 and the pemphigus vulgaris antigen (desmoglein 3) (Amagai et al., 1991; Buxton et al., 1993). These are localised in the intercellular adhesion junction structures, termed desmosomes, which mediate strong adhesion between cells in many epithelial (e.g. between keratinocytes) and non-epithelial tissues (Garrod, 1993). They may participate in the actual binding of the keratinocytes and are thought to be involved in

maintaining the mechanical strength of the epidermis under physiological conditions (Horiguchi et al., 1994).

4. Cadherins with a very short cytoplasmic domain or none e.g. truncated (T-) cadherin and liver/intestine (LI-) cadherin (Berndorff et al., 1994; Ranscht, 1994).
5. Protocadherins PC42 and PC43 which are cadherin-related molecules shown to mediate calcium-dependent, homophilic cell adhesion (Sano et al., 1993).
6. Related gene products including the drosophila fat tumour suppressor gene (Mahoney et al., 1991), the dachsous gene, and the ret-protooncogene (Hashizume et al., 1996; Aberle et al., 1996).

1.6. Classical cadherins

The intercellular adhesion molecule, cadherin, was first reported in 1977 (Takeichi, 1977). Numerous members of the classical cadherin family have been identified and E- and N-cadherin are the best characterised molecules in the family (Barth et al., 1997). Classical cadherins mediate Ca^{2+}-dependent cell-cell adhesion which are expressed on the cell surface and which function as intercellular adhesion molecules (Rasbridge et al., 1993). They play important roles in membrane integration, cytoskeletal interactions, and posttranslational modifications such as glycosylation, phosphorylation and proteolysis. Furthermore, they possess functional sites for adhesive recognition and calcium binding (Alattia et al., 1999). Cadherins play a role in the regulation of keratinocyte growth and differentiation and may be involved in controlling integrin expression (Moles and Watt, 1997). They are transmembrane proteins which are characterised by a highly conserved carboxy-terminal cytoplasmic domain (Kemler, 1992). Many studies on classical cadherins have shown that the cytoplasmic domain regulates the function of these proteins through its direct interactions with a number of cytoplasmic components called catenins or plakoglobin that link them to the actin-based cytoskeleton (Mathur et al., 1994; Cowin and Burke, 1996). Their extracellular domains, located in the N-terminal part of the molecule, consist of repeated Ca^{2+}-binding subdomains (Vleminckx and Kemler, 1999). This part is structurally determined by the binding of calcium ions which are central to the homophilic adhesion with cadherin molecules on adjacent cells (Ringwald et al., 1987; Nagafuchi et al.,

1987) (Fig. 1). Intracellularly they are attached, via linker proteins, to cytoskeletal filaments (Barth et al., 1997; Alberts et al., 1998).

Each of the classical cadherins is an integral membrane glycoprotein of 720-750 amino acids. On average, 50-60% of the sequence is identical among different cadherins. Importantly, each cadherin has a characteristic tissue distribution (Lodish et al., 1995). Suppression of cadherin expression has been found to correlate with tumour-cell invasion and metastasis (reviewed by Takeichi, 1993). However, expression of cadherins is developmentally regulated in which it facilitates several morphogenetic processes such as cell-sorting mechanisms (Nose et al., 1988), migration of cells, cell polarity, formation of boundaries between cell layers, maintenance of proliferation of cells within a cell layer and ultimately, differentiation of each cell layer into functionally distinct structures (Takeichi, 1988; 1991).

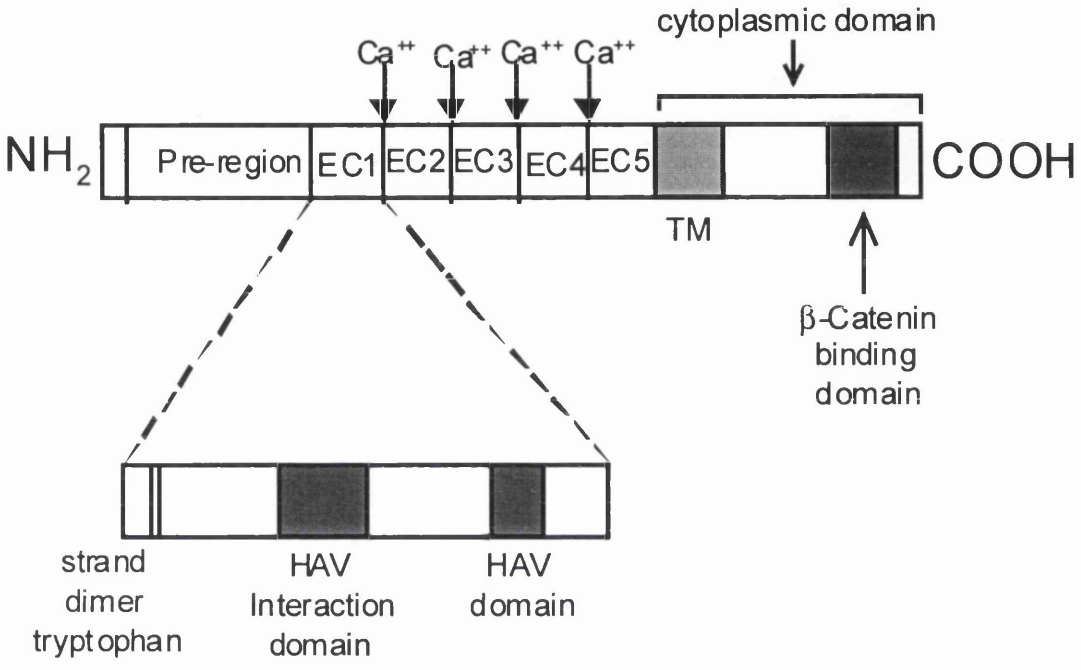

Figure 1. Classical cadherin structure (Ranscht, 1994; Wheelock et al., 1996). Mature cadherins are transmembrane proteins derived from a precursor by cleavage of the Pre-region. The membrane-spanning domain is labelled TM and the extracellular portion is labelled EC1-EC5 of approximately 110 amino acids each. Calcium ion-binding sites between adjacent EC domains are indicated. EC1 is enlarged to show the strand-dimer tryptophan, the HAV (Histidine, Alanine, Valine) domain, and the domain in which HAV interacts with its homotypic partner cadherin. The strand-dimer tryptophan near the N-terminus of EC1 plays a role in the formation of the strand dimer. The cytoplasmic region interacts with β-catenin and is highly conserved between different cadherin species.

1.7. E-Cadherin

The mechanisms of cell adhesion play a key role in many major cellular processes such as embryogenesis, tissue and organ patterning, maintenance of specific tissue architectures and tumour formation (Navarro et al., 1991).

In carcinoma, detachment of cancer cells from a primary tumour site as a result of disorganised cell-cell association is a critical step leading to tumour invasion and metastasis (Liotta, 1986; Nicolson, 1988). This phenomenon is attributable largely to the disruption of intercellular adhesion mediated by epithelial (E-) cadherin which acts as an invasion suppressor molecule (Schipper et al., 1991; Vleminckx et al., 1991; Fuller et al., 1996) (reviewed by Takeichi, 1993; Behrens, 1999).

E-Cadherin as all the members of the classical cadherins is composed structurally of a highly conserved cytoplasmic region and an ectodomain with four structural repeats containing calcium-binding motifs and a cysteine-containing region (Fig.1) (Ranscht, 1994).

The carboxy-terminal cytoplasmic domain of E-cadherin interacts directly with β-catenin (Fig. 2) and can also associate with other proteins in the complex like plakoglobin and $p120^{cas}$. E-Cadherin competes with adenomatous polyposis coli (APC), tumour suppressor gene product, for the direct binding with β-catenin; however, there are differences between E-cadherin/catenin and APC/catenin complexes and both molecules cannot be bound to β-catenin at the same time (Hulsken et al., 1994; Ilyas and Tomlinson, 1997). The binding of E-cadherin to β-catenin is positively regulated by serine phosphorylation (Cowin and Burke, 1996). β-Catenin is central to the architecture of E-cadherin/β-catenin complex, linking E-cadherin indirectly to α-catenin (Aberle et al., 1996). E-Cadherin has a regulatory role in the organisation of Ca^{2+}-induced AJ in cultured keratinocytes and in other epithelial tissues (O'Keefe et al., 1987; Lewis et al., 1995). This role may be mediated through activation of protein kinase C (PKC) (Lewis et al., 1995).

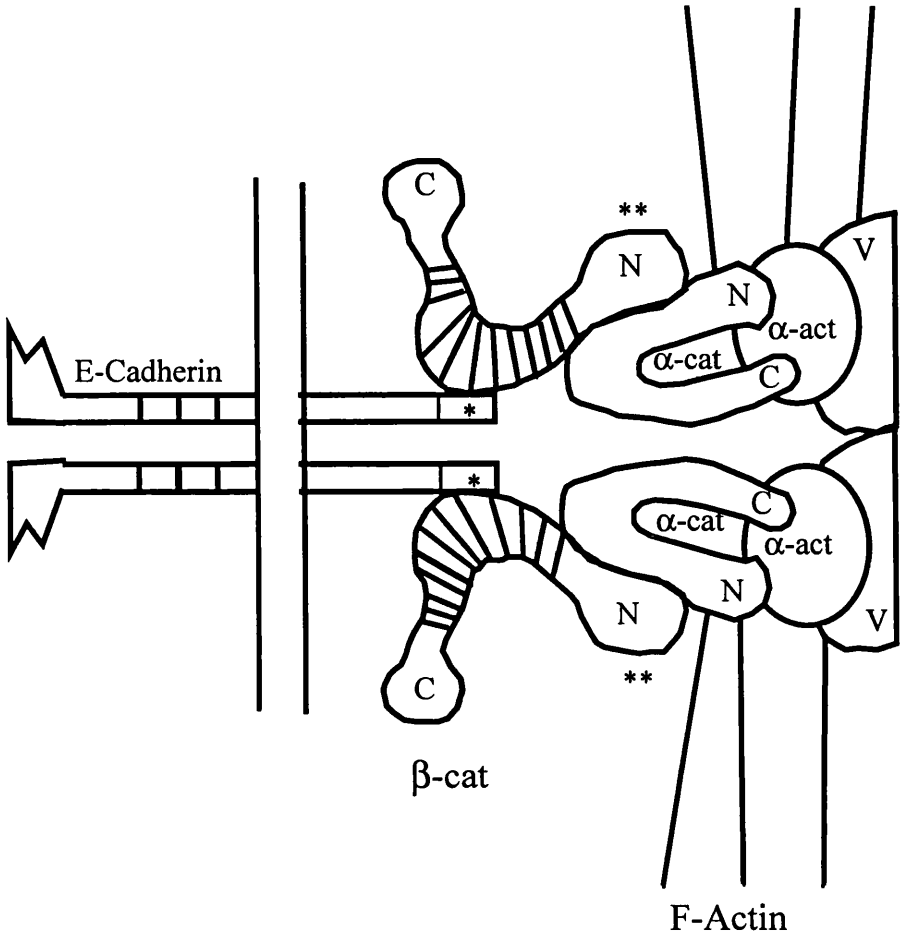

Figure 2. Connections at adherens junctions (Cowin and Burke, 1996). E-Cadherin dimers connect directly to repeats 5-8 of β-catenin (β-cat). Binding of β-catenin to E-cadherin is positively regulated by serine phosphorylation indicated with (*). The amino terminus (N) and the first repeat of β-catenin bind to the amino-terminal half of α-catenin (α-cat). Tyrosine phosphorylation (**) of the amino terminus of β-catenin negatively regulates the association between the cadherin-catenin complex and filamentous actin (F-actin); serine phosphorylation (*) of the head domain targets cytosolic form of β-catenin for degradation. α-Catenin (α-cat) associates with filamentous actin through its amino and carboxyl termini and it also binds to α-actinin (α-act). The latter binds both filamentous actin and vinculin (V).

1.7.1. Expression and gene mutations of E-cadherin in various tumours

The maintenance of epithelial differentiation requires proper formation of cell-cell junctions where destabilisation of such junctions allows initiation of the invasive process and the progression of carcinoma (Behrens et al., 1989; Frixen et al., 1991; Vleminckx et al., 1991; for review see Nicolson, 1988; Takeichi, 1993).
E-Cadherin is considered to be the major adhesion molecule found in AJ where it is expressed on most cell types that form solid tissues (Takeichi, 1988; Tang et al., 1994).

Down-regulation or complete loss of E-cadherin occurs in association with human epithelial cancers and it may convey additional signals to induce tumour-cell invasion (Fuller et al., 1996; Perl et al., 1998; Cano et al., 1996). This inverse correlation between the expression of E-cadherin and tumourigenicity has led to the proposition that E-cadherin acts as an invasive suppressor and may be a useful prognostic marker in cancer (Navarro et al., 1991; Cheng et al., 1996).
Recently, there is an accumulating experimental evidence suggesting that down-regulation or change in structure of one or more of the major components of AJ could lead to junctional disassembly and consequently to a reversion from a benign to an invasive epithelial phenotype (Perl et al., 1998; Vleminckx et al., 1991; Schipper et al., 1991; Shiozaki et al., 1994; Takayama et al., 1996).
During the past several years, various laboratories and institutions have realised the importance of characterising the structure and function of cellular adhesion molecules in human epithelium. This led to the awareness of the central role of E-cadherin in the formation of epithelial cell-cell junctions (AJ) and of the potential function of this protein as a biochemical marker for tumour progression. Because E-cadherin may function as an invasive suppressor of malignant tumours, studies have concentrated on the examination of E-cadherin expression and cellular localisation in various human and animal malignancies suggesting a correlation between reduced expression of E-cadherin and loss of differentiation and increased invasive potential of malignant tumours. These studies were carried out in a variety of tumours, including skin (Schipper et al., 1991; Pizarro et al., 1994; Fuller et al., 1996; Cowley and Smith, 1996; Cano et al., 1996), breast (Rasbridge et al., 1993; Oka et al., 1993; Glukhova et al., 1995), colorectal (Kinsella et al., 1993) and prostate cancers (Cheng et al., 1996). However, in skin,

studies on E-cadherin expression were limited, for instance, to a particular tumour such as SCC of the head and neck (Schipper et al., 1991), where it was found to be inversely correlated with the loss of differentiation. In addition, although the reduction of E-cadherin expression was related to the growth pattern and the local aggressive behaviour of BCC, it was reported to be strongly preserved in both superficial and nodular subtypes of BCC (Pizarro et al., 1991). Fuller and colleagues (1994) demonstrated that, in BCC and SCC, expression of E-cadherin was reduced, however, no correlation with tumour de-differentiation in BCC was mentioned and insufficient material was present to correlate SCC behaviour with level of E-cadherin expression.

Transformation from a normal epithelial cell to a malignant cell is a complicated process involving many steps and results from accumulation of multiple gene abnormalities. Mutations of the E-cadherin gene have recently been discovered in a variety of carcinomas including human gastric carcinoma and cell lines (Becker et al., 1994; Oda et al., 1994), carcinomas of the endometrium and ovary (Risinger et al., 1994). The truncated E-cadherin protein which is unable to associate with β-catenin is predicted to act as a dominant negative by preventing wild type E-cadherin forming normal AJ.

Since I started my work, other studies of E-cadherin expression in tumours took place such as in skin (Silye et al., 1998; Montonen et al., 1998), gastric (Guilford et al., 1998; Jawhari et al., 1999), pancreatic (Perl et al., 1998), bladder (Giroldi et al., 1999), nasopharyngeal (Zheng et al., 1999). The findings of these studies will be discussed in relation to my work in the discussion section.

1.7.2. E-Cadherin expression in normal skin and in skin tumours

In the normal epidermis, E-cadherin is expressed on the cytoplasmic membranes of human epidermal keratinocytes (Fujita et al., 1992; Horiguchi et al., 1994) where it maintains adhesion between these cells by homophilic binding (Lewis et al., 1994; Horiguchi et al., 1994). In addition, E-cadherin is expressed on the membranes of normal epidermal melanocytes, and more recently Langerhans cells, although they lack both desmosomes and AJ (Tang et al., 1994; Blauvelt et al., 1995; Seline et al., 1996). Through homophilic binding, E-cadherin may function to organise and maintain the epidermal-melanin unit (Seline et al., 1996).

E-Cadherin is distributed in the full thickness of the epidermis excluding stratum corneum (Shimoyama et al., 1989; Horiguchi et al., 1994), with an increased expression in the suprabasal layers as compared with the basal cells (Cano et al., 1996). In the hair follicle, E-cadherin is strongly expressed in the outer root sheath cells at the region of the infundibulum and weaker in the lower segment while sebaceous and sweat glands express E-cadherin in their outermost cells (Fuller et al., 1996).

Loss of E-cadherin expression in BCC and SCC, but not in premalignant lesions, was found to be consistent with the observation that the loss of E-cadherin is associated with tumour invasion (Fuller et al., 1996). In the previous study, although there was a reduction in E-cadherin expression in almost all cases of BCC, E- cadherin expression was decreased more extensively in SCC than in BCC. The reduction of E-cadherin expression was related only to infiltrating growth of BCC (Pizarro et al., 1994), whereas solid tumours such as superficial and nodular, showed preserved E-cadherin expression. Downregulation or loss of E-cadherin in BCC, SCC and malignant melanoma indicated a close association with de-differentiation and invasion potential of these tumours (Schipper et al., 1991; Montonen et al., 1998). In malignant melanoma, a differential loss of E-cadherin expression was noted (Silye et al., 1998). This was particularly observed in dermal nests of melanomas in their radial growth phase and in Clark level II and III lesions, whereas it was present in a considerable proportion of melanomas in their vertical growth phase, in Clark level IV and V lesion and in metastasising melanomas. In the previous study, membranous E-cadherin expression was detected in

all benign naevi, although heterogeneous in junctional naevus cell nests. However, Cowley and Smith (1996) reported that cadherin expression, and particularly E-cadherin expression, tended to be much greater in malignant melanomas than in benign melanocytic naevi.

E-Cadherin expression may be focally downregulated or even lost in premalignant lesions (Cano et al., 1996). This kind of observation seems to represent an earlier indication of the more pronounced changes seen at the invasive stage of the tumours and it could provide an advantage to the premalignant cells by detaching from the adjacent cells, in agreement with the proposed anti-invasive role for E-cadherin (Cano et al., 1996).

1.8. P-Cadherin

Placental (P-) cadherin, a 120 KDa glycoprotein, belongs to the subfamily of classical cadherins, glycoproteins with single transmembrane domain (Kemler, 1993). P-Cadherin has been localised in the AJ mediating intercellular adhesion through a homophilic mechanism (Jensen et al., 1997). It shares overall structural and functional similarities with E-cadherin (see above) as well as with the other members of the classical cadherin subfamily (Fig. 1) (Kemler, 1993).

Normal keratinocytes and melanocytes express both P-cadherin and E-cadherin (Horiguchi et al., 1994; Lewis et al., 1994). Furthermore, adhesion between keratinocytes and melanocytes is mediated by homophilic E- and P-cadherin binding (Tang et al., 1994). In normal murine epidermis, as in normal human skin, expression of P-cadherin follows a well defined pattern where it is restricted to the basal proliferative compartment (Hirai et al., 1989; Cano et al., 1996). It is also expressed in the keratogenous region of the precortical hair matrix and in the outer root sheath cells of the hair follicle during murine hair follicle morphogenesis and cycling (Cano et al., 1996; Muller-Rover et al., 1999). In addition to P-cadherin expression in the skin, it is found to be present in several other tissues and cell lines including the sertoli cells of

mouse testis (Lin and DePhilip, 1996), the membranes of the epithelial and lateral borders of the myoepithelial cells of the breast (Rasbridge et al., 1993; Glukhova et al., 1995) and more recently in bladder carcinoma cell lines (Giroldi et al., 1999).

The exact role of P-cadherin is less well defined than that of E-cadherin (Tang et al., 1994). Since both E- and P-cadherin are expressed in the basal layer of normal epidermis, they probably share the capability of regulating intercellular junction organisation (Jensen et al., 1997). When E-cadherin function is blocked in primary keratinocyte cultures, the calcium-induced formation of both AJ and desmosomes is delayed leading to an abnormally stratified culture (Lewis et al., 1994). Blocking P-cadherin function alone has no effect on the calcium-induced redistribution of adhesion molecules and the keratinocytes stratify normally. However, when both E- and P-cadherin are blocked, stratification does not occur and adhesion molecule concentration along the cell-cell borders is not detected (Lewis et al., 1994). Inhibition of either of the cadherins alone does not affect the initiation of stratification (Jensen et al., 1997). These findings imply that E-cadherin plays the primary role in regulating intercellular junction organisation and stratification. In addition, P- and E-cadherin may have redundant roles in the basal layer of epidermal keratinocytes with regard to the process of stratification (Jensen et al., 1997).

In cancer, E- and P-cadherin play an important role in tumour formation, progression and metastasis. Previous studies have demonstrated different expressions of E- and P-cadherins in human cancers and cell lines that vary inversely with the stage of clinical disease or even is preserved in these tumour cells (Seline et al., 1996; Shimoyama et al., 1989; Shimoyama and Hirohashi 1991; Cano et al., 1996; in abstract form, Abbasi et al., 1992). The findings of these studies along with the more recent ones will be discussed in more details in the discussion section in comparison to my work.

1.9. Catenins

The cell adhesion molecules, cadherins, form protein complexes with cytoplasmic polypeptides called catenins (reviewed by Gumbiner, 1995). Three proteins were initially identified by co-immunoprecipitation with the cytoplasmic domain of E-cadherin (Aberle et al., 1996). They also form complexes to bind other proteins of the cadherin family (Butz et al., 1992). The catenins have molecular weights of 102, 94 and 86 KDa and were termed α, β, and γ catenin, respectively and cloning of catenin genes revealed that α-catenin is homologous to vinculin, β-catenin is similar to the drosophila armadillo gene product, while γ-catenin is identical to plakoglobin (Kemler, 1993; Hashizume et al.,1996). Experimental analysis revealed the critical role of the catenins in regulating cadherin function, where deletion of the catenin-binding domain generates cadherin mutants that cannot mediate cell-cell adhesion (Hinck et al., 1994c). Catenins are implicated in many major cellular events: they mediate the connection of the E-cadherin with the actin filament network, and they link E-cadherin with other transmembrane proteins such as Na^+ /K^+ ATPase, and with peripheral cytoplasmic proteins such as fordin (Ozawa et al., 1990; McNeil et al., 1990). In addition to its adhesive role, β-catenin functions as a nuclear effector participating in many cellular processes including signal transduction and transcription regulation (Korinek et al., 1997; reviewed by Gumbiner, 1995; Willert and Nusse, 1998). These functions and properties will be discussed in more detail in the following sections.

1.9.1. β-Catenin

1.9.1.1 Structure of β-catenin and its role in cell adhesion

β-Catenin is a multifunctional 94-KDa protein that shares about 65% sequence identity with plakoglobin, an 82-KDa protein which is apparently the same as γ-catenin (Peifer and Wieschaus, 1990; McCrea et al., 1991; Fouquet et al., 1992; Knudsen and Wheelock, 1992; reviewed by Cowin and Burke, 1996). Both β-catenin and plakoglobin are members of the armadillo family of proteins in which their central region contains a 42 amino acid motif repeated 12-13 times known as the arm repeats (Piefer et al., 1992; Butz et al., 1992; reviewed by Willert and Nusse, 1998). The amino-terminal domain of β-catenin contains 130 amino acids and also contains multiple phosphorylation sites which are correlated with the downregulation of β-catenin. Its carboxy-terminus contains a transcriptional activation domain of 100 amino acids. The arm repeats (central region) interact in an overlapping and mutually exclusive manner with E-cadherin (Fig. 2), adenomatous polyposis coli (APC) tumour suppressor gene, T-cell factor (TCF) and lymphoid enhancer factor 1 (LEF1) transcription factors (Aberle et al., 1994; Hulsken et al., 1994; Huber et al., 1997; for review see Willert and Nusse, 1998). β-Catenin is also similar to the drosophila armadillo gene product which was originally identified as one of a group of proteins that regulate segment polarity (Riggleman et al., 1989).

The exact role for β-catenin in cell adhesion as a structural component of the AJ is less clear. It associates directly with α-catenin which then mediates the interaction between the cadherin-catenin complex and the actin based cytoskeleton (Ozawa et al., 1990; Gumbiner, 1995). β-Catenin binds to both the cytoplasmic domain of the cadherin molecule and the amino-terminal domain of α-catenin, whereas there is no direct interaction of α-catenin with the cadherins. However, the association of the cadherins with α-catenin through β-catenin is a crucial requirement for the adhesive function of the cadherins. Cells that express cadherin molecules but lack α-catenin are non-adherent, but become adhesive when α-catenin is expressed (Hinck et al., 1994c; Nathke et al., 1994). Experimentally, a direct mechanical fusion between E-cadherin

and α-catenin could bypass the need for β-catenin where it acts as a kind of negative regulator for the cell adhesion activity (Nagafuchi et al., 1994). It has been proposed that β-catenin plays a role in regulating adhesion, at least in part because it is a substrate for several tyrosine kinases that suppress cell adhesion (Behrens et al., 1993). β-Catenin has also been reported to form complexes with epidermal growth factor receptor in in vitro kinase assays, but the function of this intriguing interaction, mediated by the conserved central region of β-catenin, is not yet known (Hoschuetzky et al., 1994).

1.9.1.2. Expression of β-catenin in normal tissue and tumours

β-Catenin plays a major role in the cadherin-mediated cell-cell adhesion system (reviewed by Cowin and Burke, 1996; Miller and Moon, 1996). In normal epithelial cells, β-catenin is mainly localised at the membranes of the cell-cell borders (Takayama et al., 1996) where it binds to cell surface cadherins (E- and P-) and also to α-catenin to be connected to the actin cytoskeleton (Ozawa et al., 1989). Non-cutaneous normal epithelia express strong β-catenin especially on cell-cell boundaries. These include human oesophagus and stomach (Takayama et al., 1996), human colon (Takayama et al., 1996; Inomata et al., 1996) and normal breast mammary duct tissue (Hashizume et al., 1996). More recently, strong β-catenin membrane staining has been observed in rat colon (Takahashi et al., 1998), nasopharyngeal epithelium (Zheng et al., 1999), capillary endothelial cells of the thyroid gland (Garcia-Rostan et al., 1999) and human oesophagus (Kimura et al., 1999).

There is evidence of a close association between abnormal expression and/or structural abnormalities of β-catenin as well as E-cadherin (see above) and tumour development (Nakanishi et al., 1997; Jawhari et al., 1997; Valizadeh et al., 1997). This is to say that downregulation of membranous β-catenin is associated with malignant transformation as demonstrated in carcinomas of breast (Hashizume et al., 1996), oesophagus and stomach (Takayama et al., 1996) and colon (Takayama et al., 1996; Inomata et al., 1996). This may affect the E-cadherin-mediated adhesion system, as β-catenin forms a

complex with E-cadherin in normal human epithelium and cancerous tissues (Ozawa et al., 1989; Takayama et al., 1996).

Recently, it has been reported that in normal skin, β-catenin is localised in a linear pattern along the lateral and upper surfaces of basal keratinocytes at the sites of cell-cell junctions (Tada and Hashimoto, 1998; Silye et al., 1998). The normal keratinocytes in suprabasal layers express β-catenin around their peripheries. Membranous β-catenin is also present in the outer root sheath cells of the hair follicles, acinar germinative cells of the sebaceous glands and in sweat glands cells (Silye et al., 1998). However, β-catenin is absent at the basal cell surface, in stratum corneum, inner sheath cells of the hair follicle and the hair shaft (Silye et al., 1998; Tada and Hashimoto, 1998).

Since I started my work, reports on β-catenin immunolocalisation in human tumours and cell lines have been published. Abnormalities of β-catenin cellular expression have been observed in desmoid tumours (Alman et al., 1997), colon tumours (Takahashi 1998; Back et al., 1999), oesophageal cancers (Kimura et al., 1999), gastric carcinoma cell lines (Jawhari et al., 1999) and anaplastic thyroid carcinoma (Garcia-Rostan et al., 1999). However, apart from 2 recent reports in benign and malignant melanocytic lesions (Silye et al., 1998; Rimm et al., 1999), immunohistochemical studies of β-catenin expression in epidermal tumours are lacking. The findings of the published studies will be discussed in relation to my work in the discussion section.

1.9.1.3. β-Catenin's mutation and its role in signal transduction

In addition to β-catenin's role in supporting cell adhesion, another role for β-catenin in signal transduction has been proposed; however, these are separable (reviewed by Gumbiner, 1995; Cowin and Burke, 1996). β-Catenin is a key downstream component of the Wnt signalling pathway participating in signal transduction, developmental patterning and cell fate determination in both drosophila and vertebrates (Gumbiner, 1995; Brown and Moon, 1998). This role was suggested by the finding that β-catenin, as well as γ-catenin/plakoglobin, are highly homologous to the product of drosophila segment polarity gene armadillo (McCrea et al., 1991; reviewed by Miller and Moon, 1996). There are many associated and binding partners of β-catenin controlling its activity and affecting its stability and cellular localisation (reviewed by Willert and Nusse, 1998).

1.9.1.4. Role of β-catenin in the Wnt/Wingless signalling pathway

Wnt/Wingless (Wg) proteins, a large family of cysteine-rich secreted molecules associated with the extracellular matrix and cell surface, control embryonic patterning and cell fate decisions in development (van den Heuvel et al., 1989; Bradley and Brown, 1990; Papkoff and Schryver, 1990; reviewed by Morata and Lawrence, 1977; Nusse and Varmus, 1992; Klingensmith and Nusse, 1994; Moon et al., 1997; Cadigan and Nusse, 1997). Wnt family members may also modulate cell-cell interactions during morphogenetic movements by regulating the stability and accumulation of β-catenin and cadherin indirectly leading to the increased strength of cell-cell adhesion (Hinck et al., 1994b). During development there is interaction between the Wnt/Wingless and the Sonic hedgehog/Patched signalling pathways (see below) (Von Ohlen and Hooper, 1997). The Wnt signalling pathway has not been ordered fully and less is known about this pathway in vertebrates than the Wg pathway in drosophila (Peters et al., 1999). However, there are many similarities between the two signalling systems such as Wg and armadillo in drosophila are homologous to Wnt-1 and to β-catenin/plakoglobin in vertebrates respectively (reviewed by Gumbiner, 1995). Regulation of catenin function

by Wnt-1 was derived primarily from early studies on homologous proteins in drosophila (reviewed by Hinck et al., 1994a). Wnt/Wg signalling pathway regulates β-catenin negatively and is highly conserved throughout evolution from insects to vertebrates (Ben-Ze'ev and Geiger, 1998). There are different molecular interactions of β-catenin in Wnt/Wg signalling as well as in cell adhesion.

When Wnt/Wg signalling is activated (Fig. 3), a Wnt molecule binds to the extracellular cysteine-rich domain of the Frizzled (Frz) family of transmembrane receptors (Wang et al., 1996). This binding likely will lead to phosphorylation and activation of Dishevelled (Dsh) protein (Noordermeer et al., 1994; Peters et al., 1999) which is the most proximal intracellular component known in the pathway (Klingensmith et al., 1994). The way that Dsh is activated is less clear and its phosphorylation by casein kinase II does not necessarily lead to its activation (Willert et al., 1997) and it seems likely that other events are also required (Cook et al., 1996; for review see Brown and Moon, 1998). When Dsh is activated it will be recruited to the cell membrane area leading to inhibition of the serine kinase glycogen synthase kinase 3β (GSK-3β) action, possibly involving protein kinase C (PKC) (Cook et al., 1996). In the absence of Wnt/Wg signalling, β-catenin is found in a cytoplasmic multiprotein complex (Rubinfeld et al., 1996) in addition to its presence in the cell adhesion complex with cadherins at the plasma membrane. β-Catenin in the cytoplasm associates with APC, GSK-3β, Axin (reviewed by Eastman and Grossched, 1999) and the Axin homologue conductin (Behrens et al., 1998) where GSK-3β normally phosphorylates β-catenin and directs it together with APC and Axin to degradation by the ubiquitin-proteosome system (Aberle et al., 1997; Orford et al., 1997; Peifer, 1997; Polakis, 1999). Inhibition of GSK-3β by activated Dsh leads to decreased degradation of β-catenin (stabilisation) and as a result β-catenin cytoplasmic levels rise (Yost et al., 1996; reviewed by Brown and Moon, 1998). Accumulated and stabilised β-catenin appears to be translocated independently to the nucleus by less clear mechanisms (Tlsty, 1998) where it is associated with the T-cell-specific factor (TCF)/lymphoid enhancer binding factor (LEF) transcription factors (Huber et al., 1996; Molenaar et al., 1996; Fearon and Dang, 1999; Yokoya et al., 1999). This association results in increased transcriptional activation of mostly unknown target genes (Behrens et al., 1996; Korinek et al., 1997; reviewed by

Eastman and Grossched, 1999). One of these target genes recently discovered is the cyclin D1 gene which when accumulated and triggered by increased β-catenin levels may promote neoplastic conversion (Shtutman et al., 1999; Tetsu and McCormick, 1999).

In the absence of Wnt/Wg signalling (Fig. 4), Wnts are bound and inhibited by members of the Frizzed b (Frzb) family which seem to act by binding to secreted Wnt proteins and preventing productive interaction between Wnt proteins and Frz proteins (reviewed by Brown and Moon, 1998). In these circumstances, Dsh is inactive which, in turn, leads to the activation of GSK-3β (Ben-Ze'ev and Geiger, 1998). As a result, the cytoplasmic complex of GSK-3β, APC and Axin targets β-catenin for degradation by the ubiquitin-proteosome pathway. The low levels of degraded cytoplasmic β-catenin are insufficient to be translocated to the nucleus and associate with TCF/LEF transcription factors which are likely to bind to promoters in the absence of β-catenin and repress basal transcription proteins (Brown and Moon, 1998; Willert and Nusse, 1998).

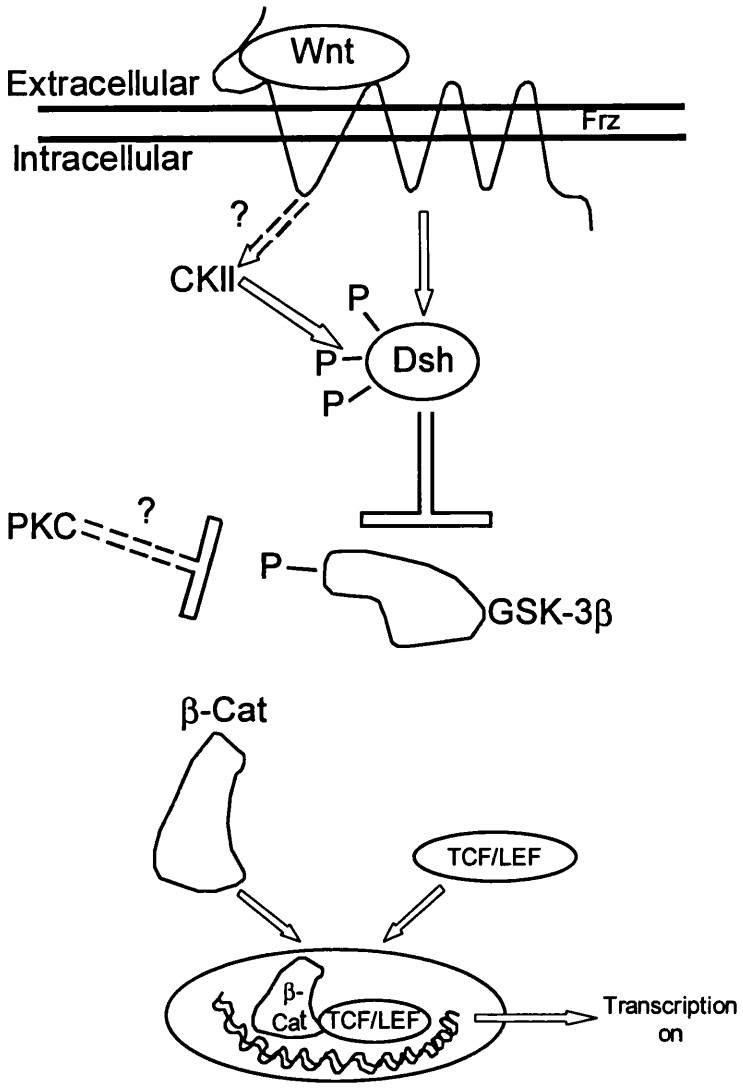

Figure 3. The Wnt signalling pathway. In the presence of a Wnt signal (Ben-Ze'ev and Geiger, 1998; Brown and Moon, 1998; Willert and Nusse, 1998; Eastman and Grossched, 1999). Binding of Wnt to the Frizzled (Frz) family of receptors leads to activation of Wnt signalling. Dishevelled (Dsh) becomes hyperphosphorylated (P) by casein kinase II (CKII), activated and recruited to the membrane area. This leads to the inactivation of glycogen synthase kinase-3β (GSK-3β) by a poorly understood mechanism that may involve protein kinase C (PKC). GSK-3β normally phosphorylates (P) β-catenin (β-cat) and targets it together with adenomatous polyposis coli (APC) and Axin for degradation. β-Catenin fails to be phosphorylated and thus is no longer targeted into the ubiquitin-proteosome pathway. Instead, β-catenin accumulates in the cytoplasm as a result of decreased rates of degradation mediated by GSK-3β and appears to be translocated to the nucleus where it interacts with T-cell-specific factor (TCF)/lymphoid enhancer binding factor (LEF) transcription factors which results in transcriptional activation.

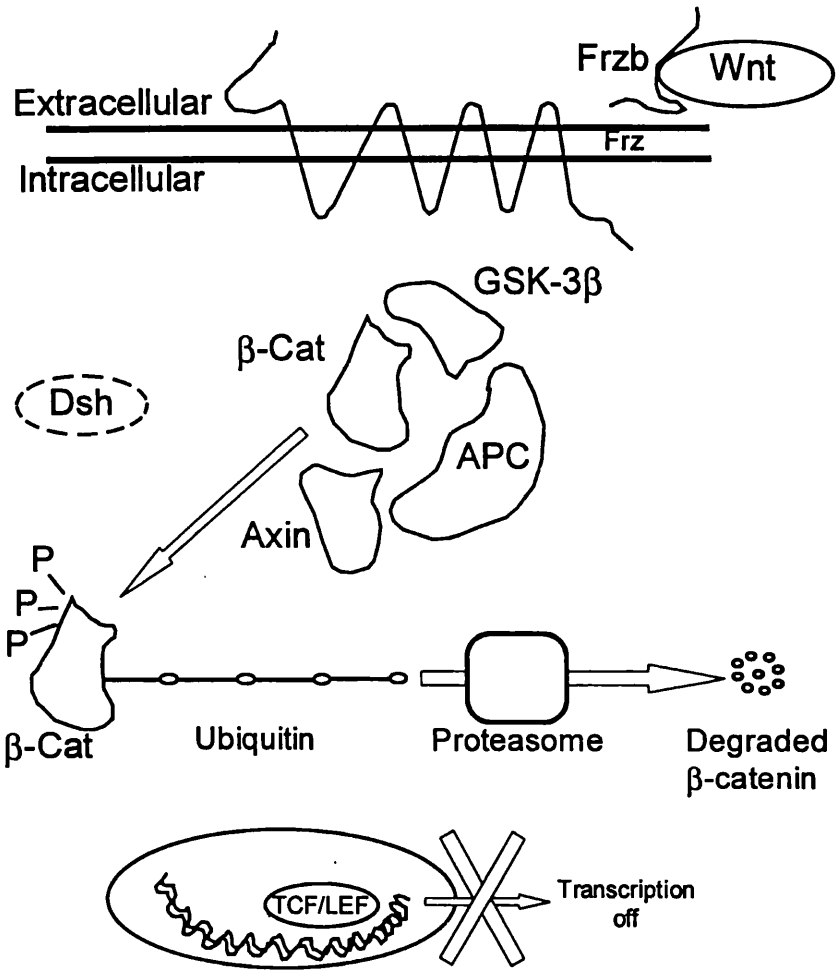

Figure 4. The Wnt signalling pathway. In the absence of a Wnt signal (Ben-Ze'ev and Geiger, 1998; Brown and Moon, 1998; Willert and Nusse, 1998; Eastman and Grossched, 1999).
Wnt is bound and inhibited by members of Frizzled b family (Frzb). Dsh is inactive and GSK-3β is active. GSK-3β phosphorylates (P) β-catenin (β-cat) and targets it together with adenomatous polyposis coli (APC) and Axin for degradation through the ubiquitin-proteosome pathway. Low levels of cytoplasmic β-catenin are insufficient to be translocated to the nucleus and associate with T-cell-specific factor (TCF)/lymphoid enhancer binding factor (LEF) transcription factors.

1.9.1.5. Interaction of β-catenin with the APC tumour suppressor protein

An inherited defect in the APC gene results in the development of colorectal polyps and colon cancer in afflicted patients, and similar APC mutations have been implicated in the majority of spontaneous non-familial colorectal tumours (Miyoshi et al., 1992).
The direct association of β-catenin with APC tumour suppressor protein (Su et al., 1993; Hulsken et al., 1994; Rubinfeld et al., 1993; 1995) suggests that it may be involved in regulating and/or mediating β-catenin signalling activity leading to regulating tumour growth (Gumbiner, 1995; Ilyas and Tomlinson, 1997). This was demonstrated by recent biochemical studies where APC, in addition to cadherin, may regulate the intracellular levels of β-catenin, thus implicating APC as a modulator of β-catenin signalling (Munemitsu et al., 1995; reviewed by Miller and Moon, 1996). In addition to GSK-3β, APC may act to antagonise Wnt signalling by targeting β-catenin for degradation through the ubiquitin-proteosome pathway and eliminating its free cytoplasmic pool (Orford et al., 1997; Miller and Moon, 1996). Mutations in the β-catenin-binding site of APC may result in cytoplasmic accumulation of β-catenin and blockade of its degradation (Munemitsu et al., 1995). Furthermore, levels of free cytoplasmic β-catenin rise in colon cancer cells with mutant APC suggesting that APC suppresses β-catenin signalling activity by maintaining low cytoplasmic levels (reviewed by Miller and Moon, 1996; Ben-Ze'ev and Geiger, 1998). Thus APC may possibly function primarily to regulate cadherin-mediated cell adhesion via β-catenin (Barth et al., 1997). However, β-catenin's signalling activities are separable from its function in cadherin-mediated cell adhesion and APC-catenin complex is distinct from the cadherin-catenin complex (Hulsken et al., 1994; Inomata et al., 1996). Recently it is known that the association of β-catenin with TCF/LEF transcription factors in the nucleus activates gene expression (Behrens et al., 1996; Korinek et al., 1997; Eastman and Grossched, 1999). APC may regulate the formation of transcriptionally competent β-catenin-TCF complexes where loss of APC function may result in uncontrolled transcriptional activation of TCF target genes contributing to colon carcinogenesis (Korinek et al., 1997). These findings strengthened the early suggestions by Gumbiner (1995) that APC has a distinct role in

the β-catenin signalling pathway initiated by Wnt growth factors controlling cell growth and tumourigenesis by transducing signals leading to changes in gene expression.

1.9.1.6. β-Catenin redistribution to the nucleus

β-Catenin translocation to the nucleus seems to be a highly specific process although its mechanism is not completely understood (Willert and Nusse, 1998). Stabilisation of β-catenin is associated with its nuclear localisation in the presence of Wnt/Wingless signals, where it binds to TCF/LEF transcription factors (reviewed by Brown and Moon, 1998; Eastman and Grossched, 1999). In the nucleus, β-catenin acts as a transcriptional co-activator leading to increased expression of TCF/LEF-regulated target genes (Fearon and Dang, 1999). Traffic of macromolecules occurs in both directions through the nuclear pores which span the nuclear envelope (Alberts et al., 1998). Large molecules (proteins) and macromolecular complexes are generally passed through the nuclear pores via an active, receptor-mediated mechanism (Yokoya et al., 1999). At elevated expression levels, β-catenin may be detected in the nucleus although it may not contain any obvious nuclear localisation signal (Funayama et al., 1995). In addition, β-catenin is very large to passively enter the nuclear envelop through nuclear pore complexes (Cowin and Burke, 1996; Yokoya et al., 1999). Recently it was found that β-catenin can be translocated to the nucleus using a permeabilised cell system (Fagotto et al., 1998). β-Catenin nuclear translocation in this particular system was independent of nuclear localisation signal sequences and probably without associating in the cytoplasm with TCF/LEF transcription factors or other cytoplasmic factors. β-Catenin, like importin-β and β-karyopherin, appears to use a domain with common nuclear pore components that are nuclear localisation signal-independent (Fagotto et al., 1998; Ben-Ze'ev and Geiger, 1998). Furthermore, Yokoya and colleagues (1999) have recently shown that β-catenin possesses the ability to migrate into the nucleus through nuclear pore complex by a facilitated mechanism without requiring importin-β-related transport factors and Ran, a small GTPase, suggesting that the nuclear levels of β-catenin could be regulated by several alternative mechanisms.

1.9.2. α-Catenin

α-Catenin, a 102 KDa cytoplasmic polypeptide, is homologous to vinculin (Herrenknecht et al., 1991; Nagafuchi et al., 1991), a peripheral cytoplasmic protein which connects membrane bound proteins to the cortical actin cytoskeleton (reviewed by Kemler, 1993; Ranscht, 1994). At AJ, the amino-terminal half of α-catenin binds to the amino terminus and the first repeat of β-catenin (Knudsen et al., 1995; Rimm et al., 1995; reviewed by Cowin and Burke, 1996). It is not known whether α-catenin can interact directly with the cadherins in the AJ or only through β-catenin (Ozawa and Kemler, 1992), however, it has been found to be associated with all classical cadherins (Kemler, 1993). In addition to associating with actin through its amino and carboxyl termini (Ozawa et al., 1990), α-catenin also connects the cadherin-catenin complex to α-actinin which binds both filamentous actin and vinculin (Knudsen et al., 1995; Rimm et al., 1995; reviewed by Aberle et al.,1996; Cowin and Burke, 1996). Apart from possible free pool of α-catenin, it can also associate with APC (Su et al., 1993) and with other, as yet unidentified, proteins (Hinck et al., 1994a; 1994c). α-Catenin is found in cells that do not express any cadherin where it is localised in the cytoplasm possibly having, as yet undetermined, biological functions (Hinck et al., 1994c; Kemler, 1993). Adhesive functions of the cadherins require the association with α-catenin in which α-catenin-deficient cells that express cadherins are non-adherent, but become adhesive when α-catenin is expressed (Ranscht, 1994). This possible modulation of cadherin adhesive functions by α-catenin was also noted in some human cancer cells which have impaired E-cadherin-mediated cell adhesiveness through the downregulation of α-catenin expression (Shiozaki et al., 1994).

Normal epithelial cells of human breast, oesophagus, stomach and colon express α-catenin in an almost identical distribution pattern to those for E-cadherin and β-catenin (Shiozaki et al., 1994; Hashizume et al., 1996). In these normal epithelial cells, α-catenin is located mainly on the cell membrane, particularly at the area of cell-cell contact. While normal epithelial cells express strong α-catenin, the reduction of α-catenin expression in cancer cells may be a characteristic feature acquired through

malignant transformation (Shiozaki et al., 1994). α-Catenin membranous expression was found to be frequently reduced in tumours of the breast, oesophagus, stomach, colon, and recently in malignant melanoma (Shiozaki et al., 1994; Hashizume et al., 1996; Silye et al., 1998). Thus functional or structural abnormalities of α-catenin might take place in tumours with reduced α-catenin expression interfering with cadherin-mediated cell-cell adhesion since the functions of E-cadherin are modulated by α-catenin (Shiozaki et al., 1994).

Since I commenced my work, studies on the distribution of α-catenin in normal and cancerous tissues have been published (Silye et al., 1998; Glukhova et al., 1995). These were done using immunostaining in normal breast and in invasive breast carcinoma, benign and malignant melanocytic lesions. The distribution of α-catenin in the normal epidermis follows the same pattern as E-cadherin and β-catenin (Silye et al., 1998). It is localised along the lateral and upper surfaces of normal basal keratinocytes at the cell-cell junctions and uniformly around the periphery of the cells in suprabasal layers. α-Catenin is also expressed in the membranes of adnexal structures. Membranous α-catenin is present in the outer root sheath cells, cell membrane of acinar germinative cells of sebaceous glands and in sweat gland cells. There is no expression of α-catenin in stratum corneum, hair follicle inner root sheath cells and hair shaft.
The findings of these studies will be discussed in relation to my own results.

1.10. Sonic hedgehog (Shh)/Patched (Ptc)-Smoothened (Smo) signalling pathway

Studies of embryonic development in drosophila, have led to the identification of many genes which are important in postnatal control of cell growth and differentiation (reviewed by Gailani and Bale, 1997). Mutations in some of the vertebrate and human homologues of these genes such as the patched gene have been implicated to cause disease and cancer (reviewed by Ingham, 1998).

Mutations in human Ptc were identified as the underlying genetic event in the autosomal dominant naevoid BCC syndrome (NBCC), some sporadic BCC (Gailani et al., 1996; Johnson et al., 1996; Hahn et al., 1996a; Aszterbaum et al., 1998; Smyth et al., 1999) and NBCC-associated medulloblastoma (Smyth et al., 1999; Vortmeyer et al., 1999). Furthermore, deletion of the Ptc gene has been shown in sebaceous naevus (Xin et al., 1999). In these studies, particularly in hereditary BCC, both alleles of Ptc are lost or mutated suggesting that Ptc is a tumour suppressor gene acting probably as the gatekeeper of basal cell carcinoma (Sidransky, 1996).

The Ptc gene is the vertebrate homologue of the drosophila segment polarity gene ptc (Goodrich et al., 1996; Hahn et al., 1996b), identified originally as a regulator of embryonic pattern formation (Nusslein-Volhard and Wieschaus, 1980). Ptc gene encodes a 12 transmembrane domain protein (Hooper and Scott, 1989; Nakano et al., 1989) that specifically and physically binds with high affinity to the vertebrate homologue of the drosophila hedgehog (Hh), sonic hedgehog (Shh) (Stone et al., 1996; Marigo et al., 1996). This provides evidence that Ptc is the Shh receptor (Stone et al., 1996; Murone, et al., 1999). The product of another drosophila segment polarity gene, Smoothened (Smo), complexes with Ptc but not directly with Shh (Stone et al., 1996; reviewed by Ingham, 1998). Smo encodes a seven-transmembrane protein which is most homologous to the Frizzled (Frz) family of serpentine proteins (van den Heuvel & Ingham, 1996; Alcedo et al., 1996).

In drosophila, it is suggested that ptc protein inhibits the Hh signalling pathway through its interaction with the Smo protein forming an inactive complex (Fig. 5) (Murone et al., 1999). Smo is downstream of ptc suggesting that its activity is regulated by Ptc (Chen and Struhl, 1996; Alcedo et al., 1996; Ingham, 1998). Based on this model, Shh might

activate the signalling pathway by relieving Ptc inhibition of Smo on binding to Ptc or following mutational inactivation of Ptc (Stone et al., 1996; Marigo et al., 1996). This alteration of the complex will free Smo to transduce the signal, which in drosophila, is associated with transcriptional activation of specific target genes including Wg, ptc, decapentaplegic, and repression of others such as Hh (Ingham et al., 1991; Ingham and Hidalgo, 1993; Capdevila et al., 1994; Basler and Struhl, 1994; Sekelsky et al., 1995). Cubitus interruptus (ci) is a drosophila transcription factor (Ohlmeyer and Kalderon, 1998) related to the human Gli family of zinc-finger transcription factors by showing significant homology (Kinzler et al., 1987; 1988). Ci is needed for the transcriptional activation of the signalling pathway target genes by mediating Hh signalling. In the presence of Hh, ci is functionally activated by loss of ptc (Aza-Blanc et al., 1997; Akimaru et al., 1997; Ingham, 1998). Thus, Ptc gene inactivation by mutation and chromosome loss may lead to the inability of Ptc to repress Smo activity, resulting in the activity of the Shh signalling pathway and causing cancer e.g. BCC (Hahn et al., 1996b; Lam et al., 1999). In accord with this, transgenic mice that overexpress Shh specifically in the skin develop BCC-like tumours suggesting that Shh may have a role in human tumourigenesis (Oro et al., 1997).

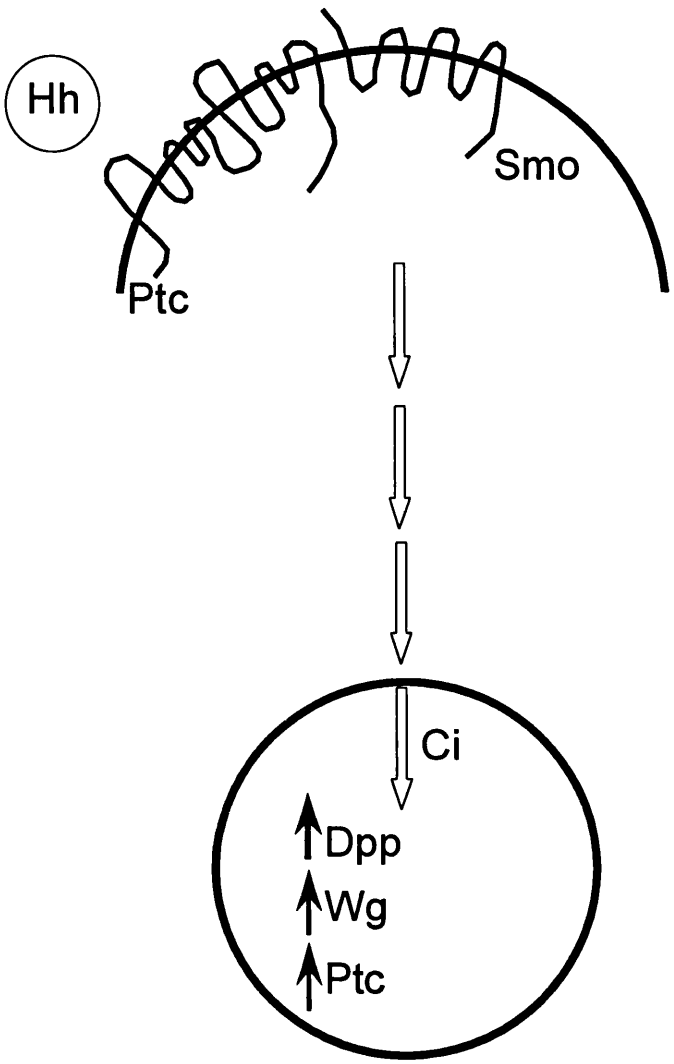

Figure 5. Interactions between patched, smoothened and hedgehog proteins (Gailani and Bale, 1997). Complementary DNA sequence predicts that patched (Ptc) is a trans membrane protein with 12-membrane spanning domains and two large extracellular loops which are required to bind hedgehog (Hh). Patched represses transcription of hedgehog target genes by inactivating smoothened (Smo). Hedgehog binds to patched, which activates smoothened and results in the increase of many genes including wingless (Wg), decapentaplegic (Dpp) and patched. In the absence of patched, smoothened may be constitutively activated which results in the overexpression of these genes. Cubitus interruptus (Ci) is a transcription factor that is activated in the presence of hedgehog.

1.11. Gli-1 role in BCC development

Up to date 3 Gli genes have been identified (Kinzler et al., 1987; Ruppert et al., 1988; 1990). Gli-1, a member of the Gli family of zinc-finger transcription factors (Kinzler et al., 1988), was originally isolated as an amplified gene in human malignant glioma (Kinzler et al., 1987). It has been identified as a candidate downstream mediator of the Shh signalling and acting as a transcriptional regulator in development (Dominguez et al., 1996; Alexandre et al., 1996; Lee et al., 1997; Hynes et al., 1997).

Since I commenced my work, two reports have investigated the expression of Gli-1 and Gli-3 in normal skin and BCC by reverse transcription-polymerase chain reaction and in situ hybridisation (Dahmane et al., 1997; Green et al., 1998). The results of these studies suggested that Gli-1 transcription factor plays a key role in BCC development. Recently, by using immunohistochemistry, Ghali and colleagues (1999) reported that Gli-1 protein is expressed in the cytoplasm of the outer root sheath keratinocytes and BCC cells. The findings of these studies will be discussed in more details in relation to my own results in the discussion section.

1.12. Vinculin and α-actinin

The focal adhesion is a complicated complex formed at sites where cells attach to the extracellular matrix, connecting actin to the plasma membrane (reviewed by Goldmann et al., 1996). Vinculin, a 130 KDa protein, is a major protein component of focal adhesion and AJ probably performing a similar function in both structures (Kaiser et al., 1993; Hazan et al., 1997). It shares 25-30% sequence homology with α-catenin (Herrenknecht et al., 1991; Nagafuchi et al., 1991; Ranscht, 1994). Both α-catenin and vinculin are capable of binding to α-actinin and actin (Ozawa et al., 1990; Knudsen et al., 1995). In addition to its adhesive functions, vinculin controls cytoskeletal remodelling and cell spreading by mechanically stabilising the molecular bridge between actin and integrins that form the core of focal adhesion (Ezzell et al., 1997).

At AJ, α-catenin connects the cadherin-catenin complex to α-actinin and filamentous actin in addition to associating with actin through both termini. α-Actinin in the complex binds both filamentous actin and vinculin (Fig. 2) (reviewed by Cowin and Burke, 1996). The self-associating properties of vinculin and the presence of actin-binding domain in its structure may contribute to the lateral clustering of the entire complex in the plane of the plasma membrane (Goldmann et al., 1996; Cowin and Burke, 1996).

α-Actinin is a major component of the AJ (Arikawa and Williams 1991; Knudsen et al., 1995; reviewed by Cowin and Burke, 1996). In normal epithelia, α-actinin is distributed largely in the cytoplasm with enrichment at the normal cell peripheries (Graig and Pardo, 1979; Geiger et al., 1979; Sanger et al., 1983; Drenckhahn and Franz, 1986).

In breast cancer cell lines, vinculin was found to play a role in the establishment or regulation of the cadherin-based cell adhesion complex substituting for α-catenin by direct interaction with β-catenin (Hazan et al., 1997).

In human skin and oral mucosa, vinculin was reported to be present in all epidermal layers except the stratum corneum (Kaiser et al., 1993). In the basal layer, vinculin is expressed in the area of the plasma membrane at sites of cell-cell contacts and the dermal-epidermal junction. Suprabasally, its plasma membrane expression is less

marked than the basal cells but strong in the granular layer with minimal or absent cytoplasmic distribution. Lifschitz-Mercer and colleagues (1997) reported that vinculin is also expressed strongly in a large variety of normal human tissues including squamous epithelia, smooth muscle, hair follicle epithelia and myoepithelial cells. However, the vinculin labelling in stratified epithelia was particularly prominent in the suprabasal cells of the epidermis which was largely cytoplasmic and was often enriched at cell borders and intercellular bridges. Furthermore, they have examined the expression of vinculin in different carcinomas including BCC, keratoacanthoma, Bowen's disease and invasive cutaneous and non-cutaneous SCC of different grades of tumour differentiation. Their results indicate that the level of expression of vinculin is inversely related to the degree of tumour-metastasising potential where most BCC, keratoacanthomas and Bowen's disease were uniformly positive whereas most invasive and metastatic SCC were either negative or highly non-uniform with extensive negative regions. These findings support the general indication that malignant transformation is commonly associated with reduced adhesiveness pointing to the role of vinculin in the formation of cell-matrix and cell-cell adhesion.

1.13. Actin filaments

Actin is a widely distributed protein found in all eukaryotic cells. The actin-based cytoskeleton is a dynamic structure, consisting of actin filaments and associated actin-binding proteins, localised in the cortical cytoplasm lying beneath the plasma membrane of cells (Cantiello and Prat, 1996). Actin filaments appear in electron micrographs as thin threads about 7 nm in diameter, and are formed from a chain of globular actin molecules (Alberts et al., 1998). The association of actin filaments with the actin-binding proteins enables them to serve a variety of functions including stability of cell shape, mechanical support, cell-cell adhesion and cell motility (Carlier, 1998; Alberts et al., 1998). Cell movement is thought to be driven largely by the polymerisation of actin monomers into filaments near the plasma membrane (Machesky and Way, 1998).

In polarised epithelial cells, AJ form a continuous belt around the apical portion of the cell and are connected to a circumferential band of actin filaments which may allow morphogenetic movements of the cells through actin filaments contraction (Kaiser et al., 1993). At AJ, the cadherin-catenin complex associates with actin filaments via the amino and carboxyl termini of α-catenin which also binds to α-actinin (Knudsen et al., 1995). The latter binds both vinculin and actin filaments (Rimm et al., 1995).

Keratinocytes in human normal epidermis have been shown to express weak staining of actin filaments which were arranged in parallel bundles beneath the cell membrane (Metze et al., 1996). Recently, it was reported that the pattern of distribution and degree of development of actin bundles vary in different parts of human hair follicles reflecting local demands of cytoskeletal activities for the growth and integrity of hair follicles (Furumura and Ishikawa, 1996).

During terminal differentiation of human epidermal keratinocytes, changes in the abundance and distribution of actin filaments and several actin-associated proteins including vinculin, α-actinin were examined by Kubler and colleagues (1991) using indirect immunofluorescent staining and confocal microscopy. The ventral cytoplasm of basal keratinocytes, located towards the centre of colonies, contained prominent bundles of actin filaments, often concentrated in the centre of the cells. In basal cells at the edges of the colonies, the actin filaments were concentrated at the leading edge of

the cells. In suprabasal cells, actin staining was concentrated at lateral cell margins, but was less pronounced and more diffuse than in the basal layer. Vinculin and α-actinin staining revealed typical focal contact structures, although α-actinin was most pronounced at the free margins of cells at the edges of colonies. The findings of Kubler and colleagues (1991) reflected differences in actin filaments distribution and cell shape between rapidly dividing keratinocytes at the basal layer and those with low probability of division at the centre of colonies as they grow by lateral migration.

Morphological changes of tumour cells due to quantitative and/or qualitative changes in actin proteins can contribute to malignant tumour progression by affecting their invasive and metastatic potential (Tsukamoto et al., 1994). Using immunoelectron microscopy and immunofluorescence staining, previous reports have suggested increased numbers of actin microfilaments in BCC and different tumour cell lines (Gabbiani et al., 1976; McNutt, 1976; Kumakiri and Hashimoto, 1978; Low et al., 1981; Jones et al., 1989). Similar microfilaments were also found in epidermal cells during wound healing (Martin and Lewis, 1992). The findings of these studies in BCC will be discussed in relation to my own results in the discussion section.

1.14. Culture of basal cell carcinoma

BCC is locally invasive (Leffell et al., 1991; Ko et al., 1992; McKee, 1996a) but rarely metastasises (Domarus and Stevens, 1984; Lo et al., 1991). In comparison with other tumours, BCC is a slow-growing tumour (Brysk et al., 1992). The cell origin of BCC remains unknown or highly controversial (Kore-eda et al., 1998; Kruger, et al., 1999).
BCC is composed of two essential components, namely epidermal downgrowths of small, dark, epithelioid cells cytologically similar to the cells of the basal layer and the abnormal connective tissue matrix or stroma surrounding these downgrowths (MacKie, 1996a). The stroma proliferates with the tumour in a symbiotic fashion (Brysk et al., 1992) and is arranged in parallel bundles around the tumour lobules (Lever and Schaumburg-Lever, 1990a). It may appear mucinous and often shows numerous fibroblasts, a mild inflammatory infiltrate and deposits of amyloid (Lever and Schaumburg-Lever, 1990a).

Biochemical and molecular studies of BCC were limited by the lack of a suitable and reproducible tissue-culture model system. Establishment of BCC cultures in the absence of the connective tissue stroma was not successful (Van Scott et al., 1961; Cooper et al., 1977). However, there are studies reporting growth of BCC cells in culture (Flaxman, 1972; Hernandez et al., 1985; Bradbeer et al., 1988; Brysk et al., 1992; Asada et al., 1992). Unfortunately, all these attempts to establish pure BCC cell lines were complicated by the presence of normal keratinocytes and they failed because the cells keratinised or differentiated and eventually died or they yielded cell cultures that are substantially unlike in vivo tumours (Brysk et al., 1992). Moreover, Grando and colleagues (1996) reported that the contamination of BCC cultures with normal human epidermal keratinocytes can suppress the growth of BCC cells.
In general there is interaction between the tumour and its adjacent connective tissue matrix in which tumours may directly alter the stroma by producing proteolytic enzymes that may degrade stromal glycoproteins and proteoglycans (Liotta et al., 1979; Kramer et al., 1982). In addition, tumours may regulate the function of the stromal cells by producing incompletely characterised soluble factors that stimulate proteolytic enzyme synthesis such as collagenase by the surrounding fibroblasts leading to indirect alteration of the stroma (Biswas, 1982; Barsky et al., 1987). These connective tissue

changes have been associated with BCC in which the tissue surrounding the tumour lobules characteristically has an increased number of fibroblasts (Lever and Schaumburg-Lever, 1990a) and increased amounts of hyaluronic acid and extractable collagenase (Liotta et al., 1979; Barsky et al., 1987). These interactions between the tumour and its stroma may play a critical role in the establishment, growth and invasive properties of the tumour (Lozzo, 1984).

The inconsistent outcomes of BCC culture attempts have cast a doubt on how genuine these results are in producing a suitable and reproducible tissue-culture model system of BCC.

1.15. Aims of the study

Aim 1a:

There is accumulating experimental evidence suggesting that down-regulation, complete loss or change in structure of one or more of the major components of AJ could lead to junctional disassembly and consequently to a reversion from a benign to an invasive epithelial phenotype. In biopsy specimens of human non-cutaneous carcinomas, the major components of AJ are frequently reduced or absent in association with malignant transformation particularly in the most differentiated tumours (Shimoyama and Hirohashi, 1991; Oka et al., 1992; Kinsella et al., 1993; Shiozaki et al., 1994). In human cutaneous tumours, studies of cell-cell adhesion have concentrated mainly on the expression of E-cadherin. E-Cadherin was found to be inversely correlated with the loss of differentiation of SCC of the head and neck (Schipper et al., 1991), but this observation was only limited to the expression of E-cadherin in this tumour. Although the reduction of E-cadherin expression was related to the growth pattern and the local aggressive behaviour of BCC, Pizarro and co-workers (1991) reported strongly preserved expression of E-cadherin in both superficial and nodular subtypes. Fuller and colleagues (1994) demonstrated that, in BCC and SCC, expression of E-cadherin was reduced, however, no correlation with tumour de-differentiation in BCC was mentioned and insufficient material was present to correlate SCC behaviour with level of E-cadherin expression. In addition, apart from melanocytic lesions, studies of P-cadherin and β-catenin expression in human cutaneous tumour tissues are lacking. Therefore, one of my aims was to investigate and define in detail the cellular distribution of AJ components in the normal human epidermis and in a variety of skin tumours including BCC, pilomatrixoma, SCC and precursor lesions and melanocytic lesions using immunohistochemical techniques. In order to do this, it was necessary to develop techniques for cadherin and catenin immunostaining of routinely processed, paraffin-embedded specimens. As there is evidence that down-regulation or complete loss of E-cadherin occurs in association with human epithelial cancers, this work aimed to assess whether the decrease in membranous distribution of the AJ major components, particularly cadherins and β-catenin, in human skin cancer progressed further in dedifferentiated or cancers with a poorer outlook.

Aim 1b:

By using immunofluorescence staining and laser scanning confocal microscopy, this part of the work was intended to characterise and define the overall structure of AJ in BCC versus the normal epidermis. Primarily, the immunofluorescence staining of the major AJ components was used to investigate the various types of adhesion deficiencies in BCC and compare it with the normal epidermis. Accordingly, double immunolabelling of the major components of AJ with filamentous actin was done to investigate the expression and the association of these components with the actin-based cytoskeleton. In my study, the distribution of filamentous actin in BCC was identified by Rhodamine-Phalloidin in contrast to a previous study which used anti-actin antibody (Low et al., 1981). Furthermore, this present work has attempted to examine the patterns of distribution of filamentous actin in the normal epidermis and BCC and how actin bundles are arranged or rearranged in the tumour cells with respect to their shape, migratory behaviour and thus invasiveness.

Aim 1c:

Although E- and P-cadherins share an overall structural similarity, they mediate the cell-cell adhesion of distinct cell types by homophilic binding. However, in the normal epidermis, both E- and P-cadherins are expressed on the cytoplasmic membranes of normal human keratinocytes following a specific distribution. E-Cadherin is distributed in the full thickness of the epidermis excluding the stratum corneum with an increased expression in the suprabasal layers as compared with the basal cells. In the hair follicle, E-cadherin is strongly expressed in the outer root sheath cells at the region of the infundibulum and weaker in the lower segment. P-Cadherin is restricted to the basal proliferative compartment and is also expressed in the keratogenous region of the precortical hair matrix and in outer root sheath cells of the hair follicle. Although E-cadherin is thought to be the major adhesion molecule found in AJ, basal cells of the normal epidermis have two cadherins regulating intercellular junction organisation. Therefore, it was decided to investigate the possible co-localisation of these cadherins in the basal and the first layer of suprabasal cells of the human normal epidermis.

Likewise, double immunolabelling of E- and P-cadherin in BCC was carried out to determine their expression, co-localisation and to test the hypothetical stepwise loss of cadherin by tumour cells.

Aim 1d:

Cultures of human keratinocytes provide a useful experimental model with which to study the factors that regulate cell proliferation, terminal differentiation and the assembly of cell-cell and cell-substrate junctions. Therefore, to extend the analysis of actin filaments, this present work attempted to compare the changes in the abundance and distribution of actin in nodular and infiltrative BCC with those in migrating keratinocytes in vitro. To achieve this aim, the surfaces of the cultured keratinocytes wells were wounded vertically and horizontally in straight lines. In addition, as the cultured keratinocytes differentiate by elevation of calcium concentration, it was of interest to compare the expression of E-, P-cadherin and β-catenin, in relation to actin, in both low and high calcium conditions with regard to the cell-cell adhesion. Thus, three days prior to the staining of cultured keratinocytes, the calcium concentration in the culture medium was shifted in half of the cultures from low (0.1 mM) to high (1.5 mM) $CaCl_2$.

Aim 2:

During development there is interaction between the Shh/Ptc-Smo and the Wnt/Wg signalling pathways (Von Ohlen and Hooper, 1997). Recently, it has been reported that the Shh/Ptc-Smo signalling pathway is dysregulated in BCC (Hahn et al., 1996a; Johnson et al., 1996). In addition to β-catenin's role in supporting cell adhesion at the cell-cell junctions by association with E-cadherin, another role for β-catenin in signal transduction has been proposed. β-Catenin is a key component of the Wnt signalling pathway possessing a nuclear function as it binds the transcription factors T-cell-specific factor and lymphoid enhancer binding factor (Huber et al., 1996; Molenaar et al., 1996). There are many striking parallels between the Wnt/β-catenin and the Wg/armadillo

signalling pathways in which they may operate via a similar biochemical mechanism. Because the activation of the Hh/ptc-Smo signalling pathway in drosophila is associated with transcriptional activation of specific target genes including Wg, it is possible that this may lead to the secondary activation of the Wnt/β-catenin signalling pathway contributing to the development of BCC. Nuclear localisation of β-catenin may indicate its activation as an oncogene reflecting activation of the Wnt/Wg signalling pathway in human cancers. In vivo, cytoplasmic and/or nuclear accumulation of β-catenin has been observed in non-cutaneous tumours including colorectal polyps and cancers, oesophageal and stomach cancers and desmoid tumours (Inomata, et al., 1996; Takayama et al., 1996; Alman et al., 1997). However, apart from a recent report on malignant melanoma (Rimm et al., 1999), nuclear localisation of β-catenin has not been demonstrated in epidermal tumours. Therefore, another aim was to investigate whether any decrease in membrane β-catenin expression in epidermal tumours is accompanied by redistribution to the cell nucleus as there is evidence that the activation of the Shh/Ptc-Smo signalling pathway plays a key role in BCC development.

Aim 3:

An investigation of the expression of the transcription factor Gli-1 in normal skin and BCC was undertaken because Gli-1 is required during the activation of the Shh/Ptc-Smo signalling pathway underlying the development of this tumour. This pathway is associated with transcriptional activation of specific target genes including Wg and Ptc (Ingham et al., 1991; Ingham and Hidalgo, 1993; Basler and Struhl, 1994).

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Aim 4:

Because of the lack of a suitable and reproducible tissue-culture model system for BCC, this part of my study attempted to grow this tumour by seeding fresh single cell suspension of BCC derived by collagenase/dispase treatment onto fibroblast-embedded collagen gel model (dermal equivalents). As controls and for comparison, seeding of suspension of cells derived by collagenase/dispase from normal foreskin onto dermal equivalents was undertaken.

Immunohistochemical methodology was used in this present work for several reasons. Immunohistochemical staining techniques nowadays are well defined and standardised. They are considered to be essential in the differential diagnosis of a large variety of skin diseases and tumours. In addition, they have given us the potential to localise antigens in cells and tissues with strongly improved diversity, selectivity and specificity of staining reactions. More importantly, immunohistochemical staining is the best methodology for subcellular localisation of antigens which will achieve my aims. Provided that a suitable, sensitive and specific antibody can be produced and is commercially available and the antigen is preserved, it seems that there is no limit to the specific antigen-antibody reaction that may be localised by using immunohistochemistry. However, as with all techniques, pitfalls and problems are possible if the limitations of the technique were not fully understood.

Chapter 2: Materials and Methods

2.1. Introduction to antigen retrieval systems

Immunohistochemical techniques are powerful tools in diagnostic and investigative studies, which have provided the potential to localise antigenic constituents in cells and tissues with strongly improved selectivity and specificity of staining reactions (De Mey and Moeremans, 1986). Although formaldehyde remains the most popular fixative used in pathology (Mason and O'Leary, 1991), the molecular mechanism of the fixation process remains obscure (Fox et al., 1985). Formaldehyde probably is not always the best fixative for the preservation of tissue antigens to be used in immunohistochemistry (Shi et al., 1991). Although fixation in formaldehyde preserves tissue structures and prevents decomposition, masking of antigenic constituents is the major artifact induced by the fixation process (Cattoretti et al., 1993). This has been thought to be due to either intermolecular cross-links formed between formaldehyde reactive sites which include cyclic aromatic rings, alcoholic hydroxyl and amide groups, primary amines and thiols or of adjacent proteins (Fox et al., 1985; Mason and O'Leary, 1991). Thus, Mason and O'Leary (1991) showed that significant denaturation of proteins occurred at temperatures of 70-90°C in unfixed, purified globular proteins, whereas these temperatures have no adverse effect on formaldehyde-fixed protein structure.

Because of the good preservation of tissue morphology, antigen retrieval techniques have mainly been applied to formalin-fixed, paraffin-embedded material since cryostat sections often showed poor cytological details (Pileri et al., 1997).

The antigen retrieval techniques include enzymatic treatment (Cattoretti et al., 1993; Pileri et al., 1997), microwave treatment (Shi et al., 1991; Cattoretti et al., 1992; Kayser et al., 1992; Taylor et al., 1994; Shi et al., 1995; in abstract form, Suurmeijer, 1992), autoclave pre-treatment (Morgan et al., 1994) and pressure cooking (Norton et al., 1994; Pileri et al., 1997).

Shi and colleagues (1991) reported for the first time the heat-based antigen retrieval of formalin-fixed paraffin-embedded tissue sections, before immunostaining, using microwave oven and heavy metal salt solutions. It was found that strong enhancement of immunostaining could be obtained with saturated lead thiocyanate, 1% zinc sulphate (Shi et al., 1991), aluminium chloride (Suurmeijer, 1992), calcium chelating agents (EDTA, EGTA and citrate buffer solution) (Morgan et al., 1994; Taylor et al., 1994),

glycine HCl or urea solution (Taylor et al., 1994). Although the mechanism of the microwave-stimulated antigen retrieval is not yet completely defined, Shi et al (1991) found this method superior to enzymatic digestion and could substantially improve the accessibility to antigens that was achievable with no treatment. In addition, similar results were reported by Pileri et al (1997) in which they found that high temperatures proved more effective than protease XIV (Sigma chemical Co). It was suggested that the heat-based antigen retrieval can affect tissue proteins either by increasing the molecular agitation or by raising their temperature (Cattoretti et al., 1993). Thus, it is possible that protein denaturation, re-exposing epitopes masked by fixation, is the most likely mechanism involved (Cattoretti et al., 1993; Norton et al., 1994). Furthermore, Cattoretti and colleagues (1993) found that enzymatic (trypsin or Pronase) and non-enzymatic (microwave oven heating) antigen retrieval are not dependent on the epitope sequence, although some antigens benefit selectively from one treatment but not from the other.

Norton and colleagues (1994) have reported that superheating in citrate buffer using pressure cooking is an effective and reliable method of antigen retrieval showing several advantages over microwave heating. They suggested that the antigen retrieval mechanism of action in this new method could be due to further protein denaturation together with rupture of some of the aldehyde cross-links. According to the findings of Norton and colleagues (1994), pressure cooking heat-mediated antigen retrieval is very easy to use; not time-consuming; the cooker accommodates large batches of slides; and it needs only extremely cheap equipment. Furthermore, it improves antigen retrieval efficiency by avoiding the occurrence of hot and cold spots especially when heating many slides (Pileri et al., 1997).

As high temperature heating of tissues remains one of the most critical factors of antigen retrieval, the choice of the retrieval fluid is also crucial (Pileri et al., 1997). Although high temperature is believed to be the primary mechanism in antigen retrieval, the type, molarity and the pH value of the buffer solutions can be involved in this process as co-factors (Cattoretti et al., 1993; Shi et al., 1995). Currently, the most widely used antigen retrieval solution is sodium citrate buffer (0.01M, pH 6.0) (Cattoretti et al., 1993; Taylor et al., 1994; Pileri et al., 1997). Shi and colleagues (1995) have shown that Tris-HCl or sodium acetate buffer pH 8-9 may be suitable for most antigens. In addition, Pileri and colleagues (1997) reported that the use of 1 mM EDTA (pH 8.0)

achieved the best results in terms of staining intensity and the number of positive cells when compared 0.1 M Tris-HCl (pH 8.0) and 0.01M citrate buffer (pH 6.0). Therefore, these findings clearly stated the importance of choosing the appropriate antigen retrieval solution and method for investigated antibody to guarantee excellent immunostaining results. I have tested several conditions for each antibody used in my experiments. The conditions chosen for applications of these antibodies in immunohistochemical staining of frozen and routinely processed tissues are shown in table 3 and appendices C and D.

2.2. Patients and tissue specimens

Tissue specimens used in this study were:

1. Paired paraffin-embedded and frozen specimens of basal cell carcinoma (BCC) (n = 12). The excised fresh specimens were obtained from the biopsy clinic, Department of Dermatology, Glasgow Western Infirmary. They were bisected; one-half was fixed in 10% formalin and then processed in paraffin for routine diagnostic histopathology (see Appendix A) and the other half immediately snap frozen in liquid nitrogen and stored in special storage vials in liquid nitrogen tanks. Serial sections of frozen BCC specimens were cut at 5-6 μm, attached to Silane-coated glass microscope slides (BDH Laboratory supplies, Poole, England) (see Appendix B) and stored at - 20°C.

2. Formalin fixed and paraffin-embedded (routinely processed) specimens of pilomatrixoma (n = 10), invasive well differentiated squamous cell carcinoma (SCC) (n = 10), moderately differentiated SCC (n = 5), keratoacanthoma (n = 3), Bowen's disease (n = 10), actinic keratosis (n = 10), psoriasis (n = 5), lichen simplex chronicus (n = 5), benign naevi (n = 6), blue naevi (n = 6), atypical naevi (n = 6), Spitz naevi (n = 6), primary malignant melanoma (n = 6), metastatic melanoma (n = 6) and further formalin fixed and paraffin-embedded BCC (n = 20) (Table 1) were obtained from the departmental archives, Department of Dermatology, University of Glasgow. Cases were not age matched. Representative tissue blocks of paraffin- embedded specimens were selected and serial sections were cut at 5-6 μm. The sections were attached to Silane coated glass microscope slides by heating overnight in an oven at a temperature of 60°C.

3. Fresh specimens of BCC (n = 8) for cell culture were obtained from the Glasgow Southern General hospital. After excision, the tumours were bisected; one-half was processed in paraffin for routine diagnostic histopathology and conformation of BCC diagnosis and the other half containing tumour was transported in a sterile universal on ice to start the preparation of cell suspension immediately.

CLINICOPATHOLOGICAL SUBTYPE	NUMBER
Superficial (multifocal)	12
Diffuse (infiltrating)	9
Nodular	8
Metatypical	2
Spindle cell	1

Table 1. Subtypes of BCC.

2.3. Antibodies

The antibodies used in this study are listed in Tables 2 and 3.

Antibody	Clone	Specificity	Isotype	Host	Source
C-β-Catenin	14	Human	IgG1	Mouse	TL
N-β-Catenin	7D11	Human	IgG2a	Mouse	Affiniti
E-Cadherin	36	Human	IgG2a	Mouse	TL
P-Cadherin	56	Human	IgG1	Mouse	TL
α-Catenin	5	Human	IgG1	Mouse	TL
Vinculin	hVIN-1	Human	IgG1	Mouse	Sigma
α-Actinin	Poly	Human	Poly	Rabbit	Sigma
Phalloidin	a fluorescent phallotoxin TRITC labelled to identify filamentous actin				Sigma
C-Gli 1	Poly	Human	Poly	Goat	Santa Cruz
N-Gli 1	Poly	Human	Poly	Goat	Santa Cruz
APC	CC-1	Human	IgG2bκ	Mouse	Calbiochem

Table 2. Properties and sources of the antibodies used in the study.

Staining in tumours was compared to normal epidermis.
C = carboxyl terminus; N = amino terminus; E = epithelial; P = placental; APC = adenomatous polyposis coli; Poly = polyclonal; TCS Bio = TCS Biologicals; TL = Transduction Laboratories.

Antibody	Tissue	PT	Fix	Dilution	Concentration	Incubation	Temp	Remarks
C-β-Catenin	Frozen	None	A	1:1000	0.25 µg/ml	O/N	4°C	
	Paraffin	PC	F	1:500	0.5 µg/ml	1 Hour	37°C	
N-β-Catenin	Frozen	None	A	1:800	0.25 µg/ml	O/N	4°C	
	Paraffin	PC	F	1:400	0.5 µg/ml	1 Hour	37°C	
E-Cadherin	Frozen	None	A	1:200	1.25 µg/ml	O/N	4°C	
	Paraffin	PC	F	1:200	1.25 µg/ml	1 Hour	37°C	
P-Cadherin	Frozen	Triton	A	1:500	0.5 µg/ml	O/N	4°C	
	Paraffin	PC	F	1:500	0.5 µg/ml	1 Hour	37°C	
α-Catenin [a]	Frozen	Triton	A	1:100	2.5 µg/ml	1 hour	RT	
	Paraffin		F					Negative
Vinculin	Frozen	Triton	F	1:400	0.2 µg/ml	2 Hours	RT	See
	Paraffin	PC	F	1:400	0.2 µg/ml	1 hour	37°C	Appx. C
α-Actinin	Frozen	Triton	F	1:400	0.2 µg/ml	2 Hours	RT	for
	Paraffin	PC	F	1:400	0.2 µg/ml	1 hour	37°C	results
Phalloidin	Frozen	Triton	F	1:50	2.0 µg/ml	30 min	RT	
	Paraffin							Not done
C-Gli1	Frozen							Not done
	Paraffin	PC	F	1:200	1.0 µg/ml	1 hour	37°C	
N-Gli1	Frozen							Not done
	Paraffin	PC	F	1:200	1.0 µg/ml	1 hour	37°C	
APC	Frozen	Triton	A[b]F[c]	1:100	1.0 µg/ml	O/N	4°C	Appx. C
	Paraffin	None	F	1:100	1.0 µg/ml	O/N	4°C	

Table 3. Final conditions chosen for applications of the antibodies in immunohistochemical staining of frozen and formalin fixed paraffin-embedded tissues.

C = carboxyl terminus; N = amino terminus; E = epithelial; P = placental; APC = adenomatous polyposis coli; PT = pre-treatment; Fix; fixative used; A = acetone; F = 10% formalin; O/N = overnight; PC = pressure cooker; RT = room temperature.

[a] Negative staining in paraffins despite using several dilutions, pre-treatments and incubation times.

[b] In immunoperoxidase staining method.

[c] In immunofluorescent staining.

2.4. Pre-treatments

Each of the following pre-treatments was carried out after the paraffin sections were dewaxed and dehydrated. The sections were cut on Silane coated slides.

A. High temperature antigen retrieval

I. Microwaving

This was carried out as described previously (Shi et al., 1991) with modifications. The slides were placed in a container containing approximately 800 ml of one of the following buffers:
10 mM citrate buffer pH 6.0. (Standard).
Tris buffer at pH 9.

The slides were microwaved on full power (950 W) for 10-60 min. The buffer was topped up with distilled water every 10 min to maintain the fluid level. Optimum times were established for each antibody.
After microwaving, the slides were left to cool at room temperature for 20 min and then washed in running water for 2 min and in TBS for 5 min afterwhich they were ready for immunohistochemical staining.

II. Microwaving using a pressure cooker

The basic protocol, as described previously (Norton et al., 1994), was used with modifications. The special plastic microwave pressure cooker (Nordic Ware, Minneapolis, USA) was filled with 1 L CB pH 6 for 1 rack of slides or 1.5 L of buffer for 2 racks of slides.
With the lid off, the pressure cooker was placed inside the microwave and brought to boil using 80% power, usually 12-15 min. The pressure cooker was then taken out carefully, the slides immersed and the lid secured.

After microwaving for 3-4 min to make sure that the cooker was under pressure, a further microwaving time of 5-6 min was allowed. Very carefully, the cooker was taken out, left to cool for 15-20 min at room temperature to reduce the pressure.
The slides were then washed in running tap water for 2 min prior to staining immunohistochemically.

B. Enzyme digestion

I. Trypsin

The sections were incubated in 0.1% Trypsin Type II-S (Sigma chemical Co) and 0.1% calcium chloride in distilled water for 30 min at 37°C, washed in running tap water to stop the enzyme digestion, then washed in TBS for 5 min before immunohistochemical staining.

II. Pronase

The sections were incubated in 0.1% Pronase Type XIV (Sigma chemical Co) in TB pH 7.6 for 15-20 min at room temperature (Pileri et al., 1980), washed in running tap water to stop enzyme digestion, then washed in TBS before immunohistochemical staining.

2.5. Immunohistochemical staining

2.5.1. Monoclonal antibodies

A. Formalin fixed and paraffin-embedded specimens

The Extra Avidin-biotin-peroxidase complex technique was used for immunohistochemical staining as described previously (Hsu et al., 1981) with modifications.

Sections (5-6 μm) on Silane coated slides were cut, incubated in xylene for 10 min, hydrated through graded alcohol, and placed in tap water.

Sections were subjected to high temperature antigen retrieval pre-treatment: sections were microwaved for 6 min in a 10 mM TB pH 9 using a special pressure cooker as described above. Endogenous peroxidase was blocked by immersing sections in 3% hydrogen peroxide (BDH Laboratory supplies, Poole, England) in distilled water for 15 min then washing in 2 changes of TBS over 5 min, before incubating with normal rabbit serum (SAPU, Carluke, Lanarkshire, Scotland) diluted 1/5 in TB pH 7.6 for 10 min. Tissue sections were incubated with the appropriate monoclonal antibodies (Table 2 and 3) diluted in TB pH 7.6. After incubation the sections were washed in 4 changes of TBS over 15 min and incubated with biotinylated rabbit anti-mouse secondary antibody (DAKO, Denmark) diluted 1/200 in TB pH 7.6 for 30 min. After washing in 2 changes of TBS over 5 min, the sections were incubated for 30 min with a 1/100 dilution of Extra Avidin-biotin peroxidase complex (Sigma, Poole, Dorset, England) in TB pH 7.6. After washing in 2 changes of TBS over 5 min, the peroxidase reaction was developed with peroxidase substrate kit (Vector VIP, Vector Laboratories, Burlingame, CA). Negative controls were established omitting the primary antibody (Fig. 83). Sections were counterstained lightly with hematoxylin 'Z' (CellPath, Hemel Hempstead Herts, UK), dehydrated and mounted with Pertex mounting media (CellPath, Hemel Hempstead Herts, UK) and viewed through a light microscope. These sections were viewed by two independent advisors.

B. Frozen specimens

Specimens were snap frozen in liquid nitrogen and embedded in water frozen by dry ice. Cryostat sections (5-6 μm) were cut, fixed in one of the following fixative protocols according to the primary antibody used (Table 2 and 3):

- Fresh acetone (BDH Laboratory supplies, Poole, England) for 1 hour at -20°C.
- 10% Neutral buffered formalin for 10 min at room temperature.
- Sequential fixation in 10% neutral buffered formalin for 7 min at room temperature, chilled methanol (- 20°C) for 5 min and chilled acetone (- 20°C) for 3 min (Choudhry et al., 1992).

For certain antibodies (Table 3), sections were extracted with 0.5% (v/v) Triton x-100 (Boehringer Mannheim Gmbh, Germany) in DPBS for 5-10 min using gentle agitation. Extraction with Triton x-100 was found to increase antibody permeablisation and reduce non-specific immuno-reactivity (Lin and DePhilip, 1996).

After washing in 3 changes of TBS over 10 min, endogenous peroxidase was blocked by immersing sections in 1% hydrogen peroxide in TB pH 7.6 for 10 min. After blocking with normal rabbit serum as described above, tissue sections were incubated with the appropriate monoclonal antibodies (Table 2 and 3) diluted in TB pH 7.6. Sections were then processed in a similar fashion to that outlined for paraffin sections.

2.5.2. Polyclonal antibodies

When a polyclonal antibody (rabbit or goat) was used as the primary reagent, both formalin fixed paraffin-embedded and frozen sections were treated as described above apart from the serum block and the biotinylated secondary antibody steps.

A. Rabbit polyclonal antibodies

After washing in 3 changes of TBS over 10 min, sections were incubated for 10 min at room temperature with normal swine serum (DAKO, Denmark) (20% (v/v) dilution in TB pH 7.6) to block non-specific antibody-binding sites. Without washing, sections were incubated with the appropriate rabbit polyclonal antibody (Tables 1 and 2) diluted in TB pH 7.6 and then incubated at room temperature with biotinylated swine-anti rabbit secondary antibody (DAKO, Denmark) diluted 1/200 for 30 min.

B. Goat polyclonal antibodies

The sections incubated with normal rabbit serum diluted 1/5 for 10 min at room temperature. Without washing, the sections were incubated with the appropriate goat polyclonal antibody (Tables 2 and 3) diluted in TB pH 7.6 and then incubated with biotinylated rabbit anti-goat secondary antibody (Vector Laboratories, CA) diluted 1/200 for 30 min at room temperature. Sections were then processed in a similar fashion to that outlined for paraffin and frozen sections.

2.6. Multiple immunofluorescence Microscopy of the Cytoskeletal-Membrane Associations

2.6.1. Double immunolabelling of monoclonal antibodies with Actin

The double immunofluorescence staining was carried out according to a standard protocol (Mies et al., 1998) with modifications. Tissue sections which were already cut and stored at -20°C, were allowed to come to room temperature, then rehydrated in diluted phosphate buffer saline DPBS (GIBCO BRL, Life technologies Ltd, Paisley, Scotland) for 2 min. The sections were fixed by immersion in neutral buffer formalin for 10 min at room temperature, then washed in DPBS changing the buffer 3 times in 10 min. Tissue sections were extracted with 0.5% (v/v) Triton x-100 in DPBS for 5-10 min using gentle agitation. After washing in 3 changes of DPBS over 10 min, the sections were incubated with normal horse serum diluted 1/5 in TB pH 7.6 for 10 min at room temperature; without washing, the sections were incubated with the appropriate primary antibody (Table 4).

The sections were then washed in 4 changes of DPBS over 15 min, and incubated with biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Peterborough, UK) diluted 1/100 in TB pH 7.6 for 30 min at room temperature. After 3 washes in DPBS over 10 min, the sections were incubated with Avidin-FITC labelled (Vector Laboratories, Peterborough, UK) diluted 1/50 in TB pH 7.6 for 30 min at room temperature, then washed in 3 changes of DPBS over a 10 min period.

For the visualisation of polymerised actin, Tetramethylrhodamine Isothiocynate (TRITC)-labelled Phalloidin was used. This poisonous alkaloid from amanita phalloides exclusively binds to filamentous actin (Verderame et al., 1980; Heidecker et al., 1995). The sections were incubated with TRITC-labelled Phalloidin (Sigma chemical CO, Poole, Dorset) diluted 1/50 in TB pH 7.6 for 30 min at room temperature. Negative controls were established omitting the primary antibody (Fig. 112). The sections were then washed in 3 changes of DPBS over 10 min and mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, UK). The cover slips were sealed with nail varnish and the slides were visualised using laser scanning confocal microscopy.

2.6.2. Double immunolabelling of polyclonal antibodies with Actin

When a polyclonal antibody (rabbit) was used as the primary reagent for the double immunolabelling with actin, the sections were treated as described above apart from the serum block and the biotinylated secondary antibody steps.

The sections were incubated with normal swine serum diluted 1/5 in TB pH 7.6 for 10 min at room temperature; without washing, the sections were incubated with the appropriate primary antibody (Table 4). The sections were then washed in 4 changes of DPBS over 15 min, and incubated with biotinylated swine anti-rabbit secondary antibody diluted 1/200 in TB pH 7.6 for 30 min at room temperature. Sections were then processed in a similar fashion to that outlined in double immunolabelling of monoclonal antibodies with actin.

Antibody	PT	Fix	Dilution	Concentration	Incubation	Temp
C-β-Catenin	Triton	F	1:500	0.5 µg/ml	1 hour	37°C
E-Cadherin	Triton	F	1:200	1.25 µg/ml	1 hour	37°C
P-Cadherin	Triton	F	1:50	1*and 5 µg/ml	1 hour	37°C
α-Catenin	Triton	F	1:100	2.5 µg/ml	1 hour	37°C
α-Actinin	Triton	F	1:400	0.2 µg/ml	1 hour	37°C
Phalloidin	Triton	F	1:50	2.0 µg/ml	30 min	RT
APC	Triton	A	1:100	1.0 µg/ml	O/N	4°C

Table 4. Applications of the antibodies used for double immunolabelling with actin in BCC frozen sections and keratinocytes chamber slides.

C = carboxyl terminus; E = epithelial; P = placental; APC = adenomatous polyposis coli; PT = pre-treatment; Fix; fixative used; F = formalin; A = acetone; RT = room temperature; O/N = Overnight
*When used in double immunolabelling with E-cadherin.

2.6.3. Double immunolabelling of P- and E-cadherins

Tissue sections which were already cut and stored at -20°C, were allowed to come to room temperature, then rehydrated in DPBS for 2 min. The sections were fixed by immersion in 10% neutral buffer formalin for 10 min at room temperature, then washed in 3 changes of DPBS over 10 min. To increase antibody permeablisation and reduce non-specific immuno-reactivity, the sections were extracted with 0.5 % Triton x-100 in DPBS for 10 min using gentle agitation.

After washing in 3 changes of DPBS over 10 min, the sections were then incubated with normal horse serum diluted 1/5 in TB pH 7.6 for 10 min at room temperature (1^{st} block) then incubated with the 1^{st} primary antibody at the minimum working dilution (P-cadherin, 1.0 µg/ml TB pH 7.6) for 1 hour at 37°C. The sections were washed in 4 changes of DPBS over 15 min, then incubated with concentrated biotinylated horse anti-mouse secondary antibody diluted 1/25 in TB pH 7.6 for 30 min at room temperature to enhance binding to all molecules of the 1^{st} primary antibody. After washing in 3 changes of DPBS, the sections were incubated with Avidin-FITC diluted 1/50 in TB pH 7.6 for 30 min at room temperature, then washed in 3 changes of DPBS over 10 min. The sections were incubated with normal horse serum diluted 1/5 in TB pH 7.6 for 10 min at room temperature (2^{nd} block), then incubated with the 2^{nd} primary antibody at the usual working dilution (E-cadherin, 1.25 µg/ml TB pH 7.6) for 1hour at 37°C. The sections were then washed in 4 changes of DPBS over 15 min and incubated with anti-mouse Texas Red (Vector Laboratories, Peterborough, UK) diluted 1/50 in TB pH 7.6 for 30 min at room temperature, then washed in 3 changes of DPBS over 10 min and mounted in Vectashield mounting medium. The cover slips were sealed with nail varnish and visualised using laser scanning confocal microscopy.

3 controls were included in this experiment, one by omitting the 1^{st} primary antibody, i.e. P-cadherin, the second by omitting the 2^{nd} primary antibody, i.e. E-cadherin, and a third by omitting both primary antibodies.

2.7. In vitro skin model

2.7.1. Preparation of dermal equivalents (collagen gels)

Materials

Type I collagen
0.34 M NaOH
Minimal essential medium (MEM)
Foetal calf serum (FCS)
Fibroblasts
Trypsin
PBS EDTA

Method

The preparation of dermal equivalents was carried out as described previously (Bell et al., 1979; 1983) with modifications. Type I collagen was extracted from rat tail tendons in 0.5M acetic acid, precipitated by the addition of an equal volume of 10% w/v NaCl, redissolved in 0.25M acetic acid and dialysed against 1/1000 glacial acetic acid, with the final solution being adjusted to 3mg collagen/ml. The actual amount of collagen was determined by freeze-drying a known volume of the solution and weighing the dried material.

The medium from the dish of fibroblasts was removed and the dish was washed gently for 2 min with Ca^{2+}- and Mg^{2+}-free PBS/1mM EDTA, then 10 ml of 0.25% trypsin/Ca^{2+}- and Mg^{2+}-free PBS/1mM EDTA was added to the fibroblasts dish and incubated for 90 seconds at room temperature, then the solution was sucked off leaving a thin layer, and incubated for 1-2 min. at 37°C.
After detaching the fibroblasts by hitting the sides of the dish, 10 ml of media containing 10% FCS MEM (GIBCO BRL, Life technologies ltd, Paisley, Scotland) was added to stop the activity of trypsin, then the fibroblasts were taken into a sterile

universal to be centrifuged and pelleted. Neat FCS (5.5ml) was added with pipetting to obtain a predominantly single cell suspension.

Collagen gels were prepared by mixing 7 volumes of collagen solution with 2 volumes of a mixture of 10x Eagle MEM/0.34M NaOH 2:1 v/v and the pH finely adjusted to 7.2 with NaOH. The Eagle MEM was supplied by GIBCO BRL, Life Technologies Ltd, Paisley, Scotland.

One volume of FCS containing approximately $7.5x10^5$ human adult forearm fibroblasts/10 ml of gel mixture was added, thoroughly mixed, and 4 ml pipetted into 35mm Petri dishes. The gels were allowed to set at 37°C for 15 min, after which 1 ml of MEM supplemented with 10% FCS was added, the gels were detached from the dishes and incubated for 9 days at 37°C in a humidified atmosphere of 5% CO_2 in air, with a medium changed every 3 days.

The gels at this stage were highly contracted, with the fibroblasts adopting a spindle-shaped morphology.

2.7.2. Culturing of BCC cells on dermal equivalents

Materials

Fresh specimen of BCC.
Minimal essential medium (MEM).
Antibiotics (streptomycin, penicillin and Fungizone).
Collagenase type XI.
0.5% w/v dispase in PBS.
Trypsin.
PBS/EDTA

Method

The fresh specimen of BCC was transported in a sterile universal container and immediately washed in serum-free MEM. Double the normal concentration of antibiotics (streptomycin 100 µg/ml, Penicillin 100 units/ml and Fungizone (Amphotericin B) 2.5 µg/ml) (all from GIBCO BRL, Life Technologies Ltd, Paisley, Scotland) was added to the specimen.
Ten mg of crude collagenase type XI (Sigma chemical Co, Poole, Dorset) was added to 5 ml of dispase (0.5 gm/100 ml sterile PBS with antibiotics) and to this mixture, double the normal concentration of antibiotics was added.

The specimen was finely chopped in a sterile dish, then the collagenase/dispase/antibiotics mixture was added to the specimen and incubated overnight at 4°C.
The mixture was pipetted up and down several times, then centrifuged (4 min, 500g) and the supernatant fluid was removed. Five ml of 0.25% trypsin/Ca^{2+}- and Mg^{2+}-free PBS/1mM EDTA was added to the specimen and incubated for 15 min at 37°C. Then 5 ml of MEM was added, and the mixture was centrifuged and the supernatant fluid removed, then 1 ml of MEM was added to the tumour cells for re-suspension. The cells were counted using a hemocytometer.

Dermal equivalents were made ready and placed in a 24 well multidish and to each, 0.5 ml of MEM was added then finally another 0.5 ml of tumour cells was added and the dish incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, with a medium change every 2 days. The wells were inspected on a daily basis and the observations recorded.

2.7.3. Culturing of keratinocytes on dermal equivalents

Materials

Human neonatal foreskins OR
Tissue from breast reduction surgery.
0.5% w/v dispase in PBS.
Trypsin.
PBS/EDTA.
Sterile gauze.
MEM containing 10% FCS.

Method

Keratinocyte culture on dermal equivalents was carried out as described previously (Bell et al., 1993). Human neonatal foreskins or breast reduction tissue were cut into 3mm strips after removal of the subcutaneous fat, suspended in 0.5% w/v dispase in PBS (Kitano and Okada, 1983), and the tissue incubated overnight at 4°C.
The epidermis was peeled off, finely chopped, and treated with 0.25% trypsin/Ca^{2+}- and Mg^{2+}-free PBS/1mM EDTA for 3 min with pipetting to obtain a predominantly single cell suspension. The cell suspension was passed through a sterile gauze, centrifuged (4 min., 400g), resuspended in Dulbecco's MEM (GIBCO BRL, Life Technologies Ltd, Paisley, Scotland) supplemented with 10% FCS, and added to contracted dermal equivalents placed in the wells of a 24 well multidish.

2.7.4. Raising collagen gels to the air/liquid interface

Materials

Contracted collagen gels.
6 cm sterile dish.
10% FCS containing MEM.
2 cm permeable glass plates.

Method

Following incubation for 5 days as submerged cultures, the gels were then raised to the air/liquid interface on sintered glass discs and incubated for a further 7 days, at which time a highly stratified epidermis had developed.

The contracted collagen gels were placed flat onto 2 cm sintered glass discs in a 6 cm sterile dish. 10% FCS containing MEM was added to the dish just to cover the glass plates and reaching to the edges of the collagen gels, taking extra care not to cover them. The dish then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, with the medium changed every 2 days. After 7 days a highly stratified epidermis had developed.

2.7.5. Keratinocyte culture on chamber slides

Materials

2 or 4 well glass chamber slides (Nalge Nunc international, IL, USA).
75 cm^2 flask of human foreskin keratinocytes.
PBS/EDTA.
Trypsin.
Foetal calf serum (FCS).
Keratinocytes growth medium (KGM).
2 sterile 75 cm^2 flasks.
Sterile universal.

Method

The flask of human foreskin keratinocytes was washed twice with Ca^{2+}- and Mg^{2+}-free PBS/1mM EDTA. Trypsin (0.25%) in Ca^{2+}- and Mg^{2+}-free PBS containing 1mM EDTA (1:1, by volume) was added to the keratinocytes and incubated for 3-4 min at room temperature with further incubation for 3-4 min at 37°C with shaking to detach most of the cells which were transferred to a sterile universal.
The cells were suspended in 1ml of FCS to stop the activity of trypsin, then a further 2 ml of trypsin/PBS/EDTA mixture (1:1, by volume) was added to the flask of keratinocytes and the procedure repeated again to remove most of the cells. The cells were pelleted and resuspended in a specific amount of keratinocyte growth medium KGM (Clonetics BioWhittaker Inc., USA) with low calcium concentration (0.1 mM $CaCl_2$), which is enough to fill the number of the chamber slides wells required for culture. As a rule instructed by the manufacturers, 1-1.3 ml is needed for each well of the 4 well glass chamber slides and 2-2.5 ml for the 2 well ones. The surfaces of the wells were coated with a layer of neat FCS for 1-2 min and then washed with KGM. This was found to enhance the attachment of the cells to the surfaces more quickly.
The chamber slides were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, with the KGM changed after 2 days to remove dead cells. After 10-12 days of incubation in the same circumstances with the medium changed every 2-3 days, a

confluent layer of cells had developed. Three days prior to staining of cells, the calcium concentration was shifted in half of the wells from low 0.1 mM to high 1.5 mM $CaCl_2$ for comparison. In addition, the surfaces of the cultured wells were scraped in straight lines, approximately 0.5mm in width, to comment on the migration of the cells attempting to fill the gaps in between.

2.8. Subpassaging of cells

2.8.1. Keratinocytes

Materials

75 cm^2 flask of human foreskin keratinocytes.
PBS/EDTA.
Trypsin.
Neat foetal calf serum (FCS).
Keratinocytes growth medium (KGM).
2 sterile 75 cm^2 flasks.
Sterile universal.

Method

The flask of human foreskin keratinocytes was washed twice with 10 ml of PBS/EDTA. Two ml of 0.25% trypsin/Ca^{2+}- and Mg^{2+}-free PBS/1mM EDTA was added to the flask of keratinocytes and incubated for 3-4 min at room temperature and further incubation for 3-4 min at 37°C with shaking to detach most of the cells which were removed to a sterile universal.
The cells were suspended in 1ml of neat FCS to stop the activity of trypsin, then a further 2 ml of 1:1 trypsin/PBS/EDTA mixture was added to the flask of keratinocytes and the process repeated again to remove most of the cells. Without washing, the cells were pelleted and resuspended in 10 ml KGM (0.1 mM $CaCl_2$).

Five ml of the cell suspension was transferred to each of the sterile 75 cm^2 flasks, with a further 5 ml KGM to each flask. The flasks then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, with the KGM changed every 2 days.

2.8.2. Fibroblasts

Materials

75 cm^2 flask of confluent fibroblasts.
PBS/EDTA.
Trypsin.
Minimal essential medium MEM.
2 sterile 75 cm^2 flasks.
Sterile universal.

Method

The flask of confluent fibroblasts was washed twice with 10 ml of PBS/EDTA. Two ml of 0.25% trypsin/Ca^{2+}- and Mg^{2+}-free PBS/1mM EDTA was added to the fibroblasts and incubated at room temperature for 1-2 min, then the trypsin/PBS/EDTA was removed leaving a thin layer. The flask was incubated for further 1-2 min at 37°C, then the cells were detached by shaking the flask and hitting the sides to remove all the cells. Ten ml of MEM (GIBCO BRL, Life technologies ltd, Paisley, Scotland) was added to the flask to stop the activity of trypsin. The cells were transferred to a sterile universal and centrifuged and resuspended in 10 ml of MEM.
Five ml of the cell suspension was added to each sterile 75 cm^2 flasks and a further 5 ml of the MEM was added. The flasks were incubated for 7 days at 37°C in a humidified atmosphere of 5% CO_2 in air, with the medium changed every 2-3 days. Following the incubation for 7 days, the fibroblasts were confluent.

2.9. Confocal laser scanning microscope

The immunofluorescence staining of BCC frozen sections and cultured keratinocytes were examined with a BioRad MRC 600 confocal laser scanning microscope (BioRad, Hemel Hempstead, UK). The confocal microscope was used to scan immunofluorescent slides in both green (excitation 488 nm, emission filter 522 nm for fluorescein) and red (excitation 568 nm, emission filter 585 nm) (Choudhry et al., 1997). Gain controls were set to give maximum sensitivity while preventing spillover between green and red channels. A neutral density filter was set to provide illumination of specimen with 3% of maximum laser output. Slit width was adjusted to give Z-axis resolution of approximately 0.5 μm with an X60 oil immersion lens. Confocal images series were collected at intervals of 1.0 μm along Z-axis. A Kallman averaging procedure was used to collect each image (5-6 scans). Images were stored in a Zip-disc, merged and the contrast and background of each colour was adjusted using the programme "Confocal Assistant" version 4.02 (Todd Clark Brelje, USA). Images were then imported and arranged with PowerPoint and printed on a colour printer.

2.10. Evaluation of immunohistochemical staining

Assessment of immunohistochemical staining was performed in normal, pseudoepitheliomatous and lesional skin samples. Where possible, the immunohistochemical staining in lesional skin was directly compared with that of the normal epidermis adjacent to the lesion as an internal positive control. The immunohistochemical staining in lesions lacking normal epidermis (3/10 cases of pilomatrixoma and 3/5 cases of metastatic melanoma) was evaluated and compared with that of normal skin attached to similar sample of the same diagnosis.

The intensity of E-, P-cadherin and β-catenin staining in melanocytic lesions were evaluated and compared with normal keratinocytes because it was difficult to relate intensity of staining to normal melanocytes due to the surrounding keratinocytes.

When the immunohistochemical staining throughout the lesion is homogeneous (particularly in frozen sections), tumour cells were evaluated as strongly positive (++) if their intensity was the same as in normal epidermal cells. In lesions in which the intensity of immunohistochemical staining was homogeneously weak in comparison with normal epidermal cells, tumour cells were evaluated as weakly positive (+/-). Negative (Neg) indicated no detectable immunohistochemical staining.

In some cases (particularly in routinely processed, paraffin-embedded sections), the immunohistochemical staining throughout the lesion was heterogeneous i.e. presence of positive and negative cells. The number of negative tumour cells < 10% was noted as strongly positive (++), > 10% was evaluated as weakly positive (+/-). Negative (Neg) indicated no detectable immunohistochemical staining.
Repetition of staining experiments was performed on sections from same blocks or specimens. Assessment of the immunohistochemical staining was carried out using light microscopy.

Chapter 3: Results

Results I

Immunoperoxidase Staining of normal epidermis and non-melanocytic lesions

3.1. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded BCC (n=32)

Normal skin adjacent to tumour

In normal epidermis adjacent to the tumour, E- and P-cadherin cell membrane staining was specific, variable and strongly preserved in all cases (Table 5). Strongly positive P-cadherin membrane staining was confined to the basal and the first suprabasal layers of the epidermis and negative in the membranes of the upper epidermal cells (Fig.6) Hair follicles showed strongly positive P-cadherin membrane staining involving more cells in the infundibulum region when compared with the basal cell layer extending to involve most of the spinous cells, while it was only positive in the outer cells of the external root sheath especially at the level of the sebaceous gland (Fig.7). Strongly positive P-cadherin membrane staining was noted in the cells of hair matrix, while there was complete absence of staining in the inner layers of the outer root sheath, dermal papilla and hair shaft. Sebaceous glands stained strongly positive for P-cadherin on the flattened germinative cells while the matured foamy cells within the gland were negative. Both secretory and excretory components of sweat glands showed strongly positive P-cadherin membrane staining similar in intensity to basal cells of the normal epidermis with variable cytoplasmic staining.

E-Cadherin was distributed in the full thickness of the epidermis excluding the stratum corneum with relatively weaker staining intensity in the membranes of the basal layer (Fig.8). In hair follicle, E-cadherin membrane staining was strongly positive on all the cells of the infundibulum and external root sheath, but was less intense in the membranes of the internal root sheath cells (Fig.10). The staining of E-cadherin in the dermal papilla and the hair shaft was uniformly negative. In sebaceous glands, E-cadherin staining was restricted to the membranes of the flattened germinative cells whereas the mature cells within were negative. In a similar staining pattern to the normal epidermis, E-cadherin staining was strongly positive in the cells of the secretory and ductal units of the sweat glands (Fig.9).

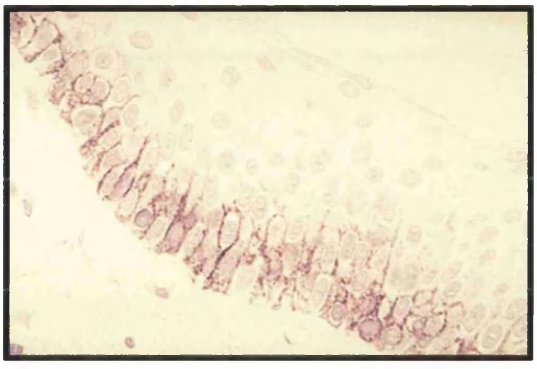


Figure 6. P-Cadherin staining in normal epidermis. Note the restriction to the basal and the first suprabasal layers.
Original magnification x500.

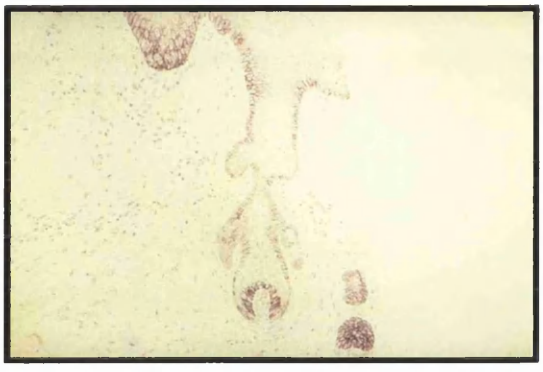


Figure 7. P-Cadherin staining in hair follicle. Positive membrane staining in ORS and hair matrix cells, negative in IRS cells and dermal papilla. x125.

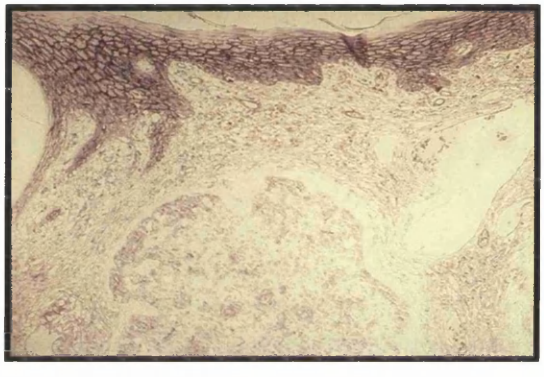


Figure 8. E-Cadherin staining in paraffin-embedded nodular BCC. Strong staining and full thickness distribution in normal epidermis; marked reduction of tumour cell membrane staining compared with epidermis. x250

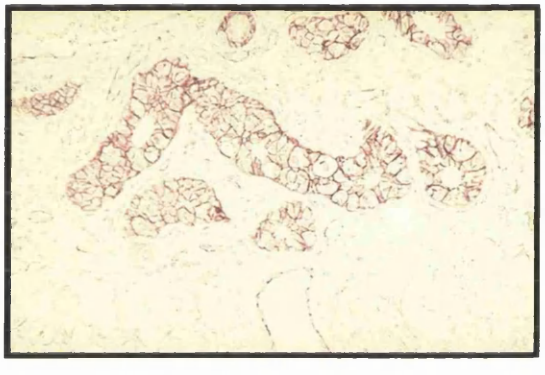


Figure 9. E-Cadherin staining in normal sweat glands. Positive membrane staining in cells of both secretory and excretory units. x400.

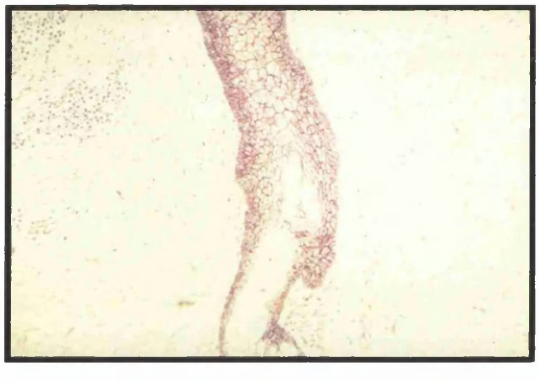


Figure 10. E-Cadherin staining in hair follicle. Strongly positive membrane staining in ORS cells, less intense in IRS cells. x320.

Figure 11. E-Cadherin staining in paraffin-embedded nodular BCC. Marked reduction of tumour cell membrane staining centrally and strong membrane staining in palisading cells. x500.

Basal Cell Carcinomas

E-Cadherin staining was present in tumour cell membranes in 30/32 of the cases of BCC but in a reduced staining intensity compared with the normal epidermis as the number of negative tumour cells was > 10% (Table 5) (Fig.8). Within tumour lobules, there was uneven membrane staining compared with the normal epidermis ranging from reduced staining intensity to complete absence. In some cases tumour lobules expressed reduced E-cadherin membrane staining centrally, while the palisading cells on the peripheries showed positive membrane staining similar to that of normal epidermis (Fig.11). This observation applied to all the different clinicopathological subtypes of BCC.

P-Cadherin staining was preserved in cell membranes within tumour lobules and at the peripheries in 21/32 of the cases (Table 5) (Fig.12) and the reduction of P-cadherin staining in the rest of the cases 11/32 was found to be mainly within tumour lobules (Fig.13). Reduction of P-cadherin staining was correlated with BCC subtypes of poorer outlook such as infiltrating and metatypical BCC (Table 6) (Fig.13). There were variable degrees of diffuse cytoplasmic distribution of both antibodies in normal and tumour cells, but no nuclear staining was seen.

	E-CADHERIN			P-CADHERIN		
	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane	32 [a]	0	0	32 [b]	0	0
Epidermal cell cytoplasm	5	8	19	0	14	18
Epidermal cell nuclei	0	0	32	0	0	32
Tumour cell membrane	2	30	0	21	11	0
Tumour cell cytoplasm	2	8	22	0	10	22
Tumour cell nuclei	0	0	32	0	0	32

Table 5♣. Distribution of E- and P-cadherin in 32 paraffin-embedded BCC.

♣Represents results of 3 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
[a] Basal and suprabasal cells.
[b] Basal cells only.

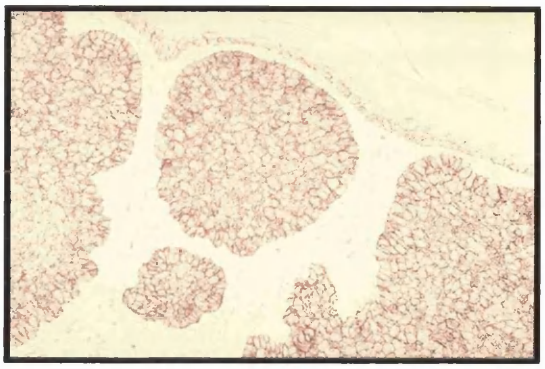


Figure 12. P-Cadherin staining in paraffin embedded nodular BCC. Strong tumour cell membrane staining. x320.

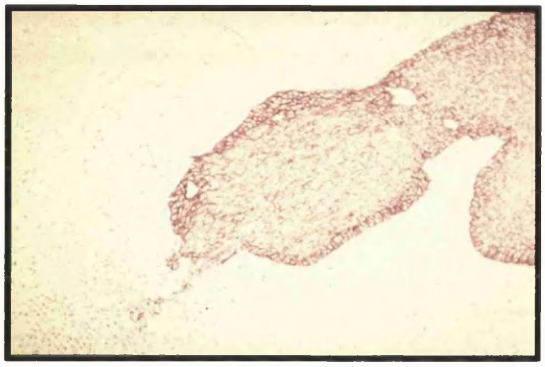


Figure 13. P-Cadherin staining in paraffin embedded infiltrative BCC. Reduction of tumour cell membrane staining and strong membrane staining in palisading cells. x320.

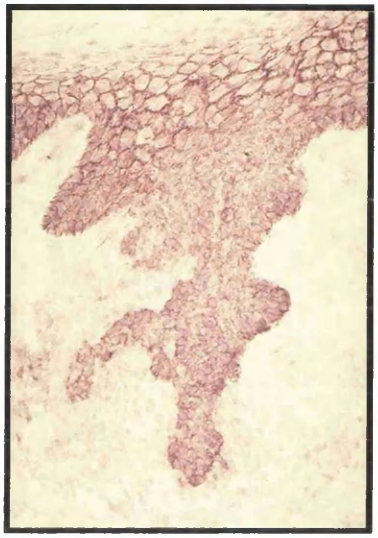


Figure 14. E-Cadherin staining in frozen superficial BCC. In comparison with the normal epidermis, tumour cells show reduction in membrane staining intensity. x500.

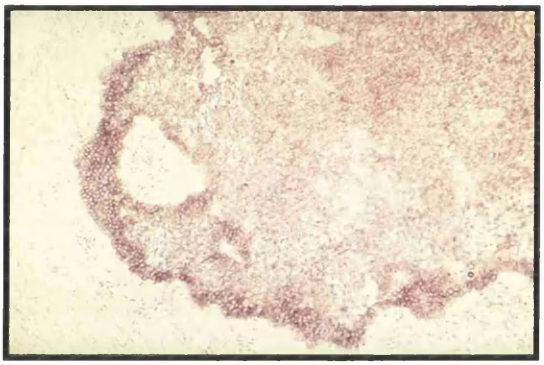
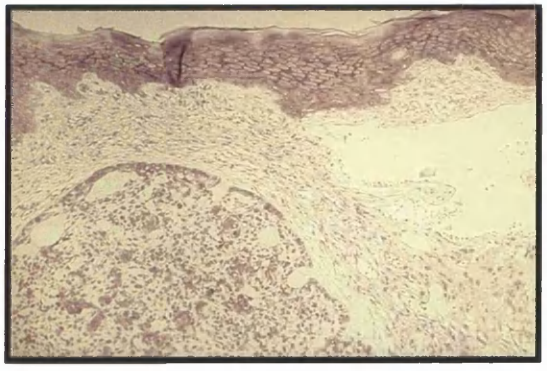


Figure 15. P-Cadherin staining in frozen infiltrative BCC. Tumour cells show reduction in membrane staining intensity in central aspect of the lobule while palisading cells show strong staining. x250.

A



B

Figure 16. β-Catenin staining in paraffin embedded nodular BCC. (A) C-terminus. (B) N-terminus. Strong staining and full thickness distribution of both antibodies in normal epidermis; marked reduction of tumour cell membrane staining compared with epidermis. x250.

Subtype	++	+/-	Neg	Total
Superficial (multifocal)	12	0	0	12
Diffuse (infiltrating)	1	8	0	9
Nodular	8	0	0	8
Metatypical	0	2	0	2
Spindle cell	0	1	0	1
Total	21	11	0	32

Table 6. Correlation of P-cadherin membrane staining with BCC subtypes.

++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.

3.2. Immunohistochemical staining of E- and P-cadherin in frozen BCC (n=12)

Normal skin adjacent to tumour

E- and P-cadherins membrane staining in normal epidermis and appendages was strongly positive and specific in all cases (Table 7) (Fig.14) following the same staining pattern in paraffin-embedded specimens.

Basal Cell Carcinomas

Staining of E-cadherin in tumour cell membranes was found to be decreased in all cases (Table 7) (Fig.14) compared with normal epidermis. The reduction of E-cadherin membrane staining was confined to the inner aspects of the tumour lobules with the cells in the palisade showing staining intensity similar to normal epidermis. P-Cadherin staining was reduced in tumour cell membranes in 10/12 of the cases (Table 7) (Fig.15), while the two examples showing strongly positive staining were non-infiltrating solid and superficial subtypes. Diffuse cytoplasmic staining was also observed in normal and tumour cells stained with both antibodies, but again no nuclear staining was seen.

	E-CADHERIN			P-CADHERIN		
	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane	12 [a]	0	0	12 [b]	0	0
Epidermal cell cytoplasm	0	6	6	0	12	0
Epidermal cell nuclei	0	0	12	0	0	12
Tumour cell membrane	0	12	0	2	10	0
Tumour cell cytoplasm	0	7	5	0	12	0
Tumour cell nuclei	0	0	12	0	0	12

Table 7♣. Distribution of E- and P-cadherin in 12 frozen BCC.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (homogeneously weak). Neg: negative
[a] Basal and suprabasal cells.
[b] Basal cells only.

3.3. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded BCC (n=32)

Normal skin adjacent to tumour

All cases have shown uniform and homogeneous membrane staining of C-terminus β-catenin in normal epidermal layers, except stratum corneum, with almost the same distribution as for E-cadherin (Table 8) (Fig.16A). There was variable cytoplasmic staining intensity within the normal keratinocytes and their nuclei were negative.

Basal Cell Carcinomas

C-Terminus β-catenin staining was present in tumour cell membranes in 29/32 of the cases of BCC but in a reduced staining intensity compared with the normal epidermis as the number of negative tumour cells was > 10% (Table 8) (Fig.16A). As for E-cadherin there was uneven membrane staining within the tumour lobules compared with the normal epidermis ranging from reduced staining intensity to complete absence. Similar to E-cadherin, C-terminus β-catenin membrane staining was preserved in the peripheral cells of the tumour lobules showing reduction within. In addition, there was variable

cytoplasmic staining ranging from weakly positive to complete absence. In thirteen of the 32 cases (Table 8), there was positive C-terminus β-catenin nuclear staining in tumour cells which showed negative membrane staining. Of these, ten were strongly positive (Fig.17A) while three showed weakly positive nuclear staining. This notable feature of tumour cell nuclear staining did not seem to be correlated with the clinicopathological subtypes of BCC.

3.4. Immunohistochemical staining of N-terminus β-catenin in formalin fixed and paraffin-embedded BCC (n=32)

Normal skin adjacent to tumour

N-Terminus β-catenin membrane staining in the normal epidermis and skin appendages was uniform and strongly positive in all cases and followed a staining pattern similar to C-terminus β-catenin and E-cadherin (Table 8) (Fig.16B). Variable degrees of diffuse cytoplasmic expression were also observed in normal cells but no nuclear staining.

Basal Cell Carcinomas

N-Terminus β-catenin staining was present in tumour cell membranes in 30/32 cases of BCC but in reduced intensity compared with the normal epidermis as the number of negative tumour cells was > 10% (Table 8) (Fig.16B). Similar to C-terminus β-catenin, there was uneven membrane staining of N-terminus β-catenin within the lobules of the tumour compared with the normal epidermis ranging from reduced staining intensity to complete absence. The preservation of N-terminus β-catenin staining in the membranes of the palisading cells at the peripheries of tumour lobules was also noted (Fig.20). Eight tumours showed positive N-terminus β-catenin nuclear staining and negative membrane staining, as for C-terminus antibody (Table 8) (Fig.17B). Of these, six were strongly positive while two showed weakly positive nuclear staining. All examples which demonstrated N-terminus β-catenin nuclear staining have also shown C-terminus

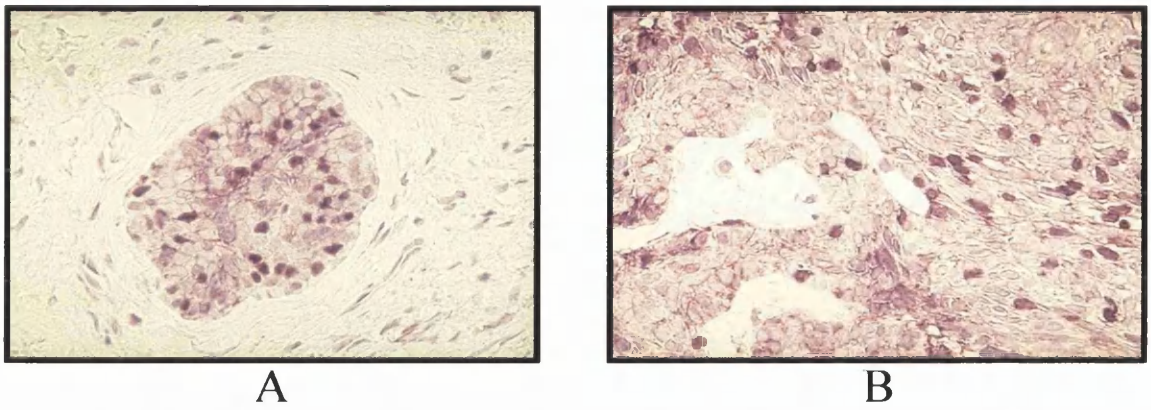


Figure 17. β-Catenin nuclear staining in paraffin-embedded BCC. (A) C-terminus. (B) N-terminus. Note the accompanying decrease in membrane β-catenin. x500.

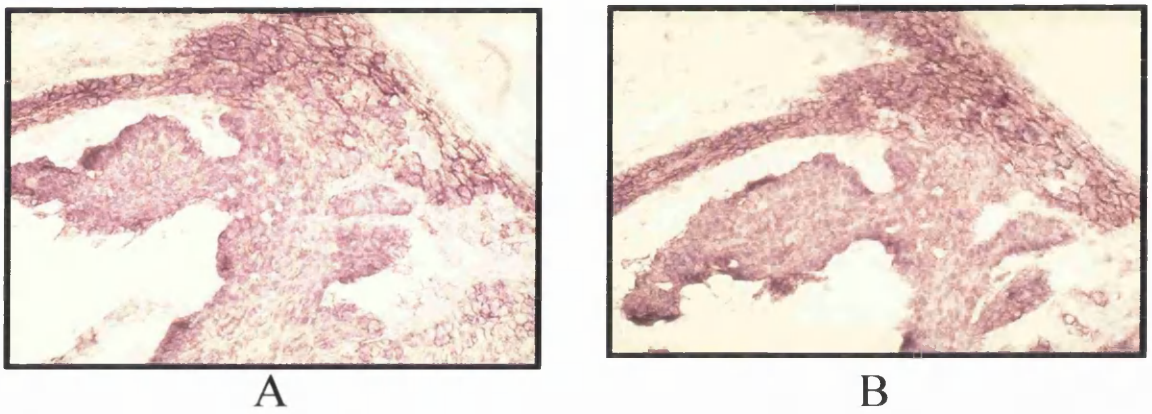


Figure 18. β-Catenin staining in frozen nodular BCC. (A) C-terminus. (B) N-terminus. In comparison with the normal epidermis, tumour cells show reduction in membrane staining intensity. x500.

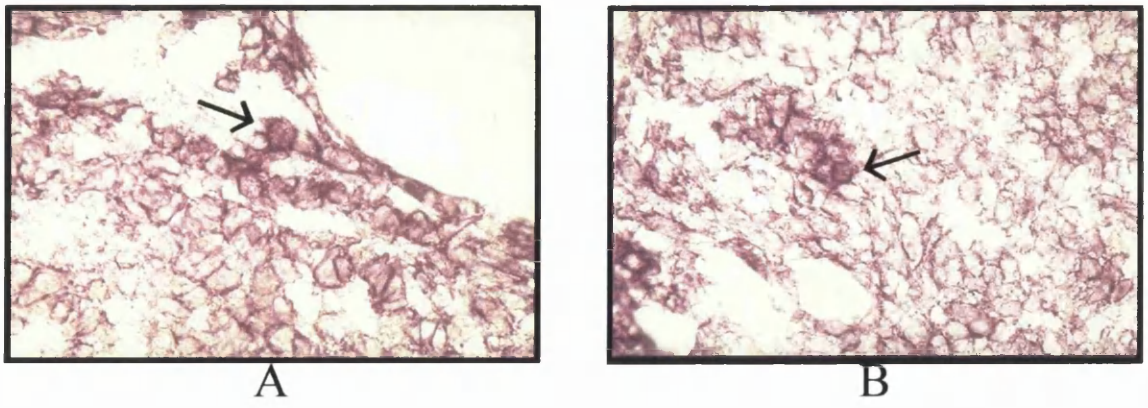


Figure 19. β-Catenin nuclear staining in frozen BCC (arrows). (A) C-terminus. (B) N-terminus. x625.

β-catenin nuclear staining. The tumour cells expressed variable degrees of cytoplasmic staining ranging from weak positivity to complete absence.

	N-terminus			C-terminus		
	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane	32	0	0	32	0	0
Epidermal cell cytoplasm	0	5	27	0	7	25
Epidermal cell nuclei	0	0	32	0	0	32
Tumour cell membrane	2	30	0	3	29	0
Tumour cell cytoplasm	0	9	23	0	12	20
Tumour cell nuclei	6	2	24	10	3	19

Table 8♣. Distribution of C and N termini of β-catenin in 32 paraffin-embedded BCC.

♣Represents results of 3 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.

3.5. Immunohistochemical staining of C-terminus β-catenin in frozen BCC (n=12)

Normal skin adjacent to tumour

There was good agreement between β-catenin staining of membranes in paraffin-embedded and frozen samples. All the 12 frozen BCC showed strong C-terminus β-catenin expression in the membranes of normal epidermis and skin appendages (Table 9) (Fig.18A) as described above in paraffin-embedded samples. The nuclei of normal cells were universally negative.

Basal Cell Carcinomas

All of the 12 cases showed some reduction of tumour cell membranes staining which was marked within the tumour lobules (Fig.18A). Most of the tumour lobules have shown positive membrane staining in the palisading cells which was almost similar to the staining intensity in normal cells. Only one case has shown weakly positive C-

terminus β-catenin nuclear staining (Table 9) (Fig.19A). Variable cytoplasmic staining in normal and tumour cells was present.

3.6. Immunohistochemical staining of N-terminus β-catenin in frozen BCC (n=12)

Normal skin adjacent to tumour

The staining of N-terminus β-catenin in normal epidermis and skin appendages was identical to the C-terminus β-catenin. It was strongly positive in the membranes and negative in the nuclei of normal cells in all the cases examined (Table 9) (Fig.18B).

Basal Cell Carcinomas

Similar to the staining with the C-terminus antibody, N-Terminus β-catenin staining was present in tumour cell membranes in all the cases of frozen BCC but in reduced intensity compared with the normal epidermis (Table 9) (Fig.18B). N-Terminus β-catenin staining reduction was mainly confined to the inner aspects of the tumour lobules. The cells in the peripheries showed staining intensity similar to normal epidermis. Uneven membrane staining of N-terminus β-catenin within the tumour lobules was also observed. One tumour has shown weakly positive nuclear staining of N-terminus β-catenin (Fig.19B). Variable cytoplasmic staining in normal and tumour cells was present.

	N-terminus			C-terminus		
	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane	12	0	0	12	0	0
Epidermal cell cytoplasm	0	5	7	0	4	8
Epidermal cell nuclei	0	0	12	0	0	12
Tumour cell membrane	0	12	0	0	12	0
Tumour cell cytoplasm	0	4	8	0	3	9
Tumour cell nuclei	0	1	11	0	1	11

Table 9♣. Distribution of C and N termini of β-catenin in 12 frozen BCC.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (homogeneously weak). Neg: negative.

3.7. Immunohistochemical staining of α-catenin in frozen BCC (n=12)

Ten of twelve specimens have shown strongly positive, uniform and homogeneous membrane staining of α-catenin in normal epidermal layers (Table 10) (Fig.21). There was relatively weaker staining intensity in the membranes of the basal and granular layer. The stratum corneum was negative. The staining was located mainly on the cell membrane, especially at the area of cell-cell contact. Hair follicles showed strongly positive membrane staining at all levels especially at the region of the infundibulum and on the external root sheath cells with consistently negative staining in the hair shaft. α-Catenin membrane staining in sebaceous glands was similar in intensity to the basal and granular layer. It was restricted to the flattened germinative cells at the peripheries of the acinus of sebaceous glands. Sweat glands stained positively for α-catenin in their membranes. The normal cells expressed variable degrees of cytoplasmic staining which were mostly negative. normal cell nuclei were universally negative.
Two of twelve cases demonstrated weakly positive α-catenin staining in cells of normal epidermis and skin appendages.

α-Catenin staining was present but in reduced staining intensity in tumour cell membranes in 11/12 of the cases in comparison with the normal epidermis (Table 10) (Fig.21). The reduction of tumour cell membrane staining of α-catenin was mainly confined to the inner aspects of the tumour lobules while the cells at the peripheries

expressed staining intensity almost similar to the normal epidermis. One tumour expressed a staining intensity identical to normal epidermis. Some tumour cells expressed obscure staining in the cytoplasm diffusely. Staining could not be detected in the tumour cell nuclei in any of the cases.

	α-CATENIN		
	++	+/-	Neg
Epidermal cell membrane	10	2	0
Epidermal cell cytoplasm	0	2	10
Epidermal cell nuclei	0	0	12
Tumour cell membrane	1	11	0
Tumour cell cytoplasm	0	3	9
Tumour cell nuclei	0	0	12

Table 10♣. Distribution of α-catenin in 12 frozen BCC.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.

++: strongly positive. +/-: weakly positive (homogeneously weak). Neg: negative.

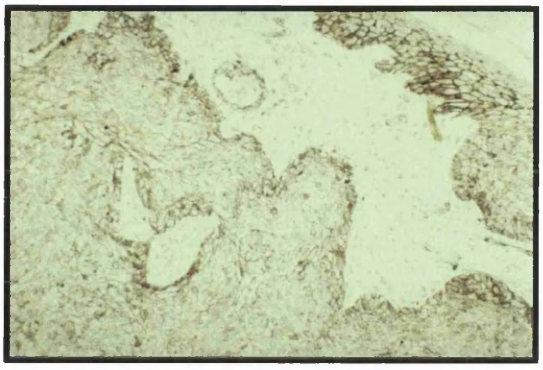


Figure 20. C-Terminus β-Catenin staining in paraffin-embedded nodular BCC. Reduction of tumour cell membrane staining centrally and strong membrane staining in palisading cells. x250.

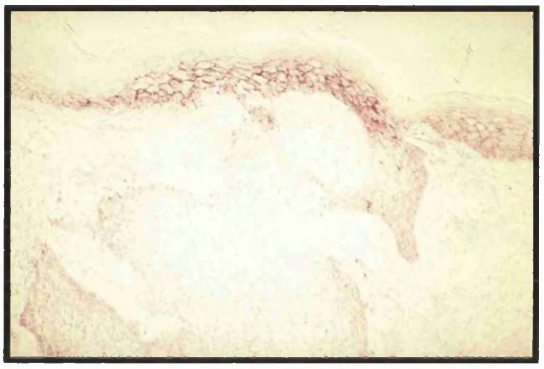
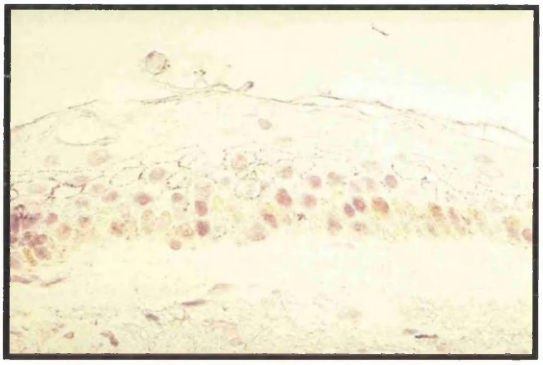
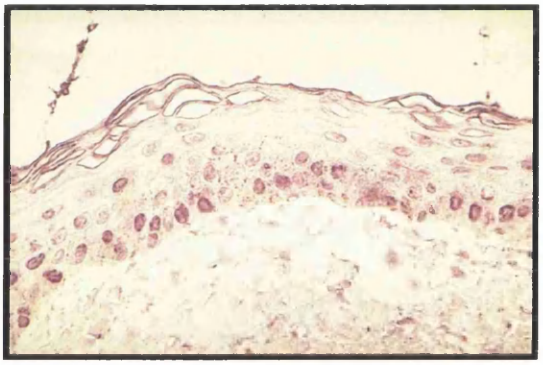


Figure 21. α-Catenin staining in frozen nodular BCC. In comparison with the normal epidermis, tumour cells show reduction in membrane staining intensity. x250.



A



B

Figure 22. Gli-1 staining in normal epidermis adjacent to BCC (paraffin). (A) C-terminus. (B) N-terminus. Note the weak membrane staining in upper cells and the nuclear staining mostly in basal cells. x400.

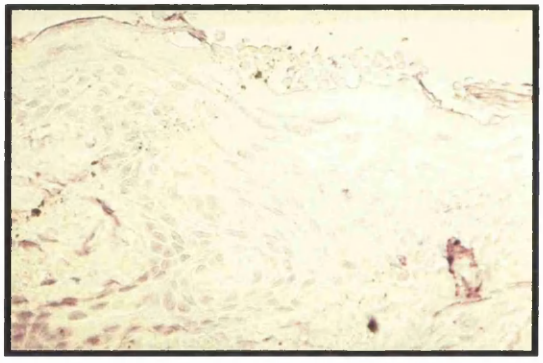


Figure 23. Gli-1 staining in epidermis overlying BCC. No staining detected. x400.

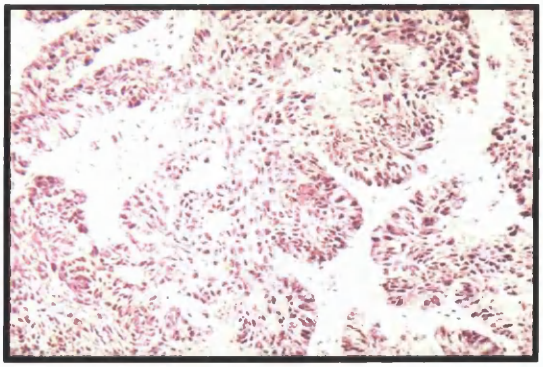


Figure 24. C-Terminus Gli-1 nuclear staining in paraffin-embedded BCC. Note the nuclear staining in almost all tumour cells. x320.

3.8. Immunohistochemical staining of C-terminus Gli-1 in formalin fixed and paraffin-embedded BCC (n=32)

Normal skin adjacent to tumour

The staining of the C-terminus Gli-1 in the normal epidermis adjacent to tumour and in skin appendages was patchy and heterogeneous in all the specimens. All specimens examined showed weakly positive staining in stratum corneum. Weakly positive and patchy staining of C-terminus Gli-1 was observed along the epidermal cell membranes in 24/32 of the cases particularly in spinous cells (Table 11). This pattern of staining was mainly observed towards the edges of the normal epidermis (Fig.22A) In some hair follicles, staining along the membranes of the outer root sheath cells was seen, but was not consistent in all normal specimens. Similarly, the pattern of staining in sebaceous and sweat glands was weak and heterogeneous. Furthermore, there was no detection of staining in the epidermis immediately above the main BCC mass in any of the specimens (Fig. 23). In addition, 8/32 of cases showed completely negative staining in the normal epidermis and appendages (Table 11). Almost all epidermal cells showed negative cytoplasmic staining. A notable feature was the presence of weakly positive nuclear staining in normal epidermal cells in 6/32 of the cases. This was particularly observed in the nuclei of basal layer cells at the edges of the normal epidermis (Fig. 22A).

Basal Cell Carcinomas

Throughout tumour lobules, the staining of the C-terminus Gli-1 was uneven at different cellular levels. In almost all cases (30/32), the C-terminus Gli-1 staining in the membranes and cytoplasm of tumour cells was negative (Table 11), although, in one, the pattern of staining along membranes of tumour cells was similar to the positive staining observed in some normal epidermal cells. In addition, there was uneven nuclear staining, in some cases, throughout the tumour lobules ranging from strongly positive staining intensity to complete absence. This was observed in both central and peripheral aspects of the tumour lobules. In 21/32 cases (Table 11), there was positive C-terminus Gli-1 nuclear staining in tumour cells which showed negative membrane

staining (Fig.24). Of these, nine were strongly positive while twelve showed weakly positive nuclear staining (Fig.26). Minimal background staining was apparent in some specimens. There was no correlation between the different clinicopathological subtypes of BCC and their pattern of C-terminus Gli-1 nuclear staining.

	N-terminus			C-terminus		
	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane	0	21	11	0	24	8
Epidermal cell cytoplasm	0	2	30	0	2	30
Epidermal cell nuclei	0	5	27	0	6	26
Tumour cell membrane	0	1	31	1	1	30
Tumour cell cytoplasm	0	1	31	0	2	30
Tumour cell nuclei	8	8	16	9	12	11

Table 11♣. Distribution of staining for C and N termini of Gli-1 in 32 paraffin-embedded BCC.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen, however, different background staining intensities were seen with each antibody.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.

3.9. Immunohistochemical staining of N-terminus Gli-1 in formalin fixed and paraffin-embedded BCC (n=32)

Normal skin adjacent to tumour

The pattern of N-terminus Gli-1 staining in the normal epidermis adjacent to tumour was similar to that of C- terminus Gli-1 in which it was patchy and heterogeneous in all the specimens. Towards the edges of the normal epidermis, there was weakly positive staining of N-terminus Gli-1 along the cell membranes in 21/32 of the cases particularly in spinous cells (Table 11) (Fig. 22B). The normal epidermis immediately above the main BCC mass was negative in all the specimens. Eleven of 32 examples showed completely negative staining in the normal epidermis and appendages. Similar to C-terminus Gli-1, weakly positive nuclear staining in normal epidermal cells was observed in 5/32 of the cases (Fig. 22B).

Basal Cell Carcinomas

The staining pattern of N-terminus Gli-1 in the tumour cells was similar to that of C-terminus Gli-1 (Table 11). Almost all cases (31/32) showed negative staining in the membranes and cytoplasms of tumour cells. Uneven nuclear staining was observed in 16/32 cases. Of these, eight were strongly positive (Fig. 25) while eight showed weakly positive nuclear staining. All these cases also showed nuclear staining using the C-terminus antibody. Similar to C-terminus Gli-1, there was no correlation between the different clinicopathological subtypes of BCC and their pattern of N-terminus Gli-1 nuclear staining.

NB: The conditions used by Ghali and colleagues (1999) for Gli-1 staining (N and C termini) were tried to compare our results. Apart from using VIP developing peroxidase substrate kit, instead of Diaminobenzidine, I have carried out 2 staining experiments on 32 routinely processed, paraffin-embedded cases of BCC using exactly the same methods published by Ghali and colleagues (1999). Unfortunately, the results were not satisfactory. The staining of both N and C terminal antibodies was very patchy and seemed to be non-specific in most of the sections because of the presence of massive background staining in collagen bundles, smooth muscles and adipose tissue.

3.10. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded pilomatrixoma (n=10)

The distribution of E- and P-cadherin staining in normal epidermis covering pilomatrixoma was identical to the staining in normal epidermis described above apart from two examples which showed weakly positive P-cadherin staining in the basal layer. There was no normal epidermis found in 3/10 of the cases examined.
E-Cadherin cell membrane staining in the tumour cells was strongly positive in 5/10 cases (Table 12) (Fig. 27). The staining was pronounced mainly in the membranes of the small basaloid cells at the peripheral aspects of the tumour which show some mitotic activity. The large cells towards the inner aspects of the tumour were also staining strongly positive. However, E-cadherin membrane staining was negative in the transformed ghost cells which are showing nuclear pyknosis. E-Cadherin membrane staining was present but in reduced staining intensity in two cases in comparison with the normal epidermis (Table 12) (Fig. 28). There was no E-cadherin staining observed in three cases examined which were composed mainly of sheets of keratinous debris in which the ghost outlines of tumour cells were faintly visible and the nuclei were lost. There were variable degrees of E-cadherin cytoplasmic staining in tumour cells ranging from weakly positive to complete absence. No E-cadherin staining could be detected in the tumour cell nuclei of all the cases.

Five of ten cases showed strongly positive P-cadherin cell membrane staining confined mainly to the small basaloid germinative cells (Table 12) (Fig. 29). However, in a few areas, P-cadherin membrane staining extended to involve some of the viable matured cells towards the inner parts of the tumour lobules (Fig. 30). P-Cadherin tumour cell membrane staining was present but in reduced staining intensity in 2/10 cases in comparison with the basal layer of the normal epidermis. As for E-cadherin, there was no P-cadherin staining observed at any cellular level in 3/10 of the cases which were composed of anuclear fully matured cells dispersed in sheets of keratinous debris. The tumour cell cytoplasms and nuclei were negative.

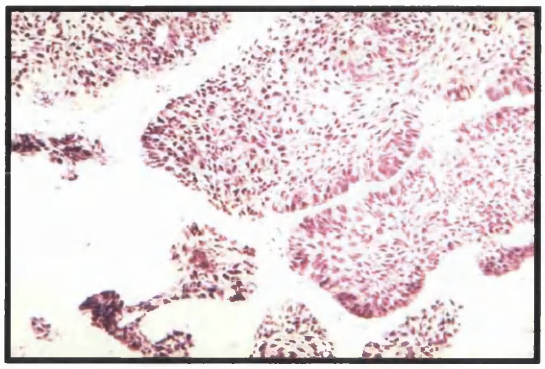


Figure 25. N-Terminus Gli-1 nuclear staining in paraffin-embedded BCC. Note the nuclear staining in almost all tumour cells. x320.

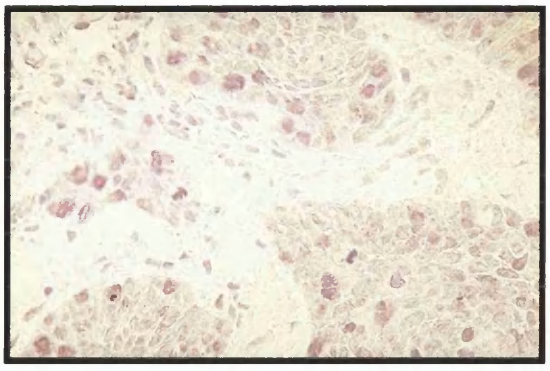


Figure 26. C-Terminus Gli-1 nuclear staining in paraffin-embedded BCC. Higher power showing weak nuclear staining in some tumour cells. x500.

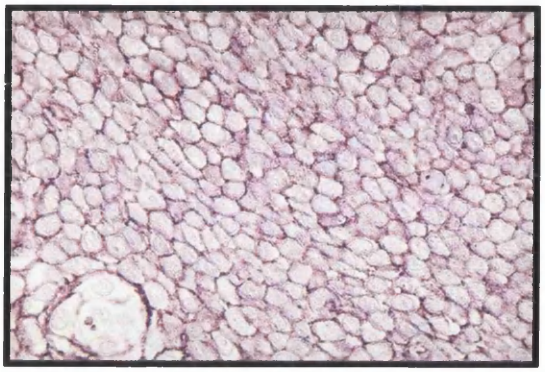


Figure 27. E-Cadherin staining in pilomatrixoma. Strong tumour cell membrane staining. x625.

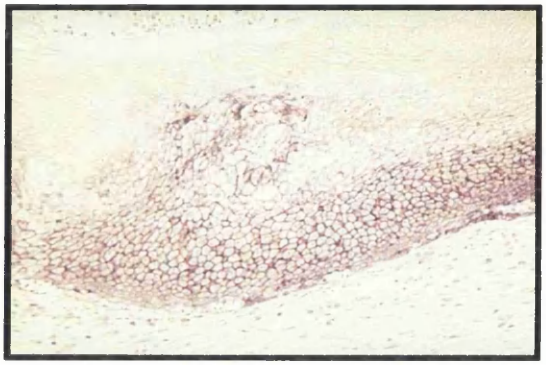


Figure 28. E-Cadherin staining in pilomatrixoma. Note the gradual decrease in membrane staining towards the central aspect of the tumour. x320.

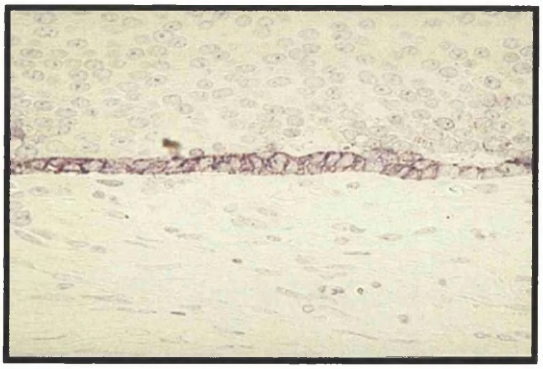


Figure 29. P-Cadherin staining in pilomatrixoma. Note the restriction to the basal cells. x500.

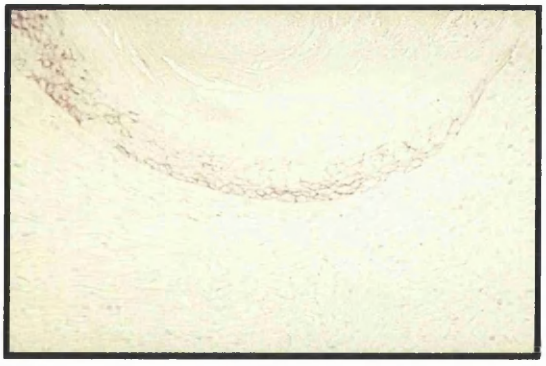


Figure 30. P-Cadherin staining pilomatrixoma. Staining accentuation towards inner aspect of tumour. x320.

	E-CADHERIN			P-CADHERIN		
	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane*	7 [a]	0	0	5 [b]	2	0
Epidermal cell cytoplasm	0	7	0	0	0	7
Epidermal cell nuclei	0	0	7	0	0	7
Tumour cell membrane	5	2	3	5	2	3
Tumour cell cytoplasm	0	7	3	0	0	10
Tumour cell nuclei	0	0	10	0	0	10

Table 12♣. Distribution of E- and P-cadherin in 10 paraffin-embedded pilomatrixoma.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative
*Three cases have no epidermis.
[a] Basal and suprabasal cells.
[b] Basal cells only.

3.11. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded pilomatrixoma (n=10)

The distribution of C-terminus β-catenin staining in normal epidermis covering pilomatrixoma was identical to the staining in normal epidermis described above (Table 13) (Fig. 31A). There was no normal epidermis found in 3/10 of the cases examined. C-Terminus β-catenin staining was strongly positive in tumour cell membranes in 3/10 of the cases (Table 13) (Fig. 32A) following almost the same pattern of E-cadherin staining in pilomatrixoma. Four of ten cases showed positive staining of C-terminus β-catenin in tumour cell membranes but in reduced intensity in comparison with the normal epidermis (Fig. 33A). There was negative staining of C-terminus β-catenin throughout the tumour in 3/10 cases. All the examples of pilomatrixoma showed highly variable degrees of cytoplasmic C-terminus β-catenin staining ranging from strongly positive staining to universally negative. Seven tumours showed positive C-terminus β-catenin nuclear staining (Table 13) (Figs. 32A and 33A). Of these, four were strongly positive while three showed weakly positive nuclear staining. Although that some of the viable large and matured cells showed nuclear staining, the majority of cells were from the small germinative basaloid population. The nuclei of the fully matured, nonviable cells were negative.

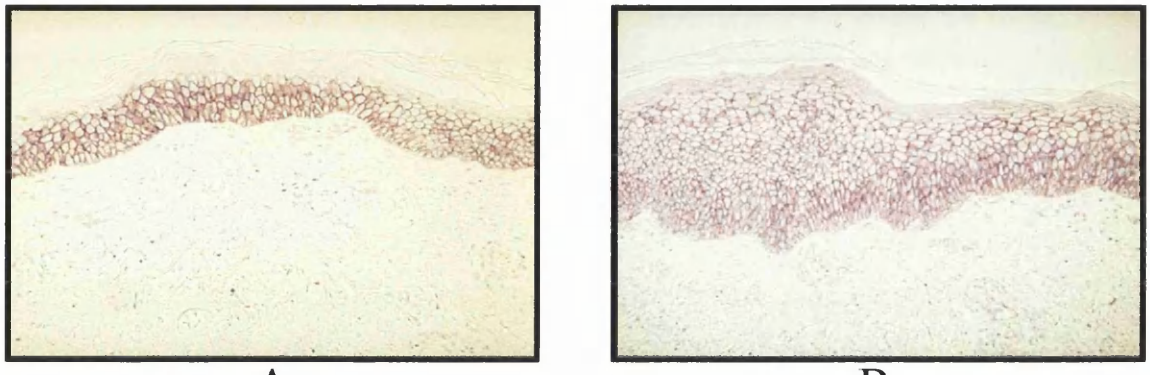


Figure 31. β-catenin staining in epidermis overlying pilomatrixoma. (A) C-terminus. (B) N-terminus. Strong staining and full thickness distribution. x250.

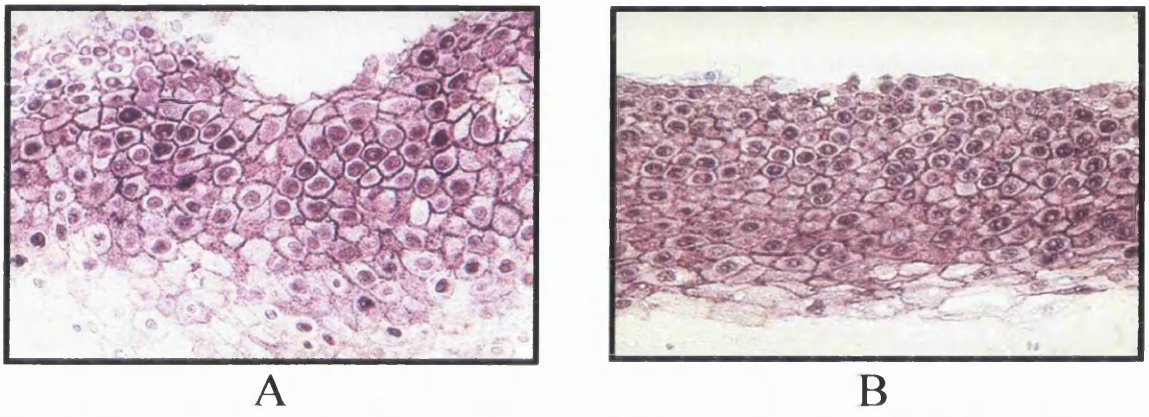


Figure 32. β-catenin staining in pilomatrixoma. (A) C-terminus. (B) N-terminus. Several tumour cells show strong membrane and nuclear staining. (A) x625, (B) x500.

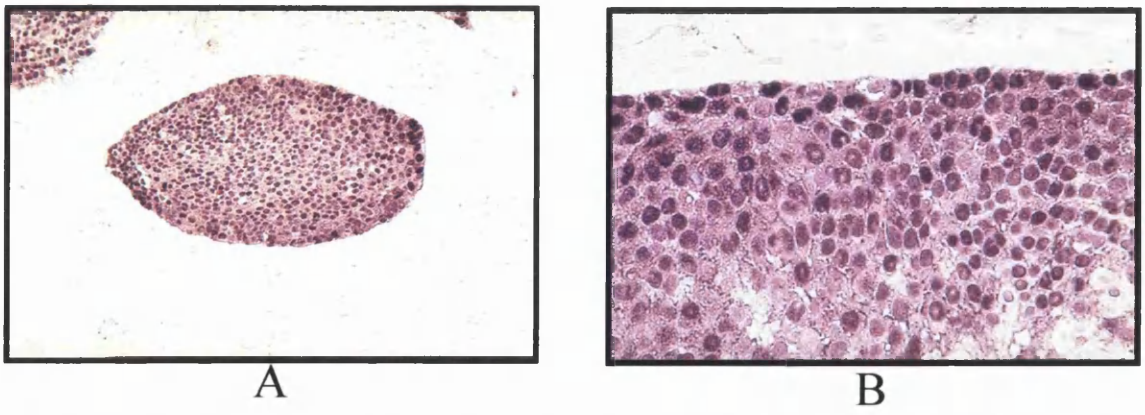


Figure 33. β-catenin nuclear staining in pilomatrixoma. (A) C-terminus. (B) N-terminus. Strong nuclear staining in almost all tumour cells. Note the accompanying decrease in membrane β-catenin. (A) x250, (B) x500.

3.12. Immunohistochemical staining of N-terminus β-catenin in formalin fixed and paraffin-embedded pilomatrixoma (n=10)

The distribution of N-terminus β-catenin staining in normal epidermis covering pilomatrixoma was identical to the C-terminus β-catenin staining in normal epidermis described above (Table 13) (Fig. 31B). There was no normal epidermis found in 3/10 of the cases examined.

N-Terminus β-catenin tumour cell membrane staining was strongly positive in 3/10 of the cases following the same staining pattern observed with C-terminus β-catenin in the tumours which showed preservation (Table 13) (Fig. 32B). Four tumours expressed reduced membrane staining of N-terminus β-catenin when compared with the normal epidermis (Fig. 33B). Three tumours gave negative staining with N-terminus β-catenin. Highly variable degrees of cytoplasmic N-terminus β-catenin staining ranging from strongly positive staining to universally negative were seen in all the tumours. Seven tumours showed positive N-terminus β-catenin nuclear staining (Table 13) (Figs. 32B and 33B). Of these, four were strongly positive while three showed weakly positive nuclear staining. The seven pilomatrixoma tumours expressed N-terminus β-catenin nuclear staining were the same examples which expressed C-terminus β-catenin nuclear staining.

	N-terminus			C-terminus		
	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane*	7	0	0	7	0	0
Epidermal cell cytoplasm	0	7	0	0	7	0
Epidermal cell nuclei	0	0	7	0	0	7
Tumour cell membrane	3	4	3	3	4	3
Tumour cell cytoplasm	4	3	3	4	3	3
Tumour cell nuclei	4	3	3	4	3	3

Table 13♣. Distribution of C and N termini of β-catenin in 10 paraffin-embedded pilomatrixoma.

♣Represents results of 3 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
* Three cases have no epidermis.

3.13. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded well differentiated SCC (n=10)

Nine cases showed strongly preserved epidermal cell membrane staining of E-cadherin either in normal or in pseudoepitheliomatous epidermis (Table 14) (Fig. 34A), although the intensity of staining tended to decrease in upper prickle and granular cell layers and was negative in stratum corneum.

P-Cadherin membrane staining was strongly positive in basal and suprabasal cells in normal and acanthotic skin in 9/10 examples (Table 14). A notable feature was the presence of strongly positive membrane staining of P-cadherin involving the basal cells and most of the spinous layer of the pseudoepitheliomatous skin over the tumour (Fig. 38).

E-Cadherin tumour cell membrane staining was present in 7/10 cases, but in reduced staining intensity (Figs. 34A and B) (particularly in parts of tumour showing poorer differentiation) when compared with normal epidermis. E-Cadherin was strongly positive in 2/10 and completely negative in one case (Table 14) (Fig. 35).

Tumour membrane staining of P-cadherin was present in 5/10 cases, but in reduced staining intensity when compared with the basal layer, strongly positive in 4/10 (Fig. 37) and negative in one case (Table 14) (Fig. 38). The adjacent normal epidermis was used as a positive control. In areas of tumour with conspicuous keratinisation, both

antibodies showed decreasing staining intensity towards the centre of whorls (Fig. 36). In contrast with both paraffin and frozen BCC, no positive staining was observed on the peripheries of the tumours. Variable cytoplasmic staining of both antibodies was noted in normal epidermal cells, but was negative in tumour cells in almost all cases with no nuclear staining observed.

3.14. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded well differentiated SCC (n=10)

The staining pattern of C-terminus β-catenin was almost similar to that of E-cadherin either in normal epidermis or in tumour cells (Table 14). Nine cases showed strongly positive epidermal cell membrane staining with decreasing intensity in granular cell layer (Table 14). In tumour cell membranes, C-terminus β-catenin staining was present in 7/10 cases, but in reduced staining intensity when compared with the normal epidermis (Table 14) (Fig. 39). Membrane staining was strongly positive in 2/10 and negative in one case. The reduction of C-terminus β-catenin in tumour downgrowths was uneven, which showed different staining intensities ranging from strong positivity to complete absence. In contrast to BCC, no positive staining was seen on the peripheral aspects of the tumour lobules. In the cytoplasm of normal epidermal cells, C-terminus β-catenin staining was variable again, but was negative in tumour cells in 9/10 cases. There was no observation of C-terminus β-catenin staining in tumour cell nuclei.

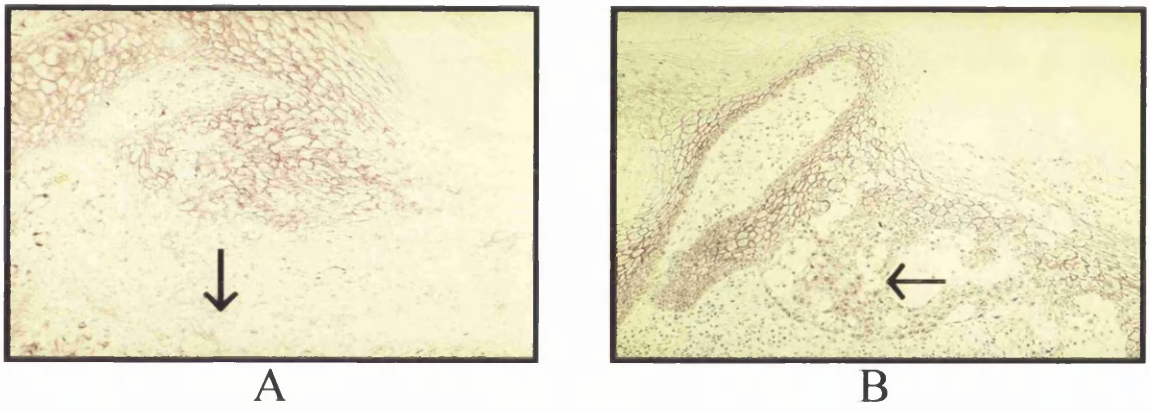


figure 34. E-Cadherin staining in well-differentiated SCC. (A) and (B) In comparison with the normal epidermis, tumour cells show reduction in membrane staining intensity (arrows). (A) x320, (B) x250.

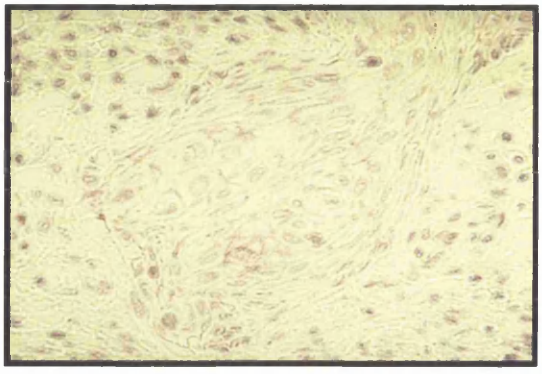


Figure 35. E-Cadherin staining in well-differentiated SCC. Complete absence of membrane staining. x500.

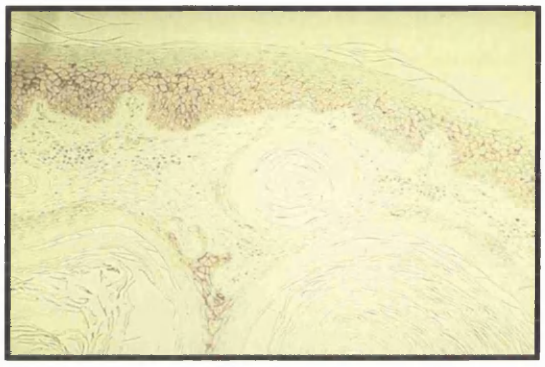


Figure 36. E-Cadherin staining in keratinising cysts of well-differentiated SCC. Weak staining in cyst peripheries and negative towards the central aspect of the whorl. x200.

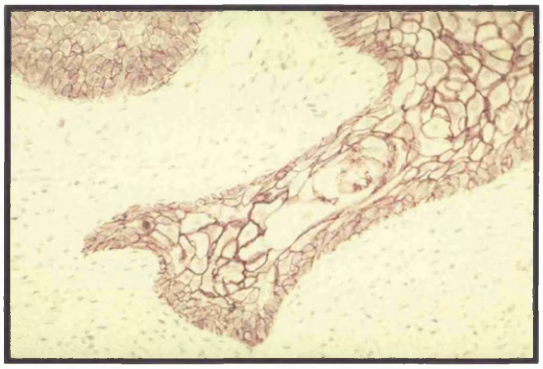


Figure 37. P-Cadherin staining in well-differentiated SCC. Strong tumour cell membrane staining. x500.

Figure 38. P-Cadherin staining in well-differentiated SCC. Tumour cells show marked reduction in membrane staining intensity in comparison with acanthotic epidermis. x320.

	E-CADHERIN			P-CADHERIN			β-CATENIN		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane*	9 [a]	1	0	10 [b]	0	0	9 [a]	1	0
Epidermal cell cytoplasm	0	8	2	0	6	4	0	7	3
Epidermal cell nuclei	0	0	10	0	0	10	0	0	10
Tumour cell membrane	2	7	1	4	5	1	2	7	1
Tumour cell cytoplasm	0	2	8	0	1	9	0	1	9
Tumour cell nuclei	0	0	10	0	0	10	0	0	10

Table 14♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 10 paraffin-embedded well differentiated SCC.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
* In normal and pseudoepitheliomatous skin.
[a] Basal and suprabasal cells.
[b] Basal cells only.

3.15. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded moderately differentiated SCC (n=5)

In normal epidermis, E- and P-cadherin pattern of staining was identical to that seen in normal epidermis of well differentiated SCC (Table 15), which was strongly preserved in all the five cases. E-Cadherin staining was reduced in tumour cell membranes in 4/5 of the cases (particularly in parts of tumour which showed poorer differentiation with conspicuous nuclear pleomorphism and mitoses) when compared with the normal epidermis and cases of well-differentiated SCC. Staining was completely negative in one case (Table 15) (Fig. 41). There was no positive E-cadherin membrane staining on the outer aspects of the tumour lobules and the concentric laminated whorls were weakly positive.

P-Cadherin staining in tumour cell membranes was present in 2/5 of the cases, but in reduced staining intensity when compared with the basal layer of normal epidermis and cases of well-differentiated SCC (Table 15) (Fig. 42). Staining was negative in 3 cases compared with the adjacent normal and pseudoepitheliomatous skin. Variable cytoplasmic staining of both antibodies was noted in normal and pseudoepitheliomatous

epidermal cells ranging from weakly positive to complete absence. The nuclei of tumour cells were universally negative with both antibodies.

3.16. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded moderately differentiated SCC (n=5)

Four of the five specimens examined showed strongly positive C-terminus β-catenin membrane staining in normal and pseudoepitheliomatous skin and one case stained weakly (Table 15) with an identical distribution as described above. The normal cells showed variable cytoplasmic diffusion and the nuclei were universally negative.

Three of five tumours showed reduction of C-terminus β-catenin staining in tumour cell membranes which was more profound in areas of the tumour which showed poorer differentiation, conspicuous nuclear pleomorphism and mitoses (Table 15). Two examples of moderately differentiated SCC showed complete absence of staining (Fig. 40). C-Terminus β-catenin membrane staining in the concentric laminated whorls, horn cysts, and the scattered individually keratinised cells was decreased more extensively than in well differentiated SCC. The tumour downgrowths were largely negative. Variable degrees of diffuse cytoplasmic distribution were observed in tumour cells and their nuclei were consistently negative.

	E-CADHERIN			P-CADHERIN			β-CATENIN		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane*	5 [a]	0	0	5 [b]	0	0	4 [a]	1	0
Epidermal cell cytoplasm	0	4	1	0	2	3	0	1	4
Epidermal cell nuclei	0	0	5	0	0	5	0	0	5
Tumour cell membrane	0	3	2	0	3	2	0	3	2
Tumour cell cytoplasm	0	4	1	0	2	3	0	1	4
Tumour cell nuclei	0	0	5	0	0	5	0	0	5

Table 15♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 5 paraffin-embedded moderately differentiated SCC.

♣Represents results of experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
* In normal and pseudoepitheliomatous skin.
[a] Basal and suprabasal cells.
[b] Basal cells only.

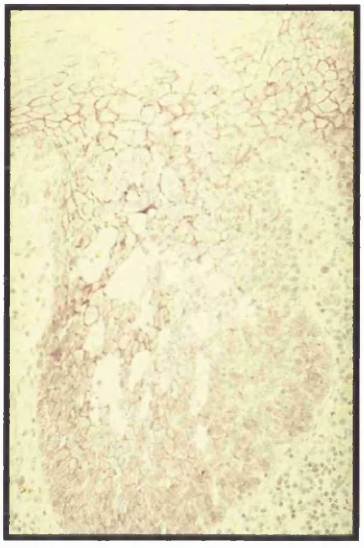


Figure 39. C-Terminus β-catenin staining in well-differentiated SCC. Tumour cells show reduction in membrane staining. x400.

Figure 40. C-Terminus β-catenin staining in Moderately differentiated SCC . Complete absence of staining. x320.

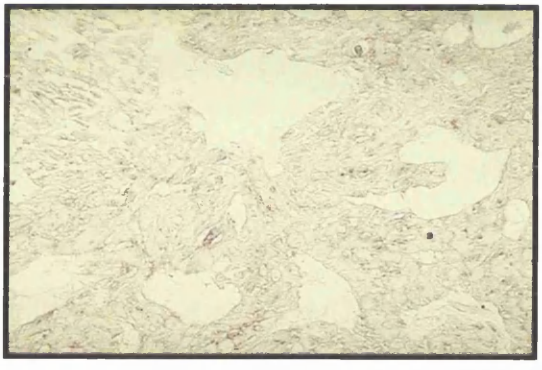
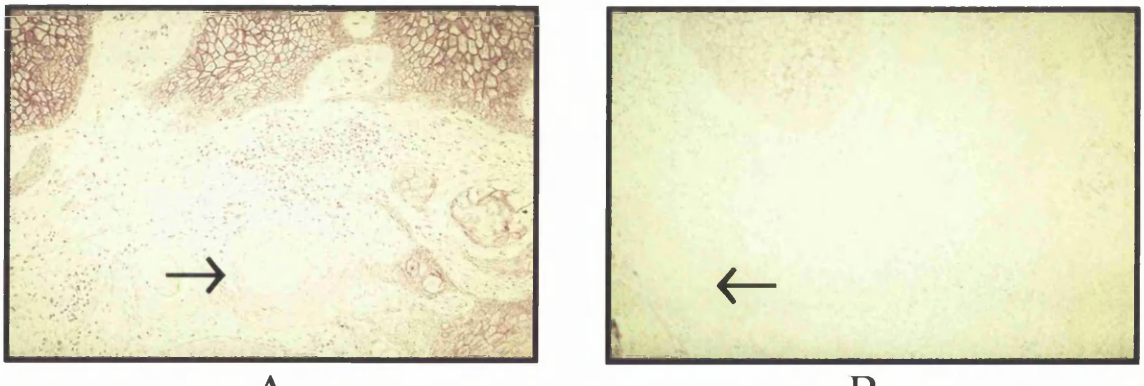


Figure 41. E-Cadherin staining in Moderately differentiated SCC. Complete absence of staining. x320.

Figure 42. P-Cadherin staining in Moderately differentiated SCC. Tumour cells show some reduction in membrane staining. x320.



A B

Figure 43. E- and P-cadherin staining in keratoacanthoma . (A) E-cadherin. (B) P-cadherin. In comparison with the pseudoepitheliomatous skin, tumour cells show reduction in membrane staining intensity (arrows). x320.

3.17. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded keratoacanthoma (n=3)

In all cases, the patterns of expression of E- and P-cadherin were identical to those observed either in normal or in pseudoepitheliomatous skin of well differentiated SCC (Table 16). E-Cadherin membrane staining in the tumour cells was weakly positive in all the cases (Fig. 43A) especially in the squamous epithelium at the lower border of the tumours. The characteristic central keratin plug (crater) was negative. The staining of E-cadherin in tumour cell cytoplasms and nuclei was negative.

Tumour cell membrane staining of P-cadherin along the tumour lower border was present in all cases examined, but in reduced staining intensity when compared with the basal layer of the normal epidermis (Table 16) (Fig. 43B). There was no staining seen in the tumour cell cytoplasms and nuclei.

3.18. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded keratoacanthoma (n=3)

C-Terminus β-catenin distribution in normal and pseudoepitheliomatous skin in keratoacanthoma was similar to that seen in well differentiated SCC. Tumour cell membrane staining of C-terminus β-catenin was present, but in reduced staining intensity in all cases in comparison with the normal epidermis (Table 16) (Fig. 44). The reduction of staining was observed in the proliferative epithelium at the floor of the lesion. The staining of C-terminus β-catenin in tumour cell cytoplasms and nuclei was negative.

	E-CADHERIN			P-CADHERIN			β-CATENIN		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane*	3 [a]	0	0	2 [a]	1	0	3 [a]	0	0
Epidermal cell cytoplasm	0	0	3	0	0	3	0	0	3
Epidermal cell nuclei	0	0	3	0	0	3	0	0	3
Tumour cell membrane	0	3	0	0	3	0	0	3	0
Tumour cell cytoplasm	0	0	3	0	0	3	0	0	3
Tumour cell nuclei	0	0	3	0	0	3	0	0	3

Table 16♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 3 paraffin-embedded keratoacanthoma.

♣Represents results of 1 experiment.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
* In normal and pseudoepitheliomatous skin.
[a] Basal and suprabasal cells.
[b] Basal cells only.

3.19. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded Bowen's disease (n=10)

In all cases, the pattern of staining in normal epidermis of both cadherins was identical to that described above except one, where P-cadherin membrane staining in normal cells was weakly positive (Table 17).

Eight of ten lesions demonstrated strongly positive membrane staining of E-cadherin which was distributed in full thickness of the dysplastic epithelium (carcinoma *in situ*) including the intraepidermal portions of the cutaneous adnexae (Table 17) (Fig. 45). The parakeratotic cells in the stratum corneum were negative. Most of the large dysplastic cells displaying mitotic figures, including abnormal forms, stained strongly positive with E-cadherin. E-Cadherin membrane staining was present in 2/10 of the cases, but in reduced staining intensity when compared with the normal epidermis (Fig. 46). The reduction was more pronounced towards the dermal aspect of the lesion. All the lesions showed weakly positive cytoplasmic staining and their nuclei were negative.

P-Cadherin membrane staining was strongly positive in 8/10 of the cases (Table 17) (Fig. 47). The staining started to involve most of the dysplastic epithelium layers (full thickness) at the demarcation between normal and dysplastic cells. The dermal aspects

(basal and suprabasal) of the lesions stained more strongly than the bulk of the dysplastic epithelium. Two of ten lesions demonstrated reduction of P-cadherin membrane staining (Fig. 48). In contrast with the strongly stained lesions, P-cadherin membrane staining was restricted to the basal cell layer whereas the disorganised, dysplastic epithelium was negative. Again, as for E-cadherin, the staining of P-cadherin in the cytoplasm was weak and completely negative in nuclei.

3.20. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded Bowen's disease (n=10)

The staining of C-terminus β-catenin in the membranes of normal cells was strongly positive in 8/10 of the cases, while two examples showed weak positivity (Table 17). In the normal cell cytoplasms, C-terminus β-catenin staining was weak and completely negative in nuclei.

C-Terminus β-catenin staining in lesional cell membranes was strongly positive in 8/10 of the cases (Table 17) (Fig. 49) with the large dysplastic cells expressing positive staining. C-Terminus β-catenin membrane staining was present in lesional cells in 2/10 of the cases, but in reduced staining intensity when compared with the normal epidermis. This was more pronounced in the dermal aspects of the lesion. All cases showed weakly cytoplasmic staining. The two examples of Bowen's disease which demonstrated reduction in their lesional cell membranes, have shown C-terminus β-catenin nuclear staining (Fig. 50).

Figure 44. C-Terminus β-catenin staining in keratoacanthoma. Tumour cells show reduction in membrane staining. x320.

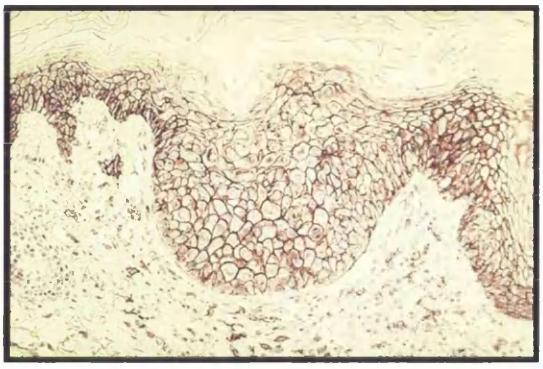


Figure 45. E-Cadherin staining in Bowen's disease. Strong staining and full thickness distribution in lesion. x320.

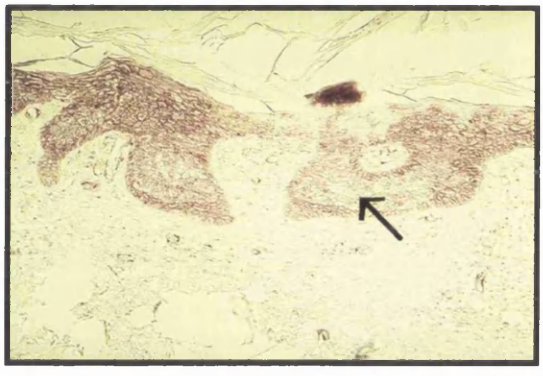


Figure 46. E-Cadherin staining in Bowen's disease. Some lesional cells show reduction in membrane staining intensity (arrow). x250.

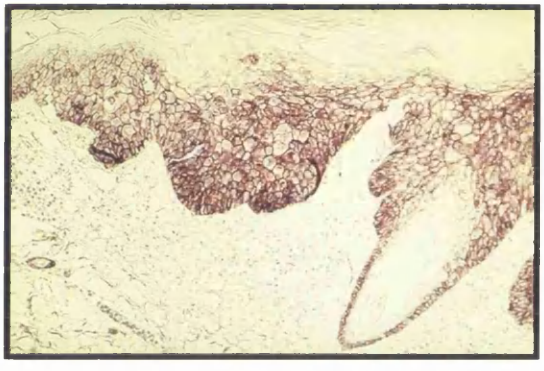


Figure 47. P-Cadherin staining in Bowen's disease. Strong membrane staining involving full thickness. x320.

Figure 48. P-Cadherin staining in Bowen's disease. Restriction of staining to the basal cells. x320.

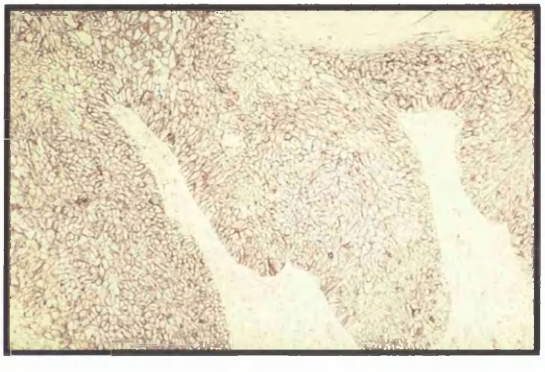


Figure 49. C-Terminus β-catenin staining in Bowen's disease. Strong staining and full thickness distribution in lesion. x320.

	E-CADHERIN			P-CADHERIN			β-CATENIN		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane	10 [a]	0	0	9 [b]	1	0	8 [a]	2	0
Epidermal cell cytoplasm	0	10	0	0	9	1	0	10	0
Epidermal cell nuclei	0	0	10	0	0	10	0	0	10
Lesional cell membrane	8	2	0	8	2	0	8	2	0
Lesional cell cytoplasm	0	10	0	0	10	0	0	10	0
Lesional cell nuclei	0	0	10	0	0	10	0	2	8

Table 17♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 10 paraffin-embedded Bowen's disease.

♣Represents results of 2 experiments apart from β-catenin which was repeated 3 times. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
[a] Basal and suprabasal cells.
[b] Basal cells only.

3.21. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded actinic keratosis (n=10)

All examples demonstrated strongly positive E-cadherin staining in normal epidermis and skin appendages with weak cytoplasmic staining and negative nuclei (Table 18). Membrane staining of P-cadherin in normal basal cells was preserved in 9/10 and weakly positive in one example. There were variable degrees of cytoplasmic P-cadherin staining in normal cells ranging from weakly positive to complete absence. The nuclei in the basal cells were negative.

There was strongly positive cell membrane staining of E-cadherin in 9/10 of the cases (Table 18) (Fig. 51) which was demonstrated in the full thickness of both the dysplastic and the uninvolved epithelia. The alternating bands of hyperkeratosis and parakeratosis which covered the normal and dysplastic epithelium, respectively, were negative.
E-Cadherin staining was present in lesional cell membranes in only one case, but in reduced staining intensity when compared with the normal epidermis (Table 18) (Fig. 52). The reduction of the staining was towards the dermal aspect of the lesion. All the cases have shown reduced cytoplasmic staining and their nuclei were negative.

Dysplastic cell membrane staining of P-cadherin was strongly positive in 8/10 of the cases (Fig. 53). A notable feature was the presence of positive P-cadherin staining in only the basal layer of uninvolved epithelium, then involvement of the full thickness of epithelium which showed dysplasia (Table 19). This pattern of P-cadherin staining in actinic keratosis seems to be correlating with the alternating bands of dysplasia (covered by parakeratotic cells) and normal epithelium (covered by hyperkeratotic cells) (Fig. 54).

Two of ten lesions showed decreased P-cadherin membrane staining in the dysplastic epidermis. There was reduced staining intensity of P-cadherin in the cytoplasms of lesional cells and the staining was completely negative in the nuclei.

3.22. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded actinic keratosis (n=10)

Eight of ten cases showed strongly positive C-terminus β-catenin in normal epidermis as previously described. The staining of C-terminus β-catenin in normal epidermis was reduced in two cases and there was variable degrees of cytoplasmic staining ranging from reduced intensity to complete absence. There was no nuclear staining observed in normal cells in all cases (Table 18).

C-Terminus β-catenin staining was strongly positive in dysplastic and normal epithelium in 9/10 of the cases (Table 18) (Fig. 55). The characteristic alternating bands of parakeratosis and hyperkeratosis were universally negative in all the cases. One case has shown presence of staining in the lesional cell membranes, but in reduced intensity when compared with the normal epidermis and other cases (Fig. 56). There was variable cytoplasmic staining in the lesional cells but the nuclei were negative.

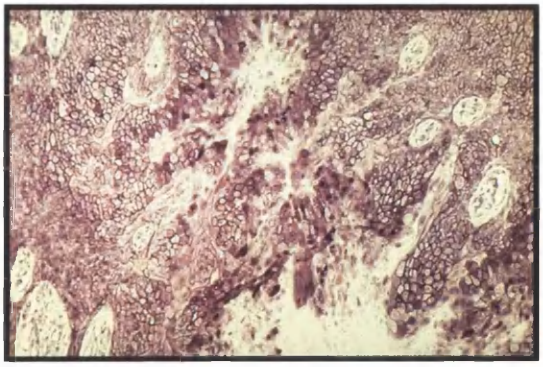


Figure 50. C-Terminus β-catenin nuclear staining in Bowen's disease. Note the accompanying decrease in membrane β-catenin. x250.

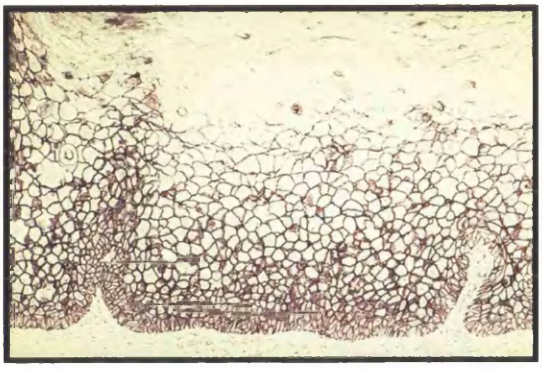


Figure 51. E-Cadherin staining in actinic keratosis. Strong staining and full thickness distribution in lesion. x320.

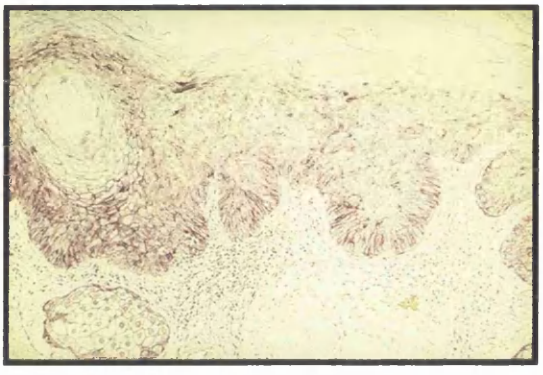


Figure 52. E-Cadherin staining in actinic keratosis. Some lesional cells show reduction in membrane staining intensity. x250.

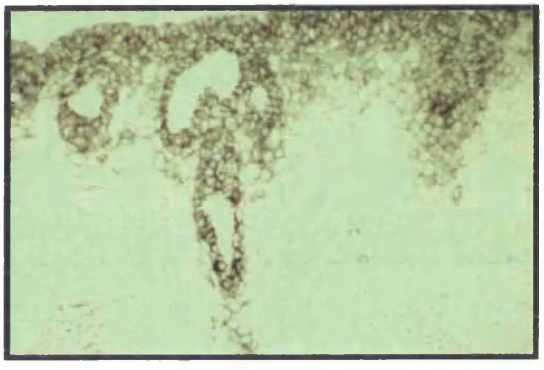


Figure 53. P-Cadherin staining in actinic keratosis. Strong membrane staining involving full thickness. x250.

Figure 54. P-Cadherin staining in actinic keratosis. Note involvement of full thickness in dysplastic epithelium and restriction to basal cells in uninvolved epithelium. x250.

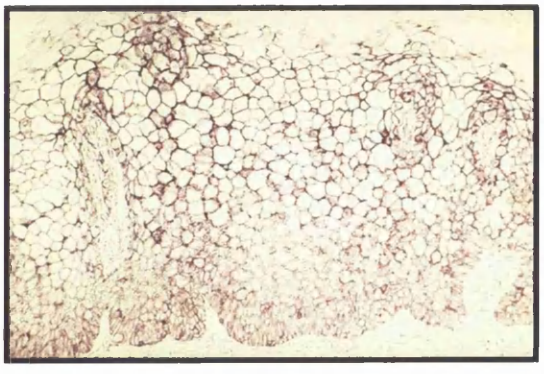


Figure 55. C-Terminus β-catenin staining in actinic keratosis. Strong staining and full thickness distribution in lesion. x320.

	E-CADHERIN			β-CATENIN		
	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane	10 [a]	0	0	8 [a]	2	0
Epidermal cell cytoplasm	0	10	0	0	7	3
Epidermal cell nuclei	0	0	10	0	0	10
Lesional cell membrane	9	1	0	9	1	0
Lesional cell cytoplasm	0	10	0	0	9	1
Lesional cell nuclei	0	0	10	0	0	10

Table 18♣. Distribution of E-cadherin and C-terminus β-catenin in 10 paraffin-embedded actinic keratosis.

♣ Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
[a] Basal and suprabasal cells.
[b] Basal cells only.

	P-CADHERIN		
	++	+/-	Neg
Epidermal cell membrane*	9 [a]	1	0
Epidermal cell cytoplasm	0	8	2
Epidermal cell nuclei	0	0	10
Uninvolved epidermal cell membrane**	10 [a]	0	0
Dysplastic epidermal cell membrane***	8 [b]	2	0
Lesional cell cytoplasm	0	10	0
Lesional cell nuclei	0	0	10

Table 19♣. Distribution of P-cadherin in 10 paraffin-embedded actinic keratosis.

♣ Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
* Adjacent to lesion.
[a] Basal cells only.
** Covered by hyperkeratosis.
*** Covered by parakeratosis.
[b] Full thickness.

3.23. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded psoriasis (n=5)

In all cases, the expression pattern of E-cadherin was identical to normal epidermis (Table 20) (Fig. 57). The basal cell layer was staining relatively weaker than the spinous layer, however, this was noted in many examples of normal skin. E-Cadherin staining was weakly positive in lesional cell cytoplasms and negative in the nuclei in all the cases examined. In addition, there was no staining seen in stratum corneum.
P-Cadherin was distributed in the full thickness of the psoriatic skin in all the five lesions examined (Table 20) (Fig. 58). Its staining was weakly positive in the lesional cell cytoplasms and negative in the nuclei.

3.24. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded psoriasis (n=5)

All examples demonstrated strongly positive C-terminus β-catenin staining in lesional cell membranes identical to normal epidermis (Table 20) (Fig. 59). Although the staining was preserved in all epidermal layers of the acanthotic lesion, it was more pronounced in the spinous cells. The orthohyperkeratotic and parakeratotic layers overlying the lesions were negative. The staining of C-terminus β-catenin was weakly positive in lesional cytoplasms and negative in the nuclei.

	E-CADHERIN			P-CADHERIN			β-CATENIN		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane	5 [a]	0	0	5 [a]	0	0	5 [a]	0	0
Epidermal cell cytoplasm	0	5	0	0	2	3	0	5	0
Epidermal cell nuclei	0	0	5	0	0	5	0	0	5
Lesional cell membrane	5	0	0	5	0	0	5	0	0
Lesional cell cytoplasm	0	5	0	0	2	3	0	5	0
Lesional cell nuclei	0	0	5	0	0	5	0	0	5

Table 20♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 5 paraffin-embedded psoriasis.

♣ Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
[a] Basal and suprabasal cells.

3.25. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded lichen simplex chronicus (n=5)

The expression pattern of E-cadherin in lichen simplex chronicus was identical to normal epidermis in all the five cases examined (Table 21) (Fig. 60). P-Cadherin staining was strongly positive in the membranes of lesional cells throughout the full thickness of the epidermis excluding the hyperkeratotic stratum corneum (Fig. 61), however, in one case, the staining was restricted to the basal cell layer (Table 21). There were variable degrees of diffuse cytoplasmic distribution of P-cadherin in lesional cells, but no nuclear staining was noted.

3.26. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded lichen simplex chronicus (n=5)

In all cases examined, there was strongly positive C-terminus β-catenin staining in lesional cell membranes identical to normal epidermis (Table 21) (Fig. 62). The staining was more pronounced in the membranes of spinous cells, weakly positive in the cytoplasms and uniformly negative in the nuclei.

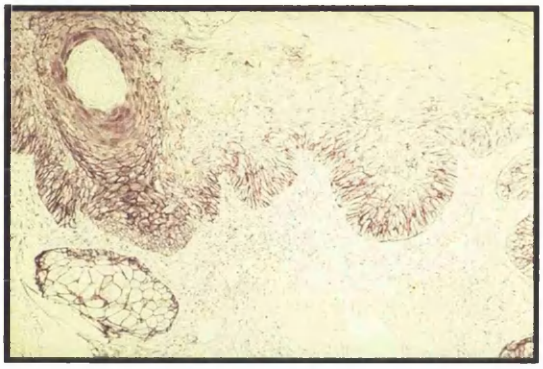


Figure 56. C-Terminus β-catenin staining in actinic keratosis. Reduction of membrane staining is more marked in interfollicular epithelium. x320.

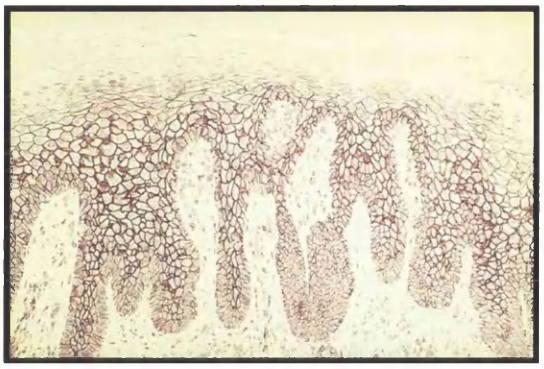


Figure 57. E-Cadherin staining in psoriasis. Strong staining and full thickness distribution in lesion. x320.

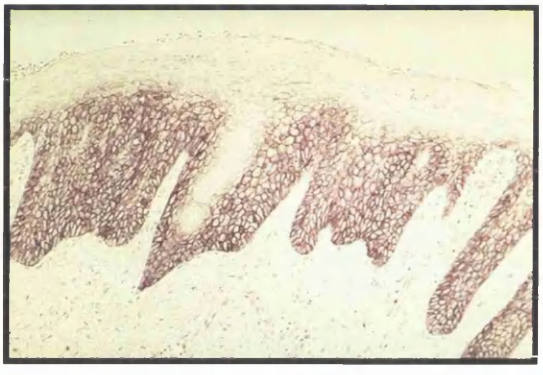


Figure 58. P-Cadherin staining in psoriasis. Involvement of full thickness. x320.

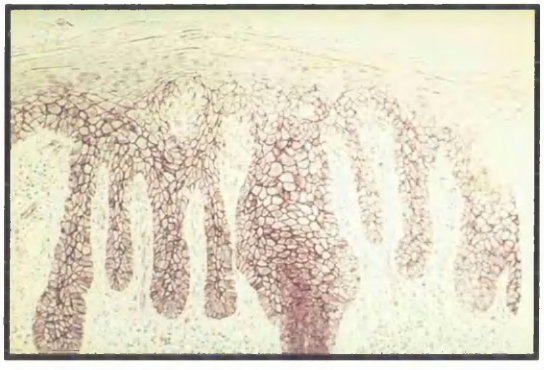


Figure 59. C-Terminus β-catenin staining in psoriasis. Strong staining and full thickness distribution in lesion. x320.

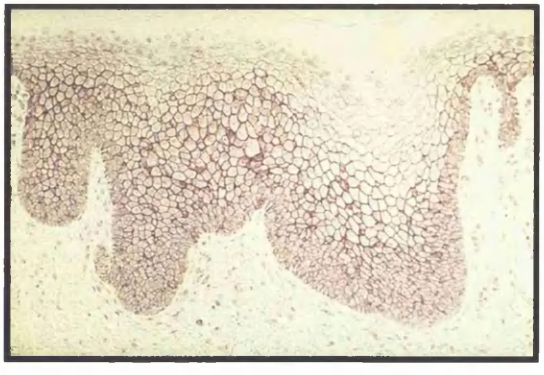


Figure 60. E-Cadherin staining in lichen simplex chronicus . Strong staining and full thickness distribution in lesion. x320.

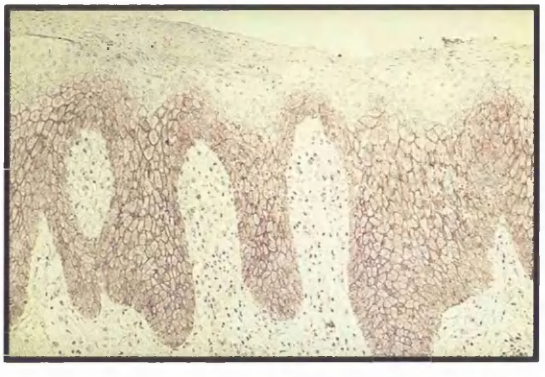


Figure 61. P-Cadherin staining in lichen simplex chronicus. Involvement of full thickness excluding parakeratotic layer. x320.

	E-CADHERIN			P-CADHERIN			β-CATENIN		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal cell membrane	5 [a]	0	0	5 [a]	0	0	5 [a]	0	0
Epidermal cell cytoplasm	0	5	0	0	4	1	0	5	0
Epidermal cell nuclei	0	0	5	0	0	5	0	0	5
Lesional cell membrane	5	0	0	5	0	0	5	0	0
Lesional cell cytoplasm	0	5	0	0	4	1	0	5	0
Lesional cell nuclei	0	0	5	0	0	5	0	0	5

Table 21♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 5 paraffin-embedded lichen simplex chronicus.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
[a] Basal and suprabasal cells.

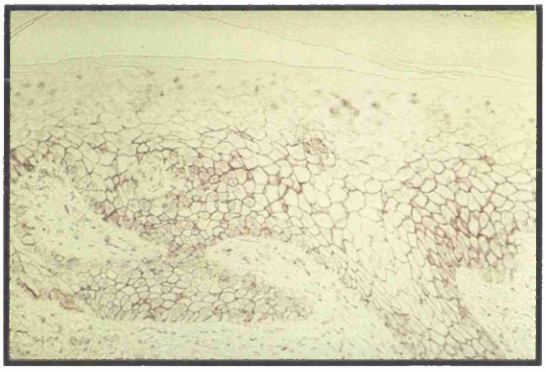


Figure 62. C-Terminus β-catenin staining in Lichen simplex chronicus. Strong staining and full thickness distribution in lesion. x320.

Results II

Immunoperoxidase Staining of melanocytic lesions

3.27. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded benign naevi (n=6)

The membrane staining of E-cadherin in melanocytic naevus cells in the epidermis was strongly positive in 4/6 of the cases (Table 22) (Fig. 63). Two of six lesions showed reduction of E-cadherin epidermal naevus cell membrane staining particularly in the junctional component of the naevus in comparison with the normal epidermis (Table 22). In these, the staining of junctional melanocytes was uneven with some melanocytes showing preserved membrane staining. E-Cadherin cytoplasmic staining of epidermal naevus cells was ranging from strongly positive to weak intensity. The membrane staining of E-cadherin in the dermal naevus cells was reduced in 5/6 of the cases (Fig. 64)and strongly positive in one example. The reduction of E-cadherin membrane staining tended to be correlated with maturation and decrease in dermal melanocytes size with depth. The minimal cytoplasm of the dermal melanocytes stained weakly positive with E-cadherin. There was no E-cadherin nuclear staining observed either in epidermal or dermal naevus cells.

P-Cadherin membrane staining in epidermal melanocytes was strongly positive in 5/6 of the cases (Table 22) (Fig. 65). The staining was relatively stronger when compared with P-cadherin membrane staining in basal normal keratinocytes. In the junctional component, there was decreased P-cadherin membrane staining in one case. Three of six benign naevi showed strong membrane staining of P-cadherin in their dermal components when compared with the normal epidermis while 3/6 of the naevi expressed reduced membrane staining (Table 22) (Fig. 65). There were highly variable degrees of P-cadherin cytoplasmic staining in both epidermal and dermal components ranging from strong staining intensity to complete absence. P-Cadherin nuclear staining was not observed in all naevi.

3.28. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded benign naevi (n=6)

C-Terminus β-catenin staining was strongly positive in the membranes of melanocytic naevus cells in the epidermis in 5/6 of the cases following the same pattern as E-cadherin (Table 22) (Fig. 66). One benign naevus showed relative reduction of membrane staining in the junctional melanocytes, although, there were some melanocytes expressing strongly positive membrane staining within the junctional component. C-Terminus β-catenin staining in the membranes of dermal melanocytes was strongly positive in two naevi, but four cases have shown gradual reduction of membrane staining which was more pronounced in the small matured melanocytes in the deeper parts of the lesions. The strongly positive membrane staining in the epidermal melanocytes was accompanied by strong cytoplasmic staining which tended to decrease in the dermal component (Table 22) (Fig. 66). The nuclei of epidermal and dermal melanocytes were negative.

	E-Cadherin			P-Cadherin			β-Catenin		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal keratinocyte membrane	6 [a]	0	0	6 [b]	0	0	6 [a]	0	0
Epidermal keratinocyte cytoplasm	0	6	0	0	4	2	0	5	1
Epidermal keratinocyte nuclei	0	0	6	0	0	6	0	0	6
Epidermal melanocytic naevus cell membrane	4	2	0	5	1	0	5	1	0
Epidermal melanocytic naevus cell cytoplasm	1	5	0	2	3	1	4	1	1
Epidermal Melanocytic naevus cell nuclei	0	0	6	0	0	6	0	0	6
Dermal naevus cell membrane	1	5	0	3	3	0	2	4	0
Dermal naevus cell cytoplasm	0	6	0	2	3	1	3	1	2
Dermal naevus cell nuclei	0	0	6	0	0	6	0	0	6

Table 22♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 6 paraffin-embedded benign naevi.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.

++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.

[a] Basal and suprabasal cells.

[b] Basal cells only.

3.29. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded blue naevi (n=6)

The overlying epidermis of all the blue naevi examined was normal with no coexistent junctional activity. E-Cadherin staining was reduced in the membranes of the dermal melanocytes in 5/6 of the cases (Table 23) (Fig. 67) and completely negative in one case. The reduction in membrane staining was associated more with the bipolar, dendritic spindle melanocytes in the deeper parts of the naevus. The cytoplasmic staining of E-cadherin in naevus cells was faint and diffuse, but their nuclei were negative.

In comparison with the basal cells of the normal epidermis, P-cadherin membrane staining of the naevus cells was reduced and largely faint in 2/6 of the cases while it was completely absent at all cellular levels in 4/6 of the cases (Table 23) (Fig. 68A).

3.30. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded blue naevi (n=6)

The dermal melanocyte membranes and cytoplasm stained weakly positive with C-terminus β-catenin in 3/6 of the cases and they were completely negative in the other 3 naevi (Table 23) (Fig. 68B). In comparison with E-cadherin, C-terminus β-catenin membrane staining in blue naevi was lighter. There was no nuclear staining observed.

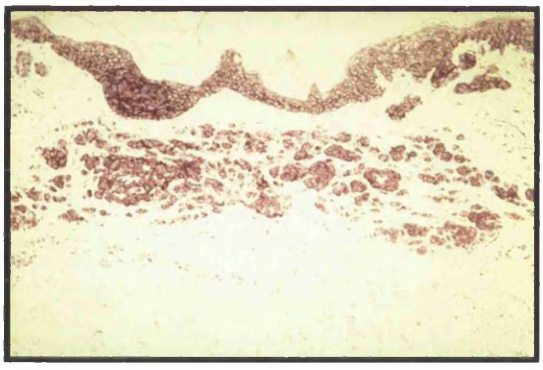


Figure 63. E-Cadherin staining in benign naevus. Strong staining in junctional and dermal naevus cells. x200.

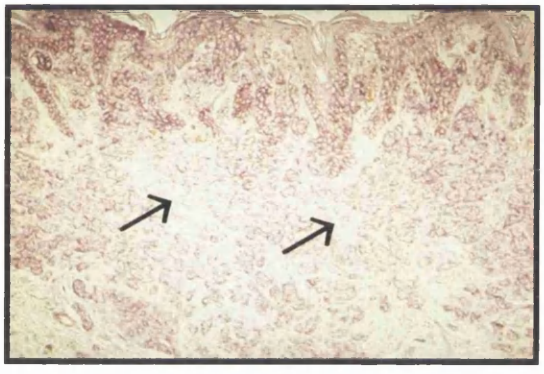


Figure 64. E-Cadherin staining in benign naevus. In comparison with the normal epidermis and junctional component, some dermal naevus cells show reduction in membrane staining intensity (arrows). x200.

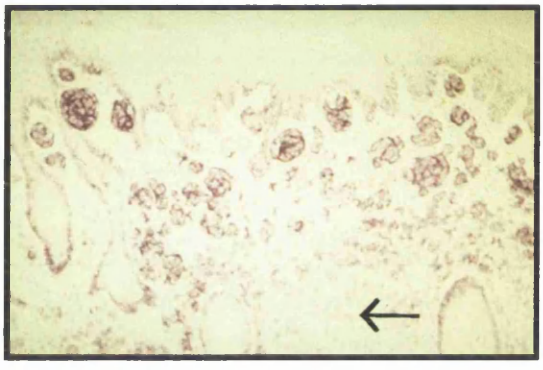


Figure 65. P-Cadherin staining in benign naevus. Positive staining in basal keratinocytes and strong staining in junctional and upper dermal nests. Note decrease in membrane staining intensity with maturation (arrow). x125.

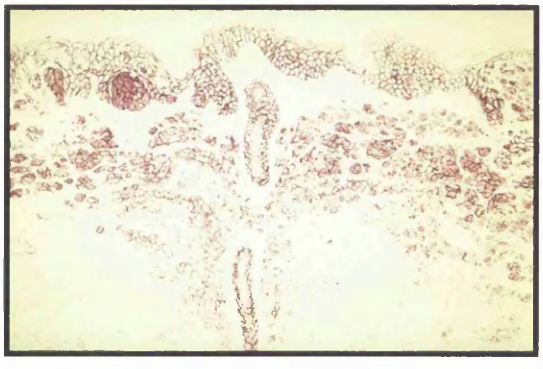


Figure 66. C-Terminus β-catenin staining in benign naevus. Strong staining in junctional and dermal naevus cells. x200.

Figure 67. E-Cadherin staining in blue naevus. In comparison with normal epidermis, dermal melanocytes show marked reduction in membrane staining intensity. x200.

	E-Cadherin			P-Cadherin			β-Catenin		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal keratinocyte membrane	6 [a]	0	0	6 [b]	0	0	6 [a]	0	0
Epidermal keratinocyte cytoplasm	0	5	1	0	3	3	0	5	1
Epidermal keratinocyte nuclei	0	0	6	0	0	6	0	0	6
Dermal naevus cell membrane	0	5	1	0	2	4	0	3	3
Dermal naevus cell cytoplasm	0	5	1	0	1	5	0	3	3
Dermal naevus cell nuclei	0	0	6	0	0	6	0	0	6

Table 23♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 6 paraffin-embedded blue naevi *.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
* No epidermal component.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
[a] Basal and suprabasal cells.
[b] Basal cells only.

3.31. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded atypical naevi (n=6)

All atypical naevi examined were compound, showing architectural dysplasia.
E-Cadherin membrane staining was reduced in epidermal melanocytes in 3/6 of the cases (Table 24) and strongly positive in other cases. In cases with epidermal melanocytes expressing reduced membrane E-cadherin staining, the reduction was noted in the atypical melanocytes at the tips of epidermal ridges showing lentiginous hyperplasia. Those cells were present singly and in small clusters, but in a rather disorganised appearance. In all the cases, a relative reduction of membrane E-cadherin in the intradermal component particularly in spindle shaped cells in the deeper parts of the lesions was seen (Fig. 69A and B). Epidermal and dermal melanocytes expressed variable E-cadherin cytoplasmic staining ranging from strongly positive to weaker intensity, but their nuclei were negative.

The staining of P-cadherin in the membranes of atypical melanocytes in the epidermis was similar to that of E-cadherin, however, it was slightly stronger in intensity when compared with basal keratinocytes. Three of six cases showed strongly positive

membrane P-cadherin staining in epidermal atypical melanocytes while there was reduction in staining in 3/6 cases (Table 24) (Fig. 70A). Atypical dermal melanocytes expressed reduced membrane P-cadherin staining in 5/6 of the cases particularly in matured deep cells (Fig. 70B). One case has shown strongly positive membrane staining of P-cadherin in the dermal component. P-Cadherin staining was variable in the cytoplasms but universally negative in the nuclei.

3.32. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded atypical naevi (n=6)

Strongly positive membrane staining was seen in 5/6 lesions with the C-Terminus β-catenin antibody. Staining was reduced in one (Table 24) in epidermal melanocytes (Fig. 71A). Intradermally, there was strong membrane C-terminus β-catenin staining in two examples and reduction in 4/6 of the cases (Fig. 71B) which was obvious in the delicate spindle shaped melanocytes. Similar to E-cadherin, epidermal and dermal melanocytes expressed variable cytoplasmic staining ranging from strongly positive to weaker intensity, and their nuclei were negative.

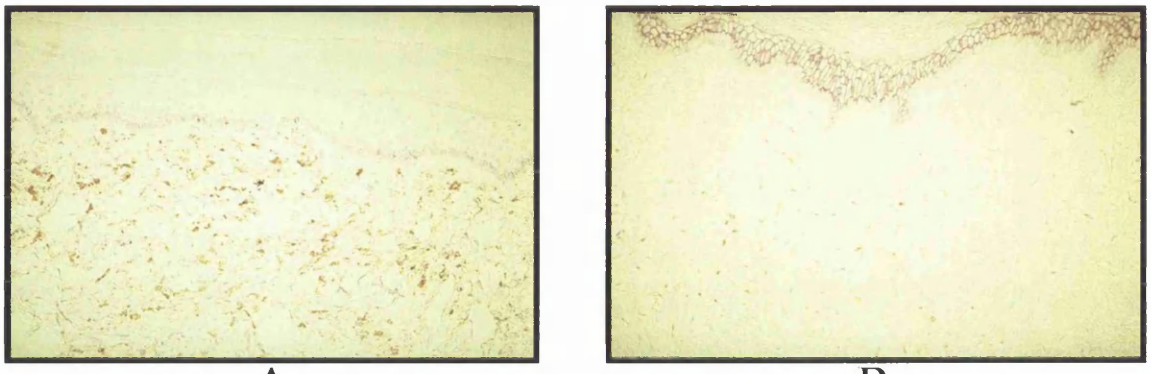


Figure 68. P-Cadherin and C-Terminus β-catenin staining in blue naevus. (A) P-cadherin. (B) β-catenin. Complete absence of staining. x200.

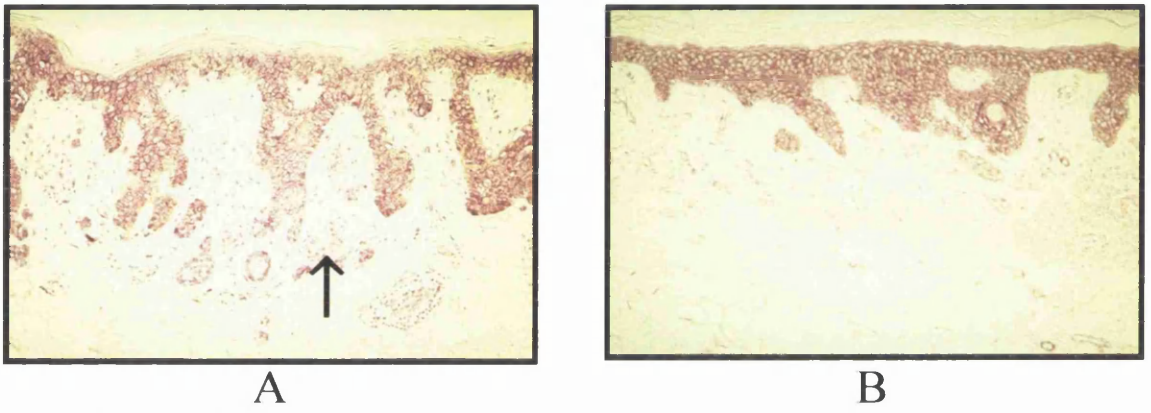


Figure 69. E-Cadherin staining in atypical naevus. (A) In comparison with normal epidermis and junctional component, some dermal melanocytes show reduction in membrane staining intensity (arrow). (B) dermal melanocytes show marked reduction in membrane staining intensity. x200.

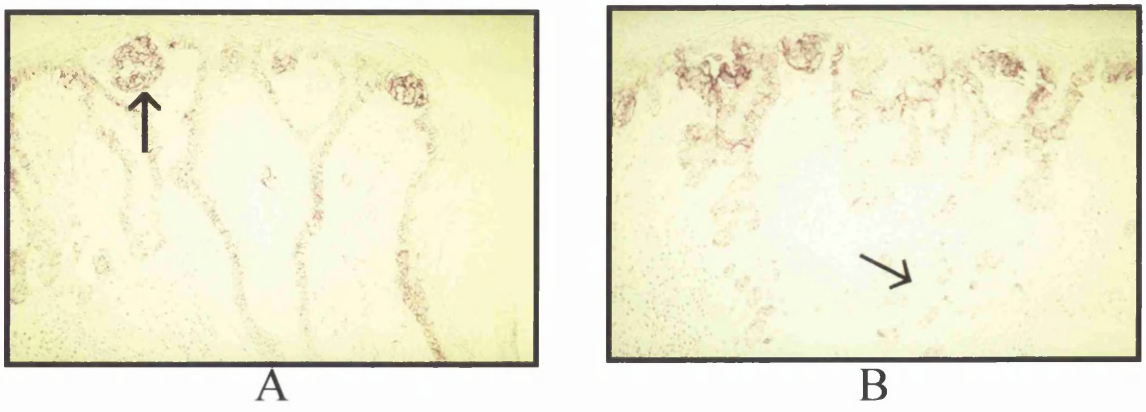


Figure 70. P-Cadherin staining in atypical naevus. (A) Strong staining in junctionally nested melanocytes (arrow).(B) In comparison with normal epidermis and junctional component, some dermal melanocytes show reduction in membrane staining intensity (arrow). (A) x250, (B) x200.

	E-Cadherin			P-Cadherin			β-Catenin		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal keratinocyte membrane	6 [a]	0	0	5 [b]	1	0	5 [a]	1	0
Epidermal keratinocyte cytoplasm	0	6	0	0	2	4	0	5	1
Epidermal keratinocyte nuclei	0	0	6	0	0	6	0	0	6
Epidermal melanocytic naevus cell membrane	3	3	0	3	3	0	5	1	0
Epidermal melanocytic naevus cell cytoplasm	2	4	0	0	3	3	2	4	0
Epidermal melanocytic naevus cell nuclei	0	0	6	0	0	6	0	0	6
Dermal naevus cell membrane	0	6	0	1	5	0	2	4	0
Dermal naevus cell cytoplasm	0	6	0	0	4	2	0	6	0
Dermal naevus cell nuclei	0	0	6	0	0	6	0	0	6

Table 24♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 6 paraffin-embedded atypical naevi.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
[a] Basal and suprabasal cells.
[b] Basal cells only.

3.33. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded Spitz naevi (n=6)

E-Cadherin staining in the membranes of melanocytic naevus cells in the epidermis was reduced in all cases (Table 25). This was observed mainly in both spindle and epithelioid melanocytes in the junctional component. Similarly, the membrane staining of the dermal melanocytes was reduced in both cell population in all the cases (Fig. 72A). E-Cadherin membrane staining reduction was more pronounced in matured melanocytes with increasing depth of the lesion. There was highly variable cytoplasmic staining of E-cadherin in Spitz naevi ranging from weakly positive to complete absence. No nuclear staining was detected either in epidermal or dermal melanocytes.

In contrast to E-cadherin, the membrane staining of P-cadherin in junctional melanocytes was strongly positive in 2/6 of the cases. Four of six Spitz naevi showed reduced P-cadherin membrane staining in both epithelioid and spindle cells in the

junctional component (Table 25) (Fig. 72B), however, the reduction was not universal. All Spitz naevi expressed uneven P-cadherin membrane staining in their dermal components with deeper areas showing marked reduction (Fig. 73). Melanocyte cytoplasmic staining of P-cadherin in both epidermal and dermal components ranged from weakly positive to complete absence. There was no P-cadherin nuclear staining in all the cases.

3.34. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded Spitz naevi (n=6)

The membrane staining of C-terminus β-catenin in the junctional component of Spitz naevi was reduced in 5/6 of the lesions and strongly positive in one naevus (Table 25) (Fig. 74). The reduction of C-terminus β-catenin was observed in the membranes of junctional melanocytes which are showing either spindle or epithelioid morphology. In addition, there were small pale non-stained naevoid cells. However, within this region there were melanocytes of both cell populations staining strongly particularly small clusters of large epithelioid cells. Within the dermis, all cases have shown reduction of melanocyte membrane C-terminus β-catenin staining increasing with depth and in cells acquiring spindle cell appearance (Fig. 74). Cytoplasmic staining was variable in epidermal and dermal melanocytes and their nuclei were negative.

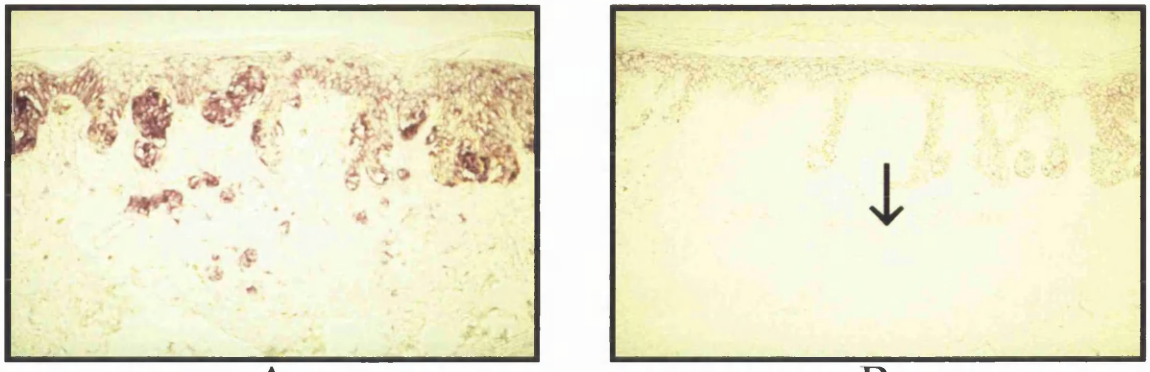


Figure 71. C-Terminus β-catenin staining in atypical naevus. (A) Strong staining in naevus cells in junctional and dermal components.(B) In comparison with normal epidermis and junctional component, dermal melanocytes show marked reduction in membrane staining intensity (arrow). x200

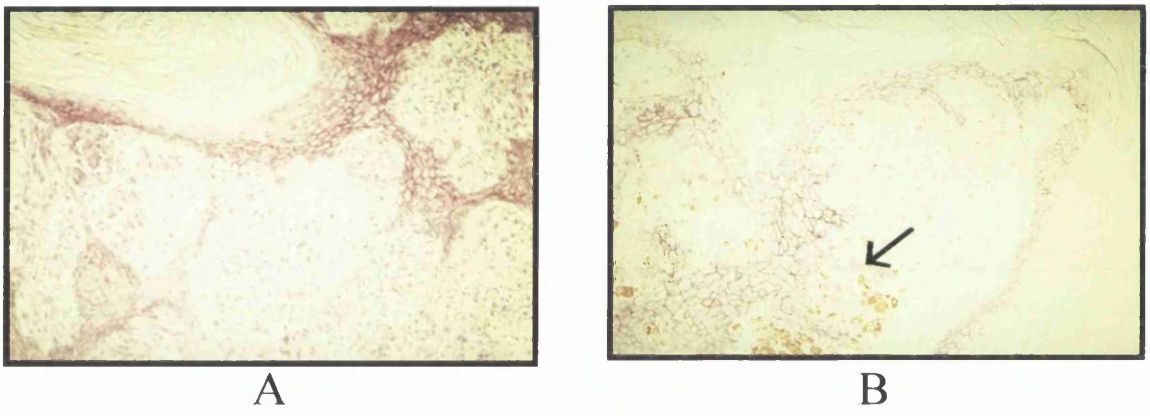


Figure 72. E- and P-cadherin staining in Spitz naevus. (A) Marked reduction of E-cadherin membrane staining in naevus cells when compared with overlying normal keratinocytes. (B) Reduction in P-cadherin membrane staining intensity in junctional component (arrow) when compared with overlying normal keratinocytes. x320.

Figure 73. P-Cadherin staining in Spitz naevus. Marked reduction of dermal naevus cell membrane staining compared with overlying normal keratinocytes. x320.

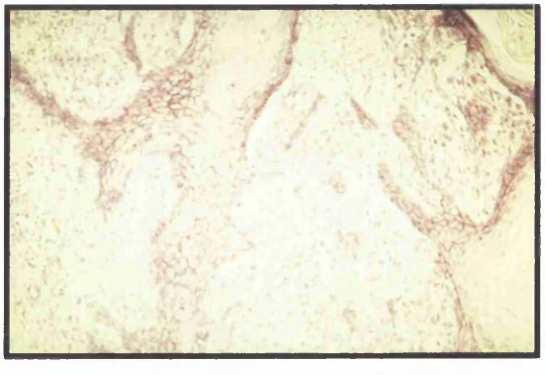


Figure 74. C-Terminus β-catenin staining in Spitz naevus. Marked reduction of β-catenin membrane staining in naevus cells when compared with overlying normal keratinocytes. x320.

	E-Cadherin			P-Cadherin			β-Catenin		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal keratinocyte membrane	6 [a]	0	0	6 [b]	0	0	6 [a]	0	0
Epidermal keratinocyte cytoplasm	0	6	0	0	4	2	0	6	0
Epidermal keratinocyte nuclei	0	0	6	0	0	6	0	0	6
Epidermal melanocytic naevus cell membrane	0	6	0	2	4	0	1	5	0
Epidermal melanocytic naevus cell cytoplasm	0	6	0	0	6	0	0	5	1
Epidermal melanocytic naevus cell nuclei	0	0	6	0	0	6	0	0	6
Dermal naevus cell membrane	0	6	0	0	6	0	0	6	0
Dermal naevus cell cytoplasm	0	5	1	0	2	4	0	6	0
Dermal naevus cell nuclei	0	0	6	0	0	6	0	0	6

Table 25♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 6 paraffin-embedded Spitz naevi.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
[a] Basal and suprabasal cells.
[b] Basal cells only.

3.35. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded primary melanoma (n=6)

Five of six melanomas have shown reduction of E-cadherin staining in the membranes of malignant melanocytes (Table 26) (Fig. 75A) which were confined to the epidermis (in situ) (Clark level I) and in those located at the junctions infiltrating the papillary dermis (Clark level II). This reduction was seen in melanomas with radial and vertical growth phases, although, it was more pronounced in melanoma cells in their vertical growth phase. One of six cases showing vertical growth phase, expressed total loss of E-cadherin membrane staining of malignant melanocytes present in the epidermis. These melanocytes were atypical with small epithelioid appearance. The reduction of membrane E-cadherin staining was not correlated with increasing Clark's level. Four of six cases showed reduction of E-cadherin membrane staining in the invasive dermal nested melanocytes. Of these, one was infiltrating the reticular dermis and subcutaneous fat (Clark level IV/V) with E-cadherin membrane staining still detected

but rather weak (Fig. 75B). E-Cadherin membrane staining was completely negative in malignant melanocytes infiltrating the papillary dermis (Clark level II) in 2/6 of the cases (Fig. 76). Of these, one was showing vertical growth phase and the other showing radial growth. Malignant melanocyte cytoplasmic staining of E-cadherin in both epidermal and dermal components ranged from weakly positive to complete absence. There was no E-cadherin nuclear staining in all the cases.

P-Cadherin membrane staining was strongly reduced in the membranes of epidermal malignant melanocytes (Clark level I and II) in 5/6 of the cases (Table 26) (Fig. 77) especially in vertical growth phase melanomas. One melanoma with vertical growth phase showed negative P-cadherin staining in epidermal malignant melanocytes and in cells just invading the papillary dermis (Clark level II). Three of six malignant melanomas (two vertical and one radial growth phase) expressed marked reduction of P-cadherin membrane staining in their solely dermal malignant melanocytes. Of these, one was showing vertical growth phase extending up to subcutaneous tissue (Clark level IV/V). There was complete absence of P-cadherin staining in dermal cells in 3/6 of the cases (Fig. 77) which were only Clark level II. Similar to E-cadherin, the cytoplasmic staining of P-cadherin in both epidermal and dermal components of melanomas ranged from weakly positive to complete absence. There was no P-cadherin nuclear staining in all the cases.

3.36. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded primary melanoma (n=6)

All malignant melanomas examined (four vertical and two radial growth phase) showed weakly positive C-terminus β-catenin membrane staining in the epidermal and junctional malignant cells (Clark level I) (Table 26) (Fig. 78A). The membrane staining of C-terminus β-catenin was markedly reduced in the dermal malignant melanocytes in 3/6 of the cases (all showing vertical growth phase). Of these, one malignant melanoma with Clark level IV/V). There was loss of C-terminus β-catenin staining in the dermal malignant cells in 3/6 of the cases (Fig. 78B). The staining of C-terminus β-catenin in

cytoplasms of epidermal and dermal malignant melanocytes was variable ranging from reduced staining intensity to complete absence and it was negative in the nuclei in all the cases.

	E-Cadherin			P-Cadherin			β-Catenin		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal keratinocyte membrane	6 [a]	0	0	6 [b]	0	0	5 [a]	1	0
Epidermal keratinocyte cytoplasm	0	6	0	0	3	3	0	5	1
Epidermal keratinocyte nuclei	0	0	6	0	0	6	0	0	6
Epidermal melanoma cell membrane	0	5	1	0	5	1	0	6	0
Epidermal melanoma cell cytoplasm	0	5	1	0	4	2	0	6	0
Epidermal melanoma cell nuclei	0	0	6	0	0	6	0	0	6
Dermal melanoma cell membrane	0	4	2	0	3	3	0	3	3
Dermal melanoma cell cytoplasm	0	4	2	0	2	4	0	3	3
Dermal melanoma cell nuclei	0	0	6	0	0	6	0	0	6

Table 26♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 6 paraffin-embedded primary melanoma.

♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.
++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.
[a] Basal and suprabasal cells.
[b] Basal cells only.

3.37. Immunohistochemical staining of E- and P-cadherin in formalin fixed and paraffin-embedded metastatic melanoma (n=6)

In three of six cases examined there was no normal epidermis attached to the tumours which were located in the dermis and/or in the subcutis.

There was strongly positive E-cadherin membrane staining in metastatic melanocytes in 1/6 of the cases (Table 27) (Fig. 79A) particularly stronger at the peripheries of the tumour mass. The metastatic melanocytes in this case were localised in the dermis in nests of different sizes and displaying epithelioid appearance with abundant cytoplasm, nuclear pleomorphism and prominent nucleoli.

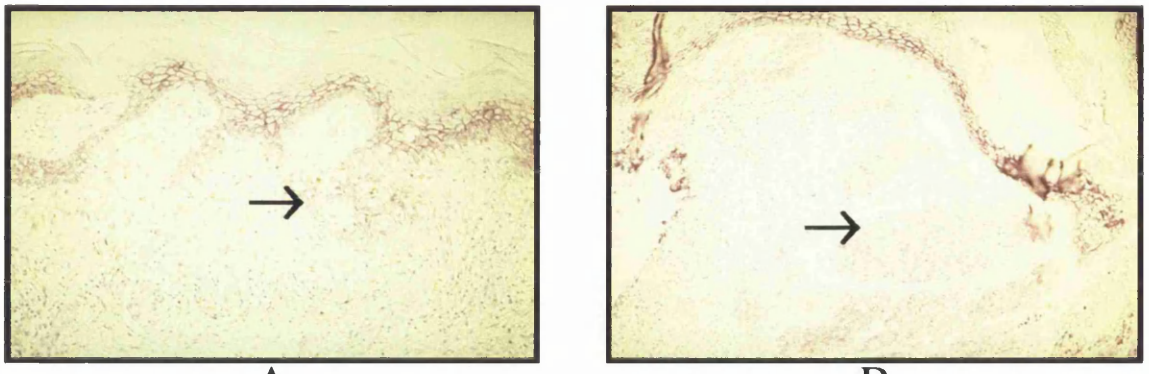


Figure 75. E-Cadherin staining in malignant melanoma. (A) In comparison with epidermis, junctional melanoma cells show marked reduction in membrane staining intensity (arrow). (B) marked reduction in staining of deeper melanoma cells (arrow). x250.

Figure 76. E-Cadherin staining in malignant melanoma. Complete absence of staining in deep dermal malignant melanocytes. x320.

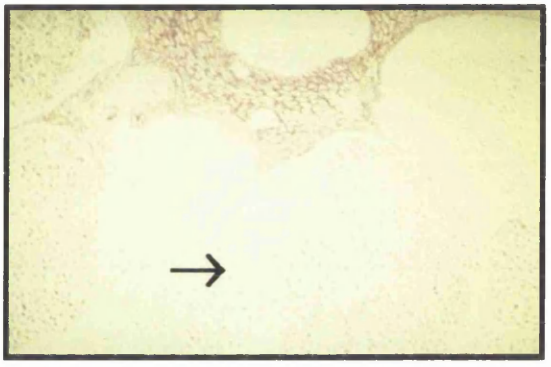


Figure 77. P-Cadherin staining in malignant melanoma. In comparison with the strong and accentuated staining in overlying normal keratinocytes, melanoma cells (arrow) show marked reduction in membrane staining intensity. x320.

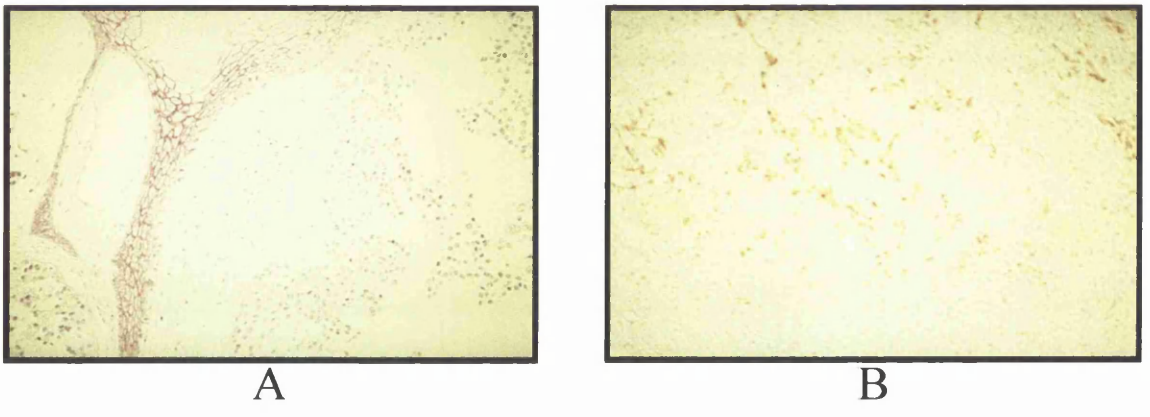


Figure 78. C-Terminus β-catenin staining in malignant melanoma. (A) Marked reduction of malignant melanocyte membrane staining compared with overlying normal keratinocytes. (B) Complete absence of staining in deeper aspects of tumour showing melanin pigment. x320.

E-Cadherin membrane staining of metastatic melanocytes was largely reduced in 3/6 of the cases. Those tumours were composed of sheets of small malignant melanocytes showing nuclear pleomorphism and mitotic activity. Two metastatic melanomas were completely negative (Fig. 79B). The staining in the cytoplasm of malignant melanocytes ranged from weakly positive to complete absence and was negative in the nuclei.

Similar to E-cadherin, there was strong preservation of P-cadherin membrane staining in the dermal metastatic melanocytes in the same case (Table 27). Two metastatic melanomas showed marked reduction of P-cadherin membrane staining especially in the inner aspects of the tumour mass where cells displaying obvious atypia. The staining of P-cadherin was completely absent in three tumours (Fig. 80). P-Cadherin staining in the cytoplasms of metastatic cells was largely reduced and universally negative in the nuclei of all cases.

3.38. Immunohistochemical staining of C-terminus β-catenin in formalin fixed and paraffin-embedded metastatic melanoma (n=6)

C-Terminus β-catenin membrane staining was strongly positive in one case following the same pattern as E-cadherin (Table 27) (Fig. 81). Two of six cases showed marked reduction of C-terminus β-catenin membrane staining particularly in melanocytes displaying apparent malignant features. There was complete absence of C-terminus β-catenin membrane staining in 3/6 of the cases (Fig. 82). All cases expressed variable cytoplasmic staining ranging from weak staining intensity to complete absence. In all cases, there was no C-terminus β-catenin nuclear staining detected.

	E-Cadherin			P-Cadherin			β-Catenin		
	++	+/-	Neg	++	+/-	Neg	++	+/-	Neg
Epidermal keratinocyte* membrane	3 [a]	0	0	3 [b]	0	0	3 [a]	0	0
Epidermal keratinocyte cytoplasm	0	3	0	0	2	1	0	3	0
Epidermal keratinocyte nuclei	0	0	3	0	0	3	0	0	3
Metastatic melanoma cell membrane	1	3	2	1	2	3	1	2	3
Metastatic melanoma cell cytoplasm	0	4	2	0	2	4	0	4	2
Metastatic melanoma cell nuclei	0	0	6	0	0	6	0	0	6

Table 27♣. Distribution of E- and P-cadherin and C-terminus β-catenin in 6 paraffin-embedded metastatic melanoma .

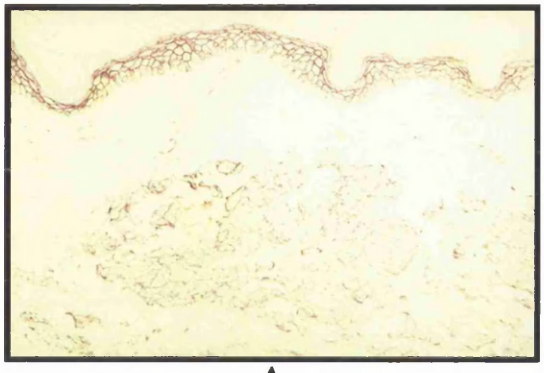
♣Represents results of 2 experiments. Results were reproducible and consistent in each specimen.

++: strongly positive. +/-: weakly positive (no. of negative tumour cells > 10%). Neg: negative.

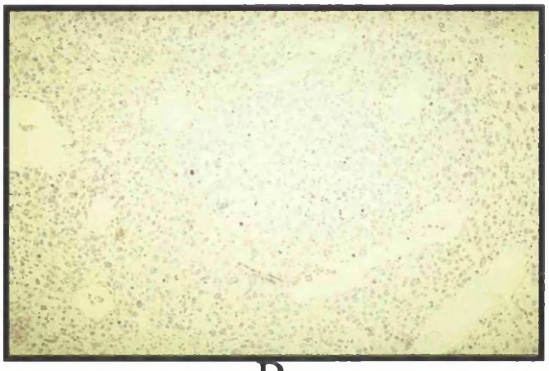
* Three cases have no epidermis.

[a] Basal and suprabasal cells.

[b] Basal cells only.



A



B

Figure 79. E-Cadherin staining in metastatic melanoma. (A) Many metastatic malignant melanocytes in the dermis show strong membrane staining similar to normal epidermis. (B) complete absence of staining. (A) x250, (B) x320.

Figure 80. P-Cadherin staining in metastatic melanoma. Complete absence of staining. x320.

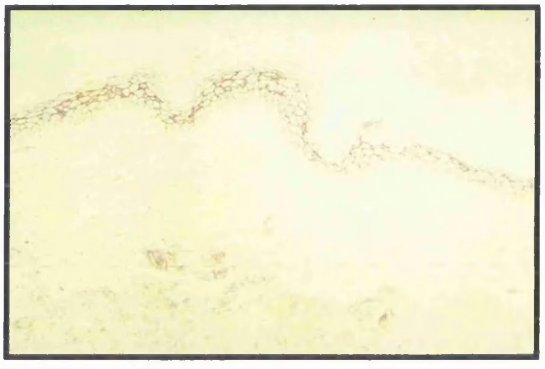


Figure 81. C-Terminus β-catenin staining in metastatic melanoma. Many metastatic malignant melanocytes in the dermis show strong membrane staining similar to normal epidermis. x250.

Figure 82. C-Terminus β-catenin staining in metastatic melanoma. Complete absence of staining. x320.

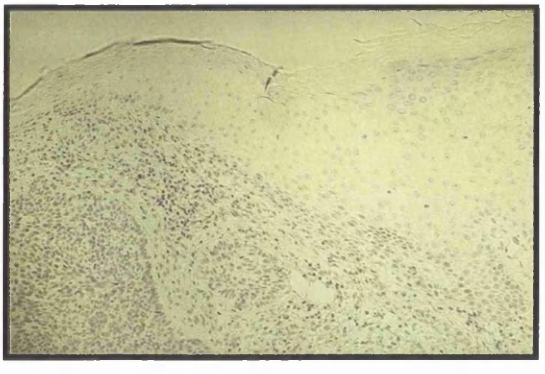


Figure 83. Negative control for immunohistochemical staining. A representative negative control. All other controls were consistently negative. x320.

Results III

Immunofluorescent staining and double immunolabelling of the major adherens junction components in Normal Skin and Basal Cell Carcinoma.

3.39. Immunofluorescence staining of major adherens junction components in normal epidermis adjacent to tumour

As summarised in Table 28, Rhodamine-Phalloidin revealed weak and diffuse staining for actin filaments in the basal and prickle cell layers, whereas in the granular cell layer a very strong binding of phalloidin was evident (Fig. 84A). However, in the normal epidermis immediately above the main BCC mass, actin bundles were distributed in all the cell layers, except stratum corneum, mainly present at the periphery of the cells (particularly granular layer which contained prominent bundles of actin filaments) (Fig. 84B). Sometimes actin staining was seen in the centre of the cells especially in the granular layer. In only one example of the normal epidermis adjacent to the tumour, bundles of stress fibers were concentrated at the apical region of the normal keratinocytes and arranged vertically so that they appear to be leading upwards (Fig. 85).

In the hair follicle (Table 29), the cells of the external root sheath were shown to express prominent actin filaments especially in their outermost cells along the basal cell surface (Fig. 86). The actin filaments in those cells were concentrated peripherally, but some were shown to have a radial arrangement transverse to the follicle axis (Fig. 86). Actin staining was weakly positive in internal root sheath cells and hair matrix, negative in hair papilla and shaft but was present in fibroblasts arranged in layers in the periadnexal fibrous tissue sheath (Fig. 86). The fibroblasts in this area showed parallel actin filaments (stress fibers) running transversally to the longitudinal axis of the hair follicle. The arrector pili muscle inserted in the periadnexal fibrous tissue sheath showed strong actin staining (Fig. 87).

Similar to the immunohistochemical staining using Extra-avidin technique, the immunofluorescence staining of E-cadherin, α- and β-catenin in normal epidermis was limited to the viable layers (Table 28) (Figs. 88, 89, 90, and 91). Their staining was uniform and exhibited preserved and even enhanced membranous reactivity. The strongest staining of E-cadherin, α- and β-catenin was observed in normal keratinocytes membranes at the level of cell-cell junctions in the spinous layer while it was generally weak in the granular layer and absent from the basal poles of basal cells.

P-Cadherin (Table 28) was expressed in the membranes of the basal layer cells at cell-cell junctions particularly at the apical poles and lateral margins of the cells in agreement with the Extra-avidin technique results (Fig. 92). In addition, P-cadherin membrane staining was observed in the external root sheath cells and hair matrix while there was complete absence of staining in the internal root sheath cells, dermal papilla and hair shaft (Fig. 93).

α-Actinin staining (Table 28) was particularly prominent in the suprabasal cells of the normal epidermis including stratum corneum. In viable cells, α-actinin staining was largely cytoplasmic and was often enriched at cell peripheries (Fig. 94). Very weak or even absent α-actinin staining was noted in the basal cell layer. No α-actinin nuclear staining was seen.

	Stratum corneum	Granular	Spinous	Basal
Actin [a]	–	++	+/–	–
E-Cadherin [b]	–	+/–	++	+/–
P-Cadherin [b]	–	–	–	++
β-Catenin [b]	–	+/–	++	+/–
α-Catenin [b]	–	+/–	++	+/–
α-Actinin [c]	++	++	++	–

Table 28. Immunofluorescence staining of major adherens junction components in layers of normal epidermis adjacent to tumour *.

* Represents a minimum of 5 staining runs showing reproducible and consistent results.
[a] Mainly present at the periphery of the cells.
[b] In cell membrane at the level of cell-cell junctions.
[c] Largely cytoplasmic with enrichment at cell peripheries.
++ : strongly positive. +/– : weakly positive (homogeneously weak). – : negative.

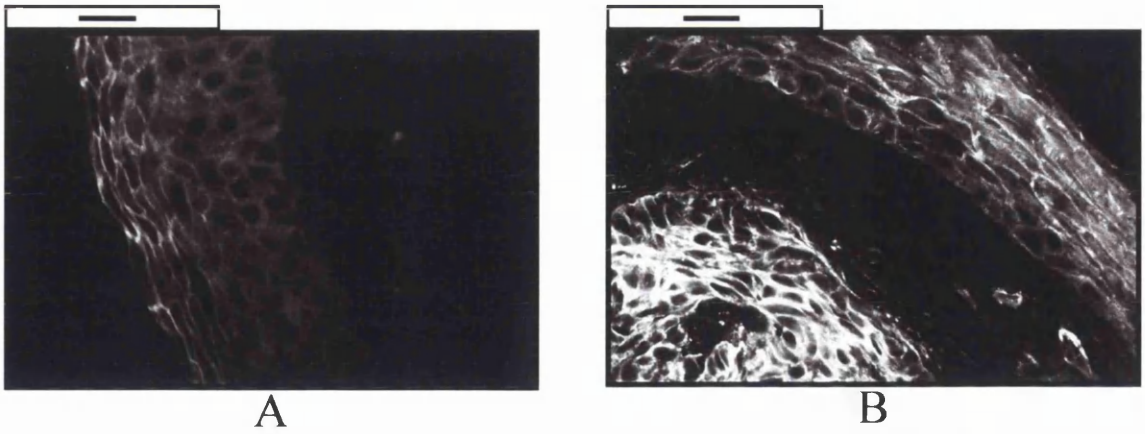


Figure 84. Immunofluorescence staining of actin in normal skin. (A) adjacent to BCC: prominent actin bundles in the peripheries of granular layer cells and weak actin bundles staining in spinous layer, basal cells are negative. (B) Immediately above BCC: Note actin bundles in all epidermal layers except stratum corneum. Scale bars = 25μm.

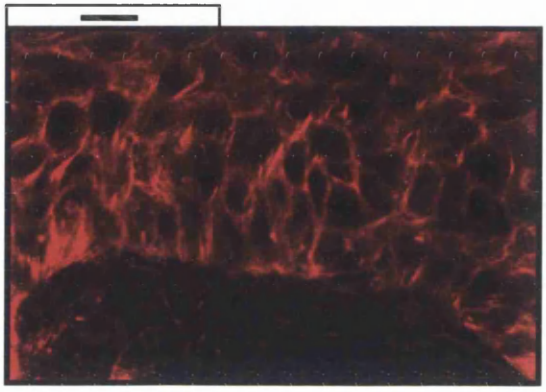


Figure 85. Immunofluorescence staining of actin in normal skin adjacent to BCC. Note concentration at apical region of normal keratinocytes and vertical arrangement. Scale bar = 25μm.

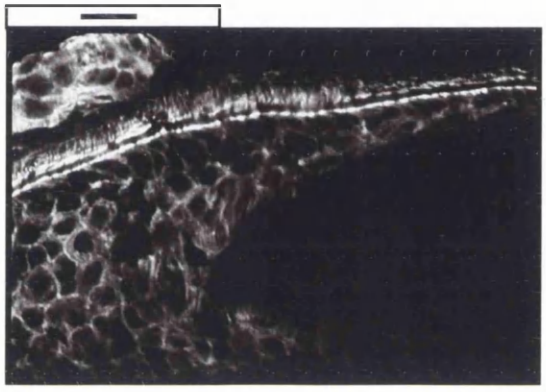


Figure 86. Immunofluorescence staining of actin in normal hair follicle. Prominent actin bundles in ORS cells with peripheral and radial arrangement. Note staining in periadnexal fibrous tissue sheath. Scale bar = 25μm.

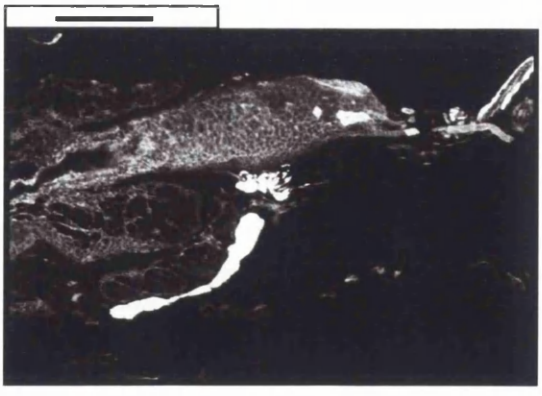


Figure 87. Immunofluorescence staining of actin in normal pilosebaceous unit. Note actin staining in ORS cells and arrector pili muscle. Scale bar = 250μm.

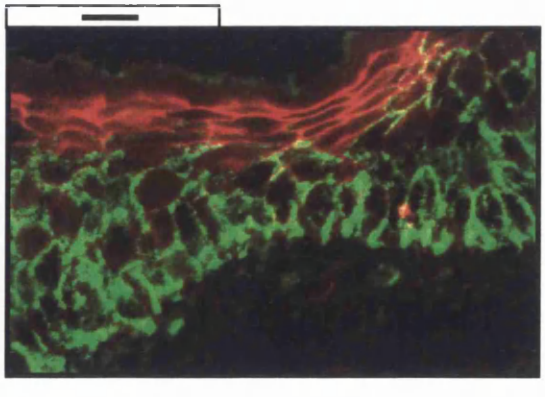


Figure 88. Double immunolabelling of E-cadherin (green) with actin (red) in normal epidermis. Note E-Cadherin staining in viable layers. No co-localisation. Scale bar = 25μm.

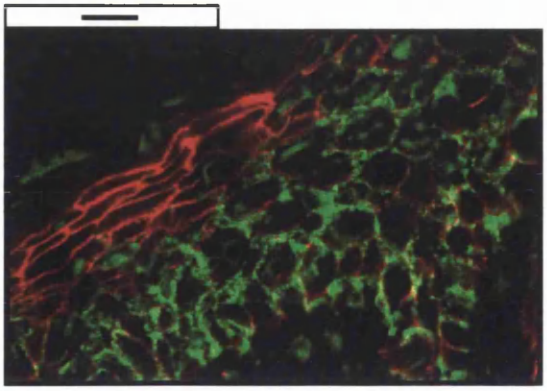


Figure 89. Double immunolabelling of β-catenin (green) with actin (red) in normal epidermis. Actin bundles staining in peripheries of granular layer cells. Note β-catenin staining in viable layers. No co-localisation. Scale bar = 25μm.

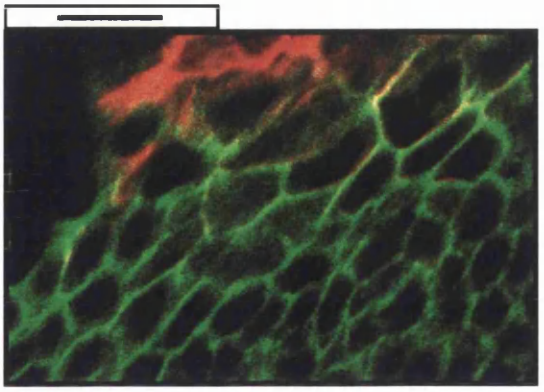


Figure 90. Double immunolabelling of α-catenin (green) with actin (red) in normal epidermis. Note α-catenin staining in viable layers. Some cells show co-localisation (yellow). Scale bar =50μm.

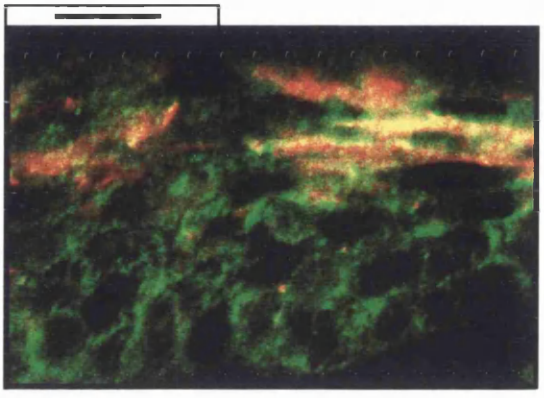


Figure 91. Double immunolabelling of α-catenin (green) with actin (red) in normal epidermis. Note the double-stained cells (yellow) in granular layer. Scale bar =50μm.

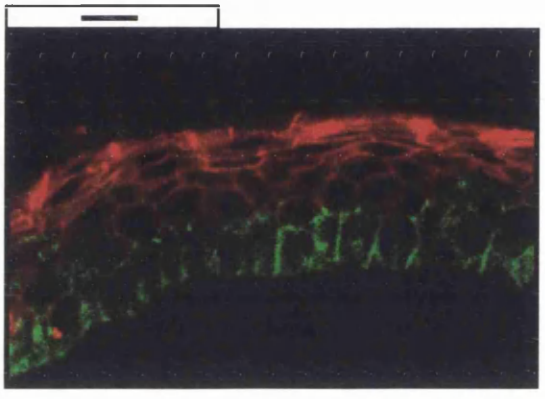


Figure 92. Double immunolabelling of P-cadherin (green) with actin (red) in normal epidermis. Note restriction of P-cadherin to basal cells. No co-localisation. Scale bar = 25μm.

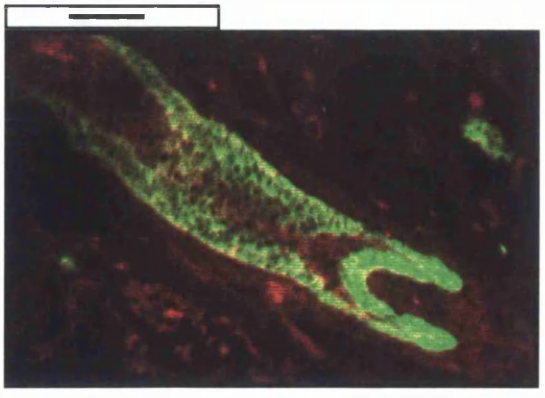


Figure 93. Double immunolabelling of P-cadherin (green) with actin (red) in hair follicle. Strong staining of P-cadherin in ORS and hair matrix cells. No obvious co-localisation. Scale bar =100μm.

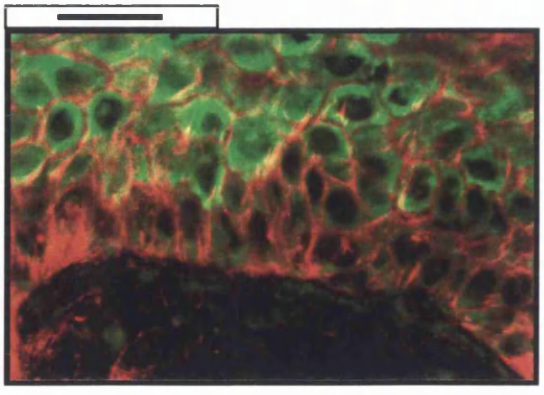


Figure 94. Double immunolabelling of α-actinin (green) with actin (red) in normal epidermis. Intense α-actinin cytoplasmic staining in the suprabasal cells. Some cells show co-localisation (yellow). Scale bar =50μm.

	Actin
External root sheath cells	++
Internal root sheath cells	+/–
Hair matrix	+/–
Hair papilla	–
Hair shaft	–
Connective tissue sheath	+/–

Table 29. Immunofluorescence staining of filamentous actin with Rhodamine-Phalloidin in normal hair follicle *.

* Represents 3 staining runs showing reproducible and consistent results.
++: strongly positive. +/– : weakly positive (homogeneously weak). – : negative.

3.40. Immunofluorescence staining of major adherens junction components in Basal cell carcinoma (BCC).

There was a dramatic increase of actin filament staining intensity in tumour cells at connection sites with the normal epidermis when compared with that of normal keratinocytes (Tables 30 and 31) (Fig. 95). At this area, however, the actin filaments (stress fibers) distribution in the tumour downgrowths was uneven in which some strands and cells showed prominent actin polymerisation but staining was less pronounced in others. This observation was particularly more pronounced in tumours with infiltrative growth pattern. In addition, the lobules of these tumours showed a distinctive pattern of actin filament staining. Actin polymerisation was more pronounced centrally accompanied by reduced or even absent E-, P-cadherin, α-, and β-catenin staining when compared with that of the normal epidermal keratinocytes (Figs. 96-98). In contrast, at the peripheries, actin polymerisation was rarely observed in the palisading cells which expressed strong E-, P-cadherin, α-, and β-catenin staining (Figs. 96-99). The spindle shaped tumour cells located towards the peripheral aspects of the lobules (not palisading cells) demonstrated thick, dense and long actin filaments (stress fibers) arranged mostly in parallel bundles traversing the longitudinal axis of the cells (Fig. 100). The staining of actin filaments in the tumour cells towards the inner aspects of the lobules was very strong in comparison with the tumour palisading cells and normal epidermis but their arrangement was variable. Such actin filaments (stress

fibers) were thick and randomly oriented. In some lobules of infiltrating growth pattern tumours, actin polymerisation was more pronounced in certain aspects (dermal or sideways) than others which seemed to be the direction of the tumour growing edge (Figs. 96 and 100).

In tumour lobules showing less aggressive behaviour (nodular) (Table 31), strong staining of actin was evident which was homogenous and evenly distributed. In these, the actin staining was located peripherally beneath the cell membrane where single actin filaments were rarely observed. This staining pattern of actin filaments was accompanied by the presence of E-, P-cadherin, α-, β-catenin and α-actinin staining but in reduced staining intensity when compared with the normal epidermis (Figs. 101-103). Staining for actin filaments was present in the loose stroma surrounding tumour lobules (Table 31) but in a reduced staining intensity and somewhat different assemblage when compared with tumour cells (Figs. 104 and 105). Actin filaments in these cells formed fine networks of stress fibers converging at several points in a disoriented arrangement. The characteristic retraction artefacts separating tumour lobules from their associated stroma were negative (Fig. 105).

	Connection with normal epidermis	Central	Peripheral	Stroma
Actin [a]	++	++	+/–	++ to +/–
E-Cadherin [b]	+/–	+/–	++	–
P-Cadherin [b]	++ to +/–	++ to +/–	++	–
β-Catenin [b]	+/–	+/–	++	–
α-Catenin [b]	+/–	+/–	++	–
α-Actinin [c]	+/–	+/–	+/–	–

Table 30. Immunofluorescence staining of major adherens junction components in tumour lobules of BCC with infiltrative growth pattern *.

* Represents a minimum of 5 staining runs showing reproducible and consistent results.

[a] Mainly thick, dense and long actin filaments arranged mostly in parallel bundles traversing the longitudinal axis of the cells

[b] In cell membrane at the level of cell-cell junctions.

[c] Largely cytoplasmic with enrichment at cell peripheries.

++ : strongly positive. +/– : weakly positive (homogeneously weak).. – : negative.

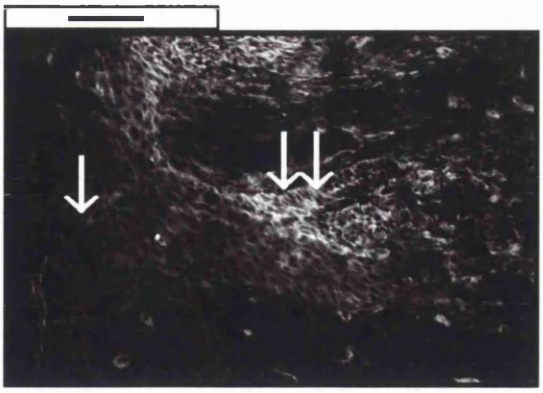


Figure 95. Actin staining at connection of infiltrative BCC with epidermis. Note the dramatic increase of actin filament staining intensity (double arrow) when compared with that of normal keratinocytes (single arrow). Scale bar =100μm.

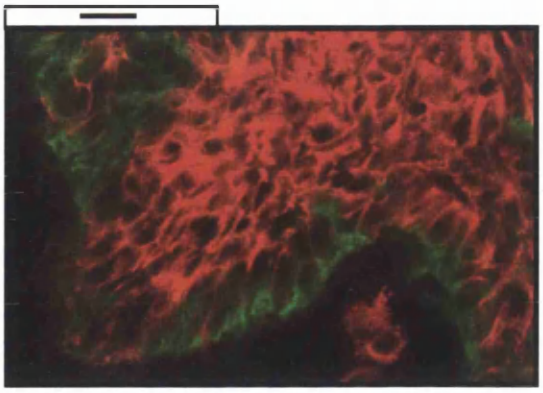


Figure 96. Double immunolabelling of E-cadherin (green) with actin (red) in infiltrative BCC. Note actin polymerisation in central aspect of lobule accompanied by reduced E-cadherin membrane staining in contrast to palisading cells. Scale bar = 25μm.

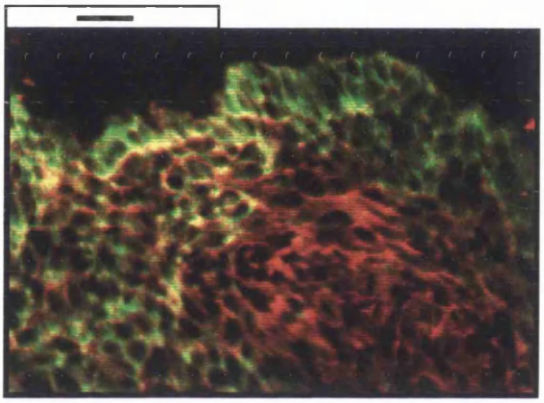


Figure 97. Double immunolabelling of P-cadherin (green) with actin (red) in infiltrative BCC. Actin polymerisation in central aspect of lobule accompanied by reduced P-cadherin membrane staining in contrast to palisading cells. Some cells show co-localisation (yellow). Scale bar = 25μm.

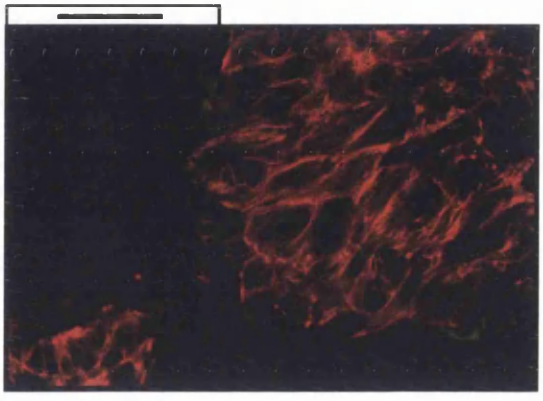


Figure 98. Double immunolabelling of β-catenin (green) with actin (red) in infiltrative BCC. Note prominent actin polymerisation in infiltrating cells accompanied by marked reduction of β-catenin staining. Scale bar =50μm.

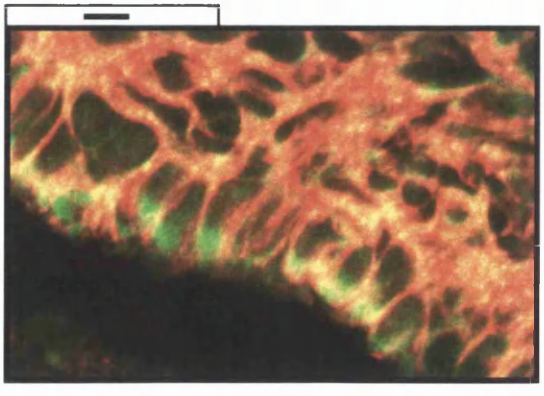


Figure 99. Double immunolabelling of α-catenin (green) with actin (red) in infiltrative BCC. Some peripheral cells show co-localisation (yellow). Scale bar =10μm.

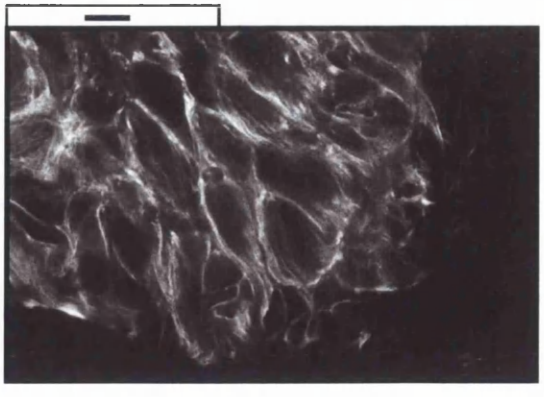


Figure 100. Actin staining in infiltrative BCC. Note the pronounced actin polymerisation in the infiltrating cells where they demonstrate thick and dense actin bundles. Scale bar =10μm.

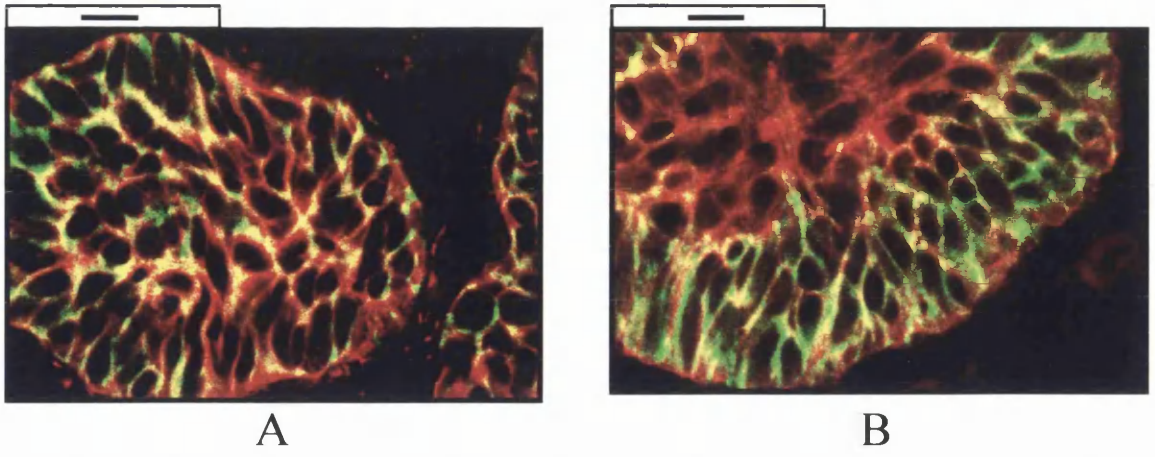


Figure 101. Double immunolabelling of E- and P-cadherin (green) with actin (red) in nodular BCC. (A) E-cadherin and actin. (B) P-cadherin and actin. Note actin staining in the peripheries of tumour cells where some show co-localisation (yellow). Scale bars = 25μm.

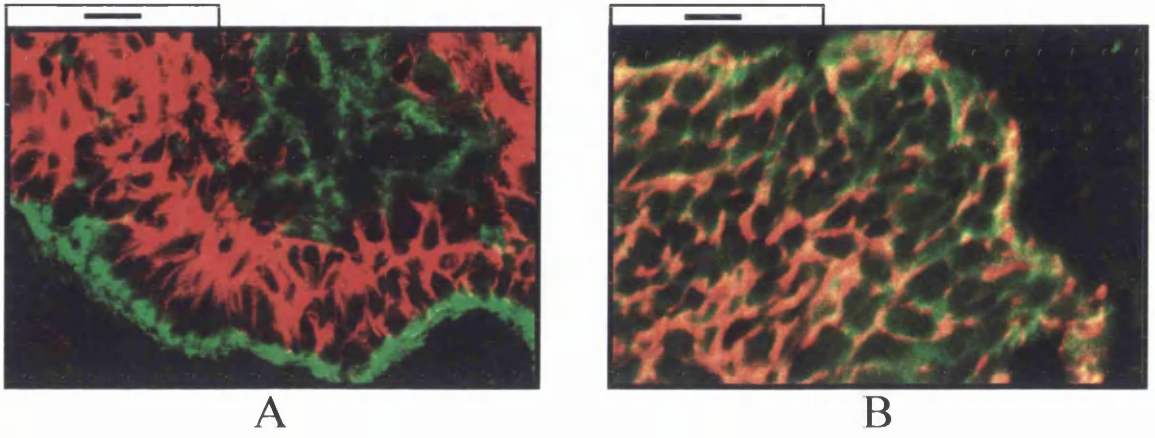


Figure 102. Double immunolabelling of β- and α-catenin (green) with actin (red) in nodular BCC. (A) β-catenin and actin: no co-localisation. (B) α-catenin and actin: some peripheral tumour cells show co-localisation (yellow). Scale bars = 25μm.

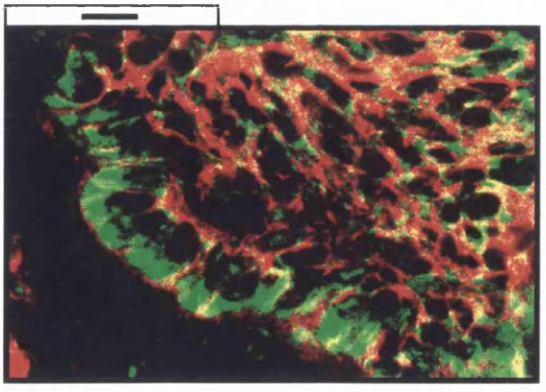


Figure 103. Double immunolabelling of α-actinin (green) with actin (red) in nodular BCC. Note actin polymerisation in central aspect of lobule accompanied by reduced α-actinin membrane staining in contrast to palisading cells which show co-localisation (yellow). Scale bar = 25μm.

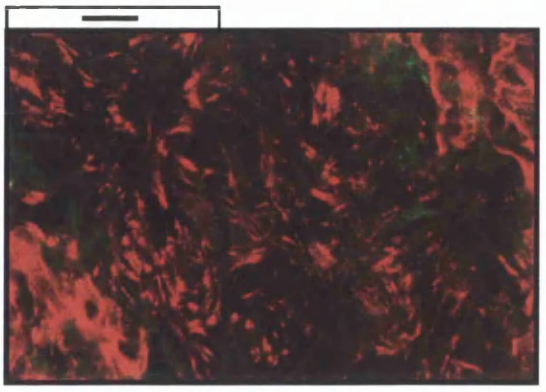


Figure 104. Double immunolabelling of E-cadherin (green) with actin (red) in stromal cells. Note strong actin staining and disoriented arrangement. No E-cadherin staining. Scale bar = 25μm.

	Connection with normal epidermis	Central	Peripheral	Stroma
Actin [a]	++	++	+/–	++ to +/–
E-Cadherin [b]	+/–	++ to +/–	++	–
P-Cadherin [b]	++ to +/–	++ to +/–	++	–
β-Catenin [b]	+/–	++ to +/–	++	–
α-Catenin [b]	+/–	++ to +/–	++	–
α-Actinin [c]	+/–	+/–	++ to +/–	–

Table 31. Immunofluorescence staining of major adherens junction components in tumour lobules of BCC with nodular growth pattern *.

* Represents a minimum of 5 staining runs showing reproducible and consistent results.
[a] Mainly present at the periphery of the cells.
[b] In cell membrane at the level of cell-cell junctions.
[c] Largely cytoplasmic with enrichment at cell peripheries.
++ : strongly positive. +/– : weakly positive (homogeneously weak). – : negative.

3.41. Double immunolabelling of major adherens junction components for actin.

Normal skin adjacent to tumour

When double immunolabelling using E-, P-cadherin, α-, and β-catenin antibodies and TRITC-labelled Phalloidin was performed, the staining of these antibodies (green) appeared to be separate from actin staining (red) particularly in the granular cell layer which showed a very strong binding of phalloidin (Figs. 88-90 and 92). There was no co-localisation observed between these antibodies and actin filament bundles in the normal epidermis. Occasionally, however, granular layer cells showed double immunolabelling of α-catenin and actin (Fig. 91) which was not seen in epidermal cells from granular layer downwards. When double immunolabelling of the α-actinin antibody and TRITC-labelled Phalloidin was performed, a portion of actin filaments staining appeared to co-localise with α-actinin staining at the plane of the plasma membrane of spinous layer cells (Fig. 94). In this particular specimen of normal skin adjacent to the tumour, strong actin filament staining was observed throughout the normal epidermis including basal layer cells. Actin filaments (stress fibers) were concentrated at the apical region of the normal keratinocytes and arranged vertically so

that they appear to be leading upwards. This observation of actin filaments arrangement and distribution was in contrast to what has been noted in many other specimens of normal skin.

Basal Cell Carcinoma

The double immunolabelling of the tumour cells showed no co-localisation of E-, P-cadherin, α- and β-catenin with actin filament bundles as the staining of these antibodies and actin was separated (Figs. 96 and 98). However, in few tumour cells, a portion of E-, P-cadherin and α-catenin staining may appeared to coincide with actin staining at the plane of plasma membrane (Figs. 97, 99, 101 and 102B). This was particularly observed in double immunolabelling of α-catenin and Rhodamine-Phalloidin where small punctate areas of co-localisation were seen in the palisading cells of tumour lobules of both infiltrative and nodular BCC (Fig. 99). Although most of the tumour cells demonstrated separate localisation of α-actinin and actin filament bundles, occasional double-stained cells were present in both the central and peripheral aspects of the tumour lobules (Fig. 103).

3.42. Double immunolabelling of E- and P-cadherin

Normal skin adjacent to tumour

Double immunolabelling of the normal epidermis adjacent to the tumour using anti-P- and anti-E-cadherin antibodies revealed the specific and normal pattern of distribution of the two antigens with areas of co-localisation (Fig. 106). E-Cadherin (red) was distributed in the full thickness of the epidermis excluding the stratum corneum while P-cadherin (green) was confined to the basal and the first suprabasal epidermal cells and negative in the membranes of the upper epidermal cells. These results are in agreement with those of Extra-avidin staining. Areas of co-localisation of E- and P-cadherin at cell-cell junction were observed in the membranes of the basal and the first suprabasal epidermal cells as indicated by bright yellow colour (Fig. 107). Similar observation was also noted in the normal hair follicle (Fig. 108). The two positive controls which were included in double immunolabelling by omitting the 1st primary antibody, i.e. P-cadherin and then omitting the 2nd primary antibody, i.e. E-cadherin yielded consistent results (Fig. 110A and B). Negative control by omitting both antibodies was negative (Fig. 111).

Basal Cell Carcinoma

Double immunolabelling of tumour lobules in nodular BCC showed preservation of membranous P-cadherin (green) in almost all tumour cells (Fig. 109A). E-Cadherin membrane staining was present, but in reduced intensity compared with normal epidermis (Fig. 109A). In many tumour cells, staining of E-cadherin appeared to coincide with P-cadherin in the plasma membrane area at cell-cell junctions cells as indicated by the yellow colour (Fig. 109A and B). However, there was no observation of tumour cells expressing only E-cadherin.

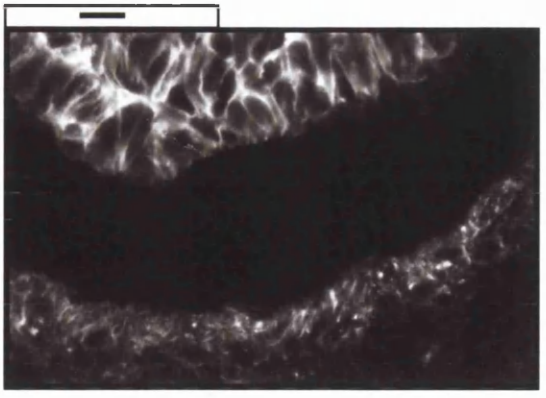


Figure 105. Actin staining in stromal cells. Less intense stromal staining when compared with that of tumour cells. Negative retraction artifact. Scale bar =10μm.

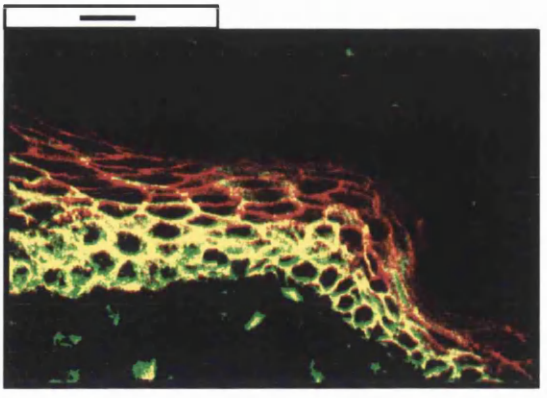


Figure 106. Double immunolabelling of P-cadherin (green) and E-cadherin (red) in normal epidermis. Note co-localisation of both antibodies in basal and first suprabasal layers of epidermis (yellow). Scale bar = 25μm.

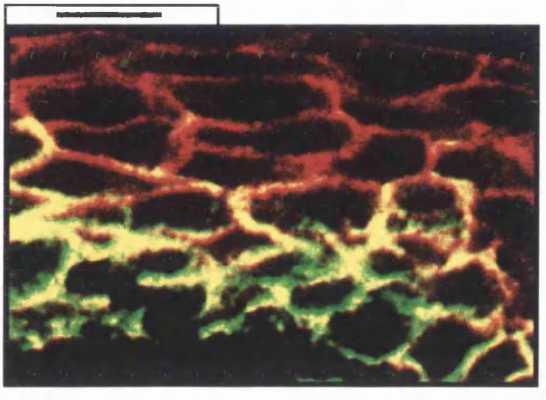


Figure 107. Double immunolabelling of P-cadherin (green) and E-cadherin (red) in normal epidermis. Higher power of basal and first suprabasal layers of epidermis showing co-localisation of both antibodies (yellow). Scale bar =50μm.

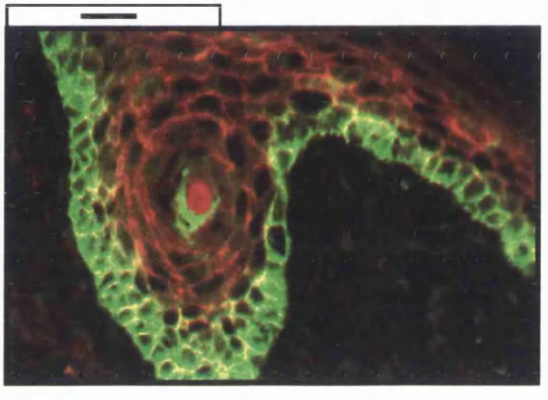


Figure 108. Double immunolabelling of P-cadherin (green) and E-cadherin (red) in hair follicle. Note the double-stained cells (yellow) in the ORS cells and in basal layer of visible normal epidermis. Scale bar = 25μm.

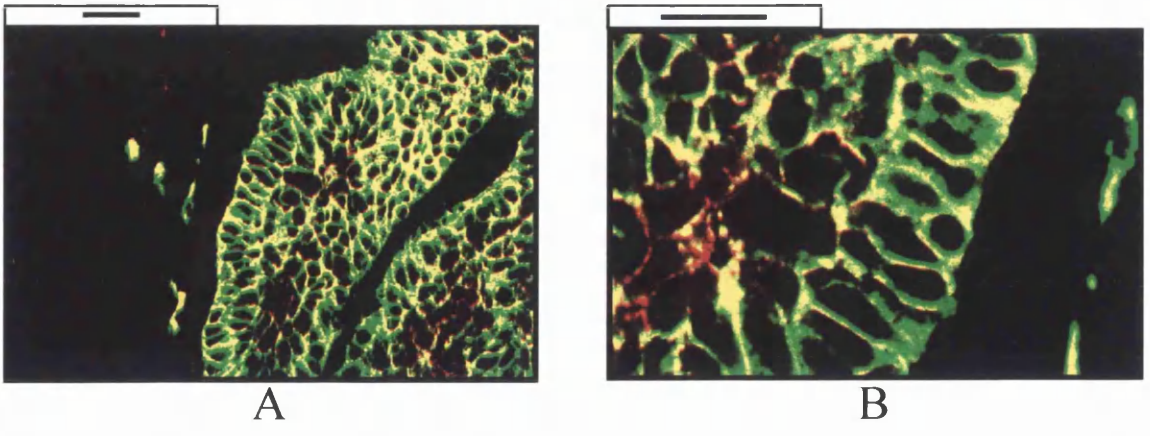


Figure 109. Double immunolabelling of P-cadherin (green) and E-cadherin (red) in nodular BCC. (A) Note strong P-cadherin membrane staining in almost all tumour cells in which several demonstrate co-localisation (yellow). (B) Higher power view showing similar observation. No tumour cell demonstrate E-cadherin only. (A) Scale bar = 25μm, (B) Scale bar =50μm.

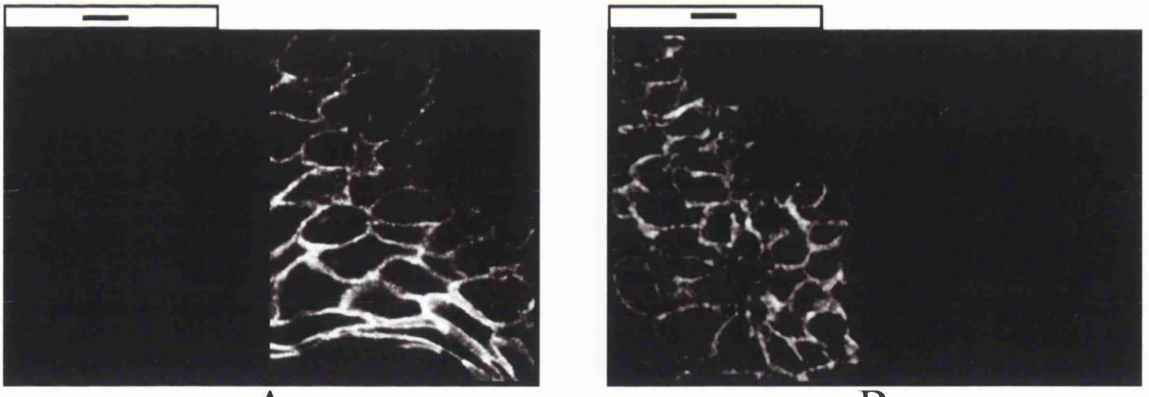


Figure 110. Positive controls of Double immunolabelling of P-cadherin and E-cadherin. (A) Immunofluorescence staining of E-cadherin only after omission of P-cadherin. (B) Immunofluorescence staining of P-cadherin only after omission of E-cadherin. Scale bars =10μm.

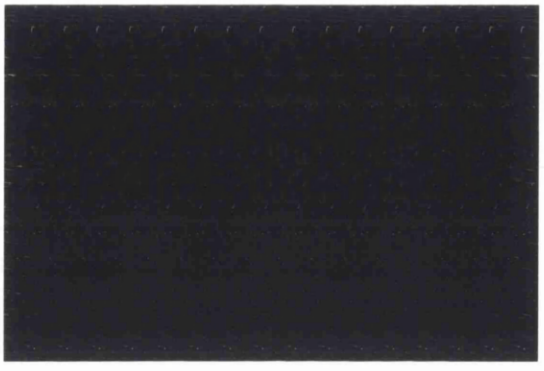


Figure 111. Negative control of Double immunolabelling of P-cadherin and E-cadherin.

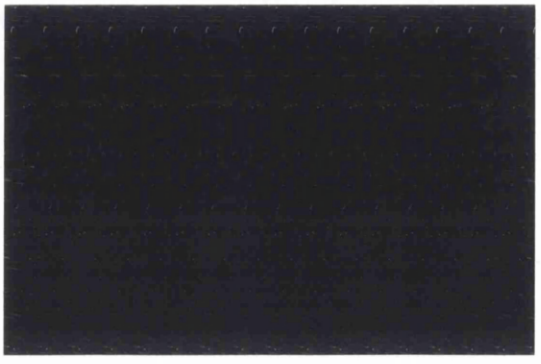


Figure 112. Negative control of Immunofluorescence staining. A representative negative control. All other controls were consistently negative.

Results IV

Immunofluorescent staining and double immunolabelling of the major adherens junction components in Keratinocyte culture.

3.43. Culture of keratinocytes in low calcium concentration

Keratinocytes grown in medium with 0.1 mM Ca^{2+} for 10 days, fixed and stained for E-, P-cadherin, β-catenin and actin, have a single-layered loose clustered morphology (Table 32) (Fig. 113). After 3 days of wounding the culture surfaces (Fig. 114), the keratinocytes grown in low concentration of Ca^{2+} migrated in a single-layered sheet and closed the gap partially. These cells were fixed in 10% formalin and stained for P-cadherin and actin which revealed weak membrane P-cadherin staining in some colonies particularly at the basal aspect (Table 32) (Fig. 115A), sometimes with punctate pattern of membranous distribution (Fig. 115B). Staining of filamentous actin with Rhodamine-Phalloidin showed uneven distribution throughout the colonies (Fig. 116). Positive actin filaments staining was diffuse throughout the cytoplasm, aligned with the longer axis of the cell (Table 32) (Fig. 117), and mainly present at the periphery and the lateral cell margins.

Similar to P-cadherin, E-cadherin and β-catenin staining in the membranes of cultured keratinocytes was weak throughout most of the colonies (Table 32). However, some over-stained small colonies revealed positive E-cadherin and β-catenin in cell membranes and intercellular bridges (Figs. 118 and 119). In addition, there was no obvious β-catenin nuclear staining.

In these cultures, the wound was only partially filled and actin filaments staining was similar to that of the cells stained with P-cadherin and actin.

	Low Calcium (0.1 mM)[a]	High Calcium (1.5 mM)[b]
Actin	Diffuse	++
E-Cadherin	+/–	++
P-Cadherin	+/–	++
β-Catenin	+/–	++
Wound*	Partially Closed**	Closed***
Status of cells	Loose	Tight

Table 32. Immunofluorescence staining of some major adherens junction components and status of cells in keratinocyte cultures of low and high calcium concentration.

[a] Keratinocytes grown in medium with 0.1 mM Ca^{2+} for 10 days.
[b] Keratinocytes grown for further 3 days in medium with 1.5 mM Ca^{2+}.
++ : strongly positive. +/– : weakly positive (homogeneously weak).
* Approximately 0.5mm wide.
** After 3 days of wounding.
*** After 3 days of wounding.

3.44. Culture of keratinocytes in high calcium concentration

When the calcium concentration in the growth medium was raised from 0.1 to 1.5 mM, more close association of the colonial cells was observed after fixation and staining for E-, P-cadherin, β-catenin and actin (Table 32). Strongly positive E-cadherin membrane staining was seen in almost all of the closely associated colonial cells when compared with the staining in low calcium cultures (Fig. 120). This was particularly observed in the areas of completely filled wound. Furthermore, there was bright staining of numerous small, pinpoint structures scattered in the background of the slide (Fig. 120). Strong actin filaments staining, although uneven, was apparent in the cells stained for E-cadherin particularly at the basal cells of the completely filled wound (Table 32). They were arranged, in some cells, in small groups of short fibres (Fig. 120). The variable and uneven staining of actin filaments and the cell adhesion molecules throughout the low and high calcium keratinocyte cultures could be due to poor antibody penetration or inadequate cell extraction using Triton x-100.

P-Cadherin membrane staining in the basal layer of the partially filled wound was stronger when compared with the staining in low calcium cultures (Fig. 121). The same

observations were also seen when staining of β-catenin was performed which showed no obvious nuclear staining (Fig. 122).

3.45. Double immunolabelling

When double immunolabelling of the keratinocytes colonies using each of the antibodies P-, E-cadherin and β-catenin with TRITC-labelled Phalloidin was performed, no double-stained cells were observed (Figs. 123-128).

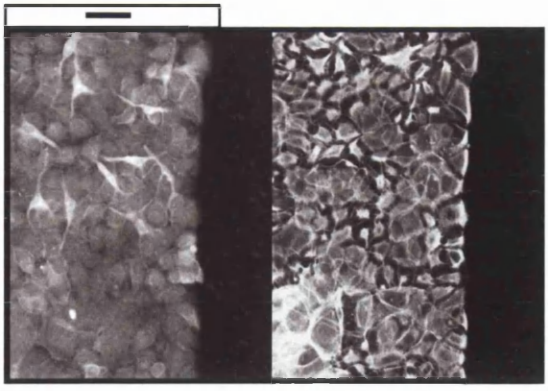


Figure 113. Immunofluorescence staining of P-cadherin (left) and actin (right) in keratinocyte colonies grown in low calcium medium for 10 days. Note the loose colony-like morphology and weak P-cadherin membrane staining. Scale bar =100μm.

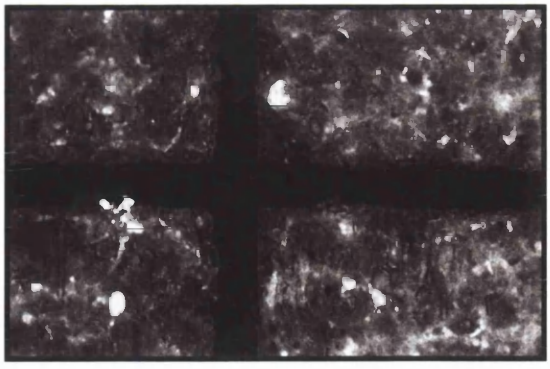


Figure 114. Vertical and horizontal wounding of keratinocyte culture.

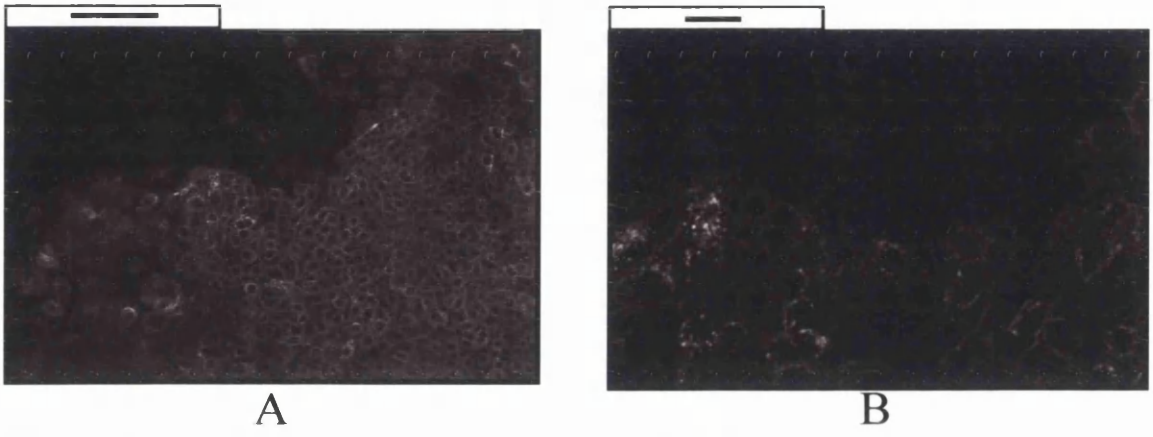


Figure 115. Immunofluorescence staining of P-cadherin in keratinocyte colonies grown in low calcium medium for 10 days. (A) weak P-cadherin membrane staining.(B) Higher power showing punctate pattern of P-cadherin membrane staining. (A) Scale bar =100μm, (B) Scale bar = 25μm.

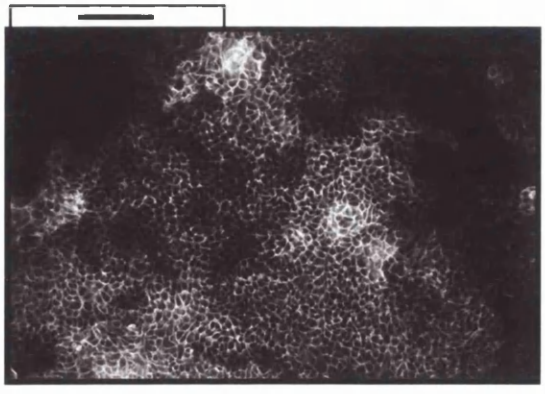


Figure 116. Immunofluorescence staining of actin in keratinocyte colonies grown in low calcium medium for 10 days. Note the uneven distribution of actin staining. Scale bar =250μm.

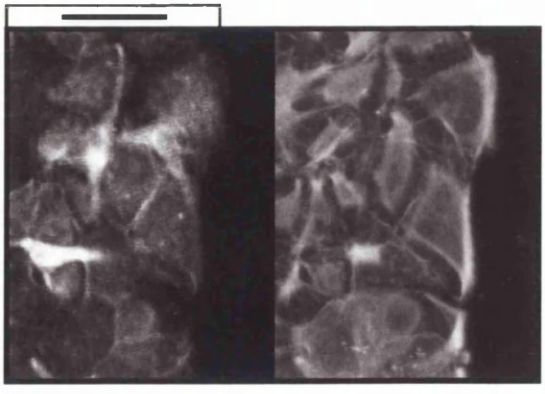


Figure 117. Immunofluorescence staining of P-cadherin (left) and actin (right) in keratinocyte colonies grown in low calcium medium for 10 days. Note the concentration of actin at cell periphery. Scale bar =50μm.

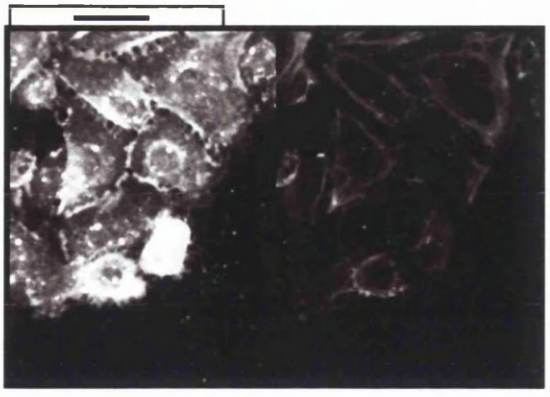


Figure 118. Immunofluorescence staining of E-cadherin (left) and actin (right) in keratinocyte colonies grown in low calcium medium for 10 days. Weak E-cadherin staining in over-stained cells. Scale bar =50μm.

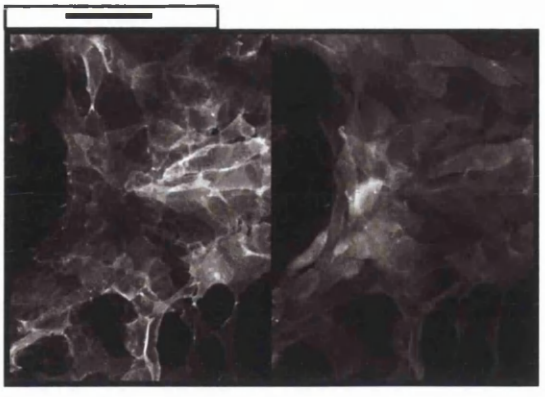


Figure 119. Immunofluorescence staining of β-catenin (left) and actin (right) in keratinocyte colonies grown in low calcium medium for 10 days. Weak β-catenin staining in over-stained cells. Scale bar =100μm.

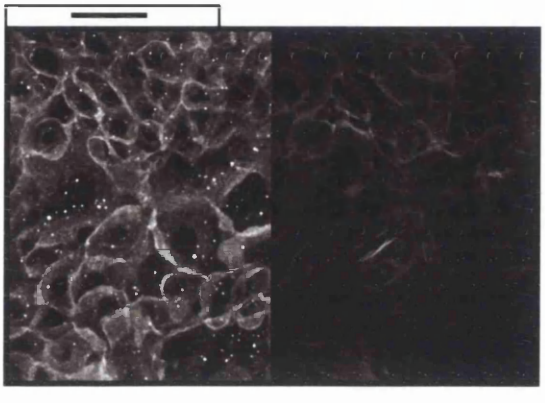


Figure 120. Immunofluorescence staining of E-cadherin (left) and actin (right) in keratinocyte colonies grown in high calcium medium. Strong E-cadherin membrane staining in completely filled wound area. Note bright staining of numerous pinpoint structures scattered in background. (right) strong actin filament staining arranged in small groups of short fibres. Scale bar =100μm.

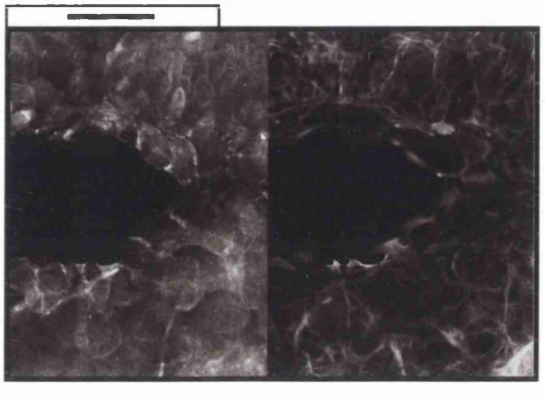


Figure 121. Immunofluorescence staining of P-cadherin (left) and actin (right) in keratinocyte colonies grown in high calcium medium. Strong P-cadherin membrane staining in partially filled wound area. Note strong actin filament staining arranged in small groups of short fibres. Scale bar =100μm.

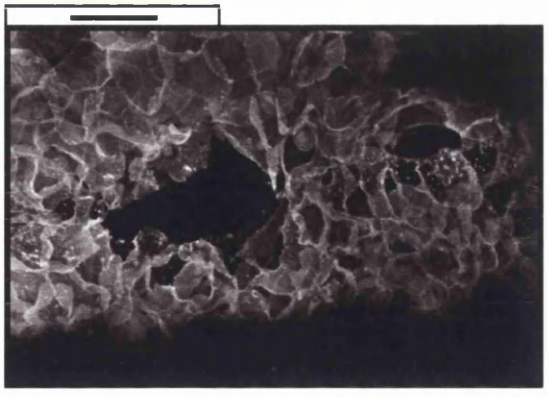


Figure 122. β-Catenin Immunofluorescence staining in keratinocyte colonies grown in high calcium medium. Stronger β-catenin membrane staining in partially filled wound area. Scale bar =100μm.

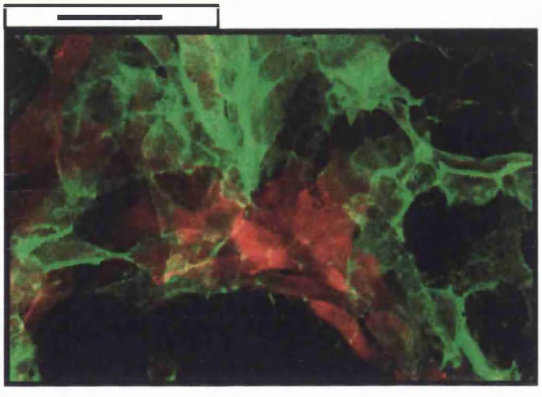


Figure 123. Double immunolabelling of E-cadherin (green) with actin (red) in keratinocyte colonies grown in low calcium medium. No co-localisation. Scale bar =50μm.

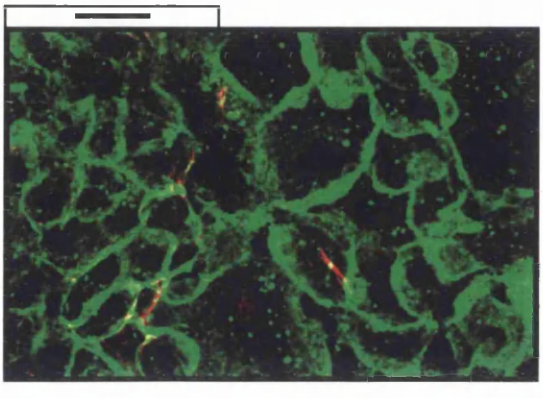


Figure 124. Double immunolabelling of E-cadherin (green) with actin (red) in keratinocyte colonies grown in high calcium medium. No co-localisation. Scale bar =100μm.

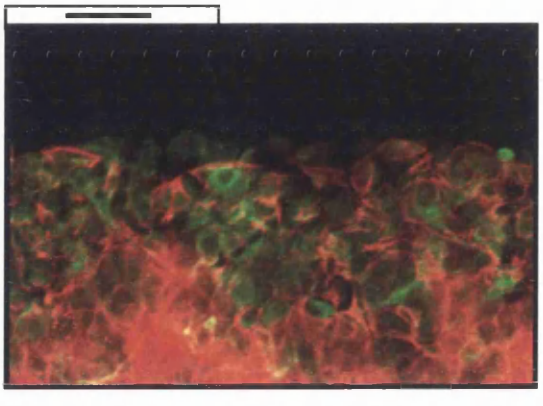


Figure 125. Double immunolabelling of P-cadherin (green) with actin (red) in keratinocyte colonies grown in low calcium medium. No co-localisation. Scale bar =100μm.

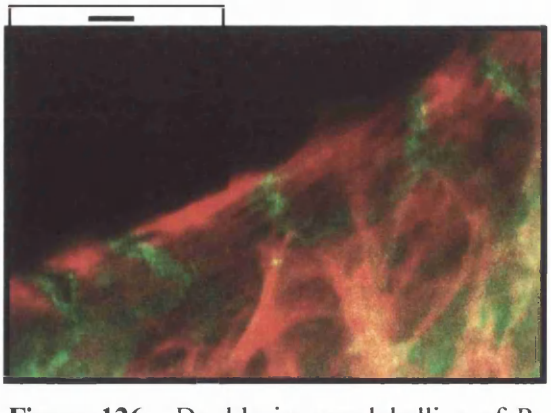


Figure 126. Double immunolabelling of P-cadherin (green) with actin (red) in keratinocyte colonies grown in high calcium medium. No co-localisation. Scale bar =10μm.

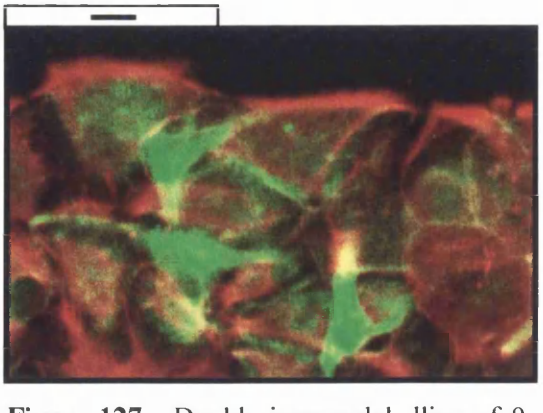


Figure 127. Double immunolabelling of β-catenin (green) with actin (red) in keratinocyte colonies grown in low calcium medium. No co-localisation. Scale bar =10μm.

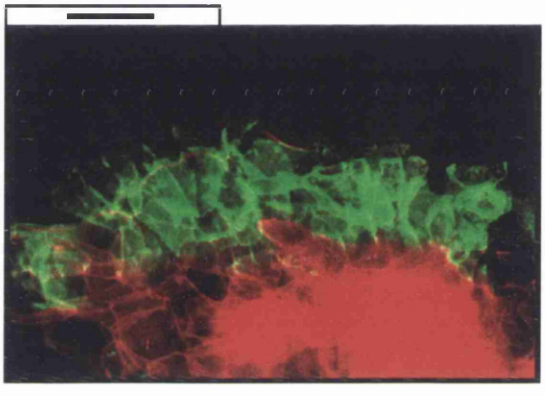


Figure 128. Double immunolabelling of β-catenin (green) with actin (red) in keratinocyte colonies grown in high calcium medium. No co-localisation. Scale bar =100μm.

Results V

BCC culture

3.46. BCC culture

Eight fresh excisional specimens of BCC (3 infiltrative, 4 nodular and 1 superficial) were used in 5 experiments.

Experiment 1: Two contracted dermal equivalents were seeded with tumour single cell suspensions derived from an infiltrative BCC as described in the methods section. The first dermal equivalent was seeded with 5 x 10^5 tumour-derived cells/ml of MEM and the second with 2.5 x 10^5. In this experiment, the MEM added to the negative control was cell-free. Both dermal equivalents showed massive degradation after 48 hours of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air (Fig. 129).

Experiment 2: Similar results were obtained when experiment 1 was repeated using cells from a second infiltrative BCC (Fig. 130A). The degraded dermal equivalents were fixed in 10% formalin and stained with H&E which showed remnants of the degraded collagen gel and some viable single cells retaining epithelioid features but mostly composed of pyknotic, non-viable cells (Fig. 130B). The cells in one dermal equivalent which was seeded with 5 x 10^5 tumour-derived cells/ml of MEM and left in culture conditions for a longer period (1 week), eventually died.

Experiment 3: This was carried out using cells from infiltrative and nodular BCC specimens. Tissue culture flasks coated with a layer of neat FCS for 2-3 min and/or with Type I collagen and seeded with the same numbers of tumour-derived cells in MEM and in KGM failed to yield cell growth. In addition, seeding of tumour cells, derived from the original tumours, mixed with or without normal keratinocytes onto fibroblast-free and standard dermal equivalents gave similar negative results (data not shown).

Experiment 4: In contrast to the above, BCC-derived cells from nodular and superficial BCC were washed and resuspended quickly in MEM for 3 times in an attempt to wash some of the exogenous collagenase. In addition, more dermal equivalents seeded with variable numbers of BCC-derived cells were used. Six dermal equivalents were seeded with the following numbers of cells:

- 4 x 10^5 tumour-derived cells/ml of MEM.
- 2 x 10^5 tumour-derived cells/ml of MEM.
- 1 x 10^5 tumour-derived cells/ml of MEM.
- 0.5 x 10^5 tumour-derived cells/ml of MEM.
- 0.25 x 10^5 tumour-derived cells/ml of MEM.
- 3 x 10^6 normal keratinocytes/ml of MEM.

Following incubation at 37°C in a humidified atmosphere of 5% CO_2 in air for 5 days as submerged cultures, no degradation of dermal equivalents was noted (Fig. 131A). After the seeded dermal equivalent were raised to the air/liquid interface for further 6 days, they were fixed in 10% formalin and stained with H&E. Most of these cultures revealed non-proliferating basaloid cells with epithelioid morphology singly scattered on the surface of the dermal equivalent and, in some, showing signs of cell death such as dyskeratosis and pyknosis (Fig. 131B). However, in the dermal equivalent seeded with 1 x 10^5 tumour-derived cells/ml of MEM, H&E stained sections showed a viable lobular mass of basaloid cells reminiscent of BCC cells with a small necrotic core (Fig. 132A).

Experiment 5: Similar results were obtained when experiment 4 was repeated using 2 nodular BCC specimens.

The dermal equivalents seeded with normal keratinocytes have developed a multilayered and stratified epidermis following incubation for 5 days as submerged cultures and raised to the air/liquid interface for a further 7 days (Fig. 132B).

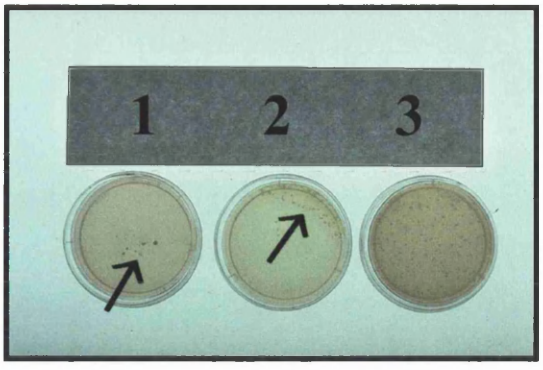


Figure 129. Dermal equivalents seeded with infiltrative BCC single cell suspension. (1) and (2) show massive degradation (arrow) after 48 hours of incubation. (3) negative control seeded with cell-free MEM.

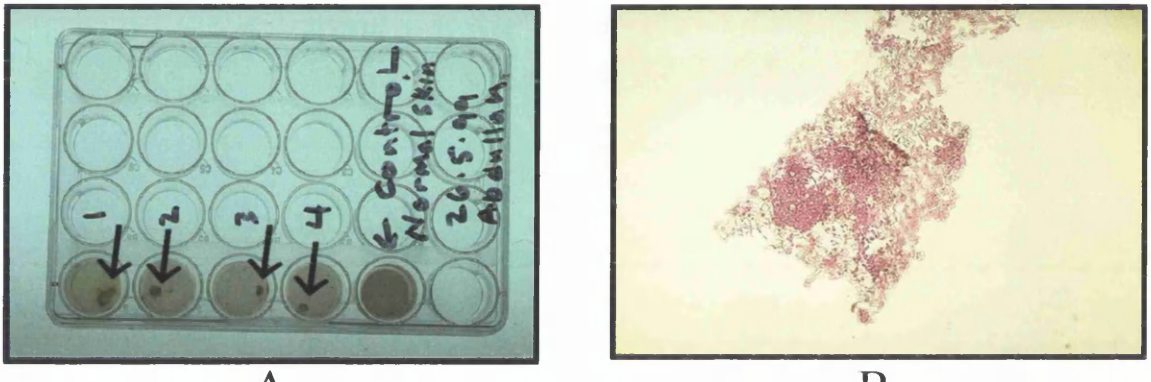


Figure 130. Dermal equivalents seeded with infiltrative BCC single cell suspension from a second specimen. (A) (1) (2) (3) and (4) (see text) show massive degradation (arrows) after 48 hours of incubation. (5) negative control seeded with neonatal foreskin keratinocytes showing no degradation. (B) H&E of degraded dermal equivalent showing mostly non-viable cells. x320.

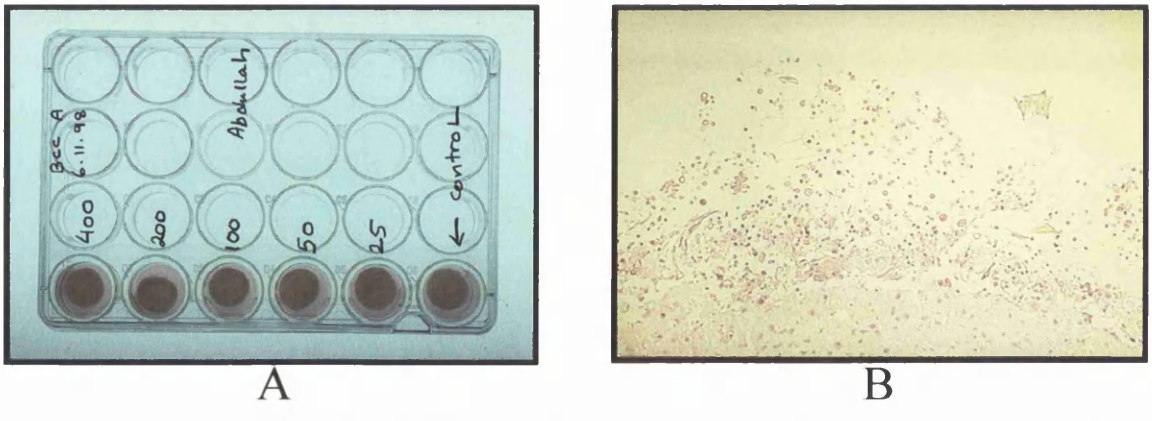


Figure 131. Dermal equivalents seeded with nodular BCC single cell suspension. No degradation after 5 days of incubation (see text). Negative control seeded with neonatal foreskin keratinocytes showing no degradation. (B) H&E of dermal equivalent after 11 days of culture showing singly scattered epithelioid non-proliferating cells. x320.

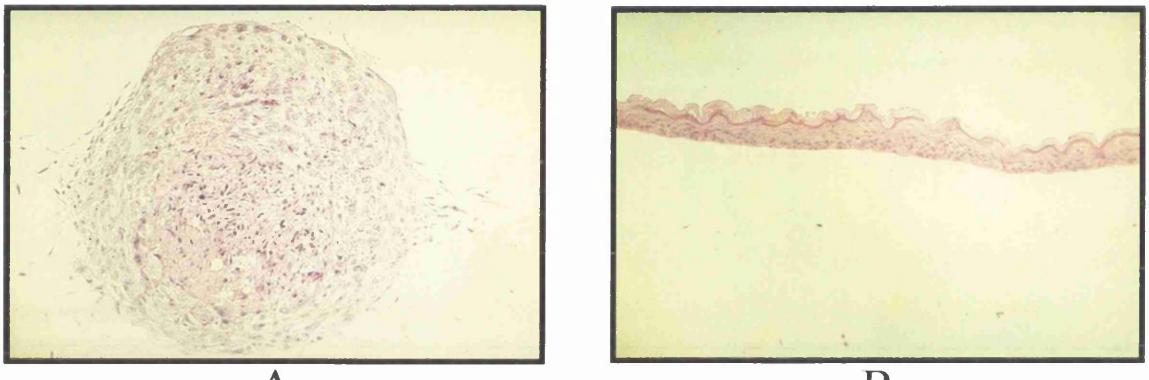


Figure 132. (A) H&E of dermal equivalent seeded with 1 x 10^5 tumour-derived cells/ml of MEM after 11 days of culture showing a viable lobular mass of basaloid cells reminiscent of BCC cells with a small necrotic core. (B) H&E of dermal equivalent seeded with normal keratinocytes. (A) x320, (B) x125.

Chapter 4: Discussion

4.1. Expression of cadherins, catenins and associated proteins in normal epidermis

In the present study, the expression of several adherens junction components and associated proteins [E- and P-cadherin, α- and β-catenin and APC (see Appendix C)] was analysed in human normal epidermis adjacent to tumours. Using frozen sections and cultured keratinocytes, previous studies have reported that normal keratinocytes and melanocytes express both P-cadherin and E-cadherin on their cytoplasmic membranes where they maintain adhesion between these cells by homophilic binding (Tang et al., 1994; Lewis et al., 1994; Horiguchi et al., 1994). This present study has confirmed and extended previous work in which it demonstrated that the expression of E- and P-cadherin in normal epidermis is specific and follows a well-defined pattern in both frozen and routinely processed paraffin-embedded specimens.

E-Cadherin was expressed normally in cellular membranes of all viable epidermal layers with an increased expression in the suprabasal layers as compared with the basal cells. It was also expressed in outer root sheath cells of hair follicles, flattened germinative cells of sebaceous glands, and in both secretory and ductal units of sweat glands. These results confirmed the findings of Fujita et al (1992) and Fuller et al (1996) in frozen tissues.

P-Cadherin distribution in normal epidermis differed from E-cadherin in that it was restricted to the membranes of basal layer cells and the outermost cells of the skin appendages. This observation is in agreement with previous reports on P-cadherin expression in frozen tissues of adult human skin (Fujita et al., 1992) and the mouse upper lip skin (Hirai et al., 1989). In addition, E- and P-cadherin were expressed on the membranes of normal epidermal melanocytes. The distribution of E-cadherin in all viable epidermal layers emphasises the possible main role of this cell adhesion molecule in the maintenance of the human normal epidermis. In addition it may function to organise and maintain the epidermal-melanin unit hence both normal keratinocytes and melanocytes express E-cadherin. This could also be applied to P-cadherin, since the normal melanocytes are usually located along the basal layer of the epidermis. Furthermore, the restricted expression of P-cadherin to the basal cells of the normal epidermis perhaps associates with the maintenance of the epidermal proliferative compartment segregating it from the upper non-proliferating strata. The dual expression

of E- and P-cadherins in basal cells may have properties in common concerning specific regulatory mechanisms. For example, their ability of regulating intercellular junction organisation in the basal layer. In addition, basal cells may require either cadherin to initiate epidermal stratification where E-cadherin may play the dominant role especially in suprabasal layers because of its broad distribution and P-cadherin is not needed due to its complete absence. Although there is a possibility of co-localisation of E- and P-cadherin in basal cell-cell junctions, either of them may still have unique function(s) in the basal cell layer.

Previous studies in many normal epithelial cells have suggested that α- and β-catenin are mainly localised at the membranes of the cell-cell borders. Strong expression of β-catenin especially on cell-cell boundaries has been demonstrated in human oesophagus (Takayama et al., 1996; Kimura et al., 1999), stomach (Takayama et al., 1996), human and rat colon (Takayama et al., 1996; Inomata et al., 1996; Takahashi et al., 1998; Back et al., 1999), normal breast mammary duct tissue (Hashizume et al., 1996), nasopharyngeal epithelium (Zheng et al., 1999) and capillary endothelial cells of the thyroid gland (Garcia-Rostan et al., 1999). Similar staining pattern was also reported for α-catenin in human breast, oesophagus, stomach and colon (Shiozaki et al., 1994; Hashizume et al., 1996; Glukhova et al., 1999).

In my work, the staining pattern of α- and β-catenin in normal epidermis was largely similar to that of E-cadherin. They were localised in a linear pattern along the lateral and upper surfaces of basal keratinocytes at the sites of cell-cell junctions where the normal keratinocytes in suprabasal layers expressed α- and β-catenin around their peripheries. In addition, membranous α- and β-catenin is also present in the outer root sheath cells of the hair follicles, acinar germinative cells of the sebaceous glands and in sweat glands cells. However, they are absent at the basal cell surface, in stratum corneum, inner sheath cells of the hair follicle and the hair shaft. These results are in agreement with recent reports on α- and β-catenin expression in normal epidermis (Tada and Hashimoto, 1998; Silye et al., 1998). The similar distribution of the catenins and E-cadherin may be explained by the direct association of these proteins in AJ at the cell membrane level where catenins mediate the connection of the E-cadherin with the actin

filament network. Furthermore, the coexistence of catenin expression and cellular localisation with E- and P-cadherin in normal epidermis is in agreement with the presence of a normally functioning cadherin/catenin complex. In this study, there was good agreement between the E- and P-cadherin, α- and β-catenin staining of the membranes in paraffin-embedded and frozen samples.

4.2. E- and P-cadherins expression in non-melanoma cutaneous tumours

Destabilisation of cell-cell junctions allows initiation of the invasive process and the progression of carcinoma (Behrens et al., 1989; Frixen et al., 1991; Vleminckx et al., 1991). The cell adhesion molecule, cadherin, was first reported by Takeichi (1977). E-Cadherin is considered to be the major adhesion molecule found in AJ where it is expressed on most cell types that form solid tissues (Takeichi, 1988; Tang et al., 1994). During the past several years, a number of reports have suggested a relationship between reduced expression of E-cadherin and metastasis in a wide variety of tumours, including skin (Schipper et al., 1991; Pizarro et al., 1994; Fuller et al., 1996; Cowley and Smith, 1996; Cano et al., 1996; Silye et al., 1998; Montonen et al., 1998), breast (Rasbridge et al., 1993; Oka et al., 1993; Glukhova et al., 1995), gastric (Guilford et al., 1998; Jawhari et al., 1999), pancreatic (Perl et al., 1998), bladder (Giroldi et al., 1999), nasopharyngeal (Zheng et al., 1999), colorectal (Kinsella et al., 1993) and prostate cancers (Cheng et al., 1996). In addition, Mutations of the E-cadherin gene have recently been discovered in a variety of carcinomas including human gastric carcinoma and cell lines (Becker et al., 1994; Oda et al., 1994), carcinomas of the endometrium and ovary (Risinger et al., 1994). These studies strongly support the proposed role of E-cadherin as an invasion suppressor molecule.

The present study investigated E and P-cadherin cellular distribution in different histological types of human cutaneous tumours. In order to do this, it was necessary to develop techniques for cadherin immunostaining of routinely processed, paraffin-embedded specimens. As down-regulation or complete loss of E-cadherin occurs in association with human epithelial cancers, the present work aims to assess whether the

decrease in membranous distribution of E- and P-cadherins in skin cancer is accentuated in dedifferentiated tumours.

The results have shown that E-cadherin staining was reduced in BCC tumour cell membranes in almost all of the routinely processed cases (93.8%) and in all the frozen samples compared with the normal epidermis. There was good agreement between results on routinely processed tissues and on freshly frozen material. Among the 10 cases of routinely processed, well-differentiated SCC, 8 cases (80%) have shown reduced E-cadherin tumour cell membrane staining. Of these, one was completely negative. E-Cadherin membrane staining was also reduced in all keratoacanthomas and moderately differentiated SCC, of which 2 cases were completely negative. In non-malignant lesions, Bowen's disease, actinic keratosis, psoriasis and lichen simplex chronicus, E-cadherin membrane staining was largely similar to normal epidermis. This was despite the epidermal dysplasia frequently seen in Bowen's disease and actinic keratosis.

Pilomatrixoma is a tumour expressing characteristics of the hair matrix where early lesions may show quite brisk mitotic activity, but this is never abnormal and is indicative of a rapid growth phase rather than malignant potential (McKee, 1996a). Downregulation or loss of E-cadherin membrane staining in some of the pilomatrixoma (5/10) was expected because they were composed mainly of fully matured cells dispersed in sheets of keratinous debris. However, in early lesions of pilomatrixoma (5/10) composed of viable cells, E-cadherin membrane staining was preserved despite the large size and numerous mitotic figures.

Although, the reduction of E-cadherin membrane staining was uneven throughout some BCC lobules, particularly in some routinely processed samples, it was observed in all different histological subtypes of BCC including the small lobules of superficial BCC projecting from the lower margin of the epidermis. However, the reduction of E-cadherin membrane staining was more pronounced in BCC cases with invasive growth pattern such as infiltrative, metatypical and spindle cell BCC.

A notable feature was the observation of preserved E-cadherin membrane staining in the palisading cells of the BCC tumour lobules expressing reduced membrane staining centrally, which was not seen in SCC variants. Fuller and colleagues (1996) reported

reduced E-cadherin expression almost in all cases of BCC (28/30) and in 12/16 cases of variable differentiation SCC of which 4 cases were completely negative. Unfortunately, in the previous study, it was not stated which variant of SCC was completely negative.

E-Cadherin plays an important role in the progression of human SCC of head and neck where downregulation of its expression is associated with de-differentiation and metastasis of tumour cells in vivo (Schipper et al., 1991). In their study, Schipper and colleagues (1991) found that E-cadherin expression is inversely correlated both with the loss of differentiation of the tumour and with lymph node metastasis. Therefore, it is suggested that the cell-cell adhesion maintained by E-cadherin as an invasion suppressor is lost in the poorly differentiated tumours. A recent study by Koseki and colleagues (1999) suggested that E-cadherin expression tended to be preserved in well-differentiated SCC of the skin and reduced or absent in poorly differentiated tumours correlating with regional lymph node metastasis. In addition, De Bruin and colleagues (1999) observed significant reduction in E-, N-, and P-cadherin protein expression in an invasive SCC cell line. An earlier study by Pizarro and colleagues (1994) on frozen specimens of BCC reported that reduction of E-cadherin expression was related to the growth pattern and the local invasiveness of BCC by showing reduced E-cadherin membrane staining in infiltrative subtypes of BCC only (10/15). In contrast to the data demonstrated in this present study, they reported that E-cadherin expression was preserved in all frozen specimens of superficial and nodular BCC.

My experiments have shown that the reduction in tumour cell membrane E-cadherin in comparison with the normal epidermis is a characteristic in all subtypes of BCC and variants of SCC, particularly those tumours with aggressive behaviour, but not in non-malignant lesions. In addition, the decrease in membranous distribution of E-cadherins in superficial, nodular BCC and well-differentiated SCC progressed further in dedifferentiated tumours such as infiltrative, metatypical and spindle cell BCC and moderately differentiated SCC. Therefore, in BCC and SCC, the downregulation or loss of E-cadherin expression is directly proportional to the loss of differentiation of the tumour. However, less aggressive tumours such as superficial BCC still demonstrate reduced E-cadherin membrane staining despite their small size, whereas other tumours larger in size, for example pilomatrixoma, show preserved E-cadherin expression. This

may support the view that BCC is a true malignancy rather than an epithelioma. Thus, E-cadherin expression in different histological subtypes of BCC and in various variants of SCC may be used as a useful prognostic marker. The preserved E-cadherin membrane staining in the palisading cells of the BCC tumour lobules may indicate that E-cadherin is one of multiple biological factors involved in the prevention of free stromal invasion and metastasis. However, this function of E-cadherin, if present, is lost in both well- and moderately-differentiated SCC hence there was no preservation of E-cadherin expression in the tumour cells at the peripheries of SCC lobules. In addition, this may explain, at least in part, the higher metastatic rate of SCC and its more aggressive behaviour in comparison with BCC. Although BCC has a low metastatic potential (Domarus and Stevens, 1984; Lo et al., 1991), it is capable of gross tissue destruction (Leffell et al., 1991; Ko et al., 1992; McKee, 1996a). Therefore the results of this present work are consistent with the observation that the reduction of E-cadherin is associated with tumour invasion at least locally. They also support the potential function of this protein as a biochemical marker for tumour progression. In addition, this study supports the suggestion of Fuller and colleagues (1996) in differentiating malignant from benign lesions by assessing their degree of E-cadherin expression as a potential clinical application.

My results have shown preservation of P-cadherin membrane staining in the majority of BCC cases and some SCC cases which simultaneously expressed reduced E-cadherin staining. In addition, I have observed that the reduction in P-cadherin tumour cell membrane staining was correlated with tumours showing aggressive growth pattern particularly in BCC (Table 6). In earlier reports of P-cadherin expression in non-cutaneous tumours, P-cadherin was detected in 7/8 adenocarcinomas of the colon (in abstract form) by Abbasi and colleagues (1992) and found to be preserved in 44 cases of carcinoma of the lung (Shimoyama et al., 1989). Furthermore, in a study by Shimoyama and Hirohashi (1991), P-cadherin was reported to be present in gastric carcinomas in more than half of the 54 cases examined. Therefore, my findings may suggest a possible contribution of P-cadherin to the maintenance of the epithelioid phenotype of the carcinoma cells, hence its continued expression. Furthermore, the downregulation or loss of E-cadherin but not of P-cadherin particularly in BCC and SCC tumours raises the possibility of stepwise loss of cadherins. Thus, during the

process of tumour progression and invasion, E-cadherin may be lost first then P-cadherin loss follows when the tumour acquires very aggressive behaviour and is about to metastasise.

Cano and colleagues (1996) detected aberrant suprabasal localisation of P-cadherin in chemically induced benign skin papillomas and in invasive cells of progressing papillomas derived from wild-type, heterozygous and homozygous p53 null mice with genetically pre-determined risks for malignant conversion. They concluded that the observation of altered localisation of P-cadherin where it is expressed suprabasally in contrast to its normal distribution could be considered as a marker for malignant transformation. However, one of the interesting observations of this present study is the strong membranous expression of P-cadherin in the full thickness of Bowen's disease, psoriasis and lichen simplex chronicus and the correlation of such staining with the alternating dysplastic bands of actinic keratosis. In addition, increased suprabasal localisation of P-cadherin is observed in the pseudoepitheliomatous epidermis adjacent to keratoacanthomas and variants of SCC. This phenomenon was observed in almost all studied cases. From my results, it is not clear what could be the biological significance of this observation. The speculation of the suprabasal expression of P-cadherin being a marker for malignant transformation in Bowen's disease and actinic keratosis could be for the first instance true as these cases may develop into an invasive SCC (Marks et al., 1988a; 1988b; MacKie, 1992). However, this is not applicable to the hyperproliferative lesions characterised by epidermal hyperplasia such as psoriasis and lichen simplex chronicus as the increased risk of cancer in these cases, particularly in psoriasis, was linked only to certain treatment modalities (Frentz and Olsen, 1999). Furthermore, in a previous report, Hirai and colleagues (1989) showed that during skin morphogenesis the expression of P-cadherin seemed to be associated with the proliferating activity of cells. Therefore, my data demonstrates suprabasal P-cadherin expression in benign hyperplasia which may indicate that this molecule is proliferation-associated rather than a marker for malignant transformation, as suggested by Cano and colleagues (1996).

4.3. E- and P-cadherins expression in melanocytic tumours

In comparison with the overlying normal epidermal keratinocytes, my results have demonstrated decreased E- and P-cadherin staining in dermal nests of primary melanomas in both radial and vertical growth phases and metastatic melanomas which ranged from reduced staining intensity to complete absence. However, in one case of metastatic melanoma, E- and P-cadherin expression was strongly preserved. This particular example could represent a reestablishment of E- and P-cadherin function in cell-cell adhesion maintenance. In other melanocytic lesions examined, E- and P-cadherin membrane staining was reduced in the dermal components, although E-cadherin staining was more marked than that of P-cadherin. Although the staining of both cadherins in dermal naevus cells was heterogeneous in distribution, it was in contrast to the membrane staining of E- and P-cadherin in melanocytic naevus cells in the epidermis.

In malignant melanoma, a differential loss of E-cadherin expression was recently noted by Silye and colleagues (1998). In their study, membranous E-cadherin expression was detected in all benign naevi, although heterogeneous in junctional naevus cell nests. My data is in agreement with that of Silye and colleagues (1998) where they reported an absence of E-cadherin staining in the membrane in dermal nests of melanomas in their radial growth phase and in Clark level II and III lesions. However, my findings are in contrast to their report of detectable staining present in a considerable proportion of melanomas in their vertical growth phase, in Clark level IV and V lesion and in metastasising melanomas. Moreover, Cowley and Smith (1996) reported that cadherin expression, and particularly E-cadherin expression, was much greater in malignant melanomas than in benign melanocytic naevi. The findings of these studies (Cowley and Smith, 1996; Silye et al.,1998) are clearly in contrast to the invasion suppressor role described for E-cadherin (Frixen et al., 1991).

In a study by Seline and colleagues (1996), human melanoma cell lines from early primary lesions were found to express more E- and P-cadherin than do melanoma lines established from patients with more advanced or metastatic disease following ultraviolet radiation.

Although the number of primary melanomas was relatively small, my data indicated that the loss of cadherin expression in melanoma lesions may allow for enhanced detachment from the epidermis with subsequent invasion and metastasis. This is also supported by my data on metastatic melanomas which showed marked reduction in cadherin expression emphasising that the decrease in membranous distribution of E-cadherins in primary melanomas progressed further in metastatic lesions.

The reduction of E-cadherin expression in the dermal components of some benign melanocytic lesions may be explained by the neural crest origin of melanocytes which failed to form cadherin maintained clusters as they migrate from the neural crest. This also may be applicable to blue naevi because they represent arrested melanocytic migration and they are confined solely to the deeper aspect of the reticular dermis.

4.4. α- and β-catenin expression in non-melanoma cutaneous tumours

Recent work has suggested that there is a close association between abnormal expression and/or structural abnormalities of β-catenin as well as E-cadherin and tumour development as demonstrated in many cancers including human oesophageal carcinomas (Nakanishi et al., 1997), gastric carcinomas (Jawhari et al., 1997), colorectal polyps (Valizadeh et al., 1997), colon tumours (Takayama et al., 1996) and rat colon tumours (Takahashi et al., 1998). On the other hand, α-catenin membranous expression was found to be frequently reduced in tumours of the breast, oesophagus, stomach, colon, and malignant melanoma (Shiozaki et al., 1994; Hashizume et al., 1996; Silye et al., 1998). The findings of these studies support the idea that downregulation of membranous α- and β-catenin is associated with malignant transformation in these cancers. This may affect the E-cadherin-mediated adhesion system, as β-catenin forms a complex with E-cadherin in normal human epithelium and cancerous tissues (Takayama et al., 1996; Jawhari et al., 1999) while α-catenin in turn, connects this membrane-associated complex to the actin cytoskeleton (Knudsen et al., 1995; Rimm et al., 1995) (Fig. 2) .

One of my aims was to investigate the cellular distribution of α- and β-catenin in different histological types of human cutaneous tumours in relation to their cadherin (E- and P) expression and whether the cadherin/catenin complex in these tumours is altered in comparison with the normal epidermis. Therefore, my study considered whether the decrease in membranous distribution of the catenins, particularly β-catenin, in skin cancer progressed further in tumours with more aggressive behaviour. Additionally, it was of interest to examine whether any decrease in membranous β-catenin was accompanied by a redistribution to the cell nucleus, where β-catenin can act as a transcriptional co-activator leading to increased expression of TCF/LEF-regulated target genes (Huber et al., 1996; Molenaar et al., 1996; Behrens et al., 1996). For instance, recent work has shown that β-catenin has a potential signalling role in colon cancer (Munemitsu et al., 1995; Korinek et al., 1997; Morin et al., 1997) as a consequence of Wnt pathway activation (Rubinfeld et al., 1997; Fearon and Dang, 1999).

My data has shown consistent and strong correlation between β-catenin and E-cadherin membranous immunostaining in the tumour cells of almost all the cutaneous tumours examined. Although, in this study, α-catenin expression was investigated only in BCC frozen specimens, the results demonstrated that the reduction in tumour cell membrane α- catenin is also associated with that of β-catenin and E-cadherin. Although β-catenin and E-cadherin expression in breast carcinoma, for example, was severely reduced, Hashizume and colleagues (1996) related these observations to the histological type and growth pattern rather than the invasiveness and metastatic potential of the tumours.

In my study, the observation of reduced membranous E-cadherin, α- and β-catenin in BCC, SCC and malignant melanoma tumour cells in comparison with the normal epidermis strongly suggests a disturbance of AJ composition in these tumours. Furthermore, my data has shown that the membranous staining of β-catenin in all the cutaneous tumours examined, is almost identical to that of E-cadherin, suggesting that β-catenin forms a complex with E-cadherin in vivo. In addition, my data also suggest that membranous β-catenin downregulation is associated with malignant transformation in cutaneous tumours probably contributing to their development and progression. This

is to say that membranous β-catenin was decreased in superficial, nodular BCC and well-differentiated SCC and decreased further in tumours with more aggressive behaviour such as infiltrative, metatypical and spindle cell BCC and moderately differentiated SCC in direct proportion to the loss of differentiation in these tumours. Therefore, the reduction in tumour cell membrane staining of β-catenin in comparison with the normal epidermis is another characteristic in BCC and SCC.

Similarly to E-cadherin, immunostaining of β-catenin in cutaneous tumours, particularly in BCC and SCC, may have prognostic value. Consistent with the report of Shiozaki and colleagues (1994) who demonstrated frequent reduction of α-catenin expression in primary tumours of oesophagus, stomach and colon, my results suggest that the reduction in tumour cell membrane α-catenin in BCC may be a characteristic feature acquired through malignant transformation. Since the functions of E-cadherin are modulated by catenins (Nagafuchi et al., 1988; Kintner, 1992; Nagafuchi et al., 1994), functional or structural abnormalities of α-catenin might take place in BCC with reduced α-catenin expression interfering with cadherin-mediated cell-cell adhesion.

4.5. β-catenin expression in melanocytic tumours

In malignant melanoma, using routinely processed, paraffin-embedded tissues, Silye and colleagues (1998) have shown diffuse cytoplasmic immunostaining for both α- and β-catenin in 35 cases with no correlation between tumour thickness or level of invasion, although, it was not stated whether this observation was accompanied by reduction in membrane staining. In a similar distribution pattern to E-cadherin, my results have demonstrated decreased membranous β-catenin staining in dermal nests of primary melanomas in both radial and vertical growth phases and metastatic melanomas when compared with the overlying normal epidermal keratinocytes. However, the staining intensity ranged from reduced to a complete absence. In addition, the other benign melanocytic lesions demonstrated reduced β-catenin staining particularly in the dermal components. The case of metastatic melanoma which showed strongly preserved E- and

P-cadherin expression also showed strong staining of membranous β-catenin suggesting possible re-establishment of AJ composition.

My data on primary and metastatic melanomas indicated that the downregulation of β-catenin expression was associated with malignant transformation and progressed further in dedifferentiated tumours, strongly suggesting a disturbance of AJ composition in these tumours. This may be preceded by an enhanced detachment from the epidermis with subsequent invasion and metastasis. Furthermore, my data has shown that the membranous staining of β-catenin in all the melanocytic tumours examined, is almost identical to that of E-cadherin, suggesting that β-catenin forms a complex with E-cadherin in vivo. However, the reduction of β-catenin expression in the dermal components of some benign melanocytic lesions could be due to the failure of the migrating melanocytes to form normally functioning AJ.

Mutations in β-catenin were found in malignant melanoma and melanoma cell lines (Rubinfeld et al., 1997; Rimm et al., 1999). These mutations that prevent phosphorylation result in activation of Wnt signalling pathway because of increased cytoplasmic β-catenin (Rubinfeld et al., 1997). Rimm and colleagues (1999) detected nuclear and/or cytoplasmic staining in 10 of 18 cytology specimens and 8 of 47 surgical specimens of primary melanoma. They have interpreted these findings as a possible activation of β-catenin reflecting focal and transient activation of the Wnt signalling pathway within the tumour. In my study, although cytoplasmic staining of β-catenin was present in primary and metastatic melanomas, nuclear staining was not observed. Although the number of primary and metastatic melanomas was relatively small, my findings do not exclude the possibility of β-catenin activation by mutations as reported by previous studies (Rubinfeld et al., 1997; Rimm et al., 1999).

4.6. Nuclear localisation of β-catenin in cutaneous tumours

Signalling via Wnt/Wg and Shh glycoproteins is critical for normal development of Drosophila melanogaster, Xenopus laevis and mammals, while dysregulation of their signal transduction pathway contributes to human cancer (Nusse and Varmus, 1992; Gumbiner, 1995; Moon et al., 1997; Cadigan and Nusse, 1997). During development there is interaction between the Shh/Ptc and Wnt/Wg signalling pathways (Von Ohlen and Hooper, 1997). There is accumulating evidence showing that the dysregulation of the Shh/Ptc signalling pathway leads to the development of hereditary and sporadic BCC (Stone et al., 1996; Gailani et al., 1996; Johnson et al., 1996; Hahn et al., 1996a; Unden et al., 1997; Oro et al., 1997; Aszterbaum et al., 1998; Smyth et al., 1999; Nagano et al., 1999). However, the role of the Wnt/Wg signalling pathway in epidermal tumourigenesis remains unclear. In addition to its function in intercellular adhesion by associating with E-cadherin (Nagafuchi et al., 1988; Ozawa et al., 1989; McCrea et al., 1991; Nagafuchi et al., 1994), β-catenin is a key component of the Wnt pathway (reviewed by Gumbiner, 1995; Miller and Moon, 1996; Willert and Nusse 1998; Polakis, 1999). Activation by Wnt redistributes β-catenin to the nucleus to form a complex with T cell factor (Tcf) and lymphoid enhancer factor (Lef) transcription factors (Huber et al., 1996; Behrens et al., 1996; Molenaar et al., 1996; Korinek et al., 1997). Mutations in β-catenin have been described in various malignancies and cancer cell lines including rat and human colon tumour tissues and cell lines (Morin et al., 1997; Takahashi et al., 1998; Mann et al., 1999), thyroid carcinoma tissue (Garcia-Rostan et al., 1999), human prostate cancer tissue and cell lines (Voeller et al., 1998) and hepatocellular carcinoma tissue (Miyoshi et al., 1998). These mutations in β-catenin resulted in frequent cytoplasmic and/or nuclear localisation of the protein which was observed in familial adenomatous polyposis tissue (Inomata et al., 1996), intestinal polyps of Peutz-Jeghers and juvenile polyposis syndromes (Back et al., 1999), anaplastic thyroid carcinoma (Garcia-Rostan et al., 1999) and rat colon tumours (Takahashi et al., 1998). Cytoplasmic and/or nuclear localisation of β-catenin may indicate its activation which appears to be frequent in melanoma (Rimm et al., 1999) and gastric carcinoma cell lines (Jawhari et al., 1999) reflecting activation of the Wnt/Wg signalling pathway in these tumours. Similarly, Kimura and co-workers (1999) demonstrated the

accumulation of free soluble β-catenin in the cytoplasm and nuclei of oesophageal cancer cells by fractionation technique. In adenomatous colonic tumours, the nuclear translocation of β-catenin and the increase in its transcriptional activity could also be due to disturbance in its metabolism due to lack of the normal APC gene product or a mutated APC gene product (Korinek et al., 1997; Back et al., 1999). Thus, β-catenin may act as an oncogene contributing to the causes and aggressive behaviour of many tumours. Furthermore, accumulation of non-membrane associated β-catenin in the cytoplasm resulting from mutations in APC or in β-catenin itself was found to be followed eventually by a later mutational inactivation of the p53 tumour suppressor during the later stages of tumour progression (Damalas et al., 1999).

Transgenic mice expressing an activated β-catenin were found to be predisposed to develop skin tumours resembling pilomatrixomas and trichofolliculomas (Gat et al., 1998). Furthermore, human pilomatrixomas and trichofolliculomas have been found to contain β-catenin-stabilising mutations which result in LEF-1 transactivation (Chan et al., 1999) (Fig. 133).

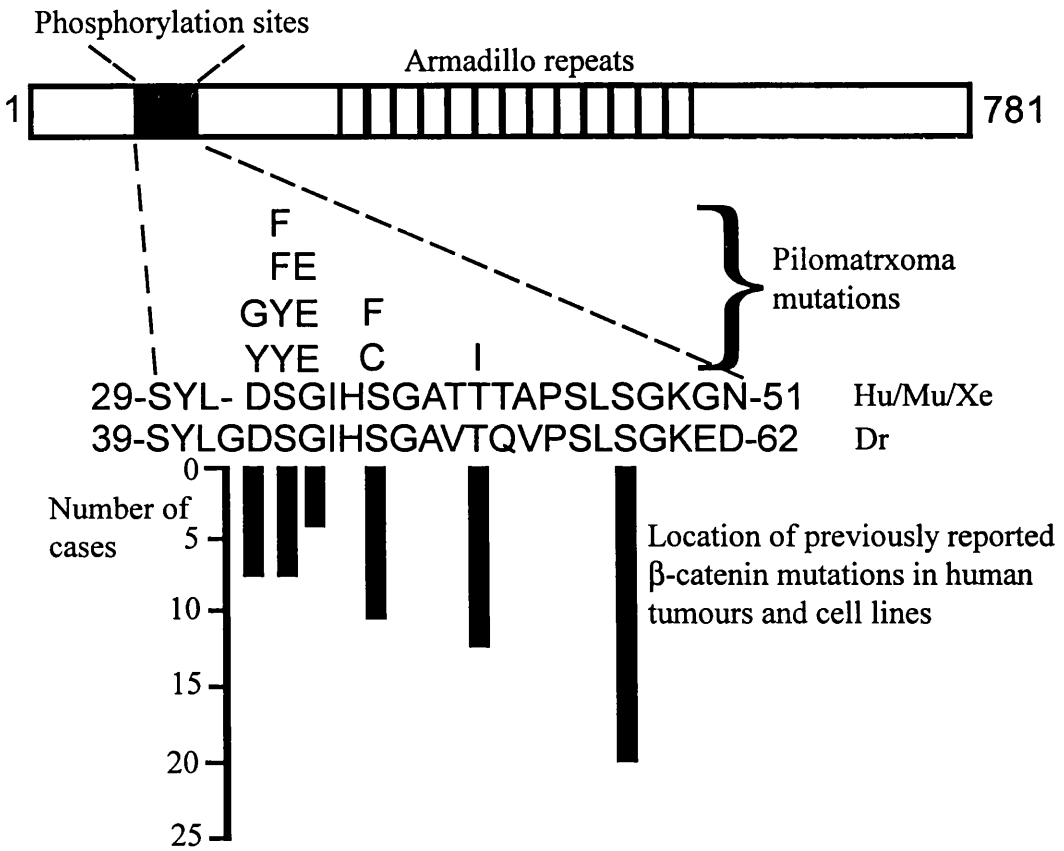


Figure 133. Schematic of β-catenin, depicting the location of the GSK3b-dependent phosphorylation sites in the N-terminal segment and the armadillo repeats. Shown are the sequences of the wild-type, conserved N-terminal segment from human (Hu), mouse (Mu), Xenopus laevis (Xe), Drosophila melanogaster (Dr) and the corresponding location of pilomatrixoma mutations and previously described mutations from other types of human tumours and cell lines (Chan et al., 1999).

My data extends the work of Chan and colleagues (1999) by showing nuclear β-catenin staining in pilomatrixoma using antibodies to the N and C terminal domains. However, my immunohistochemical analysis has also shown that β-catenin can be detected in the tumour cell nuclei of some BCC. The nuclear staining of β-catenin seen in two cases of Bowen's disease was only with C-terminus antibody. In almost all cases, the nuclear β-catenin staining was accompanied by decrease in membrane staining. The staining pattern seen with both antibodies in pilomatrixoma and frozen BCC specimens was almost identical. Positive nuclear staining was seen less frequently in paraffin-embedded BCC with the N terminal compared with the C terminal antibody. However, all paraffin-embedded BCC expressing nuclear N-terminus β-catenin have shown C-terminus β-catenin nuclear staining.

Cytoplasmic and/or nuclear accumulation of β-catenin has been observed in familial adenomatous polyposis tissue (Inomata et al., 1996), rat colon carcinoma tissue (Takahashi et al., 1998), intestinal polyps of Peutz-Jeghers and juvenile polyposis syndromes (Back et al., 1999), oesophageal cancer tissue (Kimura et al., 1999), gastric carcinoma cell lines (Jawhari et al., 1999) and anaplastic thyroid carcinoma (Garcia-Rostan et al., 1999).

The detection of nuclear β-catenin staining in Bowen's disease and BCC in addition to pilomatrixoma is novel as it suggests that dysregulation of β-catenin cellular distribution may be common to several pathways leading to epidermal carcinogenesis. However, β-catenin nuclear staining was seen in only a minority of BCC and Bowen's disease cells and was not observed in SCC, keratoacanthoma, actinic keratosis and the non-malignant lesions.

While activation of β-catenin by mutation may be a primary event in pilomatrixoma, it seems more likely that in BCC and Bowen's disease nuclear localisation of β-catenin would be secondary to activation of the Wnt pathway or possibly due to redistribution of β-catenin as a result of disruption in AJ.

4.7. Gli-1 expression in normal epidermis and BCC

Dysregulation of the Shh/Ptc signalling pathway (Fig. 5) has been shown to underlie BCC (Hahn et al., 1996a; Johnson et al., 1996; Lam et al., 1999).

Gli-1 is a member of the Gli family of zinc-finger transcription factors which includes the drosophila cubitus interruptus (ci) which shows significant homology to Gli-1 (Kinzler et al., 1987; Kinzler et al., 1988; Von Ohlen et al., 1997). Gli-1 has been identified as a downstream mediator of the Shh response (Dominguez et al., 1996; Alexandre et al., 1996; Lee et al., 1997; Hynes et al., 1997; Altaba, 1998). In drosophila, ci is needed for the transcriptional activation of the Hh signalling pathway target genes, Wg and ptc, and the repression of others, such as Hh itself (Aza-Blanc et al., 1997; Akimaru et al., 1997; Ingham, 1998).

In a study by Dahmane and colleagues (1997), ectopic expression of Gli-1 in the embryonic frog epidermis has been shown to result in the development of tumours that express endogenous Gli-1. They also showed that Shh and the Gli genes are normally expressed in hair follicles, and that human sporadic BCC consistently express Gli-1 but not Shh or Gli-3. Taken in consideration that Gli-1, but not Gli-3, mediates Shh signalling, these findings together with the fact that the Ptc gene is mutated in hereditary and some sporadic BCC raised the possibility that Gli-1 could be expressed and underlie the development of sporadic adult BCC. Furthermore, Oro et al (1997) demonstrated that overexpression of Shh in transgenic mice is sufficient to induce BCC-like tumours. Specific Ptc overexpression was also found in human sporadic BCC, suggesting that an abnormality of the Ptc-mediated signalling pathway plays an important role in the development of this tumour (Unden et al., 1997; Nagano et al., 1999). Any mutations leading to the expression of Gli-1 in basal cells are predicted to induce BCC formation (Dahmane et al, 1997). Green and colleagues (1998), have established by using reverse transcription-polymerase chain reaction (RT-PCR) that Gli-1 is differentially expressed in human BCC but not normal interfollicular skin. Using in situ hybridisation, they demonstrated Gli-1 transcripts within BCC tumour islands which would be consistent with the hypothesis that activation of Hh/Ptc signalling in keratinocytes is accompanied by the accumulation of Gli-1 transcripts. Moreover, Gli-1 was not found to be expressed in normal human skin and hair follicles (Green et al., 1998) in contrast to the

findings of Dahmane et al (1997). Thus, the differential expression of Gli-1 in BCC could be associated with the inactivation of Ptc gene in this tumour in which Gli-1 expression is increased by the activation of the Hh/Ptc signalling pathway (Green et al., 1998). Although Gli-1 has been shown to be localised to the cytoplasmic compartment in BCC (Dahmane et al., 1997; Ghali et al., 1999), it is found largely in the nucleus in COS cells transfected with the glioma-derived cDNA (Dahmane et al., 1997; Aza-Blanc et al., 1997) and in a minority of BCC cases stained with Gli-1 N and C-terminal antibodies (Ghali et al., 1999).

My immunohistochemical analysis of normal epidermis and paraffin-embedded BCC with antibodies to the N and C terminal domains has shown that Gli-1 protein can be detected in normal epidermis, although weakly heterogeneous, and in BCC using these antibodies. Although the number of cases differs slightly, the staining pattern seen with both antibodies was almost identical in which it was present in normal keratinocytes towards the edges of the normal specimens and completely negative in the epidermis immediately above the main BCC mass in all the specimens. Although some hair follicles stained weakly positive in their outer root sheath cells, this was not consistently seen reflecting possible epitope masking.

In BCC tumour cells, my results have shown positive staining with both antibodies which was almost solely nuclear in contrast with the findings of Ghali and colleagues (1999) and with a previous report using a polyclonal antibody to the DNA binding domain of Gli-1 (Dahmane et al., 1997). They have detected cytoplasmic Gli-1 staining in BCC. However, my observation of nuclear localisation of Gli-1 is consistent with previous reports of nuclear localisation in COS cells transfected with a Gli-1 construct (Dahmane et al., 1997; Aza-Blanc et al., 1997).

The similarities and differences in Gli-1 staining between my data and that of Ghali and colleagues (1999) are listed in Table 33, however, when I tried the staining protocol used in their report, the results were unsatisfactory (see results section).

	My data	Ghali et al., 1999
Tissue used	Routinely processed BCC tissue	Routinely processed BCC tissue
Staining method	Extra Avidin-biotin-peroxidase complex technique	Avidin-biotin-peroxidase complex technique
Antibody used	Goat polyclonal IgG	Goat polyclonal IgG
Antibody source	Cruz Biotechnology (Santa Cruze, CA)	Cruz Biotechnology (Santa Cruze, CA)
Method of antigen retrieval	Pressure cooking	None
Blocking of endogenous peroxidase activity	3% hydrogen peroxide in distilled water for 15 min	3% hydrogen peroxide in methanol for 10 min
Antibody dilution	1:200	1:500
Incubation	1 hour at 37°C	Overnight at 4°C
Development of peroxidase reaction	VIP peroxidase substrate kit	Diaminobenzidine
Antibody localisation in BCC	Nuclear	Cytoplasmic

Table 33. Immunohistochemical staining of Gli-1 in BCC. Similarities and differences between my data and that of Ghali et al., 1999.

In my results, the positive nuclear staining was seen less frequently with the N terminal compared with the C terminal antibody, but all BCC expressing nuclear N-terminus Gli-1 have shown C-terminal antibody nuclear staining. This may simply suggest an epitope masking phenomenon, however, inherent differences in the tumours cannot be excluded. No correlation was detected between the Gli-1 tumour cell nuclear staining and the aggressive behaviour of the tumour.

During the development of drosophila, the Gli-1 homologue, cubitus interruptus, represses Hh and other target genes in the anterior compartment to allow for anterior development (Dominguez et al., 1996; Aza-Blanc et al., 1997). As the cell origin of BCC remains controversial, the weak Gli-1 nuclear staining in the basal cells of the normal epidermis may be an early event indicating a low transient epidermal expression of Gli-1 as there could be a requirement for certain level of Gli-1 to initiate tumour formation in these cells pointing to a possible basal cell origin and a potential diagnostic tool of this common tumour.

More importantly, in contrast to the recent report of predominant cytoplasmic localisation of Gli-1 in BCC (Ghali et al., 1999), the data presented in my work of Gli-1 nuclear localisation in BCC strongly supports the evidence which indicates that Gli-1, and its drosophila homologue cubitus interruptus, may be a transcriptional activator (Aza-Blanc et al., 1997; Ingham, 1998; Alexandre et al., 1996; Von Ohlen and Hooper,

1997; Forbes et al., 1993; Akimaru 1997). While the explanation of Gli-1 functions in skin as a transcriptional activator or a repressor remains to be completely cleared, my data suggests that nuclear localisation of Gli-1 in BCC provide further evidence for the activation of the Shh/Ptc signalling pathways in this tumour. In addition, the data of Ghali and colleagues (1999) on cytoplasmic Gli-1 in BCC may also indicate activation of this pathway. However, the significance of the nuclear staining in the normal epidermis and BCC tumour cells in addition to the observation of Ghali and colleagues (1999) of cytoplasmic localisation of Gli-1 in BCC needs to be resolved by further studies as it could be important in establishing the functions of Gli-1 as a repressor or activator.

4.8. Actin and major adherens junctions components in normal epidermis, hair follicle and BCC

Distribution of actin filaments, E- and P-cadherin, α- and β-catenin, and α-actinin in BCC and adjacent normal skin was examined by immunofluorescent staining.
In normal epidermis, the results of immunofluorescent staining of E- and P-cadherin, α- and β-catenin were similar to immunoperoxidase which were consistent with the presence of a normally functioning cadherin/catenin complex in AJ.

The use of TRITC-labelled Phalloidin allowed the detection of actin filaments in human skin upon immunofluorescent staining. Keratinocytes in human normal epidermis have been shown to express weak staining of actin filaments which were arranged in parallel bundles beneath the cell membrane (Metze et al., 1996). In my work, apart from the strong immunofluorescent staining in the granular layer, actin filaments were rarely seen in the normal epidermis. These findings are consistent with previous reports (McNutt, 1976; Kumakiri and Hashimoto, 1978; Jones et al., 1989; Metze et al., 1996).

In the hair follicle, Sugiyama and colleagues (1983) have used electron microscopy to demonstrate actin-like microfilaments in the ORS cells. By using Phalloidin-TRITC, my data has demonstrated that the ORS cells contain prominent actin filaments concentrated at the periphery of the cells with occasional radial arrangement. These filaments may play a role in the cytoskeletal function to maintain the normal polygonal shape of the cell by the association with the cell membrane. Additionally, the periadnexal connective tissue sheath and arrector pili muscle also contain prominent actin filaments which could represent staining of smooth muscle actin. Similar findings in ORS cells have recently been reported by Furumura and Ishikawa (1996) and (in abstract form) by Chan and co-workers (1997).
Although actin filaments were not seen in the basal cells of the normal epidermis, such actin filaments in the hair follicle and stromal surroundings may be involved in the adhesive system between the epithelial follicle and the surrounding dermal components.
α-Actinin is a major component of the AJ (Arikawa and Williams 1991; Knudsen et al., 1995; reviewed by Cowin and Burke, 1996). Although the immunofluorescent staining of α-actinin in normal epidermal cells was largely cytoplasmic, the enrichment of this

protein often seen at normal cell peripheries is in agreement with previous immunohistochemical studies on epithelial cells (Graig and Pardo, 1979; Geiger et al., 1979; Sanger et al., 1983; Drenckhahn and Franz, 1986). The double immunolabelling results of E- and P-cadherin, α- and β-catenin with TRITC-labelled Phalloidin in normal epidermis revealed separate localisation of these proteins from actin filaments which is consistent with indirect association of these proteins with the actin-based cytoskeleton. However, my data provides evidence of co-localisation between α-actinin and actin filaments at the plane of plasma membrane in some normal cells. This co-localisation emphasises the close association of α-actinin with actin filaments and its important role for the anchorage of actin filaments to the plasma membrane.

Cell movement is thought to be driven largely by the polymerisation of actin monomers into filaments near the plasma membrane (Machesky and Way, 1998). Differences in actin filaments distribution and cell shape have been examined in cultured human epidermal keratinocytes (Kubler et al., 1991). In their study, Kubler and colleagues have shown that in the basal cells at the edges of the colonies, the actin filaments were concentrated at the leading edges of the cells whereas further from the substratum, transcellular actin filaments were not visible and instead they were concentrated at the lateral cell membranes with short bridges apparently connecting individual cells at AJ as evidenced by the presence of vinculin.

The presence of actin in human cancer cells, including BCC, has been studied previously by means of immunofluorescent staining using anti-actin antibodies and electron microscopy (Gabbiani et al., 1976; McNutt, 1976; Kumakiri and Hashimoto, 1978; Jones et al., 1989). Anti-actin antibodies detect the total actin and do not necessarily demonstrate the distribution of filamentous actin in tissue. The studies of (Gabbiani et al., 1976; McNutt, 1976; Kumakiri and Hashimoto, 1978; Jones et al., 1989) have demonstrated an increase in actin-like microfilaments in BCC tumours. Such microfilaments were also found in epidermal cells during wound healing (Martin and Lewis, 1992). Although the actin microfilaments found in BCC in the previous studies were located within fingerlike cytoplasmic extensions, they were also located at the periphery of individual cells and their density was highest at tumour borders. In addition, using anti-actin antibodies, Low and colleagues (1981) have reported no

substantial change of immunofluorescent staining for actin in BCC and other tumours cell lines compared to normal control tissues. Similar findings have been reported by Kitano (1988) using Rhodamine-phalloidin. However, in a previous study, McNutt (1976) reported that the most striking increase in actin filament is in the most infiltrative subtypes of BCC such as the morphoeic BCC.

By using TRITC-labelled Phalloidin, my data has shown that actin polymerisation into filament bundles dramatically increases in tumour cells as they penetrate into the dermis from their connections with the normal epidermis. Nodular BCC is characterised by lobular masses of various shapes and sizes embedded in the dermis. The tumour lobules of this subtype have a rounded smooth outline and well-developed palisading similar to the superficial subtype (Lever and Schaumburg-Lever, 1990a; McKee, 1996a). My data has shown actin polymerisation into filament bundles in nodular BCC, however, actin bundles were located peripherally beneath the cell membrane where single actin filaments were rarely observed. This may explain the stability of cell shape seen in nodular BCC. In contrast to nodular BCC and normal epidermis, actin filament polymerisation was strikingly increased in infiltrative subtypes. The growth pattern of infiltrative BCC is characterised by narrow tongues and aggregates of malignant epithelium that extend deeply into the adjacent fibrous stroma with spindly irregular configuration and poorly developed peripheral palisading (du Vivier and McKee, 1993a; MacKie, 1996a). These results, combined with the data on reduced E-cadherin, α- and β-catenin, and α-actinin in BCC, suggest that increased actin filaments polymerisation (stress fibers) is related to the infiltrative growth pattern. In addition, the highest density of actin stress fibers in certain lobular aspects of the infiltrative BCC may indicate the direction of the growing edge of the tumour. My data has shown the presence of bright actin filament immunofluorescent staining in the stromal cells surrounding the tumour. This provides evidence of a myofibroblast phenotype resembling that seen in the periadnexal fibrous tissue sheath pointing to a possible adhesive system between the tumour lobules and its surrounding stroma.

My data has shown differences in the actin immunofluorescent staining between normal epidermal cells and tumour cells of BCC, particularly infiltrative subtype, reflecting a dramatic increase in actin filaments polymerisation (stress fibers) in tumour cells. In

general, BCC has a low metastatic potential (Domarus and Stevens, 1984; Lo et al., 1991) and nodular BCC, in particular, retain normal basal polarity (Lever and Schaumburg-Lever, 1990a). Therefore, both the increase in actin filaments polymerisation and the decrease of E-cadherin, α- and β-catenin and α-actinin observed in this current study of BCC are probably related to invasiveness rather than rapid growth and potential for metastasis. Thus, in addition to the decrease of E-cadherin, α- and β-catenin and α-actinin, the striking increase in actin filaments polymerisation (stress fibers) in tumour cells, not seen in normal epidermis and nodular BCC, could be related to enhanced tumour cell motility as they lose cell-cell contacts and thus the invasive growth pattern seen in infiltrative BCC.

The double immunolabelling results of most tumour cells in nodular and infiltrative BCC have shown separate localisation of E- and P-cadherin, α- and β-catenin from actin filaments particularly in the central aspects of the tumour lobules. However, my data demonstrated that a portion of E- and P-cadherin, α-catenin and α-actinin was co-localised with actin filaments, especially in peripheral tumour cells, at the plane of the plasma membrane. The co-localisation of α-catenin and α-actinin with the actin filaments, seen mostly in peripheral cells, could be explained by the interactive role of α-catenin where it connects the membrane associated complex (E-cadherin and β-catenin) to the actin-based cytoskeleton by either direct association with actin filaments via its amino and carboxyl termini or indirectly by association with α-actinin, another actin binding protein (Knudsen et al., 1995; Rimm et al., 1995; reviewed by Cowin and Burke, 1996). In addition, the co-localisation of E- and P-cadherin with actin filaments in some tumour cells may indicate a direct physical association, however the functional significance of such co-localisation remains to be determined.

4.9. Co-localisation of E-cadherin with P-cadherin in normal epidermis and BCC

One of the documented features of normal human skin is the expression of two cadherins. Normal epidermal keratinocytes and melanocytes express both P-cadherin and E-cadherin on their cytoplasmic membranes where they maintain adhesion between these cells by homophilic binding (Fujita et al., 1992; Tang et al., 1994; Horiguchi et al., 1994; Lewis et al., 1994). The overall staining patterns of E- and P-cadherin observed in this current study, either by immunoperoxidase or immunofluorescent staining, were as reported previously. E-Cadherin was distributed in the full thickness of the epidermis excluding the stratum corneum (Shimoyama et al., 1989; Horiguchi et al., 1994; Moles and Watt, 1997), with an increased expression in the suprabasal layers as compared with the basal cells (Cano et al., 1996), whereas P-cadherin was restricted to the basal proliferative compartment (Hirai et al., 1989; Cano et al., 1996; Moles and Watt, 1997). In the hair follicle, both cadherins were strongly expressed in the outer root sheath cells (Fuller et al., 1996; Cano et al., 1996; Muller-Rover et al., 1999). In view of these findings, this present study considered of great interest the investigation of possible co-localisation between E- and P-cadherin in the basal and suprabasal cells of the normal epidermis and relate it to their expression and possible co-localisation in BCC by double immunolabelling.

My experiments have demonstrated that E-cadherin is co-localised with P-cadherin at the membranes of the basal and the first layer of the suprabasal epidermal cells. Similar observation was also noted in the outer root sheath cells of the normal hair follicle which was within limits of resolution.

This finding of coexpression in the proliferating region of the normal epidermis is of interest because of the evidence that cadherins are considered to be important regulators of skin morphogenesis and cell sorting (Nose et al., 1988; Hirai et al., 1989; Takeichi, 1991; Takeichi, 1995) and may regulate keratinocytes growth, differentiation (Zhu and watt, 1996) and human epidermal stratification (Jensen et al., 1997). In view of the results of these studies, the co-expression of E- and P-cadherin in epidermal basal cells suggests that cadherins in this particular region are involved not only in maintaining cell-cell adhesion and regulating intercellular junction organisation but may also be involved in other specific regulatory mechanisms. Cadherins may play a role in the

downregulation of integrin expression that is associated with terminal differentiation (Hodivala and Watt, 1994). Therefore, they may regulate integrins expression in the cell-extracellular matrix adhesion. Moreover, the co-localisation of E- and P-cadherin was taken to higher resolution which showed that they were not segregated in basal cell membranes. These results suggest that E- and P-cadherin could be in the same junction plaque.

Because the expression of P-cadherin seemed to be associated with the proliferating activity of cells (Hirai et al., 1989), it is restricted to the basal cells of the normal epidermis segregating the epidermal proliferative compartment from the upper non-proliferating strata. Additionally, although my data demonstrated co-localisation of E-cadherin and P-cadherin in the membranes of basal and suprabasal cells, either of them may still have unique function(s) in this region.

In BCC, double immunolabelling data has revealed co-localisation of both cadherins in a substantial number of tumour cells. However, the majority of tumour cells demonstrated preservation of membranous P-cadherin only. More interestingly, throughout the tumour lobules there were no tumour cells expressing only E-cadherin. E-Cadherin is considered to be the major adhesion molecule found in normally functioning AJ (Takeichi, 1988). In addition, its loss occurs in association with human epithelial cancers (Fuller et al., 1996; Perl et al., 1998; Cano et al., 1996). Therefore, in my work, the double immunolabelling data of E- and P-cadherin in BCC further support the earlier discussion, mentioned above, regarding the possibility of stepwise loss of cadherins in which E-cadherin may be lost first then P-cadherin loss follows when the tumour acquire very aggressive behaviour and is about to metastasise.

4.10. Double IF staining of E- and P-cadherin and β-catenin with actin in keratinocyte cultures

AJ of cell-cell type, distinct from desmosomes, are present in human normal epidermis and cultured keratinocytes (Kaiser et al., 1993). Normal keratinocytes express both P-cadherin and E-cadherin, which are the cell-cell adhesion molecules of the AJ, on their cytoplasmic membranes where they maintain adhesion between these cells by homophilic binding (Fujita et al., 1992; Tang et al., 1994; Horiguchi et al., 1994; Lewis et al., 1994). As revealed by electron microscopy, focal contacts representing cell-substrate adhesion are found in vitro at sites of attachment of fibroblasts to the culture surface (Abercrombie et al., 1971).

Proteins known to be associated with AJ such as E-cadherin, β-catenin, vinculin and actin can be detected by immunofluorescence at the periphery of the cells in normal epidermis and cultured keratinocytes suggesting the presence of AJ in keratinocytes in vivo and in vitro (O'Keefe et al., 1987; Kubler et al., 1991; Kaiser et al., 1993; Horiguchi et al., 1994; Haftek et al., 1996). In keratinocyte culture, the formation of AJ is rapidly induced by shifting the calcium from low to physiologic concentrations (O'Keefe et al., 1987; Kubler et al., 1991; Lewis et al., 1994; Cooley et al., 1996).

Using immunofluorescent staining and confocal laser microscopy, my experiments have demonstrated that keratinocyte cultured in low calcium (0.1 mM Ca^{2+}) grew in a loose colonial morphology and showed weak E-, P-cadherin and β-catenin membrane immunofluorescent staining and a diffuse polymerisation of actin filaments. In contrast, when the cultures were exposed to higher calcium concentration (1.5 mM Ca^{2+}), cells showed stronger staining of the cell adhesion molecules and associated more closely. However, in the high calcium condition, actin filaments, where positive, were prominent throughout the colonies and particularly in the basal cells at the borders of the wound where strong E-, P-cadherin and β-catenin membrane immunofluorescent staining was seen at or just behind the leading edge of the migrating cells. In addition, most of the cells behind the leading edge showed prominent actin filaments which were distributed at the lateral cell membrane in a pattern similar to that in nodular BCC. However, in keratinocytes grown in high calcium, the distribution of actin filaments was accompanied by strong E-, P-cadherin and β-catenin membrane immunofluorescent

staining in agreement with a recent report on E-cadherin and filamentous actin staining in keratinocytes and dendritic cells (Jakob et al., 1999). This may indicate that the cells grown in low calcium which moved individually were less motile as they migrated into the wound but failed to close it completely. In contrast, the cells grown in high calcium, particularly at the leading edge, migrated in a sheet-like colony and closed the wound completely. In addition, they showed increased actin polymerisation in which prominent bundles of actin filaments were arranged in groups of short stress fibers similar to that in infiltrating BCC cells which may indicate that the cells grown in high calcium conditions are more motile than those grown in low calcium conditions.

The strong immunofluorescent membrane staining of β-catenin in high calcium conditions and its absence in the nucleus were similar to the immunoperoxidase staining in the normal epidermis. In addition, stratification of keratinocytes was more obviously noted in colonies grown in high calcium concentration when compared with those grown in low calcium in agreement with previous reports (Hodivala and Watt, 1994; Lewis et al., 1994).

There is evidence that the proper function of classic cadherins is a prerequisite for desmosomes assembly in keratinocytes upon calcium elevation (Amagai et al., 1995). My data indicated that AJ were properly assembled in high calcium conditions hence the presence of strong E-, P-cadherin and β-catenin membrane staining when compared to the low calcium conditions. This is consistent with the notion that cadherin/catenin complex is the functional unit for the Ca^{2+}-dependent cell-cell adhesion. Cadherin/catenin complexes have specialised roles in establishing and maintaining the structural and functional organisation of polarised epithelial cells (Nathke et al., 1994). In my experiments, the tight adhesion of cells in high calcium cultures, in contrast to the loose colonial morphology in low calcium conditions, and the strong membrane staining of the cell adhesion molecules may emphasise the possible important function of the cell-cell AJ in the maintenance of the structural integrity of the epithelial sheet. In addition, the double immunolabelling results of E-, P-cadherin and β-catenin with TRITC-labelled Phalloidin in both low and high calcium keratinocyte cultures revealed

separate localisation of these proteins from actin filaments implying an indirect association of these proteins with the actin-based cytoskeleton.

E-Cadherin is considered to be the major adhesion molecule found in normally functioning cell-cell type AJ (Takeichi, 1988; Tang et al., 1994). Lewis and colleagues (1994) provided evidence that in the presence of anti-E-cadherin antibody, P-cadherin is detected at the cell-cell borders prior to any of the other cell adhesion molecules which suggested that P-cadherin may substitute for E-cadherin with regard to its regulatory role. In my work, at cell-cell contacts, the strong P-cadherin membrane staining in high calcium conditions, similar in intensity to that of E-cadherin, may suggest that it is not a minor component of the AJ. In addition, the observation of numerous small and bright structures of E-cadherin immunofluorescent staining, reminiscent of focal adhesion and not seen in a low calcium conditions, at the cell-substrate interface may suggest that the distribution of E-cadherin in keratinocyte cultures is different from that in normal epidermis. Further studies are needed since this observation may indicate an involvement of E-cadherin in cell-substrate contacts in vitro and a possible interaction with the integrins since they are expressed primarily at these junctions (Carter et al., 1990; reviewed by Hynes, 1992; Sheppard, 1996; Aplin et al., 1998) and they may be involved in cytoskeletal interactions (Tamura et al., 1990).

4.11. BCC culture

Although BCC is a slow-growing tumour which metastasises exceptionally rarely (Domarus and Stevens, 1984; Lo et al., 1991), it is capable of gross tissue destruction (Leffell et al., 1991; Ko et al., 1992; McKee, 1996a).

Unlike other cutaneous tumours such as SCC and malignant melanoma, attempts to grow BCC in primary cultures have not been found to be successful (Van Scott et al., 1961; Flaxman, 1972; Cooper et al., 1977; Bradbeer et al., 1988; Asada et al., 1992). In most of BCC culture reports, tumour cells, dispersed from BCC-containing skin, were usually seeded in serum-free keratinocyte growth medium directly into plastic tissue culture flasks, tissue culture wells or Petri dishes which may or may not be coated with type I or type IV collagen (Hernandez et al., 1985; Barsky et al., 1987; Brysk et al., 1992; Grando et al., 1996). Using a simple feeder-layer technique, Asada et al (1992) reported cultures of 73% of all BCC explants which showed characteristic and reproducible patterns of growth, keratin expression and ultrastructural differentiation distinguishing them from epidermal basal cells. Hernandez and colleagues (1985) reported an establishment of epithelial cell colonies from about 50% of cultured nodular BCC using tissue culture plates coated with type IV collagen, which enhances the attachment of the cultured cells. From our laboratory experience, coating the surfaces of the tissue culture flasks with a layer of neat FCS for few minutes was found to enhance the attachment of keratinocytes to the surfaces more quickly. By using similar materials and methods to the previous report (Hernandez et al., 1985), my preliminary data has shown that tumour cells failed to attach to culture surfaces coated with FCS and/or with collagen where they eventually died within 5-7 days of culture. This result could be due to the presence of some FCS in the culture condition as BCC cells in culture have been reported to differentiate when cultured in serum-containing media or as explant cultures (Bradbeer et al., 1988; Asada et al., 1992) probably due to an elevated Ca^{2+} concentration. Although Brysk and colleagues (1992) reported establishing undifferentiated cultures of BCC in a serum-free and low-Ca^{2+} (0.1 mM) medium, they observed the formation of cornified envelopes and the expression of a differentiated phenotype (as with normal cells) when the Ca^{2+} concentration of the keratinocyte growth medium was increased. In addition, as normal human keratinocytes may possibly suppress the growth of BCC cells (Grando et al., 1996), the presence of

these cells in addition to other dermal components in my experiments might have inhibited BCC cell attachment and/or growth.

Following these unsatisfactory results and as a new approach, my study attempted to introduce a tissue-culture model system for BCC by seeding tumour-derived cells onto dermal equivalents. Degradation of dermal equivalents which was observed in the preliminary experiments using a specimen of an infiltrative BCC occurred over a short period of time. In their study of nodular BCC, Hernandez and colleagues (1985) reported that BCC-derived epidermal cell colonies released an Interleukin-1-like mediator which may increase fibroblast proliferation and production of both collagenase and hyaluronic acid. Therefore, they suggested that BCC in vivo may regulate fibroblasts functions and mediate some of the changes in its surrounding connective tissue matrix. Previous reports have suggested that the source of high interstitial collagenase type I in BCC could be from the fibroblasts composing the abnormal connective tissue stroma surrounding the tumours (Bauer et al., 1977; 1979). However, sclerosing BCC, but not superficial and nodular, tumour cells produce type IV collagenase (Barsky et al., 1987). Therefore, the massive degradation of dermal equivalents, observed in my experiments, could be due to several reasons: firstly, due to the production of collagenase by infiltrative BCC cells, secondly due to a fibroblast-derived collagenase activity released either from BCC stromal cells or dermal equivalent fibroblasts upon interaction with BCC cells. The absence of dermal equivalent degradation using superficial and nodular BCC in comparison with the data using infiltrative BCC may support the observations of Barsky and colleagues (1987) indicating that the various subtypes of BCC differ in their degrading properties in vitro and may explain the more aggressive behaviour of infiltrating BCC clinically.

Grando and co-workers (1996) introduced a calcium shift from 0.9 to 1.6 mM to allow selective elimination of normal keratinocytes from the primary mixed-cell cultures of BCC. Although during incubation with high calcium concentration some cells flattened, elongated and stratified, they reported that the BCC cells neither cornified nor died and when returned to low calcium concentration they continued to grow. In my work, the observation of viable tumour cells after 11 days of BCC culture, although not proliferating, may allow future studies using dermal equivalents as a potential model for

BCC culture. This can be pursued further by the culture of pure BCC cells, derived primarily from superficial and nodular tumours, using dermal equivalents and the purifying technique of Grando and colleagues (1996).

Chapter 5: Conclusions

Conclusions

1. There was good agreement between the E- and P-cadherin, α- and β-catenin staining of the membranes in routinely processed, paraffin-embedded and frozen samples.

2. The restricted expression of P-cadherin to the basal cells of the normal epidermis may indicate a possible association with the maintenance of the epidermal proliferative compartment. Expansion into suprabasal layers in benign hyper-proliferation and pre-malignant lesions may indicate that this molecule is proliferation-associated rather than a marker for malignant transformation.

3. Double immunolabelling of E- and P-cadherin revealed co-localisation of both cadherins in the basal and first suprabasal layer of the normal epidermis. Such co-localisation may have properties in common concerning specific regulatory mechanisms. The co-localisation of E- and P-cadherin was taken to higher resolution which showed that they were not segregated in basal cell membranes which suggests that E- and P-cadherin could be in the same junction plaque.

4. The reduction in tumour cell membrane E-cadherin, α- and β-catenin in comparison with the normal epidermis is a characteristic in BCC and SCC, particularly those tumours with aggressive behaviour, but not in non-malignant lesions.

5. The decrease in membranous distribution of E- and P-cadherin, α- and β-catenin in superficial, nodular BCC, well-differentiated SCC and malignant melanoma progressed further in dedifferentiated and metastatic tumours in a direct proportion relationship.

6. The preserved E-cadherin membrane staining in the palisading cells of the BCC tumour lobules may indicate that E-cadherin is one of multiple biological factors involved in the prevention of free stromal invasion and metastasis. However, this function of E-cadherin, if present, is lost in both well- and moderately-differentiated

SCC hence there was no preservation of E-cadherin expression in the tumour cells at the peripheries of SCC lobules.

7. Downregulation or loss of E-cadherin but not of P-cadherin, particularly in BCC and SCC tumours with less aggressive behaviour, in addition to the double immunolabelling results of E- and P-cadherin in BCC may point to a stepwise loss of the cadherins during tumour progression in which E-cadherin may be lost first then P-cadherin loss follows when the tumour acquire very aggressive behaviour and is about to metastasise.

8. The detection of nuclear β-catenin staining in Bowen's disease and BCC in addition to pilomatrixoma is novel as it suggests that dysregulation of β-catenin cellular distribution may be common to several pathways leading to epidermal carcinogenesis.

9. The nuclear localisation of β-catenin in tumour cells of pilomatrixoma in comparison with the normal epidermis is characteristic of this tumour.

10. While activation of β-catenin by mutation may be a primary event in pilomatrixoma, it seems more likely that in BCC and Bowen's disease nuclear localisation of β-catenin would be secondary to activation of the Wnt pathway or possibly due to redistribution of β-catenin as a result of disruption in AJ.

11. Gli-1 nuclear localisation in BCC strongly supports the evidence which indicates that Gli-1 may be a transcriptional activator in which it provides evidence for the activation of the Shh/Ptc signalling pathways in this tumour.

12. The dramatic increase in actin filaments polymerisation (stress fibers) in infiltrative BCC when compared with the nodular subtype and normal epidermis indicates a relation to the infiltrative growth pattern of BCC rather than rapid growth. This was accompanied by decreased membrane staining of the cell adhesion molecules E-

cadherin, α- and β-catenin and α-actinin emphasising enhanced tumour cell motility and thus invasion.

13. The double immunolabelling results of normal skin and most tumour cells in nodular and infiltrative BCC have shown separate localisation of E- and P-cadherin, α- and β-catenin from actin filaments. However, a portion of E- and P-cadherin, α-catenin and α-actinin was co-localised with actin filaments at the plane of the plasma membrane. The co-localisation of α-catenin and α-actinin with the actin filaments could be explained by the interactive role of α-catenin where it connects the membrane associated complex (E-cadherin and β-catenin) to the actin-based cytoskeleton.

14. Results of keratinocyte culture indicate that the cells grown in low calcium were less motile (similar to nodular BCC) as they showed weak and diffuse actin staining and migrated into the wound but failed to close it completely, while the cells grown in high calcium were more motile as they showed increased actin filament polymerisation (similar to infiltrative BCC) and migrated in a sheet-like colony and closed the wound completely.

15. AJ were properly assembled in high calcium conditions hence the presence of strong E-, P-cadherin and β-catenin membrane staining when compared to the low calcium conditions.

16. The tight adhesion of cells in high calcium cultures may emphasise the possible important function of the cell-cell AJ in the maintenance of the structural integrity of the epithelial sheet.

17. The double immunolabelling results of E-, P-cadherin and β-catenin with TRITC-labelled Phalloidin in both low and high calcium keratinocyte cultures revealed separate localisation of these proteins from actin filaments implying an indirect association of these proteins with the actin-based cytoskeleton.

18. The observation of numerous small and bright structures of E-cadherin immunofluorescent staining, reminiscent of focal adhesion and not seen in a low calcium conditions, at the cell-substrate interface may suggest that the distribution of E-cadherin in keratinocyte cultures is different from that in normal epidermis.

19. The massive degradation of dermal equivalents could be due to several reasons: firstly, due to the production of collagenase by infiltrative BCC cells, secondly due to a fibroblast-derived collagenase activity released either from BCC stromal cells or dermal equivalent fibroblasts upon interaction with BCC cells.

20. The absence of dermal equivalent degradation using superficial and nodular BCC in contrast to the massive degradation using infiltrative BCC strongly suggest that the various subtypes of BCC differ in their degrading properties in vitro and may explain the more aggressive behaviour of infiltrating BCC clinically.

21. In general, the results of BCC culture were not satisfactory, however, modification of the technique used in my work may allow future studies using dermal equivalents as a potential model for BCC culture.

Chapter 6: Appendices

Appendix A

Routine tissue processing, embedding and section cutting

At the Department of Dermatology, University of Glasgow, the following steps are undertaken for routine specimen preparation, processing, embedding and cutting:

1. Tissue specimens are carefully identified and placed immediately into 10% formalin which is the routine fixative used in this department.
2. Usually, tissue specimens are left in the fixative solution for a minimum of 12 hours, but small specimens may only need 6 hours and larger specimens a longer period of fixation.
3. After fixation, the tissue specimen is removed from the container and double checked by the consultant dermatopathologist that it corresponded with the details given on the request form.
4. Tissue specimen is described macroscopically with rough diagram and details of the specimen cut up is produced according to the provisional diagnosis and macroscopic information.
5. Specimen is orientated in small plastic cassettes ready for processing.
6. Tissue processing (dehydration and clearing) is carried out overnight by the use of an enclosed pumped fluid type automated machine. Dehydration is the removal of aqueous fixative and any tissue water whereas clearing is the use of xylene which is totally miscible with both the dehydrating agent that precede it, and the embedding agent that follows it. Processor used: Shandon " Citadel 2000"

cycle used:	step 1	50% meths	1 hour
	step 2	80% meths	1 hour
	step 3	8% phenol meths	2 hours
	step 4	8% phenol meths	3 hours
	step 5	Alcohol	2 hours
	step 6	Alcohol	2 hours
	step 7	Alcohol	2 hours
	step 8	50% Alcohol/Histoclear	1 hour

step 9	Histoclear	1 hour
step 10	Histoclear	1 hour
step 11	Wax I	2 hours
step 12	Wax II	3 hours

(step 12 - Wax II is under vacuum)

7. Next morning, the tissue specimen is removed from the machine and embedded in paraffin wax at 60°C. Embedding of tissues is carried out using the Shandon Histocentre 2. Cassettes are removed from the tissue processor and placed in the holding pan of the Histocentre. There are three sizes of moulds which are used to accommodate the specimen. The mould is filled with wax and the specimen is orientated in the base of the mould. The tissue is placed with the face to be sectioned down and with the epidermis to the top (North Side) of the mould. The mould is sledded over to the cold plate and left to harden for approximately 15 minutes.
8. Paraffin sections are routinely cut within this department on a Leitz (UK) 1501 rotary microtome. Paraffin sections were cut as follows:-

- Excess wax should be removed from the tissue cassette to ensure that the specimen is held rigidly when inserted into the block holder.
- The cassette to be cut should be secured in the block holder and the section thickness required is set. For routine purposes sections are usually cut at 3 μm.
- Sections are cut by a clockwise rotation of the microtome arm, leaving either a single section or a ribbon of sections, resting on the knife.
- The sections are lifted by means of forceps and a section brush, and floated out on a water bath of fresh distilled water at 56°C.
- The sections are lifted from the water bath onto a glass slide, with the epidermis side to the right hand edge. This is accomplished by half immersing the slide in the water bath, just touching the floating section, and using an upward and slightly forward motion, lifting the section onto the centre of the slide.
- The slides are allowed to dry on a 60°C hot plate before being baked for 30 min at 80°C.

Appendix B

1. Coating slides with Silane

To prevent detachment of tissue from slides during pre-treatment and washing steps, glass microscope slides should be Silane coated. Slides were immersed in alcohol for 10 seconds then incubated in 1% Silane (Sigma chemical Co, St. Louis, Mo, USA) in acetone for 10-15 seconds and then immersed in acetone for 10 seconds. Finally, the slides were washed in running tap water tap for 1 min and incubated overnight at 37°C.

2. Staining in Vector VIP (chromagen) substrate kit for peroxidase

When the working solution of the Vector VIP substrate kit is prepared, it can be used for staining tissue sections or nitrocellulose, nylon, or other membranes. Vector VIP reagents are supplied in dropper bottles. It produces a purple reaction product which is also suitable for electron microscopy. The substrate solution should be prepared immediately before use on tissue sections in immunohistochemical staining.
The substrate solution is prepared as follows:

1. To 5 ml of TB pH 7.6, 3 drops of reagent 1 was added and mixed well.
2. Three drops of reagent 2 was added and mixed well.
3. Three drops of reagent 3 (chromagen) was added and mixed well.
4. Three drops of the hydrogen peroxide solution was added and mixed well.

Tissue sections were incubated with the substrate at room temperature until suitable staining has developed. Generally, 2-15 min provides good staining intensity. Tissue sections were then washed in running tap water for 5 min before counter-staining and mounting.

Appendix C

Preliminary experiments

Preliminary experiments with immunohistochemical staining of the antibodies used in this study (Table 2) in routinely processed, paraffin-embedded and frozen material in addition to keratinocyte culture were carried out to reach the final and the best staining results. Briefly, these were:

1. Routinely processed, paraffin-embedded tissue:

First, the immunoperoxidase staining of the antibodies used in this work was undertaken in untreated sections of normal skin. Several antibody dilutions, incubation times and temperatures were tested. The staining results were patchy with frequent over-stained cells and high background staining. Similar results were obtained when the experiments were repeated using routinely processed sections of tumours. Accordingly, the staining of the antibodies was conducted on pre-treated sections. The pre-treatment of the sections (see methods) was by one of the following methods:

- Subjecting sections to microwaving for 10, 20, and 30 min in 10 mM citrate buffer pH 6.0 or in Tris buffer at pH 9.
- Enzymatic digestion using 0.1% pronase Type XV in TB pH 7.6 for 15-20 min at room temperature or 0.1% Trypsin Type II-S and 0.1% calcium chloride in distilled water for 30 min at 37°C.
- Extraction with 0.5% (v/v) Triton x-100 in DPBS for 5-10 min using gentle agitation.

Variable concentrations of the antibodies listed in Table 2 were tried. In addition, several incubation times and temperatures and different normal sera (rabbit, horse, swine and human) were tested with each pre-treatment method. The staining quality has improved slightly particularly with the microwaving for 30 min in which more specific staining was obtained. The best results were obtained with superheating using pressure cooking which proved to be effective and reliable method of antigen retrieval showing

several advantages over other antigen retrieval methods (see table 3 for final conditions). In addition, the use of the antibodies (E- and P-cadherin, β-catenin and Gli-1) with pressure cooking followed by immunoperoxidase staining of these antibodies in routinely processed, paraffin-embedded material will allow the use of the archival tissue for future studies of these antibodies in skin and other tissues.

2. Frozen tissue and keratinocyte culture:

The immunoperoxidase staining of E-cadherin and β-catenin in untreated frozen BCC revealed good results particularly after fixation in fresh acetone for 1 hour at -20°C and incubation overnight at 4°C. Although the antibody concentrations and the incubation times and temperatures of E-cadherin and β-catenin were different in each type of tissue used (Table 3), there was good agreement between results on routinely processed tissues and on freshly frozen material of BCC. However, for E-cadherin and β-catenin staining in keratinocyte cultures, sections needed to be fixed in 10% formalin and extracted with 0.5% (v/v) Triton x-100 in DPBS for 5-10 min using gentle agitation for better results. Staining of other antibodies (P-cadherin, α-catenin, α-actinin and Phalloidin) in frozen sections of BCC and keratinocyte cultures was performed primarily in untreated sections. The results were unsatisfactory. The main pitfall was the presence of many over-stained cells. This was resolved by fixing the sections for P-cadherin and α-catenin in fresh acetone for 1 hour at -20°C and those for α-actinin and phalloidin in 10% formalin. In addition, all sections were extracted with 0.5% (v/v) Triton x-100 in DPBS for 5-10 min using gentle agitation (Table 3).

Appendix D

Staining of vinculin, α-actinin and APC

To investigate the possibility that vinculin, α-actinin and APC could be demonstrated in paraffin-embedded and cryostat sections of normal epidermis and BCC, several antigen retrieval methods were tested. These included (for formalin-fixed, paraffin-embedded sections only) pressure cooking either in a 10 mM TB pH 9 or in 10 mM CB pH 6, microwaving for 10, 20, and 30 min in the same buffers, enzymatic digestion using 0.1% pronase Type XV in TB pH 7.6 for 15-20 min at room temperature, extraction with 0.5% (v/v) Triton x-100 in DPBS for 5-10 min using gentle agitation (for both formalin-fixed, paraffin-embedded and cryostat sections) and using no pre-treatment. Variable antibody concentrations, incubation times and temperatures and different sera were tested.

The main pitfall of the immunostaining results was the associated massive background staining involving the dermal collagen bundles, smooth muscles and adipose tissue. Several attempts of technical modifications were carried out to reduce the background staining. Extended times of incubating the sections with 3% hydrogen peroxide solution and normal rabbit and horse sera at different dilutions were tried. In addition to manipulating the antibody concentrations, incubation times and temperatures; the incubation time with the peroxidase reaction developer (peroxidase substrate kit) was reduced to the minimum developing time. The same methodology was repeated again after obtaining new batches of vinculin and α-actinin antibodies.

Unfortunately, all these technical alterations were not successful in reducing vinculin, α-actinin and APC background staining. However, after 10 experiments, the best results of APC staining in formalin-fixed, paraffin-embedded BCC were obtained with no pre-treatment, using the standard Extra-avidin method, antibody concentration at 1.0 μg/ml and incubation O/N at 4°C. Although few cases were examined, cryostat sections of BCC fixed in fresh acetone for 1 hour at - 20°C and extracted with 0.5% (v/v) Triton x-100 in DPBS for 5-10 min using gentle agitation have shown similar APC staining results.

In all cases, the staining pattern of APC in normal epidermis adjacent to the tumour, was heterogeneous. All cases have shown weakly positive and diffuse cytoplasmic staining of APC throughout epidermal layers. Stratum corneum was negative.
Ten of 32 of the cases showed patchy but strongly positive APC staining which was often enriched at cell-cell junctions particularly in spinous and granular cells towards the specimen margins (Fig 134A). Immunofluorescent staining gave similar results (Fig. 135).

Similar findings have recently been reported by Midgley and colleagues (1997). In 20/32 of the examples, same pattern of APC staining was observed but in reduced intensity. Very weak cytoplasmic APC staining was noted in the basal cell layer. In addition, the staining of APC in the outer cells of sweat and sebaceous glands was largely cytoplasmic whereas outer root sheath cells showed heterogeneous staining as described above in normal epidermis. No APC nuclear staining in normal epidermal and adnexal cells was seen. The staining in the tumour cells was cytoplasmic (Fig. 134B) in almost half of the cases whereas it was negative in 17/32 of the cases.

The inconsistency and the different outcome of the immunohistochemical staining results of vinculin, α-actinin and APC in normal epidermis and BCC cast a doubt about how genuine is the staining in each experiment. Therefore it is inappropriate to draw any conclusions based on these results.

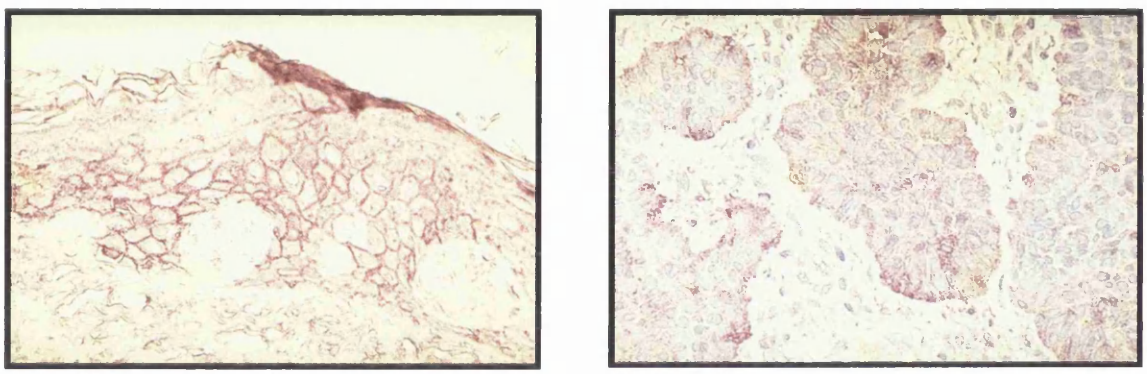


Figure 134. APC staining in normal epidermis and nodular BCC. (A) strong APC staining in normal epidermis adjacent to tumour with enrichment at cell-cell junctions particularly in spinous and granular cells. (B) Weak cytoplasmic staining of APC in tumour cells. x500.

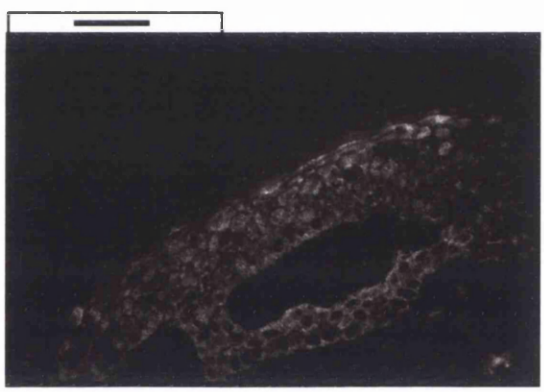


Figure 135. Immunofluorescence staining of APC in normal epidermis adjacent to tumour. Weak staining with enrichment at cell-cell junctions. Scale bar =100μm.

Appendix E

Presentations arising from this thesis

- **Poster presentations**

1. BSID meeting (April, 1998- Liverpool)

IMMUNOSTAINING OF β-CATENIN AND E-CADHERIN IN PARAFFIN EMBEDDED AND FROZEN BASAL CELL CARCINOMA. A.Al-Myouf, M.B. Hodgins, R.M.MacKie, Dept. of Dermatology, University of Glasgow.

The Sonic hedgehog (SHH)/Patched (PTC) signalling pathway is dysregulated in Basal cell carcinoma (BCC). During development there is interaction between SHH/PTC and Wingless (Wnt) pathways. β-Catenin which associates with E-cadherin in cell-cell junctions, is a key component of the Wnt pathway. Activation by Wnt redistributes β-catenin to the nucleus to form a complex with T cell factor (Tcf) and lymphoid enhancer factor (Lef) transcription factors. The aim of this study was to investigate the cellular distribution of β-catenin and E-cadherin in BCC and whether the Wingless pathway is activated.

We demonstrated by immunohistochemistry, using monoclonal antibodies and microwave antigen retrieval, the distribution of β-catenin and E-cadherin in paired paraffin-embedded and frozen specimens of 12 BCC and in a further 20 paraffin-embedded BCC. The results have shown uniform membrane staining of β-catenin and E-cadherin in normal epidermis in all cases. E-Cadherin staining was reduced in tumour cell membranes in 30/32 paraffin-embedded cases (93.8%) and in all the frozen samples. Tumour cell membrane staining of β-catenin was reduced in 29/32 paraffin-embedded cases (96.6%) and in all the frozen samples. There was good agreement between staining of membranes in paraffin-embedded and frozen samples. We observed uneven membrane staining of β-catenin and E-cadherin within the tumour lobules compared with the normal epidermis ranging from reduced staining intensity to

complete absence. We also observed variable degrees of diffuse cytoplasmic distribution of both antibodies in normal and tumour cells.

Our data has shown that reduction in tumour cell membrane β-catenin and E-cadherin in comparison with the normal epidermis is a characteristic in BCC, although it is not yet clear whether the decrease in membrane β-catenin is accompanied by a redistribution to the cell nucleus.

2. BSID meeting (April, 2000-Edinburgh) (submitted)

NUCLEAR β-CATENIN IS CHARACTERISTIC OF PILOMATRIXOMA. A.Al-Myouf, M.B. Hodgins and R.M.MacKie, Department of Dermatology, University of Glasgow.

Wnt and Sonic hedgehog (Shh) signalling are important in development of epidermis and appendages. Dysregulation of Shh has been shown to underlie basal cell carcinoma (BCC) but the role of Wnt signalling in epidermal tumourigenesis remains unclear. Recently, mutations in β-catenin were found in the hair follicle tumours pilomatrixoma (PMX) and trichofolliculoma. In addition to its function in intercellular adhesion, β-catenin is a key component of the Wnt pathway. Activation by Wnt redistributes β-catenin to the nucleus to form a complex with T cell factor (Tcf) and lymphoid enhancer factor (Lef) transcription factors. In an earlier study we found evidence for redistribution of β-catenin to the nucleus in only a small proportion of BCC.

The aim of this study was to compare the cellular distribution of β-catenin in paraffin-embedded BCC and PMX by immunohistochemistry with monoclonal antibodies to the N and C terminal domains. There was uniform membrane staining of the N and C termini of β-catenin in normal epidermis immediately above the tumour mass in all cases. BCC showed reduced membrane β-catenin compared with the normal epidermis in most cases (30/32 N- and 29/32 C- termini) and variable, weak nuclear staining in a minority (8/32 N-terminus, 13/32 C-terminus). Tumour cell membrane staining of N

and C termini of β-catenin was evenly reduced in 4/7 PMX but there was strong nuclear staining with both the N and C terminal antibodies in all 7 tumours. There was also a variable degree of diffuse cytoplasmic distribution of both termini in normal and tumour cells.

Our data has shown that nuclear localisation of β-catenin in tumour cells in comparison with the normal epidermis is characteristic of PMX. The accompanying decrease in membrane β-catenin, in some cases, may indicate a redistribution from cell membrane to the nucleus. These results provide evidence that Wnt signalling may be activated in some BCC but that this pathway is of particular importance in PMX.

- **Oral presentation**

Presented at the Skin Biology Club, Falkirk, May (1999) A. Al-Myouf, M.B. Hodgins and R.M. MacKie. Localisation of β-catenin in normal skin and basal cell carcinoma.

Chapter 7: References

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