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Neuronal Differentiation Markers in Basal Cell Carcinoma

Sinclair Gore BA (Hons) MB BChir, MRCS

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The RAFT Institute of Plastic Surgery
Mount Vernon Hospital
Northwood, Middlesex
&
The Centre for Cutaneous Research
Newark Street, Whitechapel, London

Scientific supervisor: Professor M. P. Philpott
UCL clinical supervisor: Mr. R. Grover

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

DECLARATION OF ORIGINALITY OF THIS RESEARCH

I hereby declare that the work presented in this thesis is entirely my own.

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Sinclair M. Gore
August 2006

Abstract

Basal cell carcinoma (BCC) is the most common skin cancer in humans. The demonstration of genetic and protein alterations has, so far, had limited correlation with either biological behaviour or histological classification of these tumours.

It was observed that Gli1-overexpressing keratinocytes express elevated levels of genes known to be associated with neuronal development, including *β-tubulin III*, *GAP-43*, *Arc* and *neurofilament*. It was proposed that these genes may similarly be overexpressed in BCCs and that different levels of expression may be present in different BCC subtypes

Immunohistochemistry of BCCs demonstrated that neuronal differentiation marker proteins are expressed in BCCs, but that this expression is significantly reduced in tumours that behave aggressively.

Elevated neuronal differentiation marker gene expression was shown in BCCs. Again, expression was more prominent in tumour types that behave indolently. Results were obtained for tumour samples processed by laser capture microdissection, needle microdissection and homogenised tissue.

Expression of neuronal differentiation marker genes in Gli-overexpressing keratinocytes was examined by semi-quantitiative PCR. Neuronal differentiation marker expression was associated with *Gli1* and *Gli2* over-expression in some cases (*β-tubulin III* and *Arc*). *Gli1* and *Gli2* also promoted the expression of each other in a positive-feedback loop.

Expression of these markers was examined in archival tumours for which the clinical outcome was known in terms of recurrence. In completely excised tumours β-tubulin III was significantly reduced in tumours that went on to subsequently recur. Other markers were not expressed in significantly different amounts.

In summary, I have shown that expression of markers associated with neuronal development is a feature of Basal Cell carcinoma, and that the expression of these markers correlates strongly with the tumour histological subtype but only weakly with tumour recurrence. More work will be required to discover further alterations in BCC molecular biology that impact significantly on tumour behaviour.

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Abbreviations

AGG	Aggressive
ANOVA	Analysis of Variance
APS coated	Aminopropylsilane coated
BCC	Basal Cell Carcinoma
bp, Kbp	base pairs, kilobase pairs
BT	β-tubulin III
BT-III	β-tubulin III
c(t), Δc(t)	cycle to threshold number, cycles-to threshold difference
Ca^{2+}	calcium
CDK	cyclin-dependent kinase
cDNA	complementary DNA
Ci	Cubitus interruptus
CI	Confidence interval
CO / CR / CN	Completely-excised original / recurrent / non-recurring
DAB	3-diaminobenzidinetetrahydrochloride
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EGFP	Enhanced green fluorescent protein
F	Female
FOX	Forkhead Box
g, mg, µg, ng,	gram, milligram, microgram, nanogram
GAP-43	Growth-associated protein 43
GST exzymes	Glutathione-S-transferase
h	hour
HH	Hedgehog
H_20	water
IND	Indolent
INF	Infiltrative
IO / IR / IN	Incompletely-excised original / recurrent / non-recurring
KC	Keratinocyte
kD	kilodalton
L	Large
LCM	Laser Capture Microdissection
M	Medium or Male (context dependent)
l, ml(s), µl	litre(s), millilitre(s), microlitre(s)
mm	millimetre(s)
mM / M	millimolar / molar
mRNA	messenger RNA
MN	Micronodular
MOR	Morphoeic
NBCCS	Naevoid Basal Cell Carcinoma Syndrome
NF	Neurofilament
NOD	Nodular
NS1 / NS2	Normal Skin (1 or 2)
°C	degrees centigrade
ORS	Outer root sheath
PBS	Phosphate buffered saline

PTCH / *patched*	patched
RNA	ribonucleic acid
RNase	ribonuclease
RT-	Reverse transcriptase negative
RT-PCR	reverse transcriptase polymerase chain reaction
S	Small
SCC	Squamous cell carcinoma
SE	Standard error
SHH / *Shh*	Sonic Hedgehog
SIA	Spectral image analysis
SMO / *Smo*	Smoothened
SUFU / *Sufu*	Suppressor of fused
SUP	Superficial
Tcf/ LEF	T-cell factor / Lymphoid Enhancer Factor
TGF-β	Transforming growth factor beta
ULK1	Unc51.1-like kinase
UV	Ultraviolet
v/v	unit volume per volume
VAS	Visual analogue score

Presentations and publications

I have presented work described in this thesis at the following scientific conferences:

- British Association of Plastic Surgeons, Eton, July 2005 (winner of John Calder Medal for the best scientific presentation of the conference)
- British Association of Dermatologists, Glasgow, July 2005
- British Society of Investigative Dermatology, Cambridge, April 2005
- 10th World Congress of Cancers of the Skin, Vienna, May 2005
- European Congress of Scientists and Plastic Surgeons (ECSAPS), Leuven, September 2005
- European Society of Dermatological Research (ESDR), Tubingen, September 2005

Work presented in this thesis has also been published or submitted for publication:

- "Neuronal differentiation as a feature of Basal Cell Carcinoma" submitted to Cancer Research, August 2006

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Chapter 1: Introduction

Section 1: Clinical and histological aspects of Basal Cell Carcinoma

The World Health Organisation Committee on the Histological Typing of Skin Tumours has defined Basal Cell Carcinoma (BCC) as a group of malignant cutaneous tumours characterised by the presence of lobules, columns, bands or cords of basaloid cells ("germinative cells") (LeBoit P.E. et al., 2006). Many authors have synonymously used the more benign-sounding words epithelioma and basalioma, but in this thesis the term BCC shall be used exclusively.

1.1 Histogenesis of BCC

Historically there has been much debate about the site and cell of origin of BCCs. The main debate questions whether these tumours arise from cells of adnexal structure (and particularly hair follicle) origin, or whether they arise from non-adnexal epidermal cells.

The search for the cellular origin of these tumours has focussed on stem cells which may retain the ability to develop into tumours. Several cell types have been suggested to be the precursor cells or stem cells for BCC: interfollicular basal keratinocytes, basal keratinocytes from hair follicles or sebaceous gland cells (Kruger et al., 1999; Potten and Morris, 1988; Zackheim, 1963). In general, stem cells have a relatively undifferentiated and slow-cycling phenotype, but they can be stimulated to proliferate and give rise to transient amplifying cells which have a limited proliferative potential (Miller et al., 1993b). Stem cells may be the target of carcinogens and as such play an important role in tumorigenesis. One observation suggesting that uncommitted stem cells are the most likely cells of origin for human skin cancer is the fact that sunlight exposure in childhood may contribute to tumours many decades later (Brash et al., 1991).

As first suggested by Cotsarelis and refined by Taylor et al. the ultimate source of stem cells in the skin is the bulge region of the outer root sheath of the hair follicle

(Cotsarelis et al., 1990; Taylor et al., 2000). As a result, hair follicles are likely to play an important role in skin homeostasis, wound healing and tumourigenesis. Many lines of evidence support this theory. Histologically, BCCs may resemble hair follicles (Miller, 1991) and may show characteristics from both bulge region stem cells and transient amplifying cells (Kore-eda et al., 1998). In particular, BCC can histologically resemble trichoepithelioma, a benign hair follicle tumour (Walsh and Ackerman, 1990). The suprabulbar region of the ORS of the hair follicle has an immunohistochemical profile that is almost indistinguishable from that of a BCC (Asada et al., 1993; Kruger et al., 1999). The hair follicle hypothesis is further supported by the fact that when a carcinogen is added in the anagen phase, in which the hair follicle bulge region cells undergo transient amplification, BCCs are generated more frequently (Miller et al., 1993a). Furthermore, BCCs seldomly occur on non-hairy skin (Kore-eda et al., 1998). Support for the hair follicle hypothesis can be found in the expression of the basal cell adhesion molecule (B-CAM) in normal and diseased skin as well as the ORS of the hair follicle and BCCs (Bernemann et al., 2000).

There is limited evidence to suggest that BCCs originate from interfollicular epidermis. The lack of cytokeratin-15 expression in the tumour cells suggests that BCCs do not differentiate towards a hair bulge cell fate (Kanitakis *et al.*, 1999). Markey et al have shown that keratin subtyping of BCCs is similar to the profile shown by interfollicular stem cells which are predisposed to follicular differentiation (Markey *et al.*, 1992).

Overall, the current understanding of BCC molecular genetics as outlined above tends to support the idea that the hair follicle stem cell is the progenitor cell of the BCC. In all, it seems as if the cell of origin of BCC is a hair follicle stem cell in which the normal differentiation and anagen-initiation programme has gone awry.

1.2 Incidence of BCC

As well as being the commonest form of skin cancer, BCC is assumed to be the commonest malignant tumour of all in Caucasians (Miller, 1991) but is not as news-worthy as other malignancies due to its negligible mortality rate. It has been estimated that up to 80,000 cases occur annually in the U.K. and up to 500,000 cases annually in the USA (Gloster, Jr. and Brodland, 1996; Holme et al., 2000). Some of the discrepancy between these two countries' rates may be accounted for by improved data recording in the U.S. as a result of their healthcare economy. It certainly seems that incidence rates are increasing over time, with Ko et al reporting that the UK incidence of the disease increased by 235% between 1978 and 1991 (Ko *et al.*, 1994). This has mostly been attributed to increased exposure to ultraviolet radiation and may be due to both altered personal habits and the reduced degree of environmental protection afforded to us following atmospheric ozone depletion. The cost of treatment of non-melanoma cancer as a whole has been estimated as being among the top five most expensive cancers to treat in the USA (Housman *et al.*, 2003).

1.3 Aetiology of BCC

1.3.1 Environmental risk factors

1.3.1.1 Sun exposure

There is a vast amount of aetiological evidence suggesting that sun exposure is the principal cause of Basal Cell Carcinoma. Firstly, the location of BCCs on the body matches closely with the areas that get maximum sun exposure, with 85% occurring on the head and neck, and 30% on the nose alone (Miller, 1991). Since then it has been suggested that there may be an increasing trend of BCCs occurring on the upper trunk in younger patients, reflecting altered habits of clothing and sunbathing (Marks, 1995).

Secondly, the incidence of BCC drops off markedly with increasing distance from the equator. Extremes of incidence were measured by Stone et al with rates in Hawaii (692 cases per 100,000 population per year) more than sixty times those in Iceland (10 per 100,000) (Stone *et al.*, 1986). The "world leader" is Australia, with male and

female incidence rates in Queensland measured respectively as 849 and 605 cases per 100,000 population per year in 1993 (Marks et al., 1993).

Thirdly, there is a strong association with duration of exposure to the sun. What is unclear, however, is how specific the timing of that exposure is for BCC. It was discovered that non-melanoma skin cancer is more common in outdoor workers who spend a greater proportion of time in the sun (Marks *et al.*, 1989). Recreational sun exposure seems to predispose to BCC development (Corona *et al.*, 2001). Sunlight exposure in childhood certainly seems to predispose for BCC later in life – both Marks and Gallagher demonstrated that there is significant association between childhood exposure and sunburn and BCC in later life (Gallagher et al., 1990; Marks, 1995).

1.3.1.2 Other radiation

Prior non-diagnostic X-ray treatment for skin conditions has been associated with increased risk for BCC development (Gallagher *et al.*, 1996). Nuclear radiation following atomic warfare has been shown to increase subsequent rates of BCC development, whereas no data exists to date as to whether high dose radiation from industrial accidents such as that in Chernobyl has similar effects (Sadamori et al., 1991). Exposure to UVA-treatment (PUVA) combined with use of psoralens in psoriasis patients has been shown to not significantly alter the rate of BCC development (Stern, 2001).

1.3.1.3 Chemical carcinogens

Boyd et al. recently proved that an association exists between smoking and BCC in young women (Boyd et al., 2002). Furthermore, Ergbaci et al showed that smoking is associated with the development of more aggressive morpheaform BCCs (Erbagci and Erkilic, 2002). Intake of alcohol has been reported to be associated with development of BCC in one recent study (Fung et al., 2002). Diepgen and Mahler found that other chemical carcinogens such as arsenic and coal tar products increase the risk of non-melanoma skin cancer, but these increases are mainly in development of squamous cell carcinoma (Diepgen and Mahler, 2002).

1.3.2 Constitutional Risk Factors

1.3.2.1 Skin colouring

99% of persons who develop BCC are white. Epidemiological studies suggest that it is the photoprotective effects of melanin that account for this increased protection in those with darker skin types. There is increased risk of BCC development in those with blue eyes and fair complexion, who sunburn easily, tan poorly, freckle with sun exposure, have red or blond hair, or are of Celtic ancestry (Zanetti *et al.*, 1996). BCC is rare in blacks but as in whites it occurs on the sun exposed areas. Blacks have a decreased incidence of BCC on sun exposed areas but have the same incidence of BCC on covered areas (reviewed by Gloster, Jr. and Brodland, 1996). Most BCCs in black people are pigmented which can lead to delayed diagnosis. Multiple BCC is rare in blacks and usually occurs in patients with Gorlin's syndrome.

1.3.2.2 Melanocortin gene receptor variation

The melanocortin 1 receptor (MC1R) is part of the system by which the body attempts to control photoprotection by increasing levels of melanin in the skin. Genetic variants of this receptor cause red hair and fair skin and have also been linked to an increased susceptibility for development of both melanoma and non-melanoma skin cancers (Bastiaens *et al.*, 2001; Box *et al.*, 2001). Although adding this genotypic information to phenotypic information (red hair, light skin colour) has been shown to improve the prediction of risk of skin cancer development, the improvement was too small to be of routine clinical use (Dwyer *et al.*, 2004).

1.3.2.3 Immunological factors

1.3.2.3.1 Pharmacological immunosuppression

SCC occurs more frequently than BCC in transplant patients undergoing pharmacological immunosuppression (Ondrus *et al.*, 1999) and although 40% of renal transplant recipients develop skin cancer within 20 years after grafting (Hartevelt *et al.*, 1990), no increased incidence of BCC has been described in organ recipients and it seems that immunosuppression as practised after organ transplantation does not increase the risk of developing BCC.

1.3.2.3.2 Human immunodeficiency virus (HIV)

People suffering from acquired immune deficiency syndrome (AIDS) have shown an elevated risk for the development of BCC (Franceschi *et al.*, 1998). There have been reports of BCCs metastasizing in people suffering from AIDS (Sitz et al., 1987), suggesting that immune surveillance is one of the factors determining the normally non-metastatic nature of the BCC.

1.3.2.4 Cancer Syndromes which predispose to BCC

A number of conditions exist where a genetically inherited syndrome predisposes to development of cancer and some of these particularly affect the development of BCC. The investigation of these diseases has provided great insight into the mechanism of dysregulation of cancer control.

1.3.2.4.1 Nevoid Basal Cell Carcinoma Syndrome (Gorlin's syndrome)

Gorlin's syndrome is a familial cancer syndrome that is inherited in an autosomal dominant fashion. It predisposes sufferers to the development of BCCs from a very young age, and it is not uncommon for these patients to accumulate thousands of BCCs in their lifetime. A significant number of cases have no family history and this therefore suggests a high rate of spontaneous mutation. Rather than generalised disease, segmental or unilateral disease has been described, reflecting the possibility of a single cell mutation very early in embryonic development (Gutierrez and Mora, 1986).

Gorlin and Goltz initially described the syndrome (Gorlin and Chaudary, 1960); sufferers not only have to contend with a life of BCC treatments but also they have a predisposition to other more serious cancers including medulloblastoma, meningioma and fibromas affecting the heart or ovaries (Bale, 1997). Non-malignant features include skeletal abnormalities such as odontogenic keratocysts, bifid ribs, frontal bossing and polydactyly (Gorlin, 1987). Characteristic pitting of the palms and the soles is not commonly seen but is pathognomonic for the syndrome. Expression of these features varies within families and on top of the primary genetic defect this variation probably represents modifier genes or environmental factors that differ in each patient.

The genetic mutation responsible for Gorlin's syndrome was mapped in the mid 1990's to chromosome 9q22-31 and it codes for PTCH, the human homologue of the drosophila polarity gene, patched (Farndon *et al.*, 1994). PTCH is thought to act as a tumour suppressor gene, and this mutation at this site has been shown to not only occur in syndromic BCCs but also in a significant proportion of sporadic BCCs (Gailani *et al.*, 1996). The genomic basis of this condition and its effect on BCC development will be more fully described in part two of this introductory chapter (section 1.9).

1.3.2.4.2 Xeroderma Pigmentosum (XP)

This uncommon disease (USA prevalence is estimated at 1:250,000) is inherited in an autosomal recessive fashion. Patients are extremely sensitive to sunlight and experience a 2,000 fold increase in sun-related skin cancers.

The disease is due to defective DNA repair mechanisms which result in inadequate nucleotide excision and repair following ultraviolet-induced DNA damage. Each disease subtype is determined by the specific enzyme that is deficient within the repair pathway, and this confers a spectrum of aggression of disease within the disease as a whole. It is this repair system that is responsible for the restoration of DNA structure following sunlight damage-induced pyrimidine dimer formation (as discussed in section 1.3.3.2.1).

1.3.2.4.3 Bazex syndrome

This is a rare X-linked syndrome that not only predisposes individuals to multiple BCCs at an early age, but also involves marked abnormalities of hair follicle growth resulting in hypotrichosis and hypohidrosis. So-called "ice pick scars" are pathognomonic for this disease. The link between BCC formation and hair follicle abnormalities is particularly interesting because of the possible origins of BCCs from hair follicles.

1.3.2.4.4 Rombo syndrome

Rombo syndrome is clinically very similar to Bazex syndrome, including the predisposition to BCC development. Slight differences include the striking degeneration of elastic fibrils in sunlight-exposed areas causing dramatic skin

alterations called atrophoderma vermiculatum. It appears to be inherited in an autosomal dominant fashion.

1.3.2.4.5 Muir-Torre Syndrome

This syndrome was first described by Muir in 1967 and is characterised by the presence of one or more adnexal skin neoplasms (such as BCC) in association with an internal malignancy such as colonic carcinoma (Muir et al., 1967). The clinical and pathological features overlap with the hereditary non-polyposis colorectal cancer syndrome (Kruse and Ruzicka, 2004), with characteristic microsatellite instability and mutated MSH2 mismatch repair gene present in both cases.

1.3.2.5 Skin lesions which predispose to BCC

Prior to development of definitive BCCs, there are no proven precursor (premalignant) lesions that lead to the tumours. This contrasts with the case of SCC, which is clearly preceded in many cases by actinic keratoses or Bowen's disease (carcinoma-in-situ). There are, however, two particular lesions that seem to predispose to BCC development and a number of reports of less specific associations, such as the development of BCCs in scars.

1.3.2.5.1 Fibroepithelioma of Pinkus

This was first described by Pinkus in 1953 as a "pre-malignant fibroepithelioma". It is a tumour typically composed of thin anastomosing strands of basaloid or squamous cells, sometimes only two cells thick, surrounded by abundant stroma. They appear as flesh coloured tags and are most common on the trunk and extremities. There seems to be an increased risk of BCC development in these lesions, but recent debate has taken place as to whether these lesions are really fenestrated trichoblastomas or trichoblastic carcinomas (Ackerman and Gottlieb, 2005; Bowen and LeBoit, 2005).

1.3.2.5.2 Naevus sebaceous of Jadassohn

These lesions are congenital hairless plaques that are most commonly found on the scalp, face and neck. They have the propensity to develop into basal cell carcinoma; the rates of malignant transition have been reported as ranging from 6% to 50% of cases (reviewed by Santibanez-Gallerani et al., 2003).

1.3.2.5.3 BCC formation in scars

BCCs have been reported in surgical scars, burn scars, tattoos, vaccination scars, and chronic ulcers, although it is unclear whether the scarring process has a promoting effect on BCC development (Earley, 1983; Ewing, 1971; White, 1983).

1.3.3 Ultraviolet radiation and its effects

1.3.3.1 Damage caused by UV radiation

Ultraviolet radiation is classified by its wavelength into UV-A (wavelength 320-400nm), UV-B (280-320nm) and UV-C (200-280 nm). Generally, the shorter the wavelength, the more damaging the radiation is to the human body. The majority of high energy UV-C is filtered out within the earth's atmosphere, although in certain parts of the world, the concentration of the ozone partly responsible for this is thought to be diminishing (Madronich and De Gruijl, 1994). Of the remaining ultraviolet radiation which penetrates the earth's atmosphere and which we are exposed to (UV-A and UV-B), it is generally believed that UV-B is the principal carcinogen in sunlight. However, lower energy UV-A may play a greater role than previously realised.

UV radiation is believed to have both direct and indirect mechanisms of inducing damage which can lead to malignant change. At shorter wavelengths important molecules such as nucleic acids absorb ultraviolet radiation (UV-C and UV-B). Longer wavelengths of radiation (UV-A) induce damage by more indirect means, with absorption by cellular chromophores (such as porphyrins, quinones and flavins) resulting in the production of active intermediates such as hydroxyl radicals and superoxide ions. These direct and indirect mechanisms cause damage to DNA, mitochondria and membranes. Therefore protection against ultraviolet-induced cellular damage consists of UV exposure reduction, DNA repair and antioxidant pathways.

1.3.3.1.1 DNA Damage

DNA is made up of pyrimidine bases (cytosine, "C", and thymine, "T") and purine bases (guanine, "G" and Adenine, "A"). Adenine bases are bound by intermolecular forces to a corresponding thymine and guanine is similarly linked to cytosine. These

combinations are wound in a double anti-clockwise helix. The maximum absorption of ultraviolet radiation occurs at a wavelength of 260nm. The photons absorbed tend to be absorbed at the double bond joining adjacent pyrimidines, allowing the helix to open up. At "hot-spot" sites where adjacent pyrimidines exist (C's and T's), cyclobutane dimers may result, or alternatively pyrimidine-pyrimidone 6-4 photoproducts may be produced (reviewed by Mitchell and Nairn, 1989). Both products lead to an abnormal configuration of the DNA structure.

Because of the "A-rule", where DNA polymerase typically inserts an adenine residue at bases it cannot interpret, a pairing with thymidine (T) is produced after another round of replication. The overall effect is one of a C→T substitution and ten per cent of these changes occur at adjacent cytosine molecules, giving rise to the characteristic UV signature CC→TT base substitution. This process is summarised in Figure 1.1. The result of these base substitutions is damage to genes such as *TP53*, *ptch* and *Ras*. *TP53* damage is particularly significant since this can contribute to malignant transformation - the p53 damage response protein is involved in initiation of cell cycle arrest, DNA damage repair and apoptosis; in the absence of p53 cells do not apoptose when exposed to UV radiation. It may also act as a promoter of transformation (since cell cycle arrest and DNA repair is not accomplished). This results in the accumulation of further DNA damage.

Other changes in the cell as a result of UV exposure include membrane damage (UV-A and UV-B can cause lipid peroxidation altering the cell permeability and transport systems), protein damage (including antioxidant enzymes and DNA repair enzymes), cytokine modulation and immune modulation.

1.3.3.1.2 Alteration of immune function and relation to UV susceptibility

Langerhans cells (acting as antigen-presenting cells) in the skin, together with the regional draining lymph nodes that serve them, have been labelled "skin associated lymphoid tissues" (SALT) and it is thought that UV irradiation affects this system because of direct DNA damage (Streilein, 1983). The involvement of the immune system in human skin carcinogenesis is suggested by the increased risk of malignancy in immunosuppressed patients.

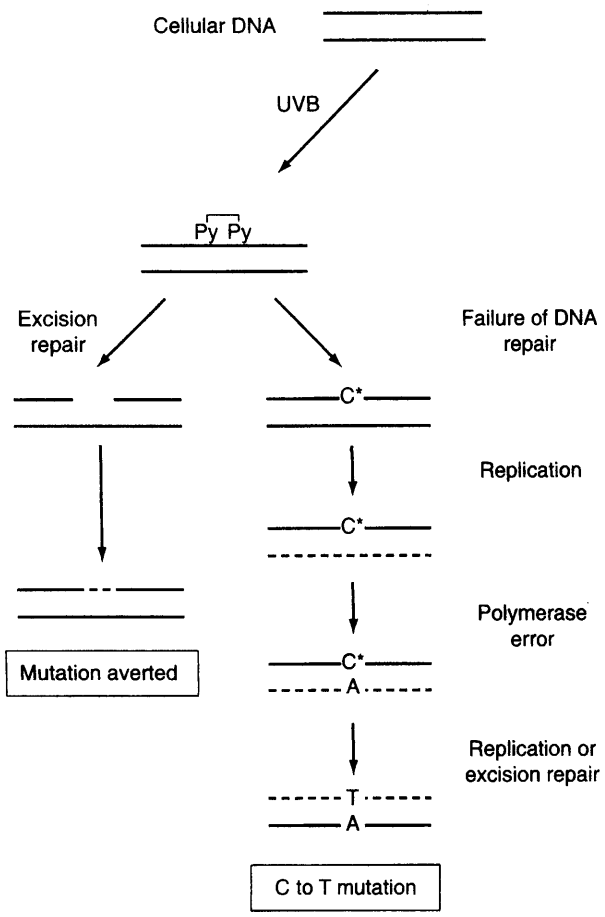


Figure 1.1. The mechanism of UV induced CC-TT substitution. Following the UV-induced creation of pyrimidine dimers, the process of excision repair attempts to excise the abnormal DNA and repair the defect with newly synthesised DNA (dotted line) with the complementary strand serving as the template. In the absence of repair, the polymerase enzyme leaves a gap opposite the lesion (asterisk) during replication. Incorrect filling in with A causes mutation in the complementary strand in the subsequent round of replication, resulting in C→T mutations (after Grossman and Leffell, 1997)

1.3.3.2 Normal defence against UV radiation damage, and how it fails

1.3.3.2.1 Normal DNA repair and how its failure promotes BCC formation

DNA repair mechanisms exist to repair the damage done to DNA (including proto-oncogenes and tumour suppressor genes) by carcinogenic insults. The variation in DNA repair mechanisms may explain part of the varied susceptibility to these tumours in certain individuals.

There are a number of principal routes of DNA repair. The **mismatch repair system** acts on single base mismatches and small displaced loops of 4-5 base pairs: colonic carcinomas in patients with Hereditary Non-Polyposis Coli Carcinoma (HPNCC) have been shown to have derangements in genes within this pathway (Chung and Rustgi, 2003). The **nucleotide excision repair system** is able to detect and repair the damage done by ultraviolet radiation such as the creation of pyrimidine dimers as described previously: this system is affected in xeroderma pigmentosum. Patients with BCCs have been shown to have a reduced capability for DNA repair compared to normal subjects as described in figure 1.2 (Grossman and Wei, 1995). This also applies to those who have a family history of BCC or SCC.

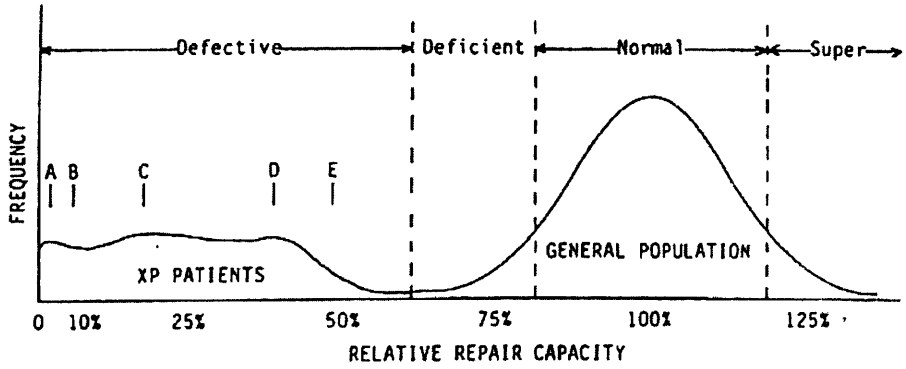


Figure 1.2. Proposed distribution of DNA repair capacity among populations (after Grossman and Wei, 1995).

1.3.3.2.2 Antioxidant pathways

Antioxidants are substances which are critical in cellular defence against reactive oxygen intermediates which arise from UV-induced damage (typically the longer

wavelengths, as opposed to those which cause direct DNA damage). Important antioxidants include vitamins A, C and E, and protein enzymes such as superoxide dismutase (which converts the superoxide ion to hydrogen peroxide), catalase (which destroys hydrogen peroxide) and glutathione enzymes, such as glutathione-s-transferase (GST).

It has been shown that these GST enzymes (which are responsible for the disposal of the potential mutagens such as lipid peroxidation and DNA hydroperoxide formation) are likely to be involved in susceptibility for BCC (Hayes and Strange, 2000). Although the exact role of these enzymes in the development of skin cancer is poorly understood, it appears that several polymorphisms in GST family members exist and that these have been associated with impaired detoxification, thus influencing the risk for several cancers, including BCC.

1.4 Clinical features of BCC

Basal Cell Carcinomas are typically recognised as skin tumours which grow slowly over a period of months or years. They can present as a simple lump, an ulcer, or a skin lesion that does not heal over a long period of time. Erythematous and crusted lesions are often misdiagnosed as other pathologies but are often found to be BCCs following definitive diagnosis.

The main methods of classifying these tumours clinically are to describe their clinical type, their relative time course (whether primary or recurrent), and their histological features. It is important to mention clinical and histological criteria when attempting to describe a BCC because often a clinical appearance cannot be adequately represented by a histological description and vice versa.

1.4.1 Clinical classifications

The clinical classification that is principally used in clinical practice today refers to the main clinical appearances of BCC, some of which are seen in Figure 1.3. In the description of clinical features which follows, pigmentation and ulceration are not discussed since these features can be seen in all of the tumour subtypes.

1.4.1.1 Nodular BCC

Nodular BCC is the most common subtype and is found in up to 45% of cases (Emmett, 1990). They typically present as a pearly nodule or papule and may later progress to central ulceration, resulting in occasional bleeding. Telangectatic vessels are characteristic of this subtype. These tumours may be pigmented, and thus may be easily be mistaken for malignant melanoma.

1.4.1.2 Superficial BCC

Superficial BCCs are usually typically flat lesions which are erythematous and have a lightly scaled surface. As a result they can often be confused for dermatitic lesions. Small superficial ulcerations are often present. Their border is often indistinct, they occur more commonly on the trunk than elsewhere and have an overall incidence of approximately 20% (McCormack et al., 1997; Sexton et al., 1990).

1.4.1.3 Infiltrative and Morphoeic BCCs

Infiltrative and Morphoeic BCCs are not as common as the nodular and superficial subtypes, and do not tend to have a characteristic physical appearance. They do however often present as lesions that are not typically recognised initially as BCCs, and can resemble scars or benign areas of atrophy. Morphoeic tumours tend to have areas of fibrosis present, and thus they may have a shiny or white patch or plaque. The disparate nature of these tumours means that it is often difficult to define exactly where the tumour margin is, and as such complete excision may be more difficult to achieve.

1.4.1.4 Basosquamous ("metatypical") BCC

These tumours are relatively uncommon and show clinical and histological features of both BCC and SCC with an intermediate degree of tumour keratinisation, aggressive behaviour and time course (Martin *et al.*, 2000). However, no clear diagnostic criteria exist as to whether these tumours should be classed as collision tumours (synchronous SCCs and BCCs), or whether they are merely variants of BCCs with a degree of squamous differentiation.

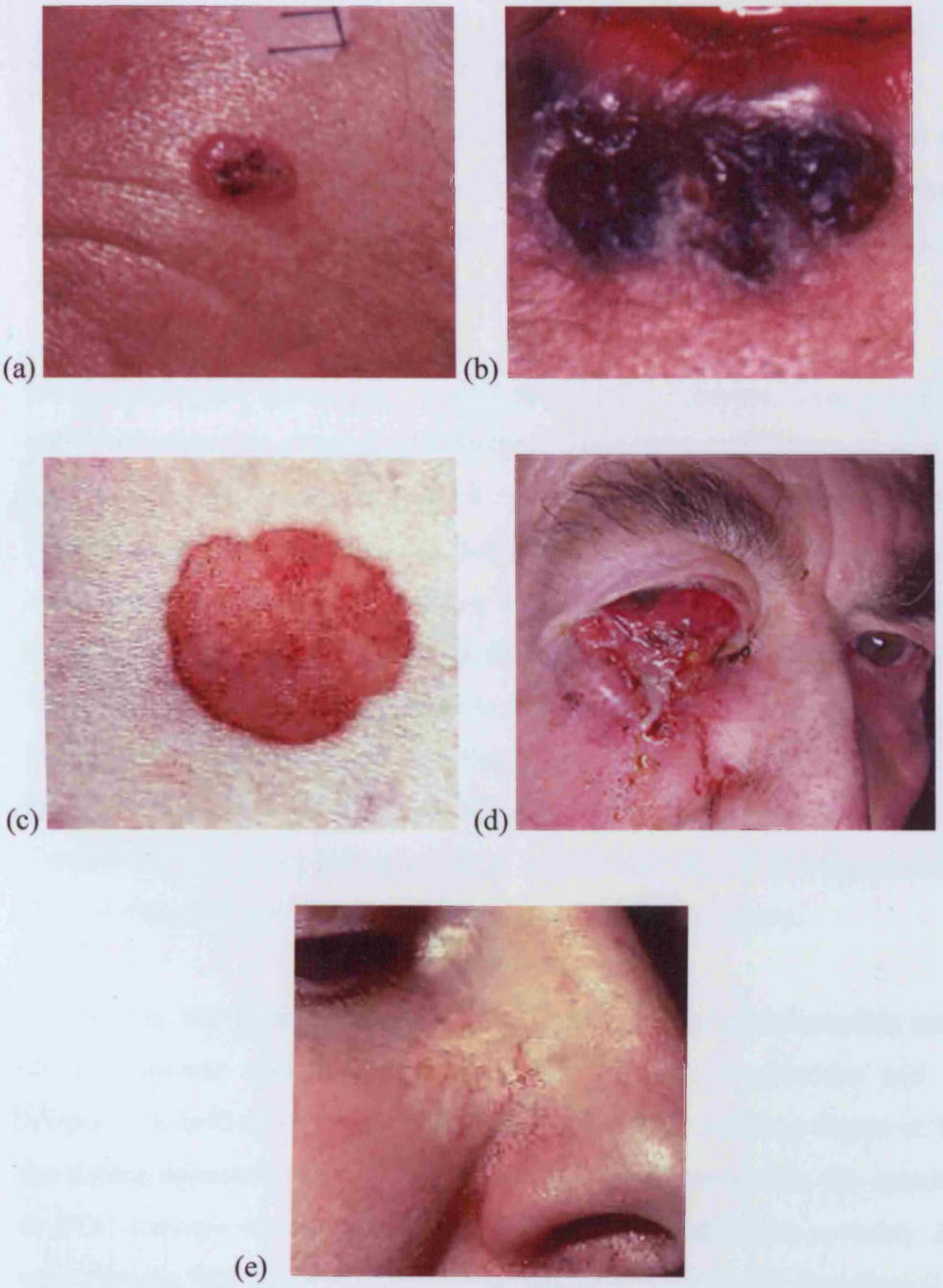


Figure 1.3: appearances of different clinical types of Basal Cell Carcinoma: (a) nodular BCC; (b) pigmented nodular BCC of the lower eyelid; (c) superficial BCC; (d) aggressive infiltrative peri-orbital BCC; (e) morphoeic BCC.

1.5 Histopathological Features of BCC

Basal Cell Carcinoma is a heterogeneous disease. This is reflected in both the variety of clinical presentations and the diversity of histological appearances of the tumours. A large number of terms and classifications have been used to describe the differentiation status, cellular features and growth patterns that exist.

Many histological features are shared across BCC subtypes. Tumours are typically composed of groups or nests of "basaloid" cells which tend to be arranged with little order centrally and a more organised "palisading" manner peripherally. The cells of these tumours do not have well defined cytoplasm and their nuclei are typically hypochromatic. Intercellular bridges are usually not seen on light microscopy. Numerous mitotic figures have been described (Kerr and Searle, 1972). Most tumour islands show a degree of attachment to the epidermis although some subtypes (such as micronodular and morphoeic tumours) tend to have a higher number of tumour islands separated by some distance from this layer. The majority of tumours remain intradermal, but some tumours invade locally and extend into surrounding tissues such as bone and cartilage. Perineural invasion, however, is rare.

Surrounding the tumour islands, BCCs are surrounded by a characteristic stroma. This stroma contains variable amounts of acid mucopolysaccharides and a patchy lymphocytic infiltrate. Morphoeic tumours particularly exhibit a degree of fibrosis in the stroma adjacent to tumour islands. Compared to normal skin, the dermis adjacent to BCC tumours shows significantly higher rates of elastosis, probably due to the solar damage that has partly caused the tumour to occur in the first place (Moon and Oh, 2001). Similarly, the overlying epidermis may show changes of solar keratosis secondary to the radiation damage it has incurred.

Regression has been recognised as a feature of some BCCs (Curson and Weedon, 1979). Clinically it masquerades as central scarring within a tumour, whereas histologically it is characterised by a dense lymphocytic infiltrate at the margins of the tumour islands and prominent apoptosis.

1.5.1 Histological classification

Following earlier classification systems based on cellular differentiation and histological growth pattern, Sloane described four histological subtypes: Nodular, Nodulo-infiltrative, Infiltrative (including sclerosing and non-sclerosing types) and Multifocal tumours (Sloane, 1977). With this classification the infiltrative and multifocal types were shown to have a higher recurrence rate than the other groups.

Five years later Jacobs produced a classification, again based on the growth pattern of the BCC, which had prognostic value (Jacobs et al., 1982). This was based on three major growth pattern types (I, II and III), each of which contained subtypes as detailed in figure 1.4.

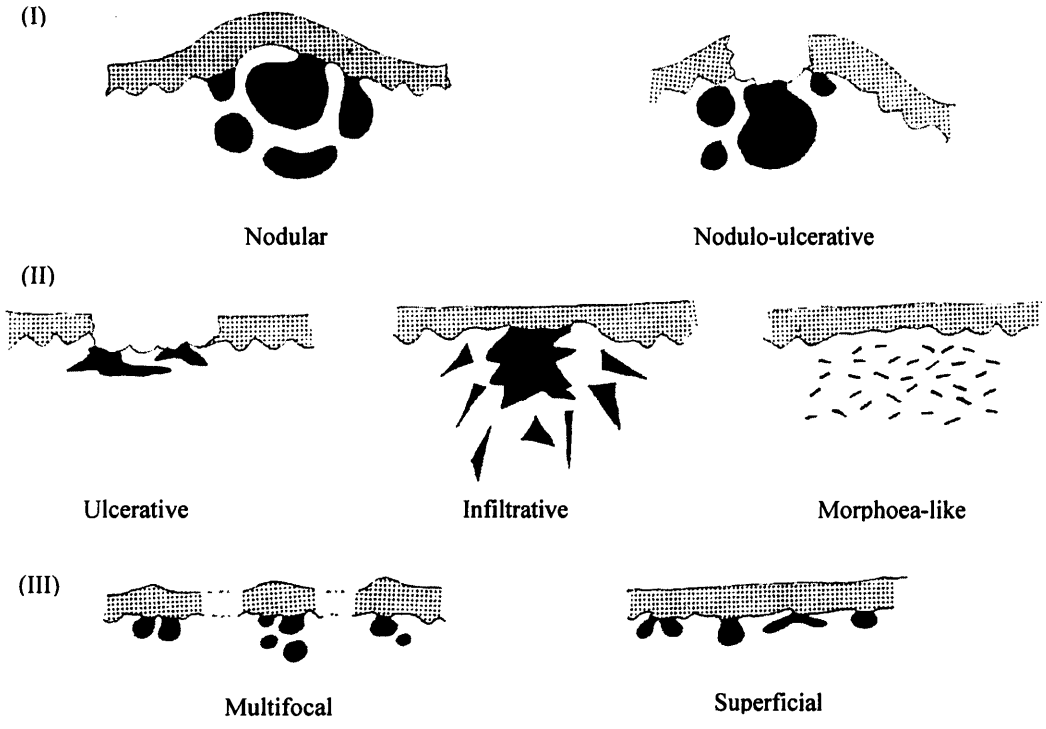


Figure 1.4. A proposed classification of BCC growth patterns (after Jacobs, Rippey et al. 1982)

The most recent, most useful, and currently most commonly used classification was published by **Sexton** et al (Sexton et al., 1990). In this, growth pattern was used as a defining feature, and it was at this stage that the concept of micronodular tumours arose. According to Sexton's classification BCCs can be grouped thus:

1) **Superficial** tumours: multiple tumour islands arise from the epidermis and/or adnexae and either abut or penetrate the papillary dermis.
2) **Nodular** tumours: a rounded mass of tumour cells, typically with a well defined edge and presence of peripheral cellular palisading.
3) **Micronodular** tumours: the multiple tumour islands are smaller than in nodular tumours (less that 0.15 mm in diameter) but are still clearly delineated from surrounding tissues.
4) **Infiltrative** tumours: irregularly shaped tumour islands are large or small and usually have little well defined peripheral palisading.
5) **Morpheic** tumours: tumour islands may be small with "cords" or "strands" extending from them. The surrounding stroma is sclerotic.

Given the current use of this system in UK histopathology laboratories and its relevance to clinical outcome I have used this classification in this thesis to define tumour subtypes.

1.6 Associations of BCC occurrence

Factors that have been investigated in BCC outcomes have included clinical factors (age, gender, anatomical site, relation to sun exposure), histological factors (subtype, cellular differentiation status), treatment factors (surgical or non-surgical, and if surgical – extent or completeness of resection) and to a small degree molecular biology factors. The outcome of any management strategy is of course a combination of the clinical behaviour of the tumour and the efficacy of the treatment chosen. It is in precisely this issue that difficulties of comparison arise, because the data recorded in many studies is not exhaustive and does not make for easy comparisons between studies.

A **primary BCC** is one that has developed in a site in which BCC has not previously been present. A **recurrent BCC** may be defined as the occurrence of a histologically-

verified BCC in exactly the same site as a previously treated BCC. In practice this means that the new BCC occurs within or adjacent to a scar from a previous procedure. Rowe stated that BCCs may recur up to ten years from the original tumour, but that 80 per cent occur in the first five years, and 66% in the first 3 years (Rowe et al., 1989). **Metastatic BCC** must originate from an original tumour that is cutaneous and not mucosal, the metastasis must be at a distant site and not merely an extension, and the original and metastatic tumours must both be of a similar histopathological subtype. Rates of BCC metastasis are extremely low, ranging between 0.0028% and 0.55% of tumours (Snow et al., 1994) with metastasis occurring on average 12 years following the original tumour.

1.6.1.1 Age and gender of BCC patients

Primary BCCs are most common in patients aged between 60 and 70 years old and 95% occur in patients between the ages of 40 and 79 years (Kopf, 1979; Roenigk et al., 1986). In most of the studies published on this subject the gender distribution of BCC patients is approximately equal. Recurrence of BCCs has been noted to be associated with age in patients who have been treated with curettage and electrodessication but not in those treated with surgery or radiotherapy (Dixon et al., 1989; Kopf, 1979; Koplin and Zarem, 1980).

It is unclear whether gender is a significant factor in recurrence. Kopf and Koplin found no association between the the gender of patients with recurrent BCCs and those primary BCCs, but Robins et al differed and concluded that tumours were more likely to recur in young women than in other groups (Robins and Albom, 1975). Although the rates of recurrence following Mohs surgery are much lower than following other treatments, a significant difference in both age and gender has been noted in patients initially treated this way, with more likelihood of recurrence in males less than 50 years of age (Rigel et al., 1981).

1.6.1.2 Anatomical position of BCCs

The head and neck was the commonest recorded site by far in two of the largest studies of this type. Kopf's data showed that 85% of tumours occurred above the clavicles, and Koplin broadly agreed, with 93% of BCCs in this area (Kopf, 1979;

Koplin and Zarem, 1980). It is clear from both studies that mid-facial tumours are most common and that the limbs and truncal areas are relatively under-represented.

There is no absolute consensus on the impact of tumour position on recurrence. Following treatment Kopf concluded that anatomical position had no statistically significant impact on the likelihood of recurrence, whereas Koplin and Zarem showed that a significantly higher number of tumours recurred above the clavicles than elsewhere (Kopf, 1979; Koplin and Zarem, 1980). Cosmetically and surgically challenging areas such as the scalp and mid-face have high rates of recurrence; whether this is due to the biological nature of these tumours or a more conservative approach to their excision remains unclear. This latter theory is supported by Breuninger and Dietz who showed that there is no significant difference in subclinical tumour extension between these sites (Breuninger and Dietz, 1991). Surgical conservatism in challenging sites has also been suggested as a cause of high incomplete excision rates on the eyelids (Rakofsky, 1973).

What small amount of data that does exist on metastatic BCCs suggests that scalp and ear tumours metastasize most commonly. It is possible that a combination of thin skin and large blood vessels may play a role in this. Tumours from the trunk and the genitalia are those that are next most likely to spread (Snow et al., 1994; von Domarus and Stevens, 1984).

1.6.1.3 Duration and size of tumour

Kopf reported that 80 per cent of patients are likely to present with a tumour less that 15mm in diameter (Kopf, 1979). He also stated that more than half the patients will claim to have had the tumour for less than a year, although extraordinarily some may claim to have known about their skin lesion for up to twenty years.

Koplin did not find tumour size and duration at presentation to be related to recurrence (Koplin and Zarem, 1980). Two other studies, however, did find that these factors were important for recurrence in tumours treated with curettage and electrodessication and in tumours treated with Mohs surgery (Kopf, 1979; Rigel et al., 1981).

It appears that there may be increased likelihood of metastasis in tumours that are large and more deeply penetrating. Three-quarters of BCCs that metastasize are stage T2 to T4, and Snow suggested that large size makes it more likely that BCCs may spread (Snow et al., 1994).

1.6.1.4 Histology

In general it appears that nodular tumours (variously including papulo-nodular, nodulo-ulcerative, nodulo-cystic) are the most common primary BCCs and that they occur in 45-60% of cases (Emmett, 1990; Jacobs et al., 1982; Sexton et al., 1990; Sloane, 1977). Superficial tumours represent approximately 8% of BCCs and the more aggressive infiltrative and morphoeic BCCs comprise 8% and 2-9% respectively. Pigmented BCCs occur in 1-6% of cases (Emmett, 1990; Hauben et al., 1982; Kopf, 1979). In none of these cases is there evidence of subtype association with age.

There have been many attempts to correlate histopathological subtype to the ultimate indicator of tumour behaviour, that of recurrence. Over the years a large number of studies have been published and all have slightly different takes on the complex interaction between histological subtype, completeness of excision and BCC recurrence.

Shannoff and Pascal both concluded that there is only limited association between histology and recurrence (Pascal et al., 1968; Shanoff et al., 1967). More recent data, however, infers some association between histological subtype and likelihood of recurrence. Emmett found that 35% of infiltrating tumours were likely to recur and Dixon reported that infiltrative and morphoeic tumours were more likely to recur than nodular or nodulo-ulcerative types (Dixon et al., 1991; Emmett, 1990).

Beyond histological growth pattern other, more "cellular", markers of growth have been correlated with recurrence. Recurrence is associated with the presence of irregularity of 75% of the peripheral palisade and a lack of lymphocytic infiltrate (Dellon et al., 1985; Sexton et al., 1990). Hauben, however, found no association between peripheral palisading or lymphocytic infiltrates or squamous differentiation and outcome but did find an increased recurrence rate in those tumours with high

mitotic activity (>10 mitoses per 5 high power fields) and the absence of ulceration (Hauben et al., 1982).

Clearly, the relationship between tumour growth pattern and tumour cell characteristics, likelihood of complete excision and tumour recurrence is a complex one. It is likely that differences in tumour excision (as determined partly by complexity of the growth pattern) may account for some of the differences between the results of these studies. Going beyond these markers using molecular biology will be discussed in the section 1.10.

1.7 How treatment of BCC affects recurrence

A variety of treatment options are used to treat BCCs. This reflects not only the variety of available options and the variety of practitioners to whom BCC patients are referred, but also the fact that no clear consensus exists for the optimal routine treatment for different types of tumour. All treatment modalities can essentially be split down to either ablative (curettage, cryotherapy, electrodessication, radiotherapy, topical chemotherapy) or excisional ("routine" surgical excision, Mohs' micrographic surgery) techniques.

1.7.1 Curettage and electrodessication

This technique involves ablation of a lesion using a curette under local anaesthesia, followed by electrodessication of the tumour bed. This is best used for soft circumscribed tumours such as nodular BCCs which are limited in size and have not been previously treated.

Five-year recurrence rates range between 3% and 18% which reflect technique and patient selection. Tumour size and anatomical site are independent risk factors for recurrence using this method (Silverman *et al.*, 1991b). Suhge d'Aubermont and Bennett excised the sites where curettage and cautery had been performed for BCC. They found high rates of residual tumour and this was more significant in facial sites compared to truncal sites (Suhge d'Aubermont and Bennett, 1984). Recurrent lesions should not be treated by this technique since re-recurrence rates may reach 40% (Rowe et al., 1989).

The main problem with this treatment method is the lack of histological information available following the procedure. Given that a watch and wait policy is advocated in every case with this technique, the first sign of a potential problematic tumour may be recurrent disease.

1.7.2 Cryotherapy

Liquid nitrogen has been used to ablate BCCs and has been reported to have good success, ranging from no recurrence to 12.9% (Kuflik, 1980). Only one study, however, has data for five-years of follow up; this demonstrates a recurrence rate of 7.5% (Fraunfelder *et al.*, 1984). Larger infiltrative tumours were shown in this study to be most likely to recur. In a similar fashion to curettage, no histological information is available and the efficacy of cryosurgery cannot be directly compared to other treatments (as reviewed by Kokoszka and Scheinfeld, 2003).

1.7.3 Radiotherapy

Radiotherapy has progressed from its initial regimens to now include fractionated conventional radiotherapy and interstitial brachytherapy. Five-year recurrence rates can be as high as 31% (Nordman and Nordman, 1978). Size of tumour before treatment has been found to be an independent prognostic marker of recurrence following radiotherapy with Silverman reporting a 4.4% recurrence rate for tumours less than 1cm rising to 9.5% for larger tumours (Silverman *et al.*, 1991a).

There is only one prospective randomised control trial comparing radiotherapy and surgery and this concluded that both recurrence and cosmesis were superior at 4 years in the group treated with surgery (Avril *et al.*, 1997). In this series recurrence over four years following surgery was low (0.7%).

Radiotherapy was initially popular because the tumour site usually healed without the need for formal surgical reconstruction. However depigmentation, atrophy, radionecrosis and induction of new malignancies are potential complications and so it is now principally used when palliation is desired for elderly patients who have large potentially incurable lesions for whom major reconstructive surgery would not be appropriate.

1.7.4 Surgical excision

The ideal end result of surgery for BCC is complete tumour excision combined with an acceptable cosmetic result. Attaining complete tumour excision is greatly dependent on the skill of the surgeon, both in appreciating that the actual extent of tumour may not tally exactly with what is seen at the tumour surface, and in having the confidence to fully excise the lesion and make the correct (not necessarily the easiest!) reconstructive choice. However, the term "complete excision" is only correctly used if one is *totally* sure that that is indeed the case. This relies greatly on the skill of the pathologist examining the surgical specimen, and requires that histological processing and analysis has been performed as well as is possible.

Routine excision is generally carried out as a one step procedure with closure or reconstruction of the surgical defect immediately following the excision of the tumour. The benefits of this obviously include a rapid procedure, no delay between excision and closure of the wound, low cost and ease of performing it due to the basic surgical material required. The major drawback with routine excision, however, is that although an effort should have been made to predict the extent of tumour by visual assessment, no microscopic assessment is possible at that time. It is therefore not uncommon to find out much later that the resection was inadequate and at that stage the decision needs to be made as to whether repeat excision needs to be performed. Some of these problems may be overcome using Mohs' surgery, discussed in section 1.7.5.

Reports of overall BCC recurrence following routine surgical excision range from 0.7% to 23.4% (Avril et al., 1997; Hauben et al., 1982) over a five-year follow-up period. The effect that histopathological subtype has on recurrence of routinely excised BCCs is closely related to the completeness of excision and this in part explains the diversity in results with this technique.

1.7.4.1 The link between tumour characteristics, surgical treatment and outcomes

Growth properties of the tumour determine whether significant **subclinical extension** is present. This may then define how large an **excision margin** is chosen (in the case

of routine excision) and this will determine the **completeness of tumour excision** (as determined by histological examination, discussed below). The completeness of excision combined with the patient's host response may then affect the likelihood of **recurrence**.

Histological reporting is essential to accurately assess the degree of completeness of excision. Random sampling is the easiest method of histological assessment, but due to the small amount of the specimen examined, this may lead to a high level of falsely negative excision margins. "Breadslicing" of tumours provides better information regarding the tumour histology and growth pattern. Mohs surgery is the best method for assessing the margins of excision specimens (as disccused in section 1.7.5); this gives unrivalled information but comes at the expense of time and cost.

1.7.4.1.1 Subclinical extension and completeness of excision

Extension of BCCs is similar to that of an iceberg: there is often much more of the tumour beneath the surface than is visible from above it. This extension is often asymmetrical, and it varies according to tumour size and subtype. It has mainly been assessed by histological assessment of completeness of excision following excision with standard margins.

Wolf assessed the adequacy of lateral margins by marking the skin with 1mm increments and found that a 4mm margin would eliminate 98% of all tumours less than 2cm diameter. He found that increased margins would have inferred little benefit, and that reduced margins of 3mm or 2mm would have achieved complete clearance in only 85% and 75% of cases respectively (Wolf and Zitelli, 1987). Epstein showed that a 2mm margin was adequate to clear 95% of tumours, but the tumours in this group were small (average diameter only 8mm) and the failures in this group had an average size of 13mm, indicating that **size of tumour** may impact on the likelihood of subclinical extension (Epstein, 1973).

However, the main determinant of the extent of subclinical extension (both lateral and deep), and therefore of incomplete excision is the **histological subtype** of the BCC. A number of studies have strongly suggested that infiltrative, morphoeic and micronodular subtypes are most likely to be incompletely excised due to their patterns

of extension (Epstein, 1973; Hendrix, Jr. and Parlette, 1996; Sexton et al., 1990). Lastly, the **chronicity** of the tumour may play a role here: recurrent tumours were also more likely to be incompletely excised than primary tumours (Breuninger and Dietz, 1991).

1.7.4.1.2 How completeness of excision affects BCC recurrence

Taking the previous discussion a step further and considering how incomplete resection translates to tumour recurrence is not so straightforward. The belief that an incompletely excised tumour should lead to recurrence relies on a number of assumptions and these can fall down at any step along the way. Firstly, **falsely positive margins** may be reported, by way of errors in histological processing. This immediately means that the link in these cases is incorrect – even if recurrence does occur, there may not have been residual tumour present after all. **False negative margins** may also be recorded, due to inadequate histological assessment. This will also skew subsequent comparisons of completely and incompletely excised tumours.

A **true positive margin** error may occur because a tumour does actually "shell out", and although there may be tumour at the absolute margin of the excised specimen (i.e. truly positive margins), there is indeed no tumour residing in the tumour bed and hence there is no recurrence. It is interesting that excision of previous sites of incomplete BCC excision show that residual tumour is only found in approximately 50% of cases (Griffiths, 1999; Suhge d'Aubermont and Bennett, 1984). Whether this is due to regression of the residue or because there was never any residue remaining is unknown.

Lastly, in the case of tumours where excision data is accurate, **follow-up data** must be adequate, and should ideally last ten years. This is necessary to ensure that the recurrence data is also accurate, and that there is no chance that tumours may be recorded as not having recurred when actually they have.

Notwithstanding all these potential difficulties, there is still strong evidence that the degree of completeness of tumour excision relates to the likelihood of recurrence. A significant increase in tumour recurrence following incomplete (or suboptimal) excision has been described by many authors (De Silva and Dellon, 1985; Gooding et

al., 1965; Lang, Jr. and Maize, 1986; Pascal et al., 1968). Richmond further explored the relative importance of clear deep or lateral margins. He followed 60 cases of incomplete BCC excision and found that all four cases which had both deep and lateral margins originally involved recurred (Richmond and Davie, 1987).

However the rate of BCC recurrence in tumours which have been supposedly incompletely excised is still lower than the absolute incomplete excision rate (Dellon et al., 1985; Pascal et al., 1968; Richmond and Davie, 1987) – hence the acceptance in many cases of incomplete BCC excision.

1.7.5 Mohs surgery

Originally described by Dr. Frederick Mohs, this technique produces flat excision margin specimens that are parallel to the tumour / stroma interface (Mohs, 1976). Following initial excision, successive saucer-shaped planes of tissue are taken and by rapidly identifying where residual tumour lies within these specimens, one can then continue the excision process only where tumour remains until one is sure that all surgical excision margins are clear of tumour. This essentially guarantees complete tumour excision (hence resulting in very low recurrence rates) and minimises the removal of uninvolved tissue.

However it is unsuitable for assessing tissues with any bony component as bone cannot be decalcified within the time frame of the procedure. It is also time- and labour-intensive, it requires specialist skills and equipment, and therefore is costly. It is best reserved for the treatment of aggressive primary or recurrent tumours in anatomical sites where as much normal tissue as possible must be spared (good examples are orbital and peri-alar BCCs).

93% and 97% cure rates have been reported for the fixed and fresh tissue methods respectively. This technique has a recurrence of 8.7 times less than that of all non-Mohs modalities for primary BCCs (Rowe et al., 1989). A recent study of peri-ocular BCC by Malhotra et al showed that there were no recurrences following Mohs resection of primary BCCs, and that the recurrence rate of previously treated tumours was 7.8% (Malhotra *et al.*, 2004). In this study previous recurrence, medial canthal

site and superficial or infiltrative subtype were all significant factors associated with recurrence.

1.7.6 Non-Surgical Techniques

There are many non-surgical techniques that have been used to treat BCC. However, at this stage none of them have the bulk of data or solid evidence base for their use that the previously mentioned treatments do.

Prophylactic use of **oral retinoids** has been shown to be of no benefit in the reduction of subsequent BCC formation (Moon et al., 1997). **Topical retinoids**, in the form of Tazarotene, have been shown to promote regression in selected cases, but are associated with high incidence of disease persistence (Bianchi et al., 2004; Brenner et al., 1993).

Intralesional interferon α2b been demonstrated to cure 67% of BCCs in the short term but long term follow-up data is limited in such studies (Kim *et al.*, 2004).

Immune modulating drugs, such as Imiquimod, induce the production of interferon and other cytokines through the innate immune system and stimulate cell–mediated immunity. They have been used topically to treat BCCs, and Geisse et al have shown that a statistically significantly higher clearance rate is achieved using this cream over control in superficial BCCs (Geisse *et al.*, 2004). The end point in this study was histological clearance rather than actual tumour recurrence.

Photodynamic therapy (PDT) with either topical or systemic haematoporphyrin derivatives is also under evaluation. Topical application of photosensitisers has the advantage of less systemic side effects but is only suitable for more superficial tumours. Rhodes et al compared the use of topical methyl aminolevulinate photodynamic therapy with routine surgical treatment and found that although cosmesis was better with PDT higher recurrent rates were present in this group (Rhodes et al., 2004). However, in all the studies performed to date, no precise rates of BCC clearance using PDT are known, and recurrence rates do seem to be higher than for conventional treatment methods (as reviewed by Marmur et al., 2004).

5-Fluorouracil has been used topically since the 1970s, but long term data with its use is lacking. Epstein found that cure rates of superficial BCCs were improved by combining its use with curettage (a drop from 21% to 6% recurrence at five years) (Epstein, 1985).

Section 2: Molecular Biology of BCC

Tumour formation results from a disruption of the normal balance between cell proliferation and cell death. As summarised by Vogelstein and Kinzler, neoplastic cells differ from normal cells by their ability to proliferate, unrestrained by normal regulatory mechanisms and by their ability to colonise territories normally reserved for other cells (Vogelstein and Kinzler, 1993). Differentiation and proliferation are controlled by certain genes which may themselves be regulated by extrinsic factors. Cancer, therefore, is essentially a genetic disease; the genetic changes predisposing us to cancer affect either a small number of crucial genes, leading to rare highly penetrant syndromes that predispose to cancer (such as Gorlin's syndrome) or a larger number of less critical genes that have a contributory effect to cancer susceptibility in the general population.

1.8 Molecular biology of cancer in general

1.8.1 Genetics of cell replication

Cell proliferation and differentiation is controlled by standard cell signalling methods involving external signals, cell surface receptors, cytoplasmic signal transduction and transcription of specific target genes. Many of the genes known to be responsible for tumour development and growth code for proteins involved in these pathways, and de-regulation of their expression results in a breakdown of normal cellular replicative function.

The regulatory components that control cellular proliferation, differentiation or death are usually external growth factors that act via signal transduction pathways, affecting the gene expression of that cell. Various components of these pathways are altered (increased, decreased or mutated) in cancer physiology. Some of these end-pathways such as Wnt and β-catenin will be discussed later as specific end results of altered cell signalling in basal cell carcinoma. For each cancer in question, there are many complex pathways which overlap to govern whether cell behaviour will conform to being safe, or whether malignant transformation occurs, including those of apoptosis, senescence, cell adhesion, and angiogenesis.

Of all the major genes that contribute to cancer development, there are three large categories of genes which affect cellular proliferation and survival: growth-promoting oncogenes, tumour suppressor genes and mutator or caretaker genes. In general (proto-)oncogenes need to be activated, and tumour suppressor genes inactivated for malignant transformation to occur.

1.8.2 Oncogenes

The normal counterparts of oncogenes, the proto-oncogenes, are crucial in regulating normal cell cycling and division, differentiation and apoptosis. When these become mutated or amplified (i.e. then properly known as oncogenes) the normal restraints of cell growth are overcome, resulting in tumour formation.

These genes act in a dominant manner, encoding proteins whose activity promotes the malignant phenotype. Cellular proto-oncogenes (c-onc) have the normal functions described above, and only upon alteration do they exert their proliferative effects as true oncogenes.

1.8.2.1 Tumour Suppressor Genes

Tumour suppressor genes are 'anti-oncogenes' – they normally inhibit cellular proliferation, and their suppressive effects on cell growth are inactivated by loss-of-function mutations during cancer development. The first evidence for these loss-of-function genetic changes was from studies in the rare childhood eye cancer retinoblastoma. From studying sporadic and familial retinoblastoma Knudson formulated his "two-hit" model of carcinogenesis in 1971 (Knudson, Jr., 1971) as illustrated in figure 1.5. In the familial form an affected parent has a 50% chance of passing the condition to any offspring and the tumours are usually bilateral, whilst in the sporadic form there is no additional risk of inheritance to any offspring, and the tumour is usually unilateral. Knudson hypothesised that this must be a two-hit event, with two rate limiting steps of tumour formation.

This is now known to be the case because in the inherited form of the disease, germline mutations in one allele of the tumour suppressor gene predispose to tumour formation. Somatic mutation of the second allele occurring during

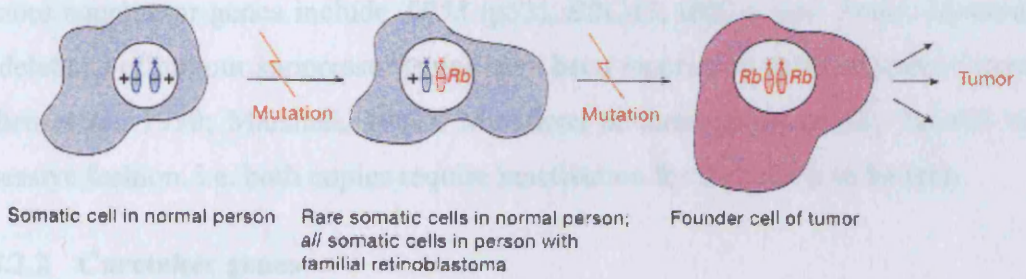


Figure 1.5: Graphical representation of Knudson's two-hit hypothesis model for acquisition of a malignant phenotype (after Strachan and Read, 2004)

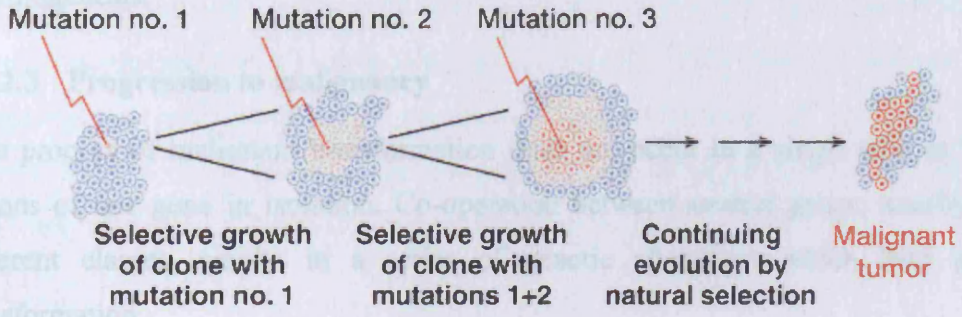


Figure 1.6: Graphical representation of sequential acquisition of genetic mutations resulting in frank malignancy (after Strachan and Read, 2004)

the lifetime of the individual then results in tumour formation. The second "hit" can occur by point mutation, allelic loss, methylation, or other methods of silencing the allele. In the sporadic form of the disease two separate sporadic 'hits' are needed in the same cell for it to develop into a tumour clone, hence this is fairly rare. Naturally, the chance of this happening in both eyes is so small that formation of sporadic bilateral retinoblastoma is incredibly uncommon. The tumour suppressor gene associated with the disease maps to chromosome 13q14. Subsequently named *RB1*, this gene encodes the cell cycle regulatory protein pRb (Friend et al., 1986). Other tumour suppressor genes include *TP53* (p53), *BRCA1*, *BRCA2* and *Ptch1*. Mutations or deletions of tumour suppressor genes have been reported in various types of cancer (Chen et al., 1990; Marshall, 1991). Mutations of these genes usually behave in a recessive fashion. i.e. both copies require inactivation for the effects to be seen.

1.8.2.2 Caretaker genes

'Mutator' or caretaker genes are principally involved in DNA repair and maintain stability of the genome. In some inherited disorders and familial cancer syndromes defects in these repair mechanisms result in genomic instability, leading to chromosomal breaks, abnormal chromosome numbers, and widespread mutations; these somatic changes often affect genes important in proliferation and carcinogenesis.

1.8.2.3 Progression to malignancy

This process of malignant transformation does not occur in a single step or by the actions of one gene in isolation. Co-operation between several genes, usually from different classes, results in a series of genetic alterations which lead to cell transformation.

This multi-step process towards malignant transformation is thought to involve a number of essential alterations in cell physiology. It is the subject of continuing debate as to how multiple mutations arise in a cell, as the apparent probability of a single cell undergoing multiple mutations is virtually nil. There are mechanisms that can explain how this process can happen. The mutated cells are subject to selection, and eventually a cell population evolves that can escape the controls of proliferation and territory. Initially mutations increase proliferation (growth), and this gives an

increased target population of cells for the next mutation. At the same time some mutations (germline or somatic) will alter the stability of the whole genome, either at the DNA or chromosomal level, increasing the overall mutation rate (Lengauer et al., 1998). This multi-step process explains why tumours develop in stages from benign growths to malignant tumour cells, at each step developing and selecting for new mutations. This is illustrated in figure 1.6.

Hanahan and Weinberg have suggested that there are six key factors that are essential for the transition of a normal cell to malignant one (Hanahan and Weinberg, 2000). They are:

1: independence from external growth signals
2: insensitivity to external anti-growth signals
3: ability to evade apoptosis
4: ability to indefinitely replicate
5: sustained angiogenesis
6: ability to invade tissue and metastasise

Obviously there does not have to be a strict order of events for a cell to mutate, but it makes sense that certain requirements are crucial early on, whereas others may come into their own later on (such as angiogenesis or the ability to invade). Metastasis may not be a requirement at all – this does not necessarily confer any survival advantage for the tumour, as the death of the organism will mean the death of the tumour. In the case of BCC, local invasion is the mainstay of tumour aggression, with metastasis only very rarely described in fewer than 1% of cases.

The first mutation in the multi-step evolution of a tumour is critical because it should confer some growth advantage on an otherwise normal cell that has all its defences intact. According to the gatekeeper hypothesis one particular gene is responsible for maintaining a constant cell number in a given renewing cell population (Kinzler and Vogelstein, 1996). Mutation of a gatekeeper gene leads to a permanent imbalance between cell division and cell death, and sets the scene for clonal expansion and the subsequent accumulation of other genetic events. Mutation of other genes, however, may have no long term effect if the gatekeeper is functioning correctly. According to this theory, the tumour suppressor genes identified by studies of cancer syndromes

inherited in a mendelian fashion are gatekeepers for the tissue involved – *Ptch* is an example of this relevant to the skin, and its dys-regulation leads to basal cell carcinoma.

1.8.3 The cell cycle

1.8.3.1 Replication

A significant proportion of the genes implicated in tumourigenesis are involved in the control of the cell cycle (figure 1.7), an essential component of cells' ability to divide and grow.

The process of nuclear division is known as mitosis or M phase. The cell then has much longer period of DNA replication and cell growth between each mitotic phase known as interphase. Interphase is divided into three phases – the S phase (in which the DNA replicates), G_1 (gap phase 1, the interval between completion of mitosis and the beginning of DNA synthesis) and G_2 (the interval between the end of DNA synthesis and beginning of the next cycle of mitosis). Cells in G_1 can pause in a specialised resting state known as G_0 indefinitely before resuming proliferation (Pardee, 1989). In G_2, between the S and M phases, the nucleus contains a tetraploid number of chromosomes (i.e. four sets as opposed to a normal complement of two). Following mitosis, the normal diploid number of chromosomes is restored. The daughter cells can then either re-enter the cycle at the G_0 / G_1 phase, proceed to differentiate, or undergo apoptosis.

1.8.3.2 Cell loss

With all of this replicative activity resulting in the net creation of cells, in the normally functioning organism there must be a balance of cell loss. Death of the cell and cell shedding are methods by which this may occur. Cells may be shed either into the local environment (e.g. exfoliation) or into the lymphatic or vascular system (e.g. metastasis). Apoptosis (planned cellular suicide) or necrosis (unplanned cell death) may result in cellular death. These two processes are distinctly different pathways, although alternate responses may result from the same stimulus (such as an event causing necrosis also triggering apoptosis in surrounding tissue because of local cell toxicity).

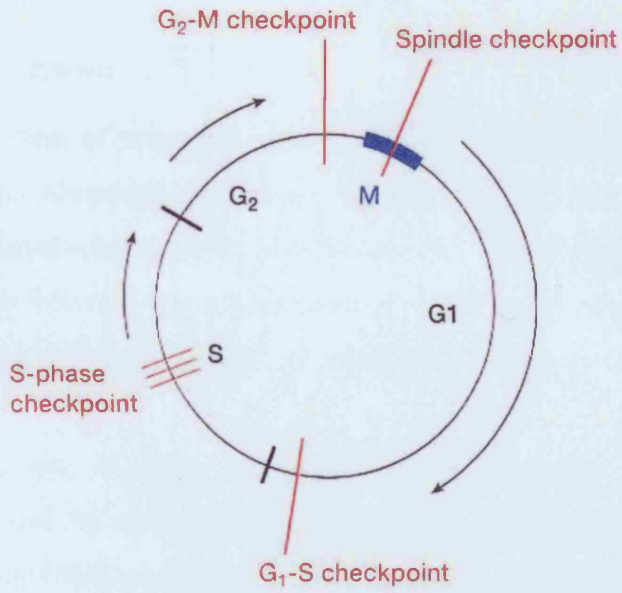


Figure 1.7: The cell cycle (after Strachan and Read, 2004)

1.8.3.2.1 Necrosis

Necrosis is an unplanned end result of severe cellular injury, such as a tumour outgrowing its blood supply and it causes cellular death. Organelle swelling and subsequent cellular disintegration ensues. Due to leaky cell membranes lysosomal enzymes (which accelerate disintegration) are released. Subsequently nuclear chromatin disappears (karyolysis) and often an inflammatory reaction occurs in the surrounding tissue: ths may result in scarring.

1.8.3.2.2 Apoptosis

Apoptosis, a form of programmed cell death, is characterized by cell shrinkage and fragmentation. Abnormal, unwanted or damaged cells are removed by apoptosis without the involvement of the immune system, but through rapid phagocytosis of apoptotic cells before lysis, which prevents inflammation. In this respect the process of programmed cell death is clearly distinguished from necrosis

Apoptosis is one of many processes that is necessary for correct embryonal development and for elimination of auto-reactive lymphocytes in development of the immune system (McDonnell et al., 1993; Raff et al., 1998). Regulation of cell death is also an essential defence against viral infection and the emergence of cancer. Too much cell death, however, can result in impaired development and degenerative disease.

Morphologically, the earliest changes are compaction of the nuclear chromatin and condensation of the cytoplasm. Continuation of this condensation is accompanied by fragmentation of the nucleus. Surface protuberances (blebs) then separate from the cell, converting the cell to into a number of membrane bound apoptotic bodies. These apoptotic bodies are absorbed rapidly by adjacent cells and are degraded by lysosomes. It is thought that the apoptotic bodies are only visible (by light microscopy) for only a few hours (Kerr et al., 1994).

Apoptosis in the epidermis is a common phenomenon. It plays a pivotal role in the morphogenesis of human fetal skin and the maintenance of adult epidermis

(Polakowska *et al.*, 1994). In fetal skin, cells undergoing apoptosis are present in several epidermal cell layers, whereas in neonatal epidermis these are found in the terminally differentiating granular cell layer, and in adult skin the spinous cells also show occasional apoptosis. In normal adult life, apoptosis occurs as a physiological response following irreparable DNA damage due to excessive UV light exposure (Tyrrell, 1996).

1.8.4 Control of the cell cycle

1.8.4.1 Cyclins and Cyclin-Dependent Kinases

For the cell cycle to occur, a number of large-scale molecular changes must take place. The chromosomes must be replicated, condensed, segregated and de-condensed and the spindle poles must duplicate, separate and migrate within the cell. A family of molecules termed the cyclin dependent kinases (CDKs) are thought to be critical for this. Expression of different members of these families and differential phosphorylation states of these members ensures that specific signals are sent at specific times in the cell cycle. Hence the cell cycle is driven from one "checkpoint" to another (Hunter and Pines, 1994). Each CDK and cyclin gene are transcribed for only a short time during the cell cycle; following a short spell of activity, the proteins are degraded. Each CDK and cyclin is only capable of binding (and activating) a specific partner. Thus tight control is exerted over the progress of the cell cycle at any time.

One of the most critical points of control in the cell cycle is known as the G_1-S checkpoint. It is at this stage, prior to the S-phase, that checks are made as to whether a cell is ready and fit to have its DNA replicated- a major event in the life of the cell and critical for normal functioning of progeny cells. Any cell in which there is unrepaired DNA damage should, in the normal situation, proceed to apoptosis. At this point D- and E- cyclins and their associated CDKs are sequentially expressed, and the key role of these macromolecular combinations is to phosphorylate (inactivate) the key negative regulator of cell cycle progression (pRb) permitting exit from the G_1 phase to the S phase.

Lastly, a number of specific Cdk-inhibitors (CdkI) exist but are not discussed in detail here. They include p21 and p27 (which are able to bind and inhibit all known cyclin-Cdk complexes) and p14 and p16 (which specifically inhibit the D-cyclin complexes).

1.8.4.2 *RB1* and pRb

The *RB1* gene was identified through its role in the childhood eye cancer, retinoblastoma (Friend et al., 1986), but it is widely expressed and helps to control the cycling of all cells. In normal cells the gene product, a 110-kDa nuclear protein is inactivated (phosphorylated) and activated (dephosphorylated) by cyclin/Cdk combinations. When phosphorylated, its inhibitory effect on the transcription factor E2F is reduced, leaving E2F free to transcriptionally activate genes critical for S-phase function. Thus pRb activity reduces cell cycling.

1.8.4.3 *TP53* and p53

Along with the pRb pathway, the p53 pathway is particularly important in cell cycle control, regulating many of the genes controlling the cell-cycle checkpoints as illustrated in figure 1.8. The tumour suppressor gene *TP53* is thought to be the most mutated gene involved in human cancer (Hollstein *et al.*, 1991). It may be knocked out by deletion or mutation, and constitutional mutations are found in families with the dominantly inherited Li-Fraumeni syndrome.

p53 has key roles in cell cycle control, apoptosis, angiogenesis, and genetic stability (reviewed by Levine, 1997). Normal functions with relevance to the cell cycle include induction of G_1 cell cycle arrest in response to DNA damage and regulation of apoptosis. In the normal situation, p53 levels in the cell are low because the protein is rapidly degraded. Upon cellular stress, however, p53 protein is activated by phosphorylation and stabilized, and it is this enhanced stability which allows the increased p53-dependent gene transcription that occurs. The cellular outcome following DNA damage may either be growth arrest (cell cycle arrest), senescence or apoptosis (Sionov and Haupt, 1999). The relative cellular content of p53 determines the response following this damage; when the content is low to moderate, cells will go into cell-cycle arrest to allow DNA repair, but when p53 levels are high, cells will progress to apoptosis.

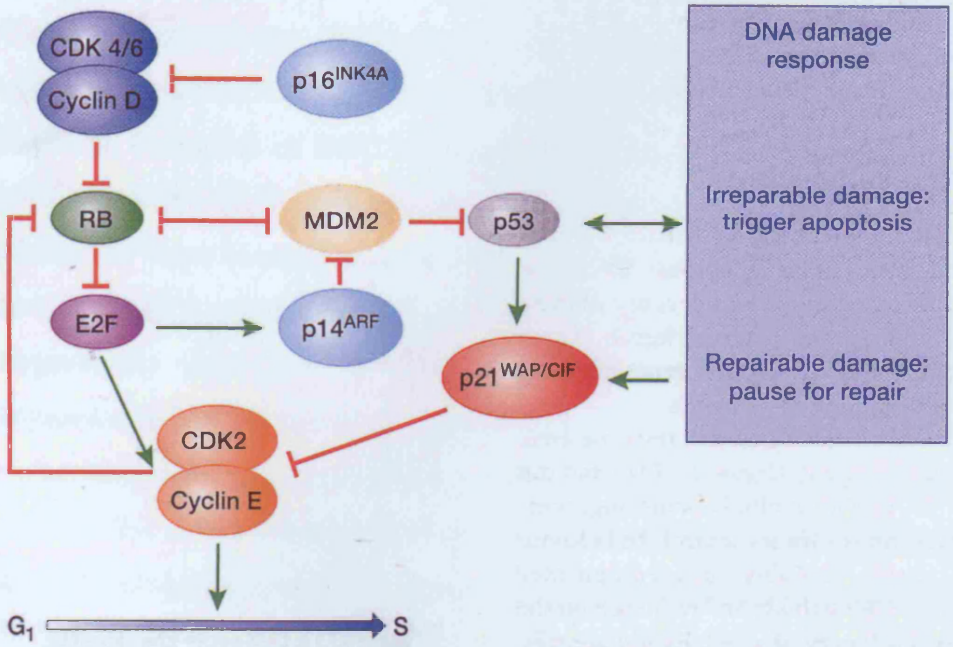


Figure 1.8: Known signalling pathways important in control of the G_1-S checkpoint (after Strachan and Read, 2004)

The timing of the p53 somatic mutation depends on tumour type. In cutaneous squamous cell cancer p53 mutation may be an early event and mutations are found in some pre-malignant lesions (about 40% of Bowen's disease) (Rees, 1994). Mutations have also been found in mild dysplasias of the oesophagus breast and larynx. In other tumours mutation of p53 may be a late event and in that case is associated with tumour progression (reviewed by Hollstein et al., 1994).

1.8.5 General cancer features in Basal Cell Carcinoma

1.8.5.1 Apoptosis in BCC

Evidence for apoptosis in BCCs comes from both morphological study and molecular biology. A high number of morphologically apoptotic cells are present in BCC (Mooney *et al.*, 1995). On a molecular level, over-expression of Bcl-2 is sufficient to enhance the formation of BCC by suppressing apoptosis (Staibano et al., 2001b). Altered expression of Bcl-2 family member proteins suggests that dys-regulation of expression of these proteins may be a possible explanation for the indolent growth behaviour of BCC (Cerroni and Kerl, 1994; Tilli et al., 2002). Bcl-2 in general is homogeneously expressed in BCC (Morales-Ducret et al., 1995; Rodriguez-Villanueva et al., 1995; Tilli et al., 2002; Verhaegh et al., 1995), while the apoptosis-inducing Bax protein is also expressed at high levels (Delehedde et al., 1999; Tilli et al., 2002). This shows that a considerable proportion of cells in BCC are in principle capable of undergoing apoptosis, confirming the earlier findings of Mooney et al.

1.8.5.2 p53 in BCCs - control of DNA repair

Principally due to UV radiation, p53 mutations have been detected in up to half of all BCCs (D'Errico *et al.*, 1997; Moles *et al.*, 1993; Rady *et al.*, 1992; van der *et al.*, 1994; Ziegler *et al.*, 1993). This results in the accumulation of p53 in cells and immunohistochemical evidence of its over-expression (Auepemkiate et al., 2002; Demirkan et al., 2000). Furthermore, Auepemkiate found that histologically aggressive BCCs are strongly associated with increased p53 expression. Other studies, however, have found varying rates of immunohistochemical p53 expression in BCCs (Barbareschi et al., 1992; Ponten et al., 1997; Shea et al., 1992) and no variation of staining between subtypes (Demirkan et al., 2000).

Much of this evidence, however, is based on immunohistochemistry and there are a number of problems associated with this. Because of the specificity of antibodies for three-dimensional antigen structure, not only does a change in the amount of antigen present affect the staining one sees, but if the protein is altered due a genetic mutation, then false negative results may occur because the antibody no longer reacts with the altered p53 epitope. False positive results may also occur due to accumulation of wild type p53 following DNA damage, where there is no evidence of mutation (Campbell et al., 1993). Additionally, false negative results have been discovered in the presence of defined DNA mutations (as assessed by DNA sequencing). Positive or negative immunostaining cannot therefore necessarily be taken to imply the presence or absence of a mutation.

However, it is still striking that patients suffering from Li–Fraumeni syndrome do not show an increased incidence of BCC. Consequently, it seems reasonable to assume that p53 mutations are secondary events in BCCs, occurring after tumour initiation. Oddly, one of the hallmarks of p53 dysfunction, aberrant mitosis, has never been observed in BCC (Pritchard and Youngberg, 1993). The relevance of p53 mutations for BCC growth remains to be demonstrated.

1.9 Hedgehog signalling and Basal Cell Carcinoma

Much of the general features of cancer development as outlined above apply to the molecular biology of Basal Cell Carcinomas. The known changes in apoptosis signalling and *p53* biology have already been discussed. However, many of the specific changes in BCC biology are not explained by these general measures, and it has been discovered that the gatekeeper theory does seem to have particular relevance for BCC. As will be outlined, alterations in Hedgehog signalling and patched membrane receptor function are widely held to be responsible for the emergence of BCC, and such genetic changes are the only changes found in BCC to have a causal link in development of the disease.

1.9.1 How hedgehog signalling was linked to BCC

The discovery that mutations of the patched receptor are responsible for BCC development is one of the classic discoveries of molecular genetics. The first breakthrough came with the identification of Gorlin's syndrome (Gorlin-Goltz

syndrome, NBCCS, as described in section 1.3.2.3.1) as a defined entity, and this was followed nearly 40 years later with the combined approaches of chromosomal loss in sporadic tumours and linkage analysis in familial tumours leading to identification of *patched* as the culprit gene.

This syndrome was first reported by a dentist called Gorlin (Gorlin and Chaudary, 1960). Over the next twenty years numerous other reports were published, and Gorlin published numerous evolving descriptions of the disease. The subsequent study of chromosomal loss in sporadic BCCs identified a large region on chromosome 9q that is lost in approximately 50% of BCCs from sporadic or Gorlin's patients (Gailani *et al.*, 1992; Quinn *et al.*, 1994). Once the region was identified through comparison of DNA from tumours and control tissue, it was possible to use the second approach – linkage analysis – to demonstrate that the gene whose mutations underlie the hereditary BCNS also mapped to the same region of chromosome 9. This culminated in the near-simultaneous discovery by Farndon and Reis (Farndon *et al.*, 1992; Reis *et al.*, 1992) that the genetic abnormality mapped to the short arm of chromosome 9, and that the responsible gene was acting as a tumour suppressor gene. Since that time its locus has been mapped to 9q22.3, and our understanding of the molecular basis of BCC has progressed significantly, although there are still many features (such as the BCC subtype differences) yet to be unwrapped.

The intracellular cellular signalling pathways involved in sporadic and syndromal BCCs in both animal models and humans are known collectively as the hedgehog signalling pathway. The main features of hedgehog signalling are described below.

1.9.2 Hedgehog (Hh) signalling

The Hedgehog signalling network was initially discovered in Drosophila, but is highly conserved between invertebrates and vertebrates (Goodrich *et al.*, 1996). It consists of a series of interacting extracellular ligands, membrane receptors, intracellular signal transducers, transcription factors and target genes; these are summarised in figure 1.9. There are many similarities between the drosophila and human networks but there are some crucial differences, particularly in the variety of down-stream transcription factors.

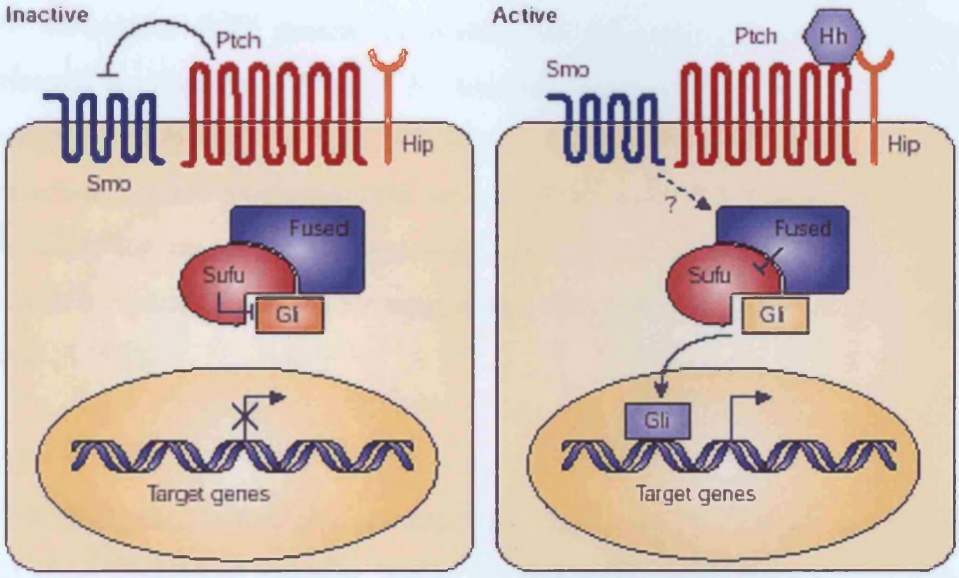


Figure 1.9: **Hedgehog signalling**. In the absence of ligand, the Hh signalling pathway is inactive (left). In this case the transmembrane protein receptor patched (Ptch) inhibits the activity of Smoothened (Smo). The transcription factor Gli is prevented from entering the nucleus through interactions with cytoplasmic proteins including fused and Suppessor of Fused (Sufu). As a consequence, transcriptional activation of Hh target genes is repressed. Activation of the pathway (right) is initiated through binding of any of the three mammalian ligands (Sonic, Desert and Indian hedgehog are represented as Hh in the figure) to Ptch. Ligand binding results in de-repression of Smo, thereby activating a cascade that leads to the translocation of the active form of the the transcription factor Gli to the nucleus. This activates nuclear gene expression including Ptch and Gli itself (after Pasca and Hebrok, 2003).

1.9.2.1 The Hh network in vertebrates

Unlike the one hedgehog gene found in Drosophila (*Hh*), three hedgehog genes, first identified in the mouse, are found in vertebrates, including humans (Marigo *et al.*, 1995). These include Desert hedgehog (*Dhh*), Indian hedgehog (*Ihh*), and Sonic Hedgehog (*Shh*). Sonic Hedgehog (the first discovered vertebrate homologue) is so-called because the hero of a popular computer game at the time of its discovery was called "Sonic the Hedgehog" (Sega, San Francisco, USA). "Sonic" has two closely set eyes with a common scleral rim, suggesting holoprosencephaly, which the murine null mutation (*Shh* -/-) also demonstrates.

Shh

In vertebrates SHH protein is synthesized as a 45kD precursor protein which is cleaved to produce a 19 kD N-terminal signal peptide and a 25 kD C-terminal fragment. Initially the SHH-N signal peptide remains tethered to the plasma membrane of the producing cell but is then transported to the responding cells locally by diffusion (regulated by interaction with heparin sulphate proteoglycans or over longer distances by multimerising within lipid rafts, rendering it more soluble (Zeng et al., 2001).

HIP

HIP (Hedgehog interacting protein, found in vertebrates but not in drosophila) binds all three Hedgehog proteins, has a binding affinity for SHH comparable to that of PTCH1 and may act as a negative regulator of SHH signalling (Tojo *et al.*, 2002).

PTCH

Human patched (*Ptch1*) is a tumour suppressor gene which maps to 9q22.3. It encodes a 12-pass transmembrane protein, PTCH1; it is the principal receptor of SHH and plays a key role as a negative regulator of SHH signaling. The PTCH protein (as depicted in figure 1.10) is expressed in the cell membrane of target tissues where, in the absence of its ligand SHH, its function is the constitutive repression of smoothened (SMO), transducer of the SHH signal (Carpenter et al., 1998).

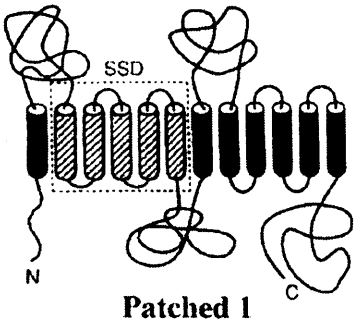


Figure 1.10: schematic representation of the PTCH1 protein (after Daya-Grosjean and Sarasin, 2000)

A second protein, PTCH2, maps to 1p32-p34, has a 54% overall identity to human PTCH1 and a 90% identity to Ptch2 in mice, but its specific role is currently unknown. PTCH1 and PTCH2 are differentially expressed during development of the epidermis, suggesting that the two proteins may have different functions.

SMO

Similar to the case in drosophila, *Smo* (which maps to 7q31-q32) encodes for a transmembrane protein called Smoothened that acts as a transducer of the Sonic hedgehog (SHH) signal. SMO protein has 7 hydrophobic membrane-spanning domains, an extracellular amino-terminal region, and an intracellular carboxy-terminal region (Murone et al., 2000).

Relationship between Patched (Ptch) and Smoothened (Smo)

Much of the evidence relating to the interaction between patched and smoothened comes from mouse model experiments. In summary, in the absence of HH, PTCH prevents SMO from signaling. When hedgehog binds to Patched, however, Smoothened is free to upregulate downstream genes in the network. Patched negatively regulates Smoothened by post-translational modification of its C-terminal domain, resulting in alteration of the phosphorylation status of Smoothened, thereby affecting its stability, activity, and subcellular localization (Alcedo et al., 2000; Ingham et al., 2000).

Sufu

Suppressor of fused (*sufu*) has been identified in chicks, mice and humans (Stone *et al.*, 1999); human *sufu* exhibits a 97% sequence identity with murine *sufu*. Stone found that human *sufu* interacts with *Gli1*, *Gli2*, *Gli3* and it is thought that SUFU protein is important for the shuttling of GLI proteins between the cytoplasm and nucleus (Murone et al., 2000).

GLI proteins

The GLI proteins are vertebrate orthologues of the Cubitus Interruptus (Ci) gene products in Drosophila. GLI proteins function as transcription factors in morphogenesis and normal development (reviewed by Matise and Joyner, 1999).

The *Gli* gene family was originally identified by the amplification of human *Gli1* in glioblastoma (Kinzler *et al.*, 1987). GLI1, GLI2, and GLI3 have similar zinc finger structures and have a high degree of sequence homology. Less is known about GLI family function in vertebrates than is known about Ci function in Drosophila. GLI1 is an activator of hedgehog target genes. Apparently GLI1 does not undergo proteolytic cleavage and does not function as a repressor in the way that Ci does. GLI2 is known to have both activator and repressor forms whereas GLI3 undergoes proteolytic cleavage, resulting in a truncated 83 kDa repressor form and possibly an activator form (Ingham and McMahon, 2001).

As can be seen, three distinct GLI proteins in vertebrates (instead of Ci in Drosophila) encompass a complex signalling network. The concerted activity of the GLI proteins is thought to control HH-dependent signalling in a highly context-dependent manner. Activation of latent GLI proteins is an intricate process that involves modifications and interactions of a number of positive and negative pathway regulators and is not fully understood. It has been suggested that the cellular response to hedgehog signaling might not simply depend on the level of ligand exposure, but also on the particular *Gli* genes expressed (Ingham and McMahon, 2001). *Gli* genes may have unevenly partitioned activator and repressor functions, may be co-expressed, possibly being competitive or working synergistically, or may be partially redundant.

1.9.2.2 Downstream of the Gli proteins

As well as positive and negative feedback mechanisms that exist to regulate the expression of the *Gli* and *Ptch* genes, other target genes of hedgehog signalling in humans include *Wnt* and β-catenin, cyclins, FOX genes and TGF-β.

1.9.2.2.1 Wnt and β-Catenin

Wnt genes encode a family of secreted glycoproteins and misregulation of *Wnt* signaling is implicated in developmental defects and oncogenesis (Taipale and Beachy, 2001). They exert their effects via two signalling pathways.

Activation of the **Wnt/Ca^{2+}** pathway results in intracellular calcium release and activation of the calcium-sensitive enzymes Ca^{2+}-calmodulin-dependent protein kinase II (CamKII) and Protein Kinase C (PKC).

In the **Wnt/β-catenin** signaling pathway, Wnt ligand binding results in the accumulation of unphosphorylated **β-catenin** (a crucial member of the E-cadherin/catenin complex) in the cell. β-catenin then complexes with members of the T-cell factor (Tcf) or lymphoid enhancer factor (LEF) transcription factor families and activates expression of downstream Tcf/LEF-regulated genes including cyclin D_1 (part of the pRb pathway – see section 1.8.4.2) and matrilysin (Tetsu and McCormick, 1999). β-catenin also forms a complex with γ-catenin (plakoglobin) and E-cadherin which plays a role in controlling cellular adhesion and motility by forming adherens junctions and functioning as an invasion suppression system. Mutation of any of the components of the complex can lead to impairment of function, and is thought to lead to cancer development and invasion.

β-catenin plays a significant role in many cancers and inter-links with many important pathways other than the Wnt pathway. Activating mutations in β-catenin have been identified in many human cancers, including, among others, colorectal cancer, endometrial cancer, hepatocellular carcinoma, ovarian cancer, uterine cancer, prostate cancer, melanoma, and pilomatrixoma. Mutations in APC, a tumour suppressor gene that is part of the Wnt/ β-catenin pathway, are known to cause colorectal polyposis and adenocarcinoma (Miller *et al.*, 1999).

1.9.2.2.2 Cyclins

Direct interaction between PTCH1 and cyclin B_1 has been proposed by Barnes et al (Barnes et al., 2001). They found that PTCH1 has a direct role in the management of the G_2/M checkpoint and thus plays a part in controlling cell division.

D-Cyclins have also been proposed as effectors of hedgehog signalling and as mediators of cell cycle control. In mice, cyclins D_1 and D_2 are expressed under the control of Sonic Hedgehog (Mill et al., 2003).

1.9.2.2.3 FOXM1 and FOXE1

It was recently shown that the transcription factors FOXM1 and FOXE1 are both upregulated in BCCs and are under the control of GLI transcription factors. These molecules are implicated in the control of cell growth, proliferation, differentiation, longevity and transformation (Eichberger *et al.*, 2004; Teh *et al.*, 2002).

1.9.2.2.4 TGF-β

There is some evidence that TGF-β expression is under control of Hedgehog signalling, although most of this work has been done in Drosophila (Heberlein et al., 1993). Immunohistochemistry of human BCCs (which are known to have dysregulated hedgehog signalling as explained below) shows reduced expression of TGF-β in the tumour islands and increased TGF-β3 and type-I and -II TGF-β receptors the peri-tumoural stroma (Furue et al., 1997; Schmid et al., 1996; Stamp et al., 1993).

1.9.2.3 The normal function of Hh in vertebrates

Sonic Hedgehog, which is involved in normal embryonic development, is expressed in the floorplate of the neural tube, the early gut endoderm, the posterior limb buds and throughout the notochord, and encodes a signal responsible for patterning the early embryo. The effect of this signal on organ development is thought to be dose-dependent, as shown by the development of pancreatic tissue only in areas with lower levels of Hedgehog expression (Apelqvist et al., 1997). Recently, the Hedgehog/Gli signaling pathway has been shown to play a prominent role in a number of different developmental processes (reviewed by Ingham and McMahon, 2001).

The normal function of hedgehog signalling has been demonstrated by the effects of absence or mutation of Sonic Hedgehog and its associated genes on development. Several human diseases, syndromes and malformations seem to be due to aberrant hedgehog signalling. **Holoprosencephaly (HPE)** is a syndrome affecting the head and face that is caused by haploinsufficiency of sonic hedgehog or mutations in other genes which affect Gli function (Roessler *et al.*, 1996).

Two other syndromes appear to be caused by the loss or dysfunction of GLI3. **Greig's cephalopolysyndactyly syndrome (GCPS)** results from haploinsufficiency of GLI3 and affects multiple tissues, including the face, limbs and central nervous system (CNS) (Vortkamp et al., 1991). **Pallister–Hall syndrome (PHS)** results from the production of a truncated GLI3 protein that is prematurely terminated at the C-terminus, just downstream of the zinc-finger domain (Kang *et al.*, 1997). PHS presents a phenotype similar to, but distinct from GCPS, affecting also multiple structures such as the face and CNS.

Hedgehog signalling has been proposed as having a role in increasing stem cell numbers, such as in the human bone marrow (Bhardwaj *et al.*, 2001). This may partly explain why HH/GLI signaling plays a prominent role in development of epidermal appendages including whiskers and hair in mammalian skin. Loss of HH-signaling in murine epidermis results in an arrest of hair follicle growth at an early stage of follicle development due to reduced proliferation, and re-expression of *Gli2* in Hh knockout mice may rescue hair follicle function (Chiang et al., 1999; Mill et al., 2003).

The transcriptional programs activated in response to HH-signaling in epidermal development and BCC are mainly controlled by GLI1 and GLI2. The skin phenotype of *Gli2-/-* mice largely resembles that of Shh deficient mice, suggesting that *Gli2* encodes the major transcriptional effector of HH-signaling during epidermal development. By contrast neither loss of *Gli1* nor of *Gli3* affects epidermal development (Mill et al., 2003; Park et al., 2000). The involvement of SHH signaling in proliferation has been elucidated from in vitro and in vivo studies that have implicated both SHH and PTCH in promoting the proliferation of human epithelial cells by direct interaction with cell cycle regulatory proteins such as cyclin B1 (Barnes et al., 2001).

1.9.2.4 Abnormal Hh in vertebrates: cancer

1.9.2.4.1 Animal model data

Elegant experiments have been performed in animal models to investigate the role of various members of the hedgehog signalling pathway on the development of cancer, and in particular BCC. These have included the injection of pathway components into model systems, such as the injection of *Gli1* into tadpoles resulting in BCC formation (Dahmane et al., 1997).

However, the mainstay method used in the relevant animal model studies has been the creation of transgenic mice with knockouts of one or both alleles of the hedgehog pathway. Over-expression of **SHH** in murine skin results in the development of BCC-like tumours (Oro et al., 1997). Regenerated human skin transgenic for SHH also displays abnormal BCC like structures when grafted onto immune-deficient mice (Fan *et al.*, 1997). The rapid and frequent appearance of BCC like tumours in the absence of induced mutations are a strong indication that activation of the SHH pathway is sufficient for BCC formation.

BCC-like tumours develop in mice overexpressing **GLI1** and **GLI2** (Grachtchouk et al., 2000; Nilsson et al., 2000). Beyond the inductive capacity of GLI1, GLI2 appears to be required for tumour survival and progression in mice: in *Gli2* knockout mice with a tetracycline-regulated *Gli* promoter, tumours were switched off and on by tetracycline administration (Hutchin et al., 2005).

Mice which are homozygous for **patched** (*Ptch*-/-) die as embryos. In heterozygote transgenic *Ptch* mice, BCC lesions are not found, although cerebellar tumours are (Goodrich *et al.*, 1997). However, these mice do develop microscopic BCC lesions upon chronic UV exposure, 40% of which contain p53 mutations (Aszterbaum *et al.*, 1999).

Similar microscopic BCC like lesions are seen in transgenic mice carrying a constitutively active mutated ***Smo*** gene (Xie *et al.*, 1998) and the mouse epidermis is hyperproliferatve, expresses BCC protein markers and gives rise to numerous down growths which invade the underlying dermis (Grachtchouk et al., 2000).

1.9.2.4.2 Hh pathway mutations in human cancers including BCC

SHH

Mutations in the Sonic hedgehog gene have been seen in tissue from sporadic basal cell carcinoma, medulloblastoma, breast cancer and from patients with Gorlin's syndrome (Oro et al., 1997), although the rare association with sporadic BCC has been questioned by Wicking who found no such modification in 36 sporadic BCCs in a later study (Wicking *et al.*, 1998). It is only from a recent study of BCCs in patients with Xeroderma pigmentosum that a role for *Shh* in human skin tumourigenesis has been established. Six novel *Shh* gene mutations were found in 33 such tumours and all were UV-specific C→T transitions or CC→TT tandem substitutions located at pyrimidine dimer sites (Couve-Privat *et al.*, 2004).

PTCH

Mutations in *Ptch1* have been identified in patients with Gorlin's syndrome, isolated basal cell carcinoma, medulloblastoma, meningioma, neuroectodermal tumor, breast carcinoma, esophageal carcinoma, squamous cell carcinoma, rhabdomyosarcoma and trichoepithelioma (reviewed by Cohen, Jr., 1999). Expression of PTCH2 and rare *Ptch2* mutations have been reported in medulloblastoma and basal cell carcinoma (Smyth *et al.*, 1999).

Ptch gene mutations occur in 12–38% of sporadic BCCs and the mutation spectrum indicates a major role for solar irradiation in tumour development, as 50% of mutations are UV-specific C→T or CC→TT transitions (Aszterbaum *et al.*, 1998).

Smoothened

Smoothened mutations have been reported in Gorlin's syndrome patients, sporadic BCCs and medulloblastoma (Reifenberger et al., 1998). In a study of 30 BCCs from XP patients, a significantly higher level (30%) of *Smo* gene alterations were noted (Couve-Privat *et al.*, 2002).

SUFU

Mutations in *Sufu* have been found in medulloblastoma tissue and BCCs (Reifenberger et al., 1998). Although not discovered yet, there is suspicion that other

cancers may be affected by such mutations because the gene locus (10q24-q25) has been found to be deleted in glioblastoma, prostate cancer and endometrial cancer.

Gli proteins

Gli3 alterations have been found in medulloblastoma tissue (Erez *et al.*, 2002). Although no definitive mutations in *Gli1* and *Gli2* have been discovered yet in BCCs, *Gli1* is expressed in almost all basal cell carcinomas due to gain-of-function or loss-of-suppression mutations upstream in the signalling pathway (Dahmane et al., 1997). The investigation of hedgehog signalling in other tumours has revealed a possible correlation between Gli levels and the grade of sarcoma (Stein *et al.*, 1999), but this is not currently a strong enough link to be used in practical staging terms.

1.9.2.4.3 Overall alterations in Hh signalling in human BCCs

Constitutive activation of the sonic hedgehog signalling pathway leads to the development of BCCs. Several studies have shown consistent over-expression of PTCH1 and SMO in human BCCs (Bonifas et al., 2001; Kallassy et al., 1997; Reifenberger et al., 1998; Tojo et al., 1999; Unden et al., 1997).

GLI1 is frequently over-expressed in BCCs where it is located principally in the cytoplasm (Bonifas et al., 2001; Kallassy et al., 1997; Reifenberger et al., 1998; Tojo et al., 1999; Unden et al., 1997). Upregulation of GLI2α/β isoforms by GLI1 in human BCCs has also been reported along with evidence for a positive feedback mechanism between GLI1 and GLI2 (Regl et al., 2002). Furthermore the latest study by this group provides evidence that GLI2 plays a dual role as activator of keratinocyte proliferation and repressor of epidermal differentiation (Regl et al., 2004). It is postulated that the hyperactivation of GLI2 by aberrant SHH signaling would then disrupt epidermal homeostasis and lead to neoplasia.

The importance of GLI2 in BCC is further consolidated by Ikram et al. (Ikram *et al.*, 2004) who show that *Gli2* is expressed in the interfollicular epidermis and the outer root sheath of hair follicles in normal skin as well in BCC tumour islands. This implies that hedgehog signalling regulates hair follicle growth and when inappropriately activated it may cause hair follicle-derived tumours, the most

clinically significant being the BCC. To date, there is no evidence that expression of GLI3 is altered in BCCs compared to normal skin.

Quantification of *Gli1* levels has been investigated in BCCs and other skin lesions, including tricoepithelioma, squamous cell carcinoma and seborrhoeic keratosis, and was found to be upregulated in BCCs and tricoepithelioma (Hatta *et al.*, 2005). The best comparison so far of *Gli* transcript levels in different tumours has been published by Grachtchouk et al (Grachtchouk *et al.*, 2003). In both mice and human tumours, a greater degree of hedgehog signalling transcripts (PTCH1 and GLI1) were seen in BCCs than in follicular hamartomas. Also, mice with limited hedgehog signalling upregulation (due to activating *Smo* mutations) developed only hamartomas. Thus, they suggested that a greater level of hedgehog upregulation is required for the induction of BCCs than other related tumors.

1.10 Variation of signalling pathways within BCC: biomarkers

Even in the face of all this information, little progress has been made over the many years of study in explaining the molecular basis for the variation of tumour phenotype that exists. Within the world of cancer biology BCC is a good example to study, for it is common and has a spectrum of behaviour ranging from indolent to aggressive and although some aspects of behaviour may be predicted by histological appearance, this is by no means absolute.

Previous attempts to link molecular biology to BCC subtype (or even better, but less commonly, risk of recurrence) have included investigation of expression of cyclins, Bcl-2, p53, Ki67 and β-catenin.

1.10.1 Cyclins

Staibano et al studied the immunohistochemical expression of Cyclin D_1 in BCCs (Staibano et al., 2001a). Their study assumed that any positivity of expression correlated with cells overexpressing cyclin D_1, which has been previously documented. They noted that aggressive BCCs (as defined by recurrence or metastasis) demonstrated a significantly higher degree of cyclin D_1 staining than

tumours with a good outcome. They proposed that BCCs likely to have an aggressive course express higher levels of cyclin D_1 and that this reflects the higher cell turnover present in the tumour cells.

1.10.2 Bcl-2

The features of Bcl-2 expression have been discussed previously (section 1.8.5.1). The diffuse staining pattern typically seen in BCCs is useful in discriminating them from tricoepitheliomas, which have a more peripheral staining pattern (Abdelsayed et al., 2000; Poniecka and Alexis, 1999; Smoller et al., 1994; Swanson et al., 1998). In contrast to BCCs, squamous cell carcinomas do not stain for Bcl-2 (Verhaegh et al., 1995). Higher levels of Bcl-2 are found in the more indolent subtypes of BCC with a concomitant reduction in the more aggressive groups (Crowson et al., 1996; Ramdial et al., 2000).

1.10.3 p53

p53 staining in BCCs shows conflicting results. This may be partly due to the specific problems with p53 immunohistochemistry mentioned previously. De Rosa et al demonstrated that there is greater degree of p53 immunohistochemistry staining in aggressive BCCs compared to non-aggressive BCCs (De Rosa et al., 1993), whereas Demirkan stated that there is no relationship between histological subtype of BCC and p53 staining (Demirkan et al., 2000). This issue has not been resolved.

1.10.4 Ki67

Immunohistochemical staining with antibodies to Ki67 has been shown to correlate with the growth fraction of a tumour, as measured by both labelled mitoses and by BrdU incorporation (McCormick *et al.*, 1993; Smith *et al.*, 1995). Baum found that Ki67 was equally expressed amongst different histopathological subtypes of BCC (Baum et al., 1993) but Healy et al went beyond histopathology, to correlate the actual recurrence of surgically excised BCCs with Ki67 expression (Healy et al., 1995). This was achieved by comparing the immunohistochemical staining of two groups of BCCs, one of which comprised of tumours that following excision, had gone on to recur, whereas the other contained tumours that had been similarly excised and had not recurred (in this study however there was no mention of what their definition of

"complete" excision was). They found that tumours which went on to recur had a higher proliferative index, as assessed by staining with Ki67.

1.10.5 β-catenin

Conflicting evidence exists as to the relevance of β-catenin and different subtypes of BCC. Boonchai et al found that there was no significant relationship between positive staining and subtype of BCC (Boonchai *et al.*, 2000), whereas El-Bahrawy et al showed that the staining pattern of β-catenin in BCCs did correlate with the histological subtype of the tumour (El Bahrawy et al., 2003). From this immunohistochemical study they concluded that infiltrative and morphoeic BCCs were more likely to have positive nuclear staining, whereas this was absent in indolent tumours. Micronodular tumours, interestingly, did not exhibit such strong nuclear staining (even though they are known to behave in an aggressive fashion) but showed strong membranous staining for β-catenin, contrasting with the absence of such localisation in other aggressive tumours.

1.11 Chapter 1 section 3: Background work leading to this study

1.11.1 Overall hypothesis

As described in this introductory chapter there is a vast amount of work that has described the different clinical, histological and molecular characteristics of basal cell carcinoma. **My overarching hypothesis is that differences in molecular biology underpin both the different growth patterns that are seen and the degree of aggression that a tumour displays**. Chapter-specific hypotheses are described in section 1.11.4 below.

1.11.2 The use of cultured cells in this study

It is well accepted that cell culture techniques have been of limited use in BCC. This is thought to be because of various specific features of BCC cells that make them less likely to grow in cell culture than other tumour cells. Reasons for this are thought to include the stromal dependency of the tumours, which may also explain the relative lack of metastasis that occurs. The resulting paucity of data from BCC cell culture means that previous studies have searched for new biomarkers in tissue from animal models or human tumours. This has its drawbacks in terms of the ease and bulk of cellular material that can be examined. A cell culture system that gives insight to the molecular mechanisms of BCC would be a significant development in the investigation of skin cancer pathogenesis.

A medline search for cell culture in Basal Cell carcinoma provides only seven published reports in English where culture of BCC cells has been successfully achieved and has yielded results in the investigation of BCC cell biology. Of note, Asada mentioned that there were only some cellular characteristics of the BCC tumours preserved in culture (Asada *et al.*, 1992). This, and the small number of reports of the technique being successful implies that the difficulty in culturing these cells is due to the fact that the cells require a milieu such as the stroma to grow efficiently, and that without it, growth is limited. As a result of such difficulties, many of these reports state that the cultures were short term only, and that although a small

number of passages may have been possible, few of these studies go beyond the technical aspects of cell culture to derive useful information from the cell analysis.

To get around this difficulty of growing and manipulating BCCs cells for the purpose of molecular biology investigation, a model system using keratinocytes has been used in the laboratory in which I performed this work. Given the importance of Gli expression in development of BCC, the group I have worked with has developed a system by which keratinocytes may be retrovirally transduced to over-express Gli transcription factor genes, as outlined in section 2.3.3.2.

1.11.3 Searching for novel biomarkers

In comparison to further development of existing theories, choosing a completely new direction of investigation is comparable to looking for a needle in a haystack. The emergence of gene array technology, however, has dramatically improved the ability to search for genes that may be responsible for differences between tumours, and I have made use of such developments in this work.

When analysing the growth features of *Gli*-transfected N-terts cells, it was noted that the growth of the cells was altered. Non-transfected (normal) keratinocytes had a uniform near-spherical pattern on development, whereas cells that over-expressed Gli1 (as measured by fluorescence of EGFP linked to the *Gli1* molecule) developed a marked fibroblastic appearance as shown in Figure 1.11.

The messenger RNA (mRNA) from these different sets of cells was analysed by gene array analysis, and a number of genes were seen to be specifically highly upregulated in the setting of *Gli* over-expression. Some of these genes are known to be associated with presence of a neuronal phenotype.

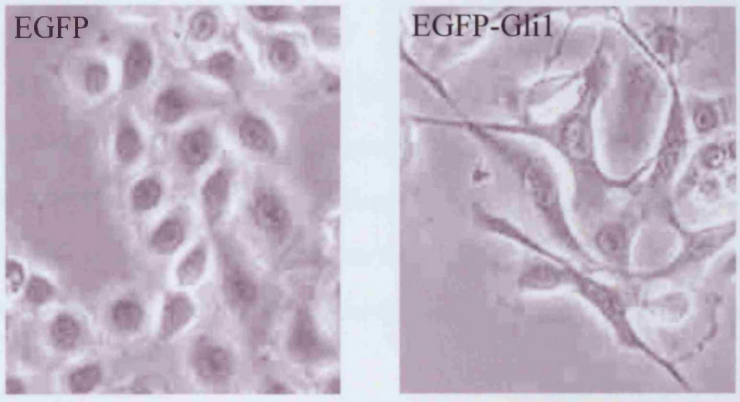


Figure 1.11: normal appearance of keratinocytes in culture, contrasting with the development of a fibroblastic appearance in cells that over-express Gli 1

1.11.4 Chapter-specifc hypotheses and experimental aims

1: The hypothesis that I test in chapters three and four is that markers of neuronal differentiation are upregulated in human basal cell carcinoma compared to control non-tumour skin tissue, and that tumours with different histological features express these markers differentially. This is examined in chapter three using archival BCC tissue using immunohistochemistry techniques, and in chapter four using freshly-biopsied BCC tumour and skin tissue using PCR-based methods of gene expression quantification.

2: In chapter five my hypothesis is that expression of neuronal markers in a keratinocyte cell culture model results in elevated levels of neuronal marker gene expression. This is examined using real-time PCR-based semi-quantitative comparision of gene expression in Gli-overexpressing keratinocytes.

3: In chapter six my hypothesis states that there is a differential level of neuronal marker protein expression between tumours that go on to recur and those that do not. This is examined using immunohistochemisty techniques on archival BCCs, the clinical outcome of which is known following extensive follow-up.

Chapter 2: Materials and Methods

2.1 Introduction

The principal aim of this study was to determine whether neuronal differentiation markers are a feature of BCCs and what impact this has on their biological growth. This was investigated by comparing BCCs of differing histological subtype to control tissues (immunohistochemistry and genomic experiments) and by comparing BCCs of differing clinical outcomes (immunohistochemistry of recurrent and non-recurrent tumours). Both archival and fresh tissue BCC was used: archival tissue has the advantage of being plentiful and easily handled and was used in these experiments for investigation of protein expression. Fresh tissue was more difficult to come by (limited amounts of tissue could be taken from individually excised tumours because of the need to avoid disrupting the tumour margins for the purposes of histological examination) but is more reliable when it comes to examination of mRNA expression. The three main types of material used for these experiments are detailed below.

2.2 Tissue used

2.2.1 Archival tissue for immunohistochemistry

2.2.1.1 Primary Basal Cell Carcinomata

The histopathology records of the Royal London Hospital were used to determine a series of BCC tumours which had been excised during the calendar year of 2003. A consecutive series of tumours was selected and the slides corresponding to these tumours were retrieved from the archived collections. These slides were examined by me and by a consultant dermatopathologist (Dr. Rino Cerio) and the following data points were noted in each case

- Presence of Basal Cell Carcinoma
- Dominant histopathological subtype
- Gender of patient

Given that the aim of experiments using this tissue was to examine properties of tumours of certain histopathological subtype without recourse to patient outcome, other clinical variables were not recorded in these cases. Inclusion criteria were merely those tumours that were clearly basal cell carcinomata which had been

surgically excised and for which histological slides and blocks were available. Exclusion critieria included those tumours that did not clearly exhibit the architectural features of BCC, and those that consisted of more than one obvious BCC subtype.

Following review of these tumours a sub-series of tumours that best represented the classical appearance of the five main histological subtypes were chosen for further investigation.

All cases were given a unique code which was only known to me. All downstream use of the tissues was referred to by this code such that individual patient identification would not be possible by other people.

2.2.1.2 Recurrent Basal Cell Carcinomata

The pathology archives of Mount Vernon Hospital contain a huge amount of patient material spanning many years of plastic surgery at this centre. All records are either computerised or stored on microfiche. For this part of the study, BCCs were specifically sought that had recurred following either complete or incomplete surgical excision. These were compared with tumours that were known to have not recurred following surgical excision.

2.2.1.2.1 Recurrent tumours

All histopathology reports from the years 1996 (the advent of computerisation in this department) to 2001 were merged and converted to Microsoft Word format (Microsoft, Seattle, USA). Word searching techniques were used to find reports that included the words "recurrent" or "recurrence". All these cases were recorded, and further searching through patients' computer records, notes and microfiche files was used to try and confirm whether the tumours were truly episodes of recurrence or not. For those tumours that were confirmed to be recurrences, the slides for the original tumours and the recurrences were obtained for examination. Data recorded for each of these cases includes

- Site of original tumour or possible recurrent tumour
- Histological subtype diagnosis of BCC (if described)
- Follow-up duration, either at Mount Vernon Hospital or GP
- Lateral surgical excision margins
- Deep surgical excision margin

- Sex
- Age
- Duration of follow-up

Exclusion criteria included

- tumours which were not definitive BCCs in both original and recurrent tumours
- tumours which occurred at different sites
- tumours where secondary tumours did not arise contiguous to the original excision scar

BCCs that were found to have recurred following excisional surgery were classified into two groups: those that recurred following complete surgical excision (as defined by excision margins of at least one millimetre) and those that recurred following incomplete surgical excision (where the original tumour had positive or very close (<0.2mm) excision margins). All specimens considered had been examined with multiple sectioning techniques with the aim of determining the margin of excision as closely as possible without recourse to Mohs surgery.

Thus, four groups of tumours were formed – **C**ompletely excised **O**riginal BCCs, **C**ompletely excised **R**ecurrent BCCs, **I**ncompletely excised **O**riginal BCCs and **I**ncompletely excised **R**ecurrent BCCs (see table 2.1 below).

The tumours that went on to recur were specifically classified by the following methods

- Age
- Sex
- Body site (Trunk, Head and Neck, Upper limb, Lower limb)
- Histological growth pattern subtype class
    - Indolent (Nodular and Superficial tumours)
    - Micronodular tumours
    - Aggressive (Infiltrative and Morphoeic)

2.2.1.2.2 Non-recurrent tumours

The databases were then searched for tumours that were known to have not recurred. Essential to this was the knowledge that the patients in question had not suffered a recurrence of their tumour at the same site, and that they were alive and available for

follow up. Review of patients' case notes and consultation with the patients' General Practitioners was used to confirm whether this was the case with each patient. Patients who had died, or who had had BCCs in a similar site to their previous tumours were excluded at this point. In addition, patients who had had a BCC incompletely excised but who did not have further surgery (either because of their personal preference or because of surgical advice) were only included if it was clearly documented in the patients' notes that this was the reason why no further surgery was performed.

Tumours from these cases were then also classified by criteria as described above for the recurrent tumours. Cases were chosen such that each group of non-recurrent tumours (complete and incomplete excisions) matched the relevant recurrent groups in terms of body site, sex, average age and histological growth pattern class (Indolent, Micronodular or Aggressive). This was done so that any differences in immunohistochemical staining between the groups would be attributable to the biological differences between these groups and not other characteristics that may affect staining patterns such as histological subtype, sex, age or body site. Two groups were created by this method: **C**ompletely excised **N**on-recurrent BCCs and **I**ncompletely excised **N**on-recurrent BCCs. The six groups of tumours are described in table 2.1.

	Original tumour which goes on to recur	Recurrent tumour	Original tumour which does Not go on to recur
Complete excision	CO	CR	CN
Incomplete excision	IO	IR	IN

Table 2.1: Summary of groups of recurrent and non-recurrent tumours

All cases were allocated a secret code (according to the groups described above) the key for which was known only to me. All downstream use of the tissues was referred to by this code such that individual patient identification would not be possible by other people.

2.2.1.3 Control tissues

2.2.1.3.1 Normal skin

Blocks of wax-embedded normal skin samples were obtained from the department of Pathology, Royal London Hospital. These sections had been removed as a result of excess of skin being removed, such as in facelift surgery, or in breast reduction surgery and had been routinely preserved and fixed.

2.2.1.3.2 Appendix tissue

Blocks of wax-embedded appendix tissue were available from the Department of Histopathology, Royal London Hospital. They had been excised during appendicectomy procedures and the remaining tissue was available for histological examination. This tissue was used because the nerve fibres in the muscle coat act as good neuronal marker positive controls (as described in section 2.4.1.2).

2.2.2 Fresh tissue used for mRNA isolation

2.2.2.1 BCC Tumour tissue

Fresh biopsies of Basal Cell Carcinoma tissue were obtained from patients undergoing excisional surgery at Mount Vernon Hospital. All patients that had been booked to undergo excision of a BCC at Mount Vernon Hospital during April and May 2005 were sent consent forms and information sheets about this study. The patients were then contacted on the day of their surgery before their operation to discuss further the issues regarding the donation of their tissue. At this stage a clinical assessment of the tumour was performed by myself - given that the different subtypes of BCC can have markedly different clinical appearances any tumour that resembled any subtype of BCC was considered. Patients with tumours in excess of 0.8cm diameter who agreed to donate tissue and had signed consent forms prior to their procedure were considered eligible.

Recording of patient data was performed by entering patient details onto a spreadsheet (Excel, Microsoft, Seattle, USA) and allocation of a unique letter and number code to each patient tumour. This code was known only to me and as such any handling of tissues downstream was referred to in this anonymous fashion.

As part of my ethical application for this part of the study, an agreement was reached with the West Hertfordshire Hospitals Trust Local Research Ethics Committee and the Consultant Histopathologists as to how samples of tissue should be taken. This technique is described in detail below.

2.2.2.2 Control tissue

Donation of non-tumour normal skin tissue was obtained in a similar manner from patients undergoing excisional surgery at Mount Vernon hospital. These patients included those that were undergoing facelift surgery or breast reduction surgery. Consent was sought in a similar manner to those undergoing surgery for BCC. Larger samples were obtained from these patients because of the lack of subsequent histological examination required. Coding, data recording and processing was performed as for BCC tumour cases.

2.2.3 Cultured cells

2.2.3.1 Primary keratinocytes

Keratinocytes were prepared from fresh foreskin samples obtained from patients undergoing circumcision surgery at the St. Bartholomew's and Royal London Hospitals Trust as previously described (Rheinwald and Green, 1975). These samples were kept initially in transport medium, minced and incubated in trypsin (0.25% v/v) for 2 hours. The cell suspension was filtered and centrifuged. Following washing of the cell pellet the re-suspended cells were stored at -80°C. Preparation of primary keratinocytes was principally carried out by Dr. Luke Gammon (Centre for Cutaneous Research, QMUL. London).

2.2.3.2 SH-SY5Y cells

These are immortalised cells which originally derived from neuroblastoma tumour tissue and they are available commercially (LGC Promochem, Teddington, UK, ATCC number CRL-2266). RNA from these cells (kind gift from Dr. Graham Neill) was available for reverse transcription and use in PCR experiments. Because of their origin they are suitable for use in these studies as a neuronal differentiation positive control (Biedler *et al.*, 1978).

2.2.4 Ethics

Ethical approval was granted for collection of each type of tissue required. Details of the approvals are given in table 2.2. Specific consent was sought and received from all patients who donated both fresh tissue and archival recurrent BCC tissue.

Tissue	Hospital	LREC	Reference
Archival primary BCCs & skin	RLH	East London and the City	T/01/028
Archival recurrent BCCs	MVH	West Hertfordshire	04/Q0203/37
Fresh BCCs and skin	MVH	West Hertfordshire	T/01/037

Table 2.2: Details of Ethics Committee approvals for archival and fresh tissue collection. RLH = Royal London Hospital; MVH = Mount Vernon Hospital; LREC = Local Research Ethics Committee.

2.3 Harvesting, processing and storing of tissue

2.3.1 Wax-embedded archival tissue

2.3.1.1 Block retrieval, Sectioning, Labelling, storage

Following identification of patients and tumours to be used, the blocks corresponding to the tumour samples were retrieved from the archives of the Royal London Hospital and Mount Vernon Hospital. The blocks were re-labelled as per the coding system described earlier, and 5 μm sections were cut and mounted onto APS coated slides (VWR, Leuven, Belgium), which had been pre-labelled with the corresponding code. After a period of drying at room temperature, the slides were baked at 45°C overnight to ensure no moisture remained. Thereafter slides were stored at room temperature.

2.3.2 Fresh frozen human tissue – BCC and normal skin

2.3.2.1 Harvesting and biopsy storage

Upon excision of the putative BCC the fresh specimen was handled carefully and depending on its size either a punch biopsy cutter (Stiefel, High Wycombe, UK) or a scalpel was used to cleanly remove a piece of tissue from it. Care was taken not to include the lateral edges or the deep margin of the tumour so as not to affect the subsequent histological examination of the margins of excision. This biopsy specimen was then snap frozen in liquid nitrogen so as to maximise preservation of the RNA in

the sample. In the majority of cases only one biopsy was possible from each tumour without affecting the excision margins of the tumour.

Samples of normal skin were also obtained as described previously, from patients undergoing procedures resulting in excision of excess skin. These samples were cut into small pieces (approximately 5 x 5 mm) and these were snap-frozen as described above.

With all samples having already been assigned a letter and number code a 1.5 mls cryotube (Corning, New York, USA) was labelled with this code. It was then used to store this sample in a box of dry ice (Global Ice, Middlesex, UK) prior to longer term storage in a freezer at -80°C.

2.3.2.2 Embedding

All biopsy samples were transported on dry ice from location of freezer to site of subsequent processing. At all times the biopsies were kept as cold as possible to avoid degradation of RNA.

Embedding was performed using "Cryo-M-bed" (Bright Instruments, Huntingdon, UK). Although a liquid at room temperature, this material forms a solid block around a small piece of tissue allowing attachment to cryostat rotors and stabilisation of the tissue during sectioning. In this case a mould of Aluminium foil (Aluchef foil, Terinex, Bedford, UK) was filled with liquid Cryo-M-bed and the tumour sample placed within it. Great care was taken to ensure that the orientation of the sample was correct such that when sectioned the correct cross section of tissue was presented to the blade. This mould and contents were then immersed in liquid nitrogen and the foil removed prior to mounting the frozen block on a small cork board. These blocks were stored at -80°C.

2.3.2.3 Sectioning, confirmation of biopsy architecture and quality scoring

Sections were cut using a cryostat (Bright Instruments) with the cabinet maintained at –35°C. For each tumour, a sharp blade was treated with "RNaseZap" (Ambion, Huntingdon, UK) and cooled prior to cutting. Tumour and normal skin blocks were mounted onto carriers for the cutting arm of the cryostat by using embedding

compound and freezing them in liquid nitrogen. 5μm sections were cut in each case and were mounted on APS-coated glass slides.

These sections were dehydrated, stained with haematoxylin and eosin and rehydrated prior to mounting and coverslipping with Depex mounting medium (Fisher Scientific, Loughborough, UK). Microscope examination of these sections confirmed the diagnosis of BCC and was compared to the histological examination of the original tumour mass. At this point an assessment was made of the biopsy's relative size (small, medium, large and very large) and the quality of the biopsy in terms of how the tissue architecture represented the predominant tumour subtype (no tumour, poor quality, medium quality, high quality).

Further sectioning of these blocks for mRNA examination occurred as described, with the exception that the slides were quickly returned to dry ice to keep them cold. The slides needed to be at room temperature initially so that the sections would melt onto the slides in a flat fashion, but once this had occurred they were chilled so as to preserve the integrity of the RNA. Slides were pre-labelled with the number and letter code assigned to each tumour.

2.3.2.4 Laser Capture Microdissection

Laser Capture Microdissection (LCM) is a technique that has recently been promoted as a way of detecting specific gene expression in small distinct areas of tumours and non-malignant tissues. The specificity it can achieve in terms of cellular harvest within a chosen area of a tissue slide is unrivalled compared to other techniques but the quantities of genomic material gained are small (in the order of hundreds of cells, and nanogram/microlitre RNA extracts). I have used this technique to examine gene expression in BCCs. The advantage in this case over conventional techniques is the ability to specifically pick up the target tumour cells and leave all other cellular material behind. This is particularly attractive in the case of infiltrative and morphoeic BCCs. The microscope, laser and processing kit I used were all manufactured by Arcturus (Mountain View, California, USA).

2.3.2.4.1 Sectioning

Sectioning was carried out as described previously. For sectioning of samples for RNA work the freshly sharpened blade was treated with "RNaseZap" to minimise RNA loss from RNAse activity. Following the sections being cut and being "melted" onto the slides, the slides were rapidly placed into the cryostat cabinet to ensure that the tissue warmed up as little as possible so that RNase activity was minimised. A maximum of four sections were placed on any one slide so as to minimise the period of time that the slide would spend at room temperature. Up to five slides (i.e. up to twenty sections of one sample) were cut at the same session and stored on dry ice.

2.3.2.4.2 Dehydration and staining of sections

Slides were removed from the dry ice box and immediately placed (in batches of up to four) in a series of vials containing RNase free graded alcohols, nuclear stain and xylene ("Histogene" kit, Arcturus, Mountain View, USA). It was found that epidermal capture from normal skin was very difficult, and so a trypsin step was included during this processing step to reduce the intercellular bonds (Agar et al., 2003) and personal communication with author). Trials of 20 seconds, 2 minutes and 5 minutes using 0.25% v/v trypsin were performed. On the basis of these trials, it was decided that for normal skin samples a two-minute 0.25% v/v trypsin step gave the best results and did not seem to affect downstream processing. The slides were then briefly air-dried and placed in a box with desiccant before proceeding to Laser Capture Microdissection.

2.3.2.4.3 Laser Capture Microdissection

Slides are removed from the dry box and placed onto the stand of the Laser Dissection microscope. Laser dissection caps are placed into the fixed-location holder on the side and the swing arm is moved around to pick up the cap. As soon as the optically-clear cap has been placed directly *onto* the tissue, the laser beam can then be focused onto the tissue.

Firing of the laser (in single or rapid fire mode) distorts the plastic cap in such way that a carpet layer of cells becomes attached to the undersurface of the cap. It is easy for moisture to be noticed here – cell attachment does not occur in the event of the slides being anything other than totally dry.

Following attachment of the cap, it is then removed from the slide, akin to "ripping" cells from the section. Such a series of slides is shown in figure 2.1, with the final selection of cells shown in figure 2.1 (c). The cap containing these cells is then placed onto a 0.5ml microcentrifuge tube containing "picopure" lysis buffer (see the following section). The tube is inverted and kept on wet ice until the next processing step.

2.3.2.4.4 RNA extraction

"Picopure" is the RNA extraction kit produced by the makers of the Laser Capture Microdissection Microscope (Arcturus, Mountain View, USA). It is designed for use with small cell populations such as those produced with this method, and consists of a series of solutions and spin columns.

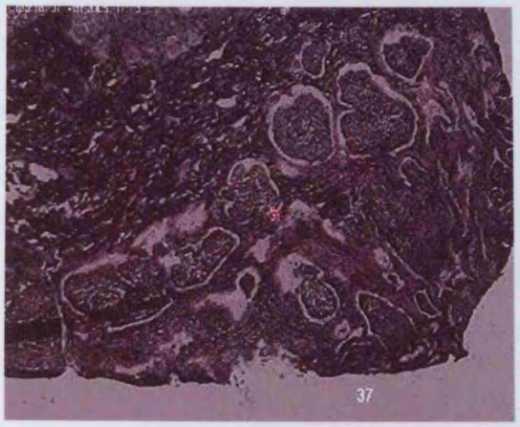
Once the cap with the captured cell population is immersed in extraction buffer, a series of heating and centrifugation steps takes place prior to adding the mixture to a specifically designed RNA purification column. Various conditioning buffers and wash buffers are used in conjunction with certain centrifugation steps. The final step involves addition and centrifugation of between 11 and 30 µl of elution buffer, resulting in a similar volume of RNA solution for onward use. In all cases the manufacturer's recommendations were adhered to.

2.3.2.5 Routine BCC RNA extraction

2.3.2.5.1 Needle Microdissection (NMD)

Following the sectioning required for Laser Capture Microdissection, 15µm sections were cut and kept in similar conditions as above. Tumour areas were then microdissected with a 25G (orange) needle (Becton Dickinson, Oxford, UK) and the resulting cell populations were placed into 1ml Trizol (Invitrogen, Paisley, UK) in a 1.5ml microcentrifuge tube kept on wet ice.

A:



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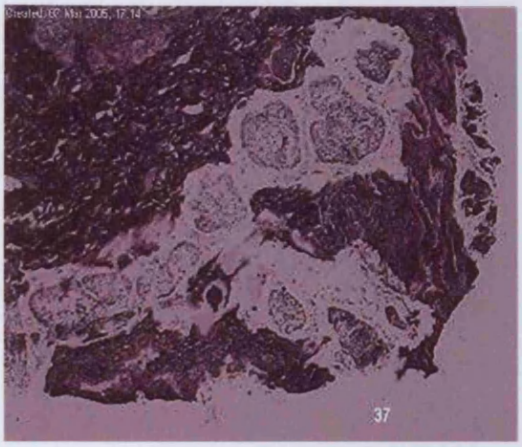

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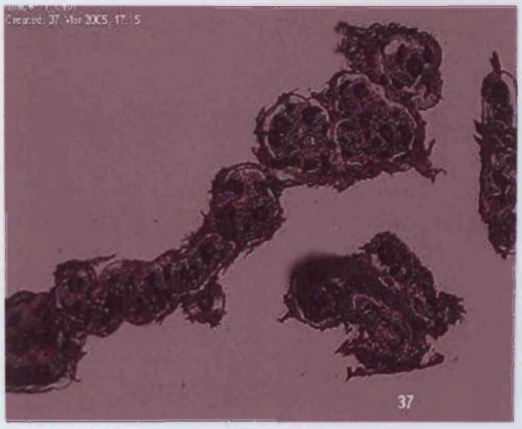


Figure 2.1: Sequential photographs of a micronodular BCC section (a) before and (b) following capture, and (c) the resected cells for RNA processing.

2.3.2.5.2 Whole Tissue Homogenisation (WTH)

Lastly, the remaining embedding compound was removed by scalpel leaving the remaining section of biopsy tissue intact. This was then also placed into 1ml Trizol in a 1.5ml microcentrifuge tube kept on wet ice. An ultra-turrax T15 tissue homogeniser (IKA, Wilmington, USA) was used to homogenise the tissue in Trizol: 3 x 30 second bursts were used, with the samples being kept on wet ice between bursts.

2.3.2.5.3 RNA extraction

The manufacturer's standard protocol for Trizol RNA extraction was used. This resulted in 30µl aliquots of needle microdissection RNA solution and whole tissue homogenate RNA solution for each BCC processed.

2.3.3 Cultured cells

Retroviral transduction of cells was used to produce cells that over-expressed the genes *Gli1* and *Gli2*. Two separate transduction experiments were performed, each under identical conditions, six weeks apart.

2.3.3.1 Cell culture techniques

Frozen populations of primary keratinocytes were thawed, and placed into flasks at a density of 1.0×10^5 cells per 6 cm diameter culture dish in defined serum-free medium (Invitrogen, Paisley, UK). These were cultured for 18-24 hours prior to retroviral transduction. All cell culture procedures were carried out in a ventilated cabinet in a category II containment suite (Laminair HBB2448, Hearoes Instruments, UK). Cells were cultured in a water-saturated atmosphere of 4.9% CO_2 at 37°C in an incubator (Sango, Loughborough, UK).

2.3.3.2 Retroviral transduction of cells and RNA extraction

Retroviral particles encoding *EGFP*, *EGFP-Gli1*, and *EGFP-Gli2* were produced and retroviral transduction of keratinocytes was achieved as described previously (Deng et al., 1997), except the more efficient Phoenix amphotropic packaging line (gift from Dr. Gary Nolan) was used as described by Regl et al (2002). Efficient transduction was confirmed by fluorescent microscopy, as illustrated in figure 2.2. RNA was harvested 96 hours post-transduction using a commercial "RNeasy Miniprep" spin-

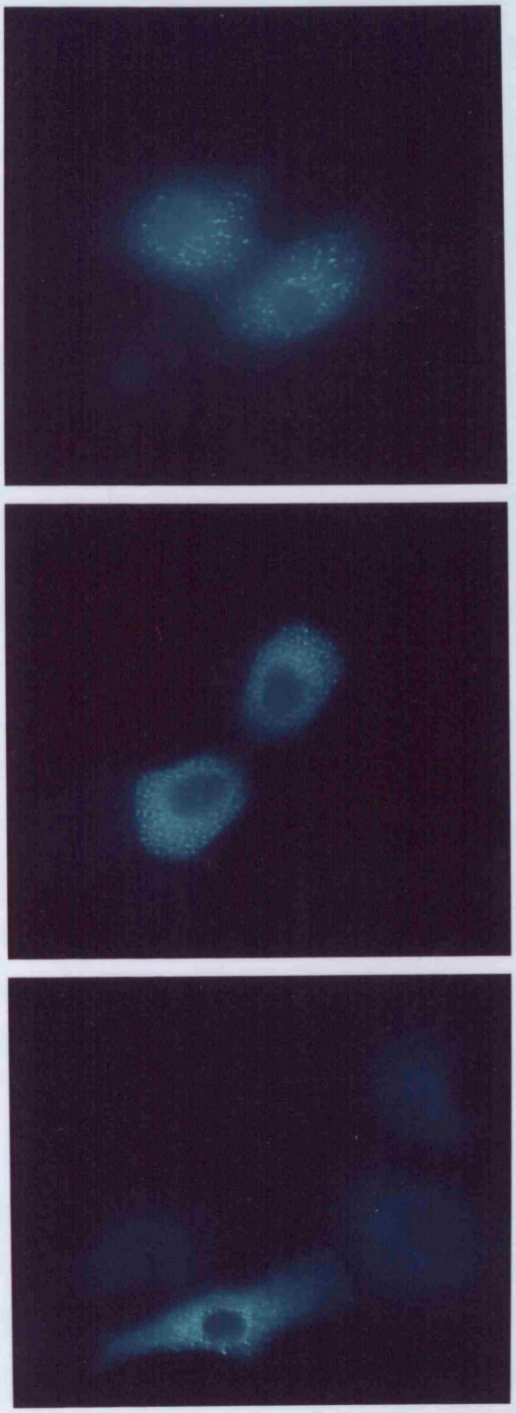


Figure 2.2: Appearance of Gli-transfected cells following retroviral transduction.

column RNA extraction kit (Qiagen, Crawley, UK). The resulting RNA solution was kept at -80°C until required for reverse transcription. Retroviral transduction and RNA extraction using the spin-column kit was principally carried out by Dr. Graham Neill, Centre for Cutaneous Research, QMUL, London. All subsequent procedures using RNA from transduced cells were carried out by me unless otherwise stated.

2.4 Proteomic and Genomic investigation

2.4.1 Proteomics: Immunohistochemistry

Immnohistochemistry is a technique that is widely used to demonstrate protein expression in both fresh and fixed tissue. Both monoclonal and polyclonal antibodies have been developed to a huge array of antigens, enabling detection of a vast number of proteins.

Although fresh frozen material can provide good immunohistochemical data this tissue is less plentiful than fixed archival tissue. Relative loss of antigenicity is, however, a drawback: antigenic sites tend to become "hidden" by protein cross-linking following aldehyde fixation. Unmasking of hidden epitopes as described by Shi and later Catoretti has improved the ability to demonstrate protein expression in fixed tissues (Cattoretti et al., 1993; Shi et al., 1991).

Following tissue section preparation I used an immunoperoxidase immunohistochemistry staining system. The "Vectastain Elite ABC" immunohistochemistry reagent kit (Vector Laboratories, Burlingame, USA) includes a suitable blocking serum, biotinylated secondary antibody and preformed Avidin-Biotin horseradish peroxidase macromolecular Complex ("ABC") tertiary antibody. Following consecutive incubations, a complex of these molecules is formed which results in peroxidase localisation to the site of primary antibody binding. Upon addition of hydrogen peroxide and an electron donor (a substrate), the substrate becomes oxidised as it catalyses the conversion of hydrogen peroxide to water. In my study the electron donor (the substrate, or chromogen) used was 3-diaminobenzidinetetrahydrochloride (DAB). This produces an insoluble brown pigment which is easily recognised by light microscopy.

2.4.1.1 Experimental design

For both the tumour groups (subtype-categorised BCCs and recurrence-categorised BCCs) the study designs were similar. In each case four different primary antibodies were used (β-tubulin III, GAP-43, Arc and Neurofilament). All samples tested within one tumour group were exposed to the same antibody in the same run. Included in each run were normal skin samples and control tissues (as described below).

2.4.1.2 Controls

Positive controls consisted of 5μm sections of appendix tissue. The nerve fibres in the muscle coat of the appendix are an excellent positive control for detection of neuronal antigens. Negative controls consisted of 5μm sections of tumour and appendix, which were not exposed to primary antibody, but were exposed to all other stages of immunohistochemical processing. Normal skin samples (from non-BCC patients as described previously) were used to compare tumour staining with that of normal epidermis.

2.4.1.3 Technical steps

In all cases the "Vectastain Elite ABC" immunohistochemistry reagent kits were used (Vector Laboratories, Burlingame, USA). This provided blocking sera, secondary and tertiary antibodies for each type of antibody used.

2.4.1.3.1 Initial treatment

Labelled sections were de-waxed in xylene and rehydrated through decreasing concentrations of alcohol (100%, 90% and 70% v/v), immersing them in each solution for three minutes. Endogenous peroxidase activity was blocked using a solution of 0.3% v/v hydrogen peroxide in methanol for 15 minutes at room temperature.

2.4.1.3.2 Microwaving

Slides were then washed well in water before immersion in 1000ml of 10 mM citrate buffer (adjusted to pH 6 as required with 2M NaOH) in a microwave pressure cooker device. The sections were microwaved (Hinari, 900W) on high power for five minutes. The cooker was removed from the microwave and the slides were left to stand in the cooling fluid for 15 minutes before being washed well in running water.

2.4.1.3.3 Blocking serum and primary Antibody

After microwave pre-treatment the slides were removed from the running water and rinsed in Phosphate-buffered saline (PBS, pH 7.6). A wax pen was used to ring the area of tissue on the slide, thus minimizing the volume of antibody reagents used. Blocking of non-specific binding was carried out using serum from the same species in which the biotinylated secondary antibody was raised. This was available by diluting the blocking serum (1:100 using PBS) provided within the immunohistochemistry reagent kits.

Following application of this blocking serum for ten minutes, it was tipped off and primary antibody (diluted with PBS) was applied as described in table 2.3. The slides were left in this state overnight at 4°C. The next morning three PBS washes (one minute each) were used to rinse the primary antibody away.

Antibody	Company	Dilution used	Antigen retrieval	Duration of 1° antibody	Raised in	Blocking serum used	Vectastain kit used
Beta Tubulin III	Chemicon	1:800	Y	Overnight	Mouse	Horse	Universal
Arc	Sigma	1:200	Y	Overnight	Mouse	Horse	Universal
GAP-43	Chemicon	1:3,200	Y	Overnight	Mouse	Horse	Universal
Neurofilament	Dako	1:200	Y	Overnight	Mouse	Horse	Universal

Table 2.3: Antibodies used in this study – see section 2.4.1.4

2.4.1.3.4 Secondary and tertiary antibodies

According to the Vectastain elite protocol, a solution comprising of secondary antibody (horse anti-mouse antibody, dilution 1:50) and blocking serum (horse serum, dilution 1:50) was applied to the slides for 60 minutes at room temperature. Following this period PBS was used to wash away the secondary antibody. Tertiary antibody (preformed Avidin-Biotin horseradish peroxidase complex, dilution 1:100) was then applied to the slides for 30 minutes at room temperature. PBS washes were then used to rinse off any remaining antibody.

2.4.1.3.5 Chromogens, dehydration and mounting

As described, the DAB chromogen (3-Diaminobenzidinetetrahydrochloride) was used to visualise the binding of the antibody complexes to the tissue sections. Using a kit system (Liquid DAB Substrate Pack, Biogenex, San Ramon, USA), the DAB solution

was made up according to the manufacturers instructions and applied to the sections until the positive control sections were uniformly stained (typically two minutes).The sections were then rinsed under running tap water for five minutes before lightly counterstaining with Haematoxylin and dehydrating in graded alcohols and xylene.

Mounting was achieved by use of DePex (BDH Laboratory Supplies; Poole, UK) mounting medium applied to coverslips which then covered the stained tissue sections adequately.

2.4.1.3.6 Solutions used

PBS:

1 Sachet of 10mM Phosphate buffered saline (PBS) powder mixed in 1000mls distilled water. For rinsing of sections.

Ethanols and Xylene:

Absolute ethanol used as 100%; lesser dilutions made up with appropriate volumes of distilled water. Xylene used neat following dehydration of sections (ethanol and xylene from Fisher Scientific, Loughborough, UK).

H_2O_2 in Methanol:

30mls 3% v/v Hydrogen peroxide (Sigma, Steinheim, Germany) mixed in 270mls absolute methanol at room temperature. For tissue section fixation.

Citrate buffer:

Citric Acid (monohydrate) 7.56g, tri-sodium citrate 47.56g, EDTA disodium 7.4g, distilled water 2000mls. This stock solution was then diluted 10:1 with distilled water for use in microwave treatment of tissue sections.

2.4.1.4 Antibodies used in this study

The antibodies used were chosen because of their specific relevance to neuronal differentiation and the previous results of gene array experiments.

2.4.1.4.1 Beta-tubulin III

Beta-tubulin III is a well-known marker of neuronal differentiation and was highlighted in our gene array data as being up-regulated by *Gli1* over-expression. It has previously been used as a marker to demonstrate the ability of dermal papilla pluripotent stem cells to undergo neuronal differentiation (Fernandes et al., 2004) .

2.4.1.4.2 GAP-43

Growth-associated protein (GAP-43) is a membrane protein that is expressed at high levels during neuronal development, and is newly produced in injured and regenerating adult nerve tissue. It is considered to be a marker for sprouting, and is usually associated with physiological events such as neuronal growth and synaptic plasticity (Gispen et al., 1991; Hoffman, 1989). In my study it was used as an independent marker of neuronal differentiation; the *GAP-43* gene had not been noted to be elevated in the gene array data.

2.4.1.4.3 Arc

The activity-regulated, cytoskeletal-associated gene, *arc*, is an immediate-early gene associated with neuronal development. It is translated to become a cytoskeletal protein expressed not only in the nucleus of neurons but also in their dendrites. It was discovered as a gene that was rapidly induced in active neurons in models of adult and developmental plasticity (Lyford et al., 1995) and is a known marker of neuronal differentiation. It was seen to be massively up-regulated in the initial gene array data from *Gli1*-transfected cells.

2.4.1.4.4 ULK-1

ULK1 (Unc51.1-like kinase 1) is the human homologue of Unc51.1, one of the earliest genes involved in neuronal differentiation (Kuroyanagi et al., 1998). Whereas UNC-51 is specifically detected in the nervous system of C. elegans, it appears that ULK1 is ubiquitously expressed in adult human tissues such as skeletal muscle, heart, pancreas, brain, placenta, liver, kidney, and lung. It was also greatly up-regulated in the gene array data, and with its precursor's specificity to the nervous system it was used to correlate gene array findings with those in BCC.

2.4.1.4.5 Neurofilament

Neurofilament is a routinely used marker of neuronal differentiation in many histological laboratories. There are a number of isotypes referred to as H, M, and L (heavy, medium, light chains). In this case the antibody used was "Neurofilament M+H" – an antibody to the medium and heavy chains.

2.4.1.5 Quantification of results

2.4.1.5.1 Visual Analogue Scoring

Immunohistochemical slides were visualised using a Leica DM RXA optical microscope and digital imaging system (Leica Microsystems AG, Wetzlar Germany).

Assessment of intensity of staining was carried out using a visual analogue scale familiar to histopathologists, described in table 2.4. Two independent examiners (myself and Dr. Rino Cerio, consultant dermatopathologist, Royal London Hospital) observed each section and a score was allocated to it. An average was taken of the two scores and this was recorded as the intensity of staining for each section with each antibody.

Score	Description	Example tissue
0	No staining at all	Negative control sections
1	Weak staining	
2	Moderate staining	
3	Strong staining	Positive control nerve fibres

Table 2.4: visual analogue scoring method of immunohistochemistry staining

Similarly, an assessment of the intracellular staining localisation was performed by each examiner by classifying the staining intensities of the cytoplasmic, membranous, and nuclear compartments. Again, for each compartment this was performed by allocation of a visual analogue score (as above) to each compartment for each tumour.

Direct visual observation is clearly the simplest technique of immunohistochemical quantification but its power of discrimination is limited. The human eye-brain combination is an excellent sensor for observing and identifying particular morphological features but is limited in its ability to quantify the intensity of staining. Most 'measures' have thus been limited to the adoption of some arbitrary scale such as the visual analogue scoring method described here. A more sensitive method of assessing these sections could give one a better understanding of the differences between certain groups. Spectral Imaging Analysis is such a method.

2.4.1.5.2 Spectral Imaging Analysis (S.I.A.)

Spectral Imaging Analysis is a technique in which a computer is used to assess the staining intensity of a tumour. Briefly, it does this by picking out the specific colours of whatever chromogens it is set to recognise. It will then calculate the intensity of these colours that are present in the section examined. The result of this is a histogram, with, among other values, a mean staining intensity for the area examined. This value is not an integer, but a number anywhere on the continuous scale of 0 (no staining) to 1 (very dense staining).

The Spectral imager works in a way analogous to a spectrophotometer, where light over a range of wavelengths is shone through a sample and the amount of light transmitted through the sample is measured. In this case, each pixel ("picture element") of a digital image is assessed in this way. It is of course also important that any sections which are to be compared should be of the same thickness, so as not to affect the optical density measurements. The Gray Cancer Institute spectral imager is an accessory to a standard microscope camera and is used to acquire a number of images that are processed to derive spectral information. The camera used merely measures the light intensity of the image pixels, whereas wavelength selectivity is provided by the filter which allows a number of wavelength–specific images to be generated. In the case of DAB, 12 different images across the spectrum of visible light were assessed.

The culmination of this is that a 3-dimensional data set (multiple pixel intensities at n wavelengths) is eventually generated by the software. Normalisation of the images is performed by a 'black' (i.e. zero light) image being acquired by energising a shutter in front of the camera, and then a "white" image is acquired with the shutter open but with no sample present. The resulting light intensities in each pixel in this normalised data set will have values ranging 0 to 255 (8-bit resolution giving a maximum of 256 possible values). Therefore each pixel can be described in gradation so of up to 256 degrees of intensity, which is markedly better than the eye-brain combination, which at best, can distinguish some 30-60 intensity levels.

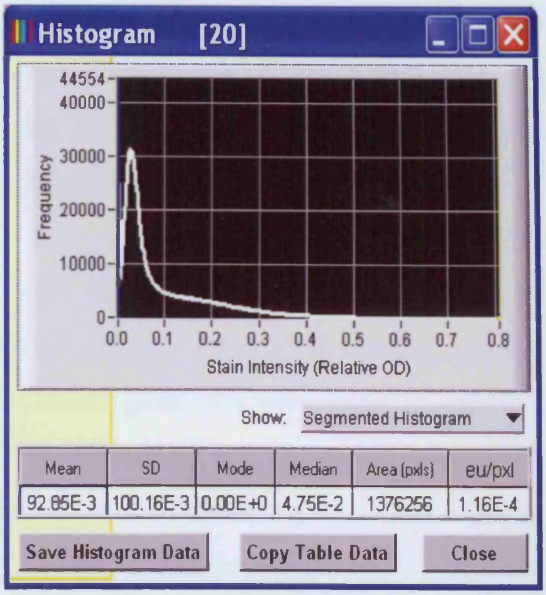


Figure 2.3: Histogram of DAB staining intensity in a uniformly stained BCC

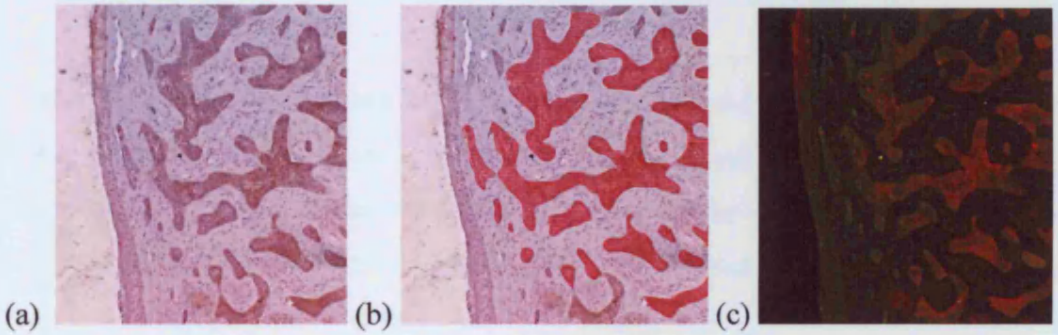


Figure 2.4: (a): original image of micronodular BCC; (b): Image Masker™ is used to highlight tumour areas in red; (c): DAB staining represented by red tones, Haematoxylin represented by green tones.

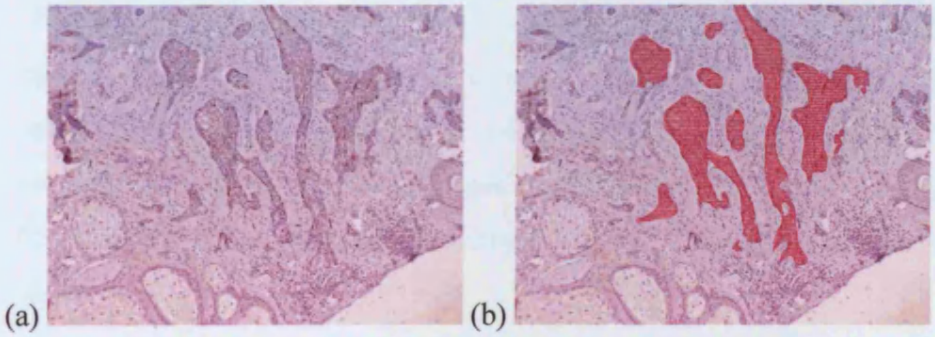


Figure 2.5: (a): an infiltrative BCC prior to tumour selection; (b): with the tumour islands highlighted in red.

To get an idea of the spread of staining intensities rather than just the average value, a convenient method is to use some form of histogram analysis. A histogram of the staining intensity describes the frequency distribution of the staining intensity, as illustrated in figure 2.3.

As well as the average intensity being calculated, the *shape* of the histogram illustrates the distribution of staining intensity. For example, in most cases, a single, albeit broad, peak will be present and the mode, mean and width of the peak may be used to indicate heterogeneity. Of course the distribution could also be bi-modal, i.e. with two peaks, indicating that there are clusters of staining intensity, e.g. distinct weakly and strongly stained regions. Additionally, a confounding factor is that, in general, some degree of background staining is likely to be present. Some tissue areas, e.g. stroma, may thus appear to contain some concentration of the chromophore and it is likely that one would wish to exclude these from the analysis.

Specific selection of tumour areas for analysis using this method was achieved by the use of an additional piece of software (Image Masker^{TM,} Gray Cancer Research Laboratory) which enables a free-hand selection of the area of interest to be analysed (figure 2.4). With this, the whole area of the tumour tissue that one wishes to examine can be marked out on the computer screen and highlighted with a red overtone (as seen in figure 2.4b). This area can be added to or subtracted from until the representative area is selected. If a tumour does not stain at all (i.e. to the same degree as a negative control) then that is reflected accurately in the resulting histogram..

This method of assessment is particularly useful in the assessment of disparate tumours, as in figure 2.5. In the case of nodular BCCs it may be easy to compare staining intensities with the naked eye, but with increasingly disparate tumours (such as infiltrative or morphoeic subtypes) this software enables one to selectively assess the staining of tumour islands, without the "background noise" of the surrounding tissue.

With regards to tumour biology this system has the power to be more sensitive than the human eye in picking up differences between comparison groups. With predominantly weakly staining sections, it may be difficult with the results from

visual analogue scoring to tell if there is a significant difference between groups (as they will tend to be classified by visual analogue scoring as 0's or 1's). With this system the continuous nature of the data is more reflective of the spectrum of staining that occurs. As a result the statistical comparison of groups is more likely to be truly representative of the actual differences present.

2.4.2 Genomics

Since messenger RNA (mRNA) is translated for protein synthesis, mRNA levels are commonly used as a surrogate for protein expression; a high level of any particular mRNA species typically reflects a high degree of protein expression. RNA is however very prone to degradation and cannot easily be quantified directly, but viral reverse transcriptase can be used to transcribe mRNA into complementary DNA (cDNA). This stable cDNA template can then be quantified by modifications of the polymerase chain reaction (PCR). In this study real-time semi-quantitative PCR and standard RT-PCR were used following reverse transcription.

2.4.2.1 Experimental design

Tumour samples of RNA were prepared as described previously. In all cases samples of BCC tissue were compared with technical negative controls (as described below), samples of non-tumour skin and, in some cases, positive controls. This was the case when using both agarose gels and real-time PCR.

2.4.2.2 Controls

Technical negative controls were developed at all stages of RNA processing. During reverse transcription an "RT-" cDNA sample was created by including BCC RNA for reverse transcription but without including reverse transcriptase in the reaction. Similarly at the stage of a PCR reaction, a sample was created where a "water" negative control was used by adding a similar volume of DNA-free water to a PCR reaction mix instead of the usual volume of BCC cDNA.

Positive controls were used in both agarose gel and real-time PCR experiments. In the real time PCR experiments on cell culture material one of my principle aims was to determine the expression of neuronal markers secondary to the expression of *Gli1* and *Gli2* genes. Thus, checking the expression of *Gli1* and *Gli2* genes were in essence a

positive control in these cell groups. In terms of the neuronal marker primers used in this study, I used the cDNA prepared from the SH-SY5Y cells previously described.

2.4.2.3 Quantification of RNA extraction

Quantification of RNA was achieved using a "Nanodrop" spectrophotometer (Nanodrop, Wilmington, USA). This can measure the concentration of both RNA and DNA in a sample by analysing the amount of light of specific wavelengths that is absorbed when passed through it. With an RNA concentration for each sample known to me, I was in the position to proceed to reverse transcription using a standard quantity of RNA in each case. This principle was used when preparing cell culture RNA for reverse transcription. In the case of fresh BCC tissue samples (Laser Capture Microdissection, Needle Microdissection and Whole Tissue Homogenate samples) RNA was quantified in this way, but typically the concentrations of RNA were low and variable such that creating reverse transcription reactions with identical amounts of starting RNA was not possible. In these cases reference to internal control genes was the mainstay of quantity comparison.

2.4.2.4 Reverse transcription of mRNA

Reverse transcription is the process by which RNA may be converted into DNA (known as complementary DNA, cDNA). It occurs by virtue of reverse transcriptase, an enzyme which converts an RNA nucleotide sequence to the corresponding DNA nucleotide sequence. Resulting cDNA can be amplified by the polymerase chain reaction and quantified. In my case all reverse transcription experiments were performed using the Promega "Reverse transcription kit" according to the manufacturer's recommendations (Promega, Madison, USA).

The steps involved are:

1: RNA denaturation by heating to 70°C for 10 minutes using a PCR block set to this temperature (Thermo Hybaid, USA).

2: Creation of a reverse transcription solution master mix including the substances listed below, and then addition of RNA and reverse transcriptase in the following amounts:

- 2µl reverse transcription 10x buffer
- 0.5µl recombinant RNasin ribonuclease inhibitor
- 0.5µl random primers
- 0.5µl oligo-dt primers
- 4µl magnesium chloride (25mM)
- 2µl dNTP mixture (10mM)
- 0.6µl AMV reverse transcriptase
- 10µl RNA solution (containing 5µg RNA, made up to volume with nuclease-free water)

3: A negative control sample which had distilled water as opposed to reverse transcriptase included was included at this stage.

4: These samples (total mixture volume 20.1µl each) were then placed onto a PCR block with the following settings:

- 42°C for 60 minutes
- 99°C for 5 minutes.
- Hold at 4°C

Following this reaction samples were stored at -20°C until PCR processing was used.

2.4.2.5 RNA amplification

Given the small amounts of RNA generated from Laser Capture Microdissection, amplification of this RNA was attempted using a commercially available kit designed for this purpose (RiboAmp RNA amplification kit, Arcturus, USA). This process involves reverse transcription of RNA to cDNA, amplification of cDNA and lastly transcription back to RNA. This resultant RNA may then be used for further reverse transcription. It is claimed that this method may amplify the amount of mRNA by a factor of up to 1,000-fold with each round of amplification, whislt retaining specificity of the sample. The manufacturers' instructions were followed meticulously in every case.

2.4.2.6 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an in vitro method that uses enzymatic synthesis to amplify specific DNA sequences. Devised by Mullis et al in 1985 (Mullis et al., 1986), the technique is powerful enough to amplify one copy of a specific DNA sequence millions of times. It exploits the unusual ability of thermophilic bacterial DNA polymerase to withstand high temperatures without denaturing. Taq polymerase from thermus aquaticus is such an enzyme (Chien et al., 1976). DNA polymerase starts copying DNA from the point where a short complementary nucleotide sequence

known as a primer binds (or "anneals"). Primers can be designed to anneal to both ends of the target sequence on the complementary DNA strands, and to differentiate the two primers for any target molecule they are known as 5' and 3' primers. The primers are generally around 20 nucleotides long in length, sufficiently long to be unique within the genome.

The PCR reagents including the enzyme, template and primer oligonucleotides are heated to a high temperature such that the DNA strands separate. They are then cooled to a chosen temperature to allow the primers to anneal to the specific target sequence in the template, and then heated to 72 °C for optimal DNA polymerase activity. Since DNA polymerase synthesises DNA in a 5' to 3' direction, this annealing pattern allows the target sequence to be copied in both directions. Lengthy DNA strands are generally not synthesised because taq polymerase stalls at DNA sequence mismatches, the enzyme having no repair activity. On cooling, the primers anneal to the newly synthesised DNA strands. Taq polymerase then copies the target sequence again or the newly synthesised product. Thus the cycling rapidly replicates the target sequence alone, as illustrated in figure 2.6. As the cycles proceed, so the amount of target DNA generated increases essentially exponentially. The process of using cDNA copied from mRNA by reverse transcriptase (RT) as the DNA template for PCR is known as RT-PCR (Strachan T. and Read A.P., 2004).

2.4.2.6.1 Primer design

The ideal characteristics of PCR primers include a length of 15-30 base pairs (bp), 40-60% G+C content with no complementary sequences between or within the primers. Additionally, they should span a gene intron and represent a unique sequence in the genome. These characteristics prevent primers from self-complementation with each other, maintain the highest possible specificity and aid detection of genomic DNA contamination.

Primer sequences were designed using the Discovery Studio primer design software package (v1.5, Accelrys, USA). The target sequence of the gene in question was entered into the computer and from this the program calculates possible primer pairing sequences, and gives each pair a score of likelihood of being unique to that

gene. From this list pairs were selected and suitability for use in terms of G/C content and primer size was assessed.

Once the sequences of a set of primers had been defined, primers were obtained by ordering directly from MWG (MWG, Ebersberg, Germany). These primers were provided desiccated, then diluted to a 100 μM concentration in nuclease-free distilled water. Aliquots of this stock solution were then used for further use at 5 μM concentration.

2.4.2.6.2 Primers and PCR conditions

Properties of the primers used in these experiments are detailed in table 2.5.

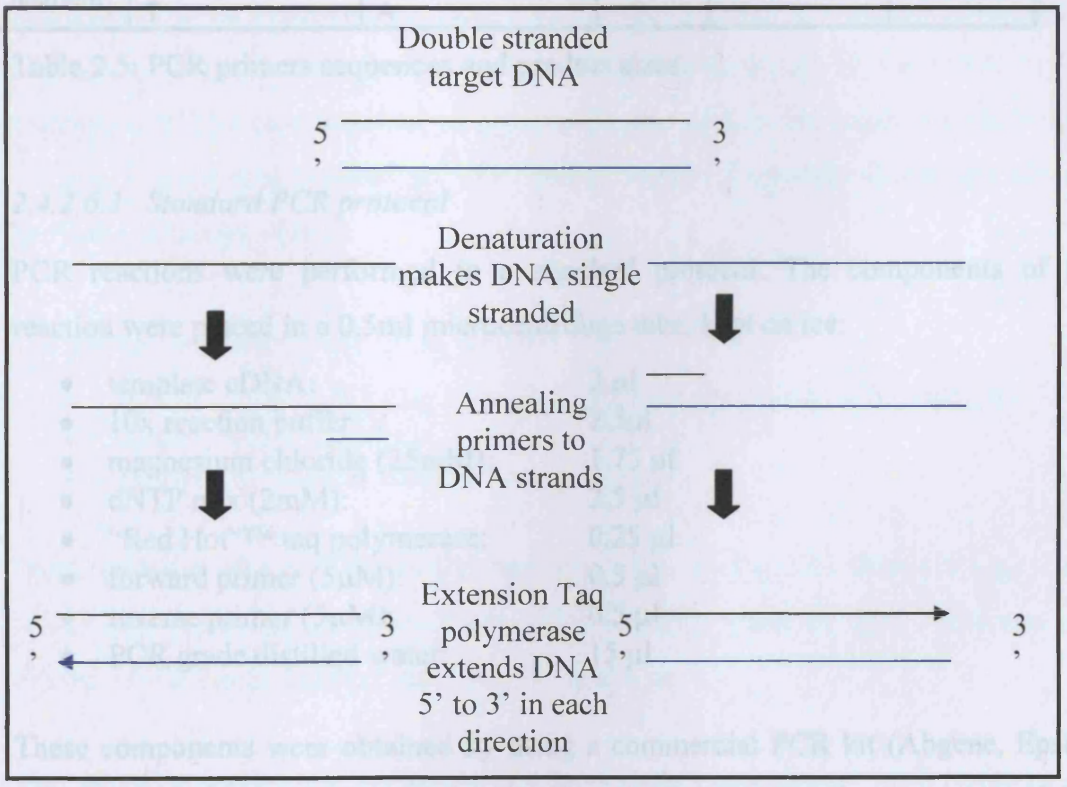


Figure 2.6: The polymerase chain reaction: Denaturation separated the DNA strands; cooling to a specific annealing temperature allows the primers to bind to the DNA strand. In the final step *Taq* polymerase can then synthesise new DNA strands extending from the bound primers. This cycle can be repeated many times allowing amplification of the target sequence.

PCR target	Sequence 5'- 3': Forward Primer	Sequence 5'- 3': Reverse Primer	Intron span	Annealing temperature	Product size (bp)
B-Actin	GTT TGA GAC CTT CAA CAC CCC	GTG GCC ATC TCT TGC TCG AAG TC	No	60°C	320
Gli-1	GAA GAC CTC TCC AGC TTG GA	GGC TGA CAG TAT AGG CAG AG	No	60°C	246
Gli-2	GGG TCA ACC AGG TGT CCA GCA CTG T	GAT GGA GGG CAG GGT CAA GGA GTT T	Yes	57°C	194
B-tubulin III	GGC CTC TTC TCA CAA GTA CG	ACC ACA TCC AGG ACC GAA T	No	60°C	200
Arc	GCT CAG GGT TCA TCG TTC TGC CTT G	AAA GCC TGT GCC AGC CTT GAG GAT T	Yes	65°C	146
GAP-43	ACC CTC TTC TCA GCT CCA CTC	GCC ACA CGC ACC AGA TCA AAT A	Yes	60°C	267
Neuro-filament	ATC TCC TGG TCG TAC GCG T	TCC TCC TCC TAT AAG CGC A	No	60°C	361

Table 2.5: PCR primers sequences and product sizes.

2.4.2.6.3 Standard PCR protocol

PCR reactions were performed to a standard protocol. The components of this reaction were placed in a 0.5ml microcentrifuge tube, kept on ice:

- template cDNA: 2 µl
- 10x reaction buffer 2.5µl
- magnesium chloride (25mM): 1.75 µl
- dNTP mix (2mM): 2.5 µl
- "Red Hot"™ taq polymerase: 0.25 µl
- forward primer (5µM): 0.5 µl
- reverse primer (5µM): 0.5 µl
- PCR grade distilled water: 15 µl

These components were obtained by using a commercial PCR kit (Abgene, Epsom, UK). After a final pulse spin (to pool the reagents) the samples were placed in a Hybaid PCR block (Hybaid, Thermo, USA). Standard PCR conditions are as follows:

- 95°C x 3 minutes (1 cycle)
- 95°C x 30 seconds
- X°C x 30 seconds (30-40 cycles)
- 72°C x 40 seconds
- 72°C x 10 minutes (1 cycle)

- 4°C x Hold

(X = annealing temperature, see table 2.5)

A negative control PCR reaction was included in each PCR run. This consisted of a PCR reaction of the relevant volume, including all relevant reagents, but no DNA. The reaction volume was made up using PCR grade distilled water.

2.4.2.6.4 Agarose gel electrophoresis.

Agarose gel electrophoresis is used to separate DNA molecules according to their size. Agarose gels are made up of a polysaccharide matrix in a buffer. When an electric current is passed through the gel the negatively charged DNA molecules will migrate through the matrix towards the positively charged anode. The matrix offers greater resistance to larger DNA molecules, thus a population of different DNA molecules can be separated by their size. Ethidium bromide chelates DNA and its addition to the gel and running buffer allows visualisation of the DNA by UV fluorescence. The concentration of agarose directly affects the resolution qualities of the gel. A more concentrated gel will resolve smaller fragments, due to the reduced pore size of the gel matrix.

1% agarose gels were made up as follows:

- agarose (Fisher UK) 1.0g added to TBE electrophoresis buffer 100mls
- solution boiled for 30sec
- addition of ethidium bromide 1mg/µl, 1µl

Stock solution of 10x TBE buffer was made by mixing Tris Base (108 g), Boric Acid (55g), 0.5M EDTA (20mL) and adding distilled water to 1.0 L. This was then diluted 10-fold with distilled water for use as above.

This was then cooled in a gel casting system (Gibco, Gaithersburg, USA) with a comb to create the wells. When cool, the comb was removed to allow filling of the wells.

In each case 5µl of PCR product was loaded with 2 µl blue/orange loading dye (Promega, Madison, USA). 3µl of 1Kbp ladder (Promega) used in a control lane to estimate fragment size. Bands were viewed using a ultraviolet imaging system

(Uvitec, Cambridge, UK). Ultraviolet-fluorescent images of agarose-PCR product gels were photographed and printed out using a printer attached to the camera above (Mitsubishi P91E, Japan).

2.4.2.7 Real-Time PCR

A polymerase chain reaction classically amplifies DNA without offering any accurate index of the amount of DNA template present. The use of a chelating fluorescent dye, however, allows indirect assessment of the total amount of DNA present by fluorescence measurement at the end of the DNA polymerase phase of each cycle; the fluorescence of the chelating dye is greatly enhanced by binding to double-stranded DNA. Thus the exponential rise in PCR product over the series of cycles can be monitored in a "real time" manner. The point on the fluorescence plot at which the PCR product starts to rise most rapidly ("lift off") is related to the amount of template cDNA present in the original aliquot.

The log plot of this rise can be extrapolated back to the base line to estimate this "cross-over point", and using calibration data the number of cDNA molecules in the original template can be calculated. This is known as quantitative real-time PCR. If absolute quantification is not required, comparison between samples can be used to produce relative quantification data. In this case reference to a constant housekeeping gene is required to normalize the data. In this study β-Actin was used as it is a good marker for epidermal cell populations, whether of tumour or non-tumour origin.

Real-time PCR was performed in this study using cDNA that had been prepared from the RNA of cells transfected with Gli genes or controls (as described in section 2.3.3.2). The fluorescent dye used in these studies was SYBR green chelating dye, which was included as part of the SYBR-green PCR kit (SYBR green qPCR kit, Finnzymes, Finland) and all reactions were carried out using the same machine (Opticon II Quantitative PCR machine, MJ Research, USA). Reactions were carried out according to the manufacturer's directions. Each reaction mix comprised of

- 10μl SYBR green quantitative PCR master mix
- 0.5μl Forward primer
- 0.5μl Reverse primer
- 1μl cDNA (of a 20.1 μl RT reaction mix containing 5 μg RNA)
- 8μl PCR grade distilled water

The same primers and parameters were used as for RT-PCR and in all cases experiments were performed in triplicate. Positive and negative technical controls were included.

The Opticon II program cycled as indicated below (X = annealing temperature, see table 2.5):

- 95°C x 10 minutes (1 cycle)
- 94°C x 10 seconds
- X°C x 10 seconds (35 cycles)
- 72°C x 20 seconds
- 72°C x 5 minutes (1 cycle)
- 65→95°C melting curve
- 4°C x Hold

2.5 Data storage

All categorical and numerical experimental data was recorded in Microsoft Excel spreadsheets (Microsoft, Seattle, USA). Identification of tissue or product samples was achieved using the number-and-letter code assigned to each tumour.

2.6 Data analysis

2.6.1 Gene array data

Array data produced by Regl et al in Salzburg was analysed using a spreadsheet programme (Excel, Microsoft, Seattle, USA). This data had been generated following hybridisation of Gli-transfected keratinocyte cDNA to nylon gene array membranes as described previously (Aberger *et al.*, 2001).

Prior analysis of hybridisation intensities had included normalisation for total signal intensity and with two copies of each gene present on the array. Both copies had to be registered as having hybridized with the membrane to be included in this data.

The data available to me was presented in a spreadsheet fashion and referred to hybridisation intensities of each gene present on the nylon gene array membrane. Two values of hybridisation intensity were recorded for each gene on the array. An average

was taken of these two values to represent the level of gene expression for each cell group. By comparing the control keratinocyte sample data with the data generated from Gli-transfected keratinocytes, it was possible to generate fold-change data, representing up- or down-regulation of gene expression in Gli-transfected keratinocytes compared to control keratinocytes.

The data in this spreadsheet was then ordered according to the level of up-regulation of gene expression in Gli-transfected cells compared to controls. The genes with the top 100 levels of up-regulation due to Gli transfection were focussed on and a record made of the known function of such genes.

2.6.2 Immunohistochemistry data

Comparison across groups was performed using statistical analysis software (SPSS, Chicago, USA) using tests as outlined in table 2.6. Multiple groups were considered by a One Way Analysis of Variance (ANOVA). Specific related groups were compared using a paired t-test. An example in this study is the comparison of recurrent tumours with their original counterparts (e.g. groups CO vs CR). Specific unrelated groups were compared using an unpaired t-test. Such examples are the comparison of tumours that do go on to recur vs those (from other patients) that do not (groups CN vs CO) and the comparison of separate tumour class groups (e.g. indolent vs aggressive).

Type of comparison	Groups compared	Example in this study	Statistical test used
Multiple groups	Skin and primary BCC subtypes	Skin and Nodular, Superficial, Infiltrative, Micronodular and Morphoeic BCCs	ANOVA
Related groups	Recurrent tumours vs their original counterparts	CO vs CR IO vs IR	Paired t-test
Unrelated groups	Tumours that recur vs those that do not recur	CN vs CO IN vs IO	Unpaired t-test
	Primary BCC histological classes	Indolent vs Aggressive	Unpaired t-test

Table 2.6: Statistical methods used in this thesis.

2.6.3 mRNA expression data

2.6.3.1 Real-time PCR

Real time PCR data was recorded initially as the number of cycles required to hit a certain threshold for each group of cells. Details of the melting curve was also recorded and examined to ensure the quality of the reaction.

Within each transfection / primer / cell / Gli-status combination used, the triplicate values were averaged to give a representative figure for the cycles-to-threshold value. Within each transfection experiment, there were 3 main groups to compare (cells transfected with *EGFP-Gli1*, *EGFP-Gli2*, or *EGFP* alone). Comparison of these independent groups was performed by assessing the Analysis of Variance (ANOVA) with differences between specific groups considered using post-hoc Bonferroni comparisons.

2.6.3.2 RT-PCR

In this case no statistical analysis was performed, with the main comparison between groups being direct visual comparison between bands seen on agarose gels.

Chapter 3: Immunohistochemical expression of neuronal markers in Basal Cell Carcinoma

3.1 Introduction

The expression of neuronal markers in Basal Cell Carcinoma has not previously been described in the medical or scientific literature. Following the analysis of gene array data derived from Gli-transfected N-terts cells, I wished to investigate whether this would be a feature of human Basal Cell Carcinomata. Not only did I wish to ascertain whether these markers were expressed at all in BCCs, but I specifically wished to determine whether a differential degree of expression was present in the various histopathological subtypes of BCC.

3.2 Methods

An immunohistochemical analysis of neuronal differentiation marker staining in BCCs was performed using the panel of primary BCC tumours described in chapter 2. Thirty-two tumours were selected for examination, as described in table 3.1. All these tumours had characteristic appearances of a particular histological growth pattern subtype. Positive and negative controls were included as well as normal non-tumour skin samples.

BCC growth pattern subtype	Tumours examined
Nodular	7
Superficial	4
Micronodular	9
Infiltrative	8
Morphoeic	4
Total	**32**

Table 3.1: Histopathological subtypes of BCC examined in this study

Five antibodies (as described in table 3.2) were applied to all the sections. The same techniques were employed for all antibodies and all sections, as described previously in chapter 2. Staining was analysed by both visual analogue scoring and by spectral image analysis in all cases. Comparisons were made using both of these methods between histological subtypes and growth pattern class within each antibody group.

Antibody	Raised in	Manufacturer	Dilution used
ULK-1	Goat	Santa Cruz	1:50
β-Tubulin III	Mouse	Chemicon	1:800
GAP-43	Mouse	Chemicon	1:3,200
Neurofilament-M+H	Mouse	Dako	1:200
Arc	Mouse	Sigma	1:200

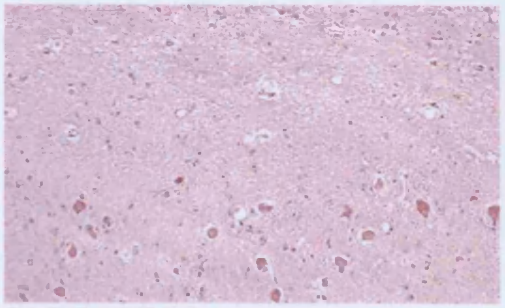
Table 3.2: Antibodies used in immunohistochemistry study of neuronal differentiation marker expression in BCCs.

3.3 Results

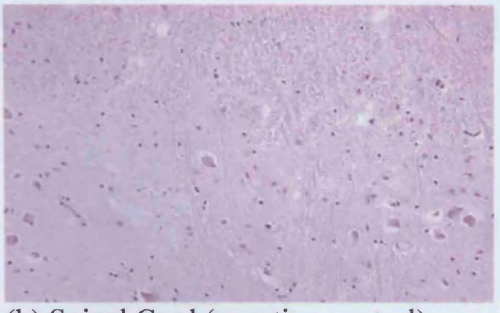
3.3.1 ULK1 expression

Multiple human tissues were used as controls for the expression of ULK-1 as has been described. These included normal kidney, rhabdomyosarcoma, normal skin, bowel, appendix, placenta and spinal cord. The intensity of staining in all of these tissues was zero, with the exception of spinal cord tissue (representative slides are shown in figure 3.1). In this case a subsection of neuronal cell bodies stained positively for ULK-1, and this staining was specific to the use of primary antibody (the negative control in which the primary antibody was omitted did not stain at all). In the case of appendix tissue which was used as a positive control with the other antibodies used, no staining of the nerve fibres was seen.

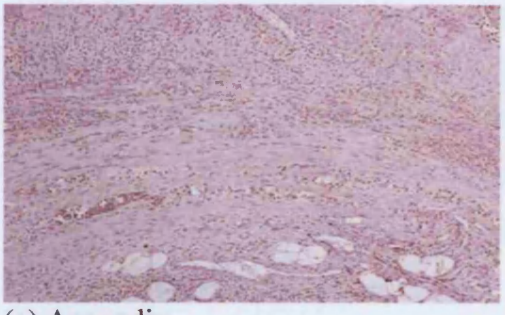
In only one out of thirty-two BCCs was there any staining above the baseline level. This staining was seen to be very weak and was not specific to any part of the tumour. No further formal quantification of staining with this antibody was undertaken due to the negative technical findings.



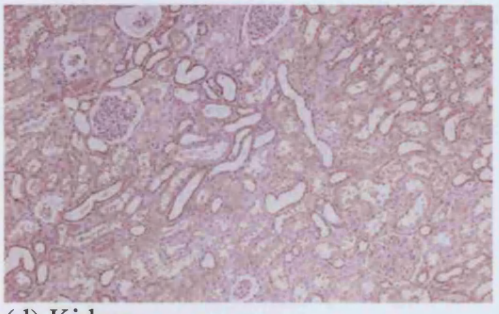
(a) Spinal Cord



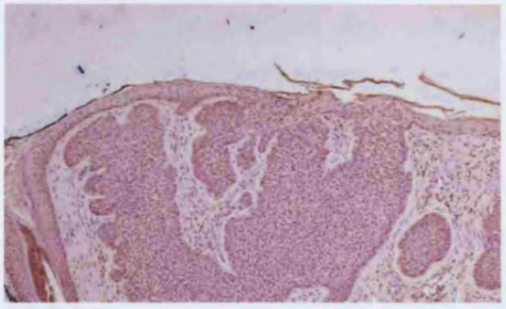
(b) Spinal Cord (negative control)



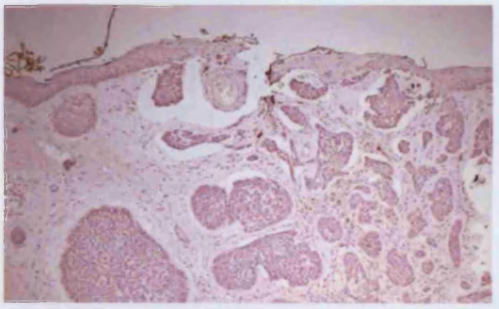
(c) Appendix



(d) Kidney



(e) BCC



(f) BCC

Figure 3.1: Essentially negative staining with ULK-1 antibody in multiple control tissues: appendix, kidney and BCC. Minimal positive staining was seen in some cells of the spinal cord section.

3.3.2 β-tubulin III expression

Images representing the staining present in each type of tissue and BCC are shown in figure 3.2. Neuronal tissue within the human appendix positive control sections stained strongly. Negative controls did not stain at all.

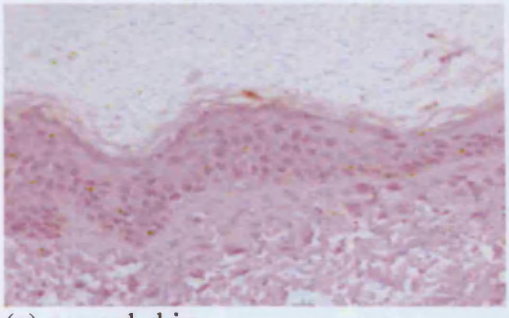
3.3.2.1 β-tubulin III: Visual Analogue Scoring

Results of the visual analogue scoring of the staining of these tumours were analysed by plotting the mean staining results (with 95% confidence limits) by subtype. The results are depicted in figure 3.3 and show a much lower level of staining in the aggressive and morphoeic groups than in the other groups. ANOVA analysis of these figures demonstrated that the differences in staining are significantly different between these groups and that they do not represent the same population of tumours ($p < 0.001$). The wide variation in confidence limits with the superficial tumours is likely to due to both the heterogeneity of staining seen, and the small number of tumours examined.

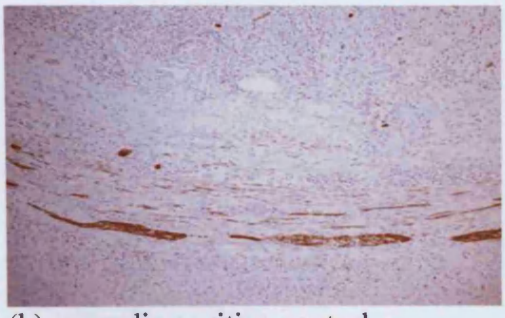
Further ANOVA analysis by tumour growth class (Indolent, Micronodular, Aggressive) demonstrated a marked difference between these groups ($p<0.001$). These results are shown in figure 3.4. Using a Bonferroni multiple comparisons analysis it was evident that there was a highly significant difference between the intensity of staining of the aggressive tumours and the other classes. Infiltrative and morphoeic tumours (which tend to behave more aggressively) stain less than those that behave indolently (typically superficial and nodular tumours) or those have a micronodular growth pattern.

3.3.2.2 β-tubulin III: Spectral Image Analysis

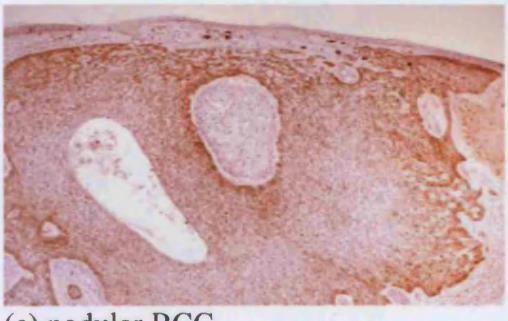
Results of the computerised scoring of the staining of these tumours was analysed by plotting the mean staining results (with 95% confidence limits) by subtype. The results are depicted in figure 3.5 and concur with the previous results - a lower level of staining is seen in the aggressive and morphoeic groups than in the other groups. Although there are differences between the means and confidence limits in this data compared to the visual scoring data, the results of statistical comparisons are similar.



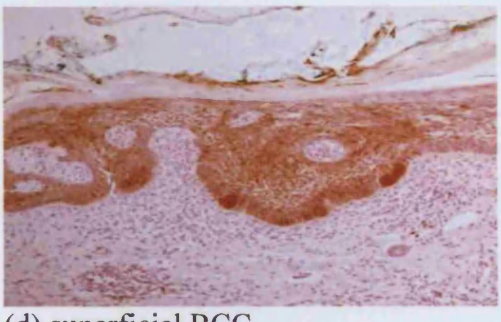
(a) normal skin



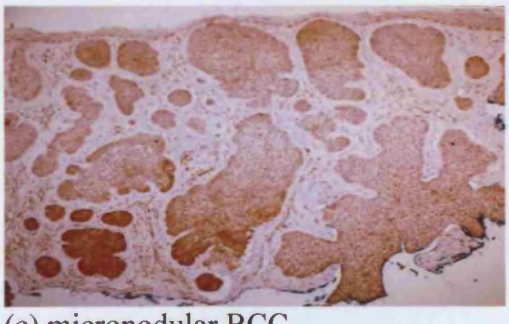
(b) appendix positive control



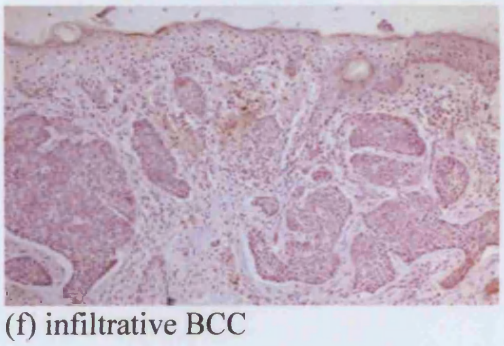
(c) nodular BCC



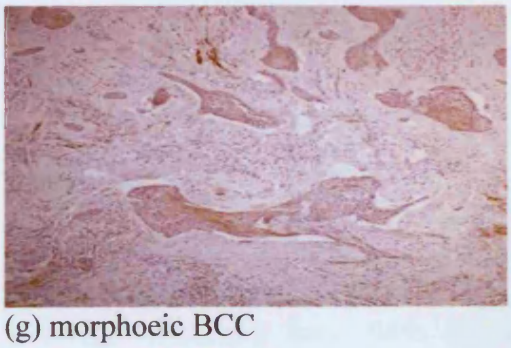
(d) superficial BCC



(e) micronodular BCC



(f) infiltrative BCC



(g) morphoeic BCC

Figure 3.2: Representative examples of β-tubulin III staining in control tissues and BCC tumours classified by histological growth pattern subtype

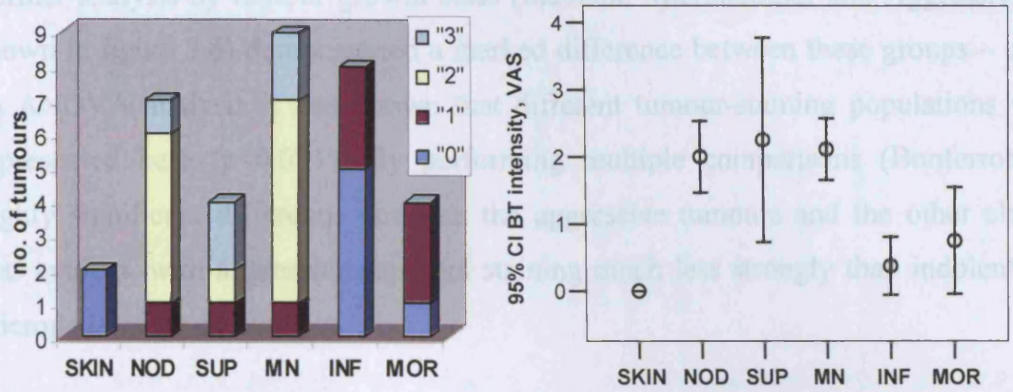
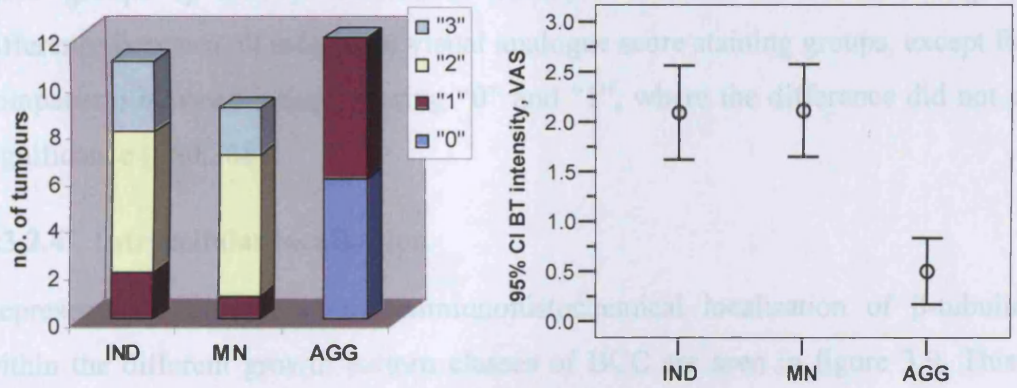


Figure 3.3: β-tubulin: means of visual analogue scoring staining values with error bars (95% CI) by tumour subtype. ANOVA analysis demonstrated highly significant differences between these groups (p> 0.001).



Class types compared	p value.
Indolent & Micronodular	>0.999
Indolent & Aggressive	<0.001
Micronodular & Aggressive	<0.001

Figure 3.4: β-tubulin: means of staining values (as determined by visual analogue scoring) with error bars (95% CI) by tumour class. Significances of Bonferroni multiple comparisons are shown.

ANOVA analysis again demonstrated that the differences in staining between these groups are highly significantly ($p< 0.001$).

Further analysis by tumour growth class (Indolent, Micronodular and Aggressive, as shown in figure 3.6) demonstrated a marked difference between these groups – using an ANOVA analysis it was shown that different tumour-staining populations were represented here ($p<0.001$). By performing multiple comparisons (Bonferroni) a highly significant difference between the aggressive tumours and the other classes was evident, with aggressive tumours staining much less strongly than indolent and micronodular types.

3.3.2.3 Comparison of visual scoring with Spectral Image Analysis

Graphs plotting the visual analogue score on the x-axis and the computerised score on the y-axis are shown in figure 3.7. It can be seen that there is a near-linear correlation between these values with little overlap between staining classifications and measurements. ANOVA analysis showed a highly significant difference between these groups ($p<0.001$). Bonferroni multiple comparisons reveal a significant difference between all individual visual analogue score staining groups, except for the comparison between groups scoring "0" and "1", where the difference did not attain significance ($p=0.268$).

3.3.2.4 Intracellular localisation

Representative images of the immunohistochemical localisation of β-tubulin III within the different growth pattern classes of BCC are seen in figure 3.9. This was assessed by visual examination as described in chapter 2 and the results were plotted on a graph as shown in figure 3.8.

Inspection of the data showed that a higher proportion of membranous staining was seen in micronodular tumours compared to all other types. In no case was nuclear staining seen to be present.

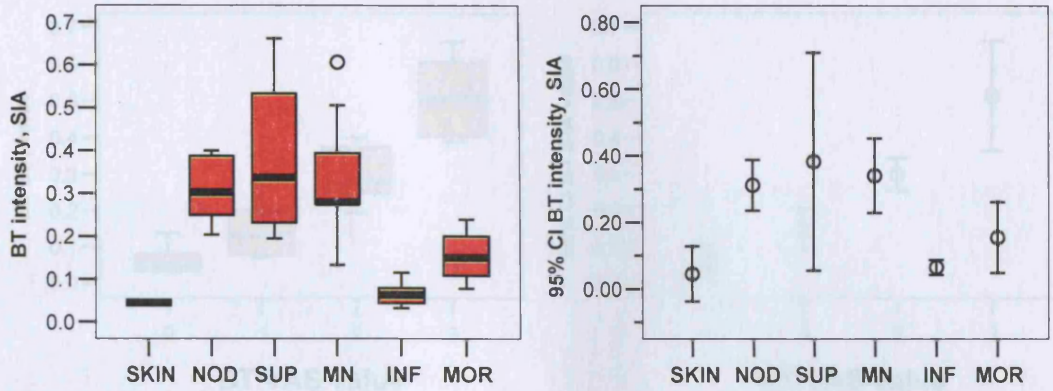
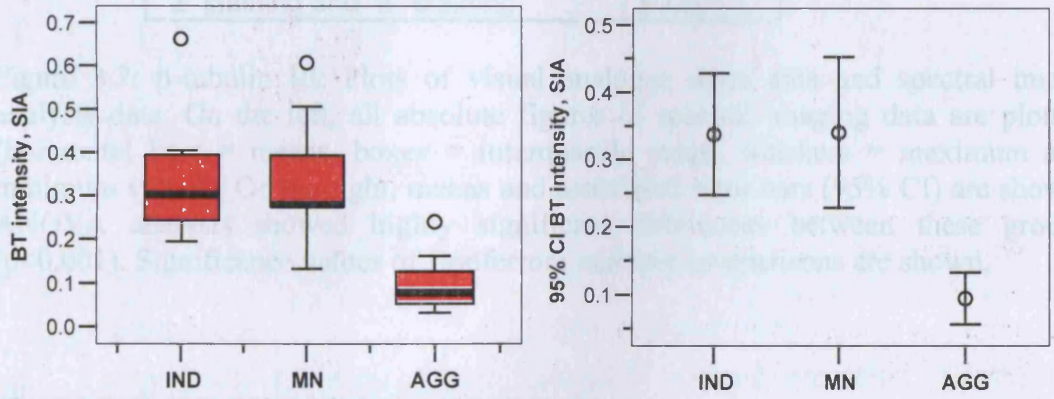
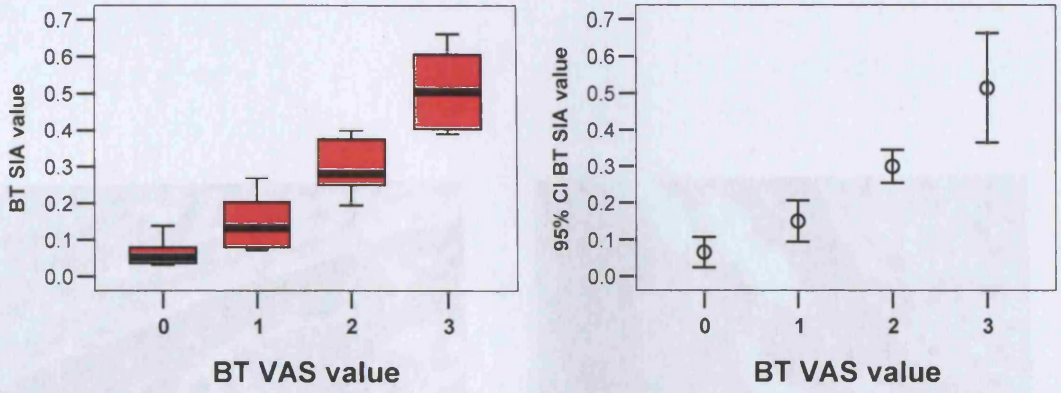


Figure 3.5: β-tubulin: means of spectral image analysis staining values with error bars (95% CI) by tumour subtype. ANOVA analysis demonstrated highly significant differences between these groups ($p < 0.001$)



Class types compared	p value.
Indolent & Micronodular	>0.999
Indolent & Aggressive	<0.001
Micronodular & Aggressive	<0.001

Figure 3.6: β-tubulin: means of staining values (as determined by spectral image analysis) with error bars (95% CI) by tumour class. Significances of Bonferroni multiple comparisons are shown.



Visual score groups compared	p value.
"0" staining and "1" staining	0.268
"0" staining and "2" staining	<0.001
"0" staining and "3" staining	<0.001
"1" staining and "2" staining	<0.001
"1" staining and "3" staining	<0.001
"2" staining and "3" staining	<0.001

Figure 3.7: β-tubulin III: Plots of visual analogue score data and spectral image analysis data. On the left, all absolute figures of spectral imaging data are plotted (horizontal bars = means, boxes = interquartile range, whiskers = maximum and minimum values). On the right, means and associated error bars (95% CI) are shown. ANOVA analysis showed highly significant differences between these groups ($p<0.001$). Significance values of Bonferroni multiple comparisons are shown.

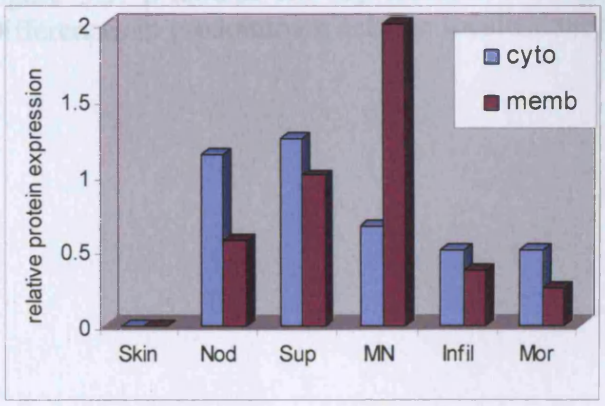
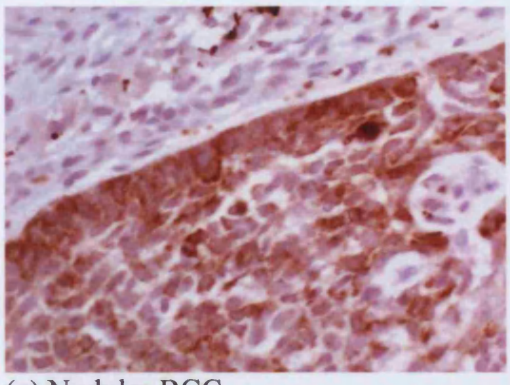
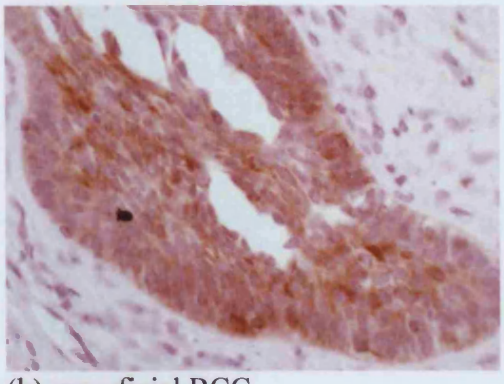


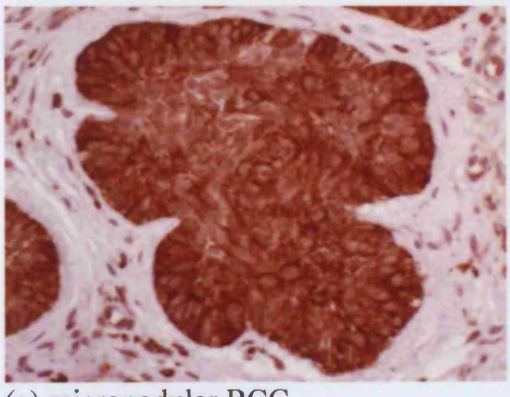
Figure 3.8: β-tubulin III: Relative intensity of membranous and cytoplasmic staining seen in BCCs according to subtype.



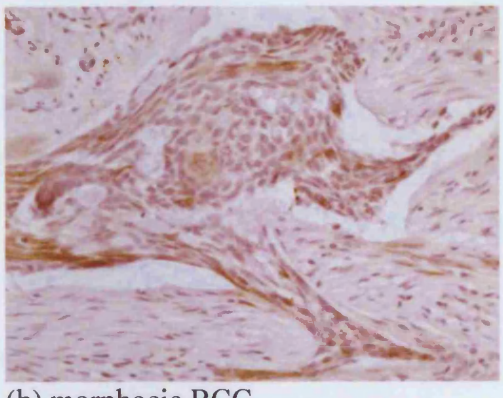
(a) Nodular BCC



(b) superficial BCC



(a) micronodular BCC



(b) morphoeic BCC

Figure 3.9: β-tubulin III: representative images of staining according to subtype. Differences in predominant cellular localisation are seen.

3.3.3 Arc expression

Images representing the staining present in each type of tissue and BCC are shown in figure 3.10. Neuronal tissue within the human appendix positive control sections stained strongly. Negative controls did not stain at all.

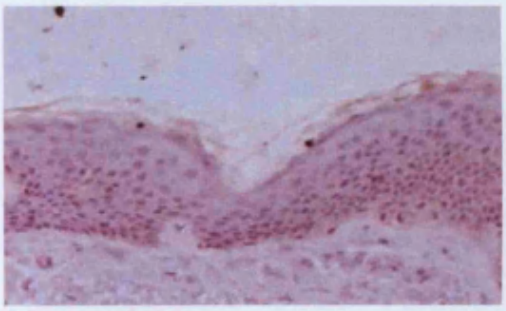
3.3.3.1 Arc: Visual Analogue Scoring

A lower level of staining was seen in the aggressive and morphoeic groups than in the other groups. These results are depicted in figure 3.11. ANOVA analysis of these figures demonstrated that the differences in staining are significantly different between these groups and that they do not represent the same population of tumours ($p=0.001$). There is a variation in confidence limits with many of these groups: a degree of heterogeneity of staining was seen with this antibody across the groups.

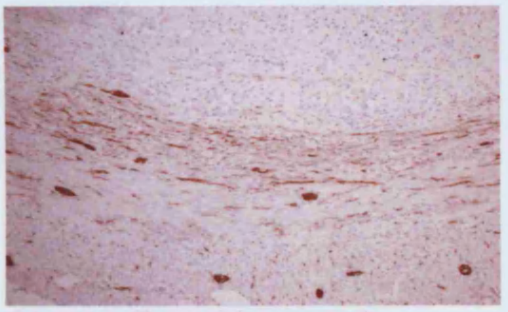
Further ANOVA analysis of the staining as classified by tumour growth class (as shown in figure 3.12) demonstrated a difference between these groups ($p=0.001$). Using a Bonferroni multiple comparisons analysis it was evident that there was a highly significant difference between the aggressive tumours and the other classes, with the aggressive tumours staining less that the other groups.

3.3.3.2 Arc: BCC Spectral Image Analysis

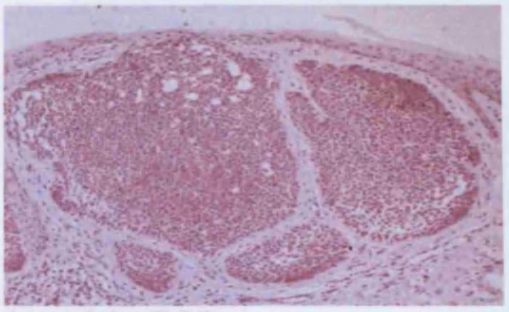
Results of the computerised scoring of the staining of these tumours was analysed again by plotting the mean staining results (with 95% confidence limits) by subtype. The results are depicted in figure 3.13 and concur with the previous visual analogue results - a much lower level of staining is seen in the aggressive and morphoeic groups than in the other groups. Although the differences between the means and the confidence limits are slightly different, the results of statistical comparisons are the same as those derived with visual analogue scoring. ANOVA analysis again demonstrated that the differences in staining between these groups are highly significant ($p=0.001$).



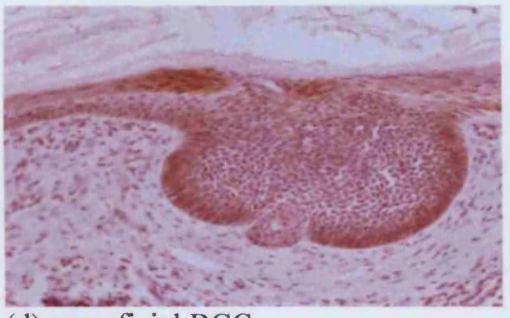
(a) normal skin



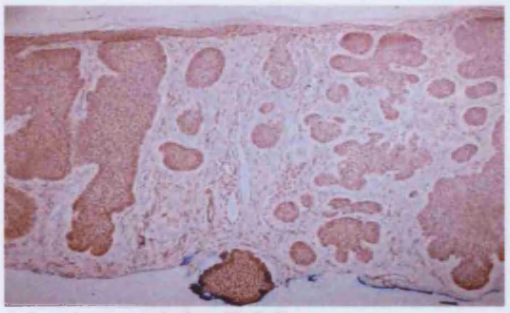
(b) appendix positive control



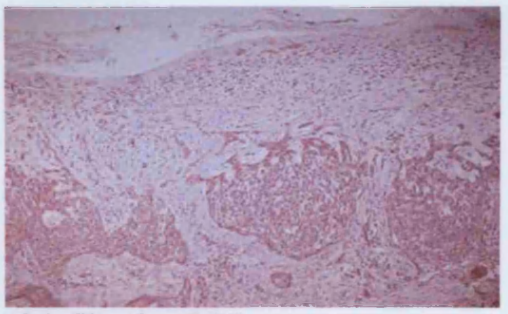
(c) nodular BCC



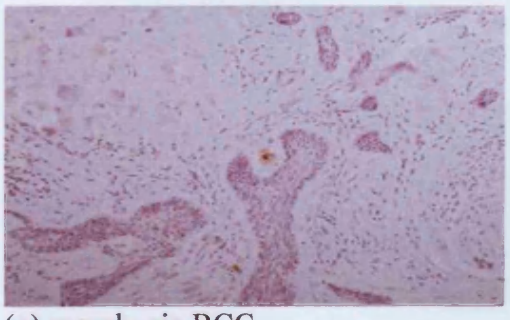
(d) superficial BCC



(e) micronodular BCC



(f) infiltrative BCC



(g) morphoeic BCC

Figure 3.10: Representative examples of Arc staining in control tissues and BCC tumours classified by histological growth pattern subtype

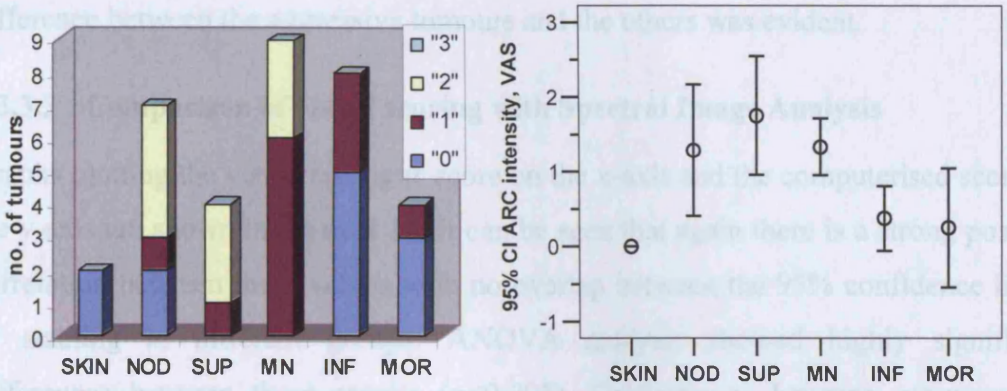
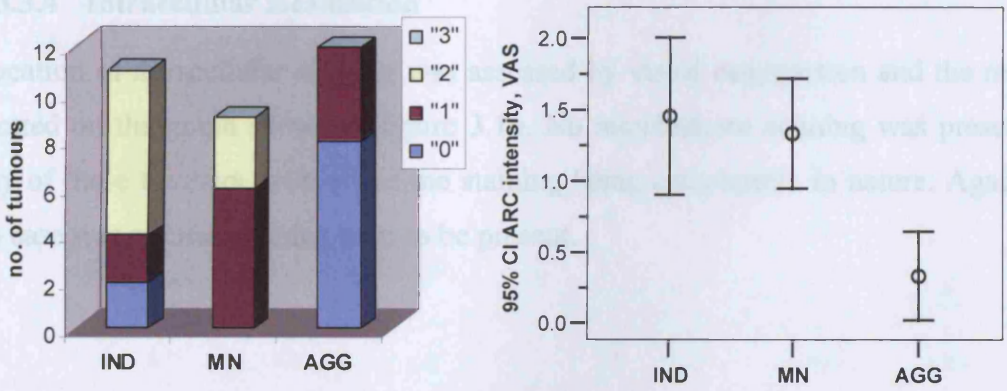


Figure 3.11: Arc: means of visual analogue scoring staining values with error bars (95% CI) by tumour subtype. ANOVA analysis demonstrated highly significant differences between these groups (p=0.001).



Class types compared	p value.
Indolent & Micronodular	>0.999
Indolent & Aggressive	<0.001
Micronodular & Aggressive	<0.001

Figure 3.12: Arc: means of staining values (as determined by visual analogue scoring) with error bars (95% CI) by tumour class. Significances of Bonferroni multiple comparisons are shown.

ANOVA analysis by tumour growth class (Indolent, Micronodular and Aggressive, as shown in figure 3.14) demonstrated a marked difference between these groups ($p=0.001$). Using a Bonferroni multiple comparisons analysis a highly significant difference between the aggressive tumours and the others was evident.

3.3.3.3 Comparison of visual scoring with Spectral Image Analysis

Graphs plotting the visual analogue score on the x-axis and the computerised score on the y-axis are shown in figure 3.15. It can be seen that again there is a strong positive correlation between these values with no overlap between the 95% confidence limits of staining in different groups. ANOVA analysis showed highly significant differences between these results ($p<0.001$). Comparisons between groups using Bonferroni multiple comparisons showed that the differences between each of the groups was different to each of the others. In the case of the visual analogue "0" and "1" tumours, the difference between the staining in each group was marginally insignificant ($p=0.051$). Potential factors affecting this are discussed at the end of this chapter.

3.3.3.4 Intracellular localisation

Location of intracellular staining was assessed by visual examination and the results plotted on the graph shown in figure 3.16. No membranous staining was present in any of these tumours, with all of the staining being cytoplasmic in nature. Again, in no case was nuclear staining seen to be present.

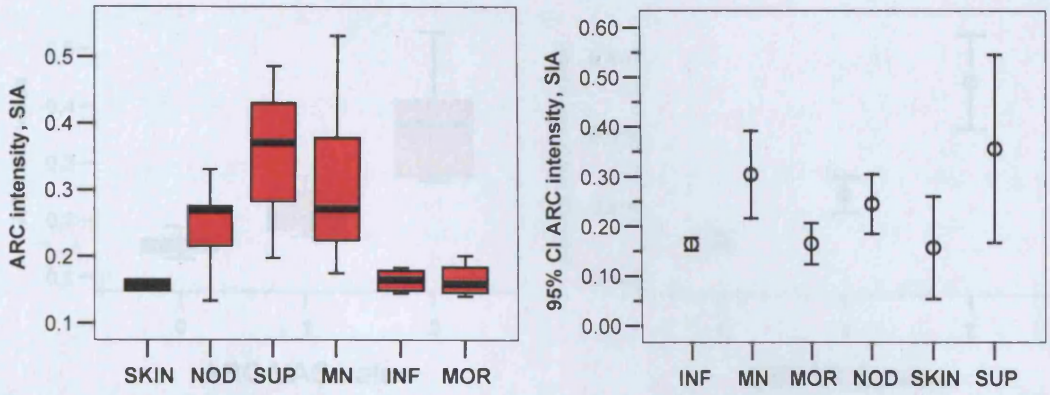
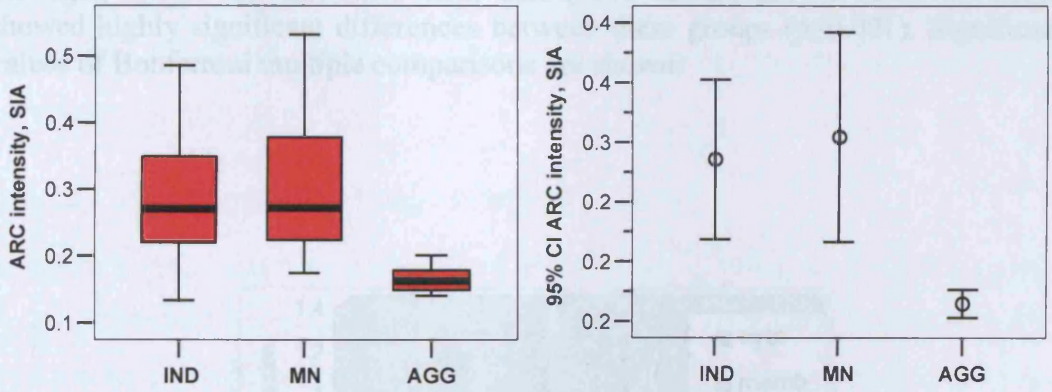
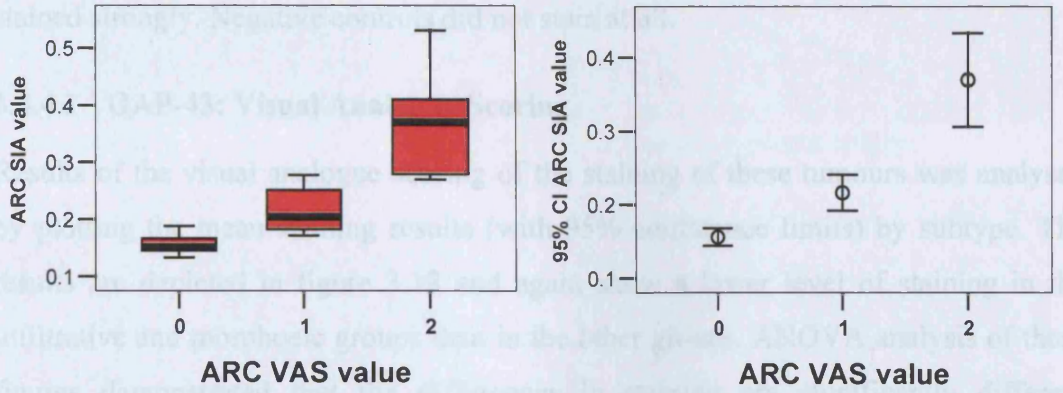


Figure 3.13: Arc: means of spectral image analysis staining values with error bars (95% CI) by tumour subtype. ANOVA analysis demonstrated highly significant differences between these groups (p=0.001).



Class types compared	p value.
Indolent & Micronodular	>0.999
Indolent & Aggressive	0.006
Micronodular & Aggressive	0.002

Figure 3.14: Arc: means of staining values (as determined by spectral image analysis) with error bars (95% CI) by tumour class. Significances of Bonferroni multiple comparisons are shown.



Visual score groups compared	p value.
"0" staining and "1" staining	0.051
"0" staining and "2" staining	<0.001
"1" staining and "2" staining	<0.001

Figure 3.15: Arc: Plots of visual analogue score data and spectral image analysis data. On the left, all absolute figures of spectral imaging data are plotted (horizontal bars = means, boxes = interquartile range, whiskers = maximum and minimum values). On the right, means and associated error bars (95% CI) are shown. ANOVA analysis showed highly significant differences between these groups ($p<0.001$). Significance values of Bonferroni multiple comparisons are shown.

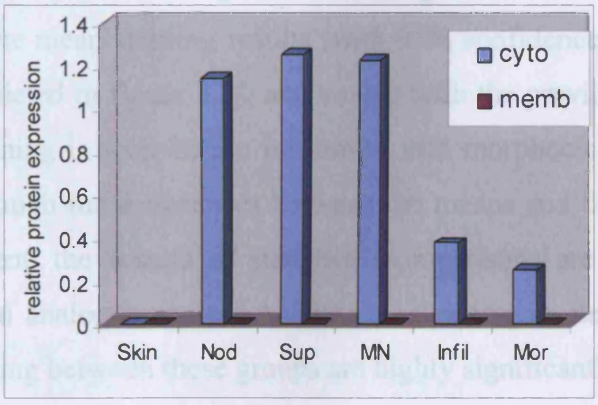


Figure 3.16: Arc: Relative presence of membranous and cytoplasmic staining seen in BCCs according to subtype.

3.3.4 GAP-43 expression

Images representing the staining present in each type of tissue and BCC are shown in figure 3.17. Neuronal tissue within the human appendix positive control sections stained strongly. Negative controls did not stain at all.

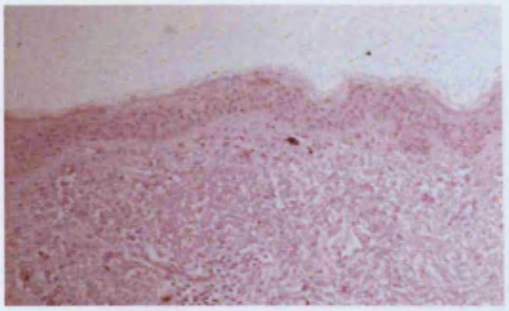
3.3.4.1 GAP-43: Visual Analogue Scoring

Results of the visual analogue scoring of the staining of these tumours was analysed by plotting the mean staining results (with 95% confidence limits) by subtype. The results are depicted in figure 3.18 and again show a lower level of staining in the infiltrative and morphoeic groups than in the other groups. ANOVA analysis of these figures demonstrated that the differences in staining are significantly different between these groups and that they do not represent the same population of tumours ($p<0.001$).

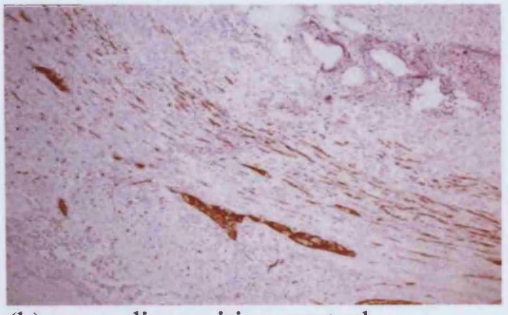
Further ANOVA analysis of staining by tumour growth class (Indolent, Micronodular, Aggressive) as shown in figure 3.19 demonstrated a marked difference between these groups ($p<0.001$). Using a Bonferroni multiple comparisons analysis it was evident that there was a highly significant difference between the aggressive tumours and the others.

3.3.4.2 GAP-43: Spectral Image Analysis

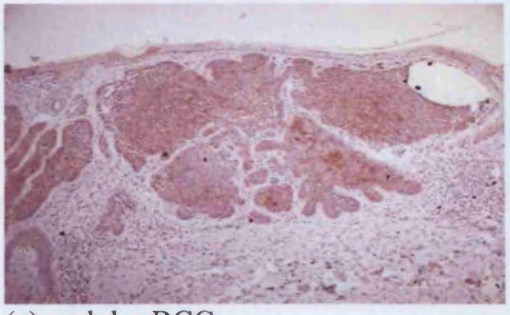
Results of the computerised scoring of the staining of these tumours was analysed again by plotting the mean staining results (with 95% confidence limits) by subtype. The results are depicted in figure 3.20 and concur with the previous results - a much lower level of staining is seen in the infiltrative and morphoeic groups than in the other groups. Although the differences between the means and the confidence limits are slightly different, the results of statistical comparisons are the same as those derived from visual analogue scores. ANOVA analysis again demonstrated that the differences in staining between these groups are highly significantly ($p<0.001$).



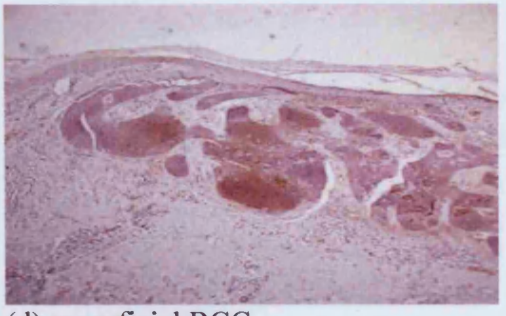
(a) normal skin



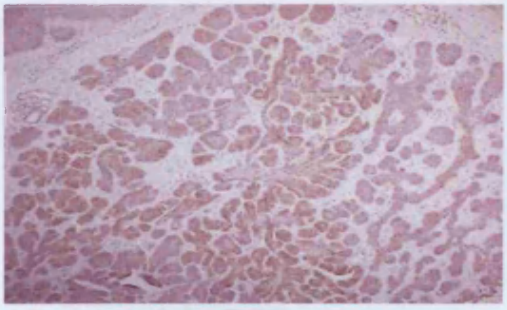
(b) appendix positive control



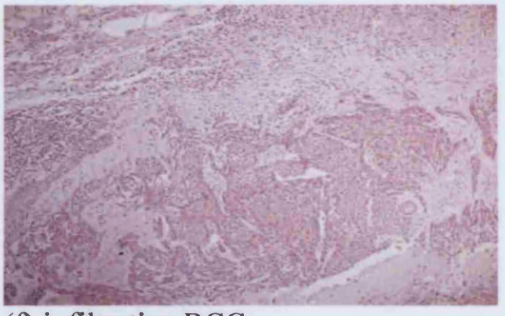
(c) nodular BCC



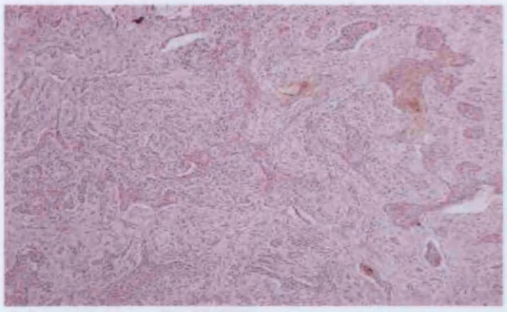
(d) superficial BCC



(e) micronodular BCC



(f) infiltrative BCC



(g) morphoeic BCC

Figure 3.17: Representative examples of GAP-43 staining in control tissues and BCC tumours classified by histological growth pattern subtype

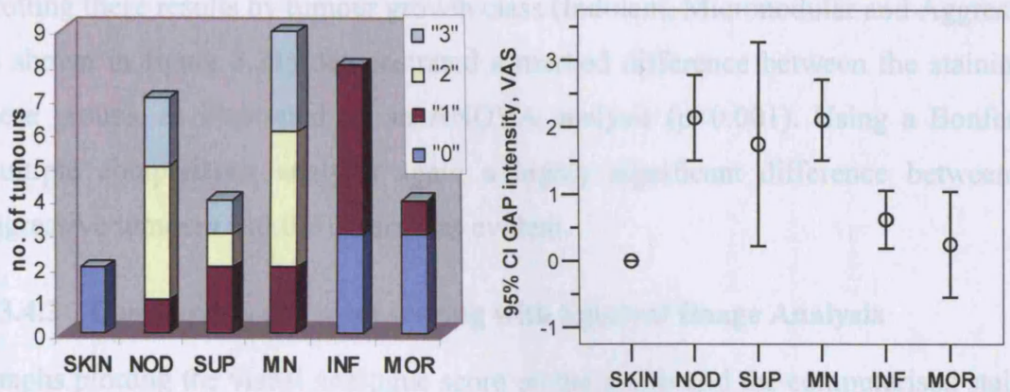
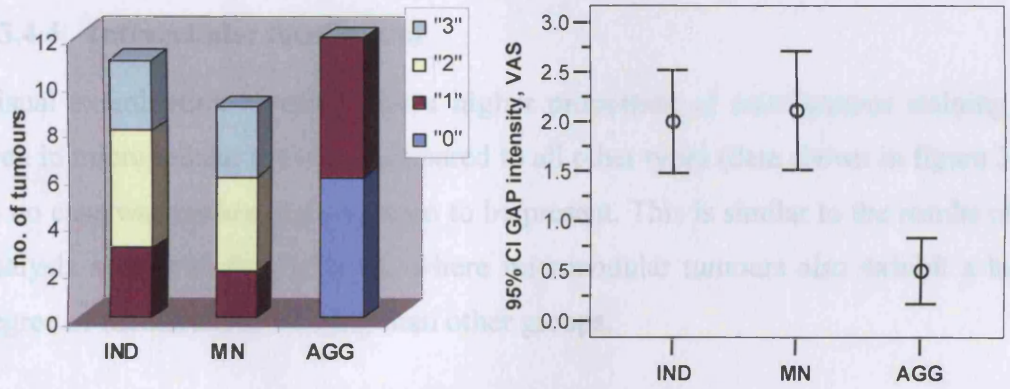


Figure 3.18: GAP-43: means of visual analogue scoring staining values with error bars (95% CI) by tumour subtype. ANOVA analysis demonstrated highly significant differences between these groups ($p<0.001$).



Class types compared	p value.
Indolent & Micronodular	>0.999
Indolent & Aggressive	<0.001
Micronodular & Aggressive	<0.001

Figure 3.19: GAP-43: means of staining values (as determined by visual analogue scoring) with error bars (95% CI) by tumour class. Significances of Bonferroni multiple comparisons are shown.

Plotting these results by tumour growth class (Indolent, Micronodular and Aggressive, as shown in figure 3.21) demonstrated a marked difference between the staining of these groups, as illustrated by an ANOVA analysis ($p<0.001$). Using a Bonferroni multiple comparisons analysis again a highly significant difference between the aggressive tumours and the others was evident.

3.3.4.3 Comparison of visual scoring with Spectral Image Analysis

Graphs plotting the visual analogue score on the x-axis and the computerised staining score on the y-axis are shown in figure 3.22. It can be seen that there is a strong correlation between these values; the main overlap between raw values occurs in the strongly staining groups (Visual score 2 or 3). ANOVA analysis showed highly significant differences between these groups ($p<0.001$) and individual comparison (Bonferroni multiple comparisons) demonstrated a significant difference between all these groups.

3.3.4.4 Intracellular localisation

Visual examination revealed that a higher proportion of membranous staining was seen in micronodular tumours compared to all other types (data shown in figure 3.23). In no case was nuclear staining seen to be present. This is similar to the results of this analysis seen with β-tubulin III, where micronodular tumours also exhibit a higher degree of membranous staining than other groups.

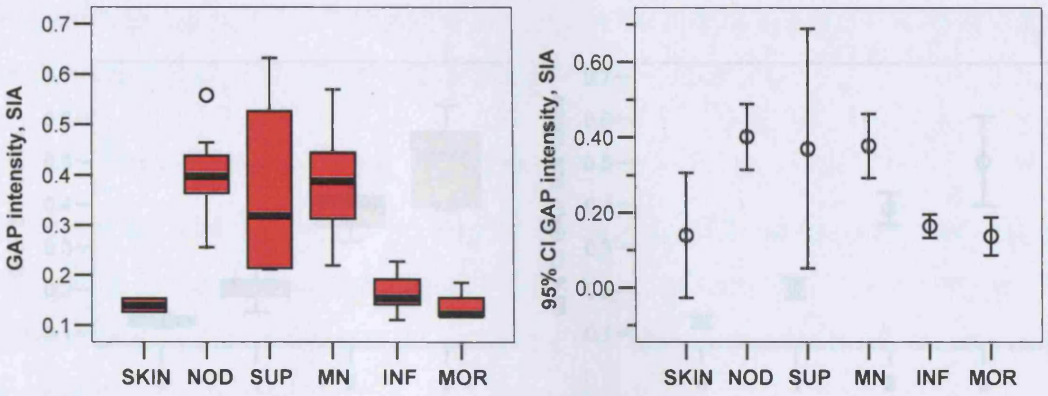
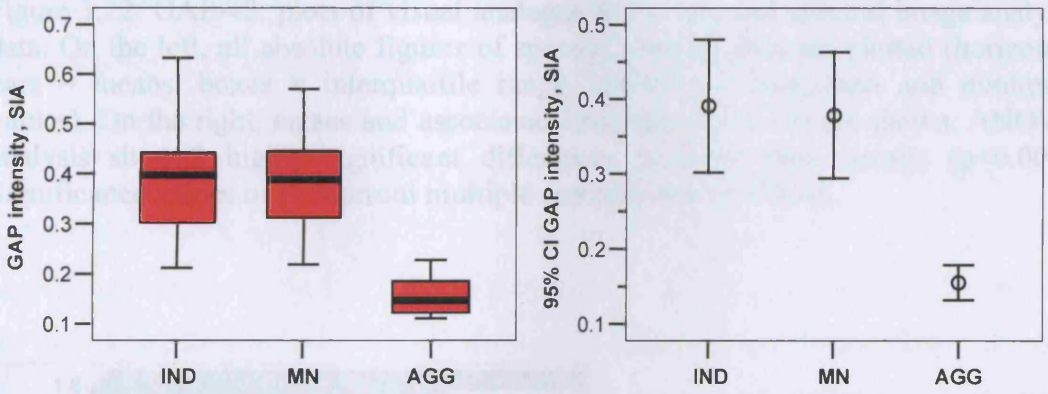
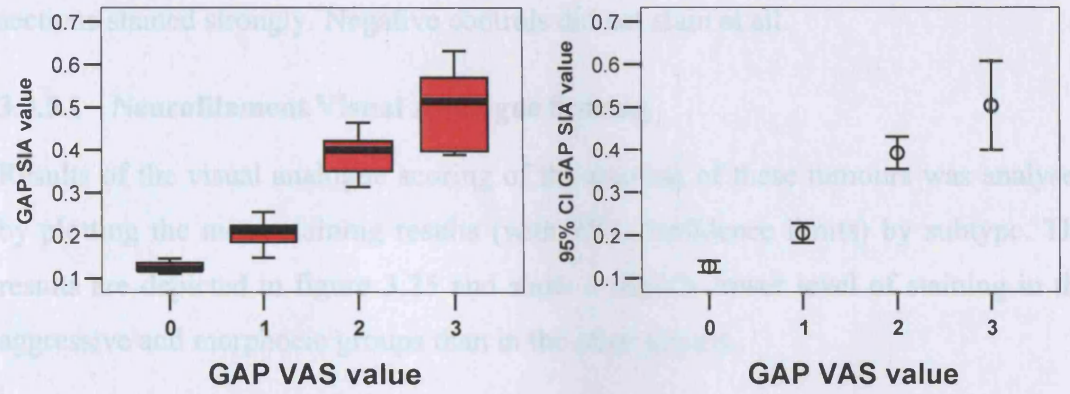


Figure 3.20: GAP-43: means of spectral image analysis staining values with error bars (95% CI) by tumour subtype. ANOVA analysis demonstrated highly significant differences between these groups ($p<0.001$).



Class types compared	p value.
Indolent & Micronodular	>0.999
Indolent & Aggressive	<0.001
Micronodular & Aggressive	<0.001

Figure 3.21: GAP-43: means of staining values (as determined by spectral image analysis) with error bars (95% CI) by tumour class. Significances of Bonferroni multiple comparisons are shown.



Visual score groups compared	p value.
"0" staining and "1" staining	0.045
"0" staining and "2" staining	<0.001
"0" staining and "3" staining	<0.001
"1" staining and "2" staining	<0.001
"1" staining and "3" staining	<0.001
"2" staining and "3" staining	0.003

Figure 3.22: GAP-43: plots of visual analogue score data and spectral image analysis data. On the left, all absolute figures of spectral imaging data are plotted (horizontal bars = means, boxes = interquartile range, whiskers = maximum and minimum values). On the right, means and associated error bars (95% CI) are shown. ANOVA analysis showed highly significant differences between these groups ($p<0.001$). Significance values of Bonferroni multiple comparisons are shown.

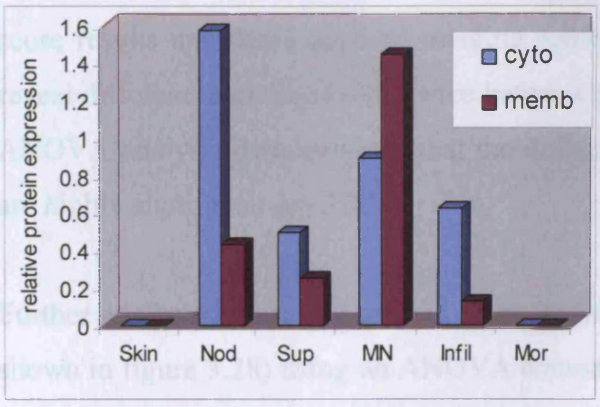


Figure 3.23: GAP-43: Relative presence of membranous and cytoplasmic staining seen in BCCs according to subtype.

3.3.5 Neurofilament expression

Images representing the neurofilament staining present in each type of tissue and BCC are shown in figure 3.24. Neuronal tissue within the human appendix positive control sections stained strongly. Negative controls did not stain at all.

3.3.5.1 Neurofilament Visual Analogue Scoring

Results of the visual analogue scoring of the staining of these tumours was analysed by plotting the mean staining results (with 95% confidence limits) by subtype. The results are depicted in figure 3.25 and show a slightly lower level of staining in the aggressive and morphoeic groups than in the other groups.

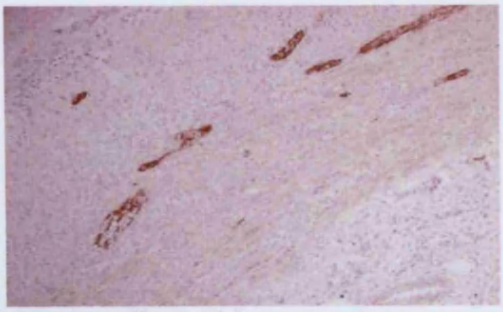
Analysis by tumour growth class (Indolent, Micronodular, Aggressive) as shown in figure 3.26 demonstrated a marginally significant difference between these groups (ANOVA $p=0.048$). Using a Bonferroni multiple comparisons analysis it was evident that there was no significant difference between any of the tumour groups.

3.3.5.2 Neurofilament: Spectral Image Analysis

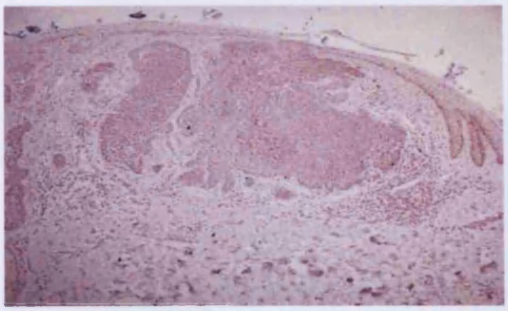
Results of the computerised scoring of the staining of these tumours was analysed again by plotting the mean staining results (with 95% confidence limits) by subtype. The results are depicted in figure 3.27 and demonstrate a lower level of staining in all groups compared to previous antibodies. The mean staining intensity in the aggressive and morphoeic groups is lower than in the other subtypes. The differences between the means and the confidence limits are slightly different between the visual analogue score results and these spectral imaging results. As a result, statistical comparisons reveal different degrees of difference between groups when using the two techniques. ANOVA analysis demonstrated that the differences in staining between these groups are highly significant ($p=0.003$).

Further analysis by tumour growth class (Indolent, Micronodular and Aggressive, as shown in figure 3.28) using an ANOVA comparison did not demonstrate a significant difference between these groups ($p=0.12$). Using a Bonferroni multiple comparisons analysis, however, a significant difference was shown between the staining of the

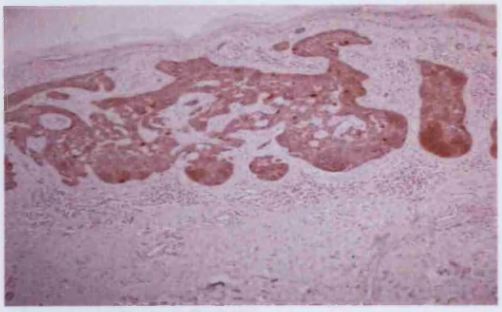
(a) normal skin



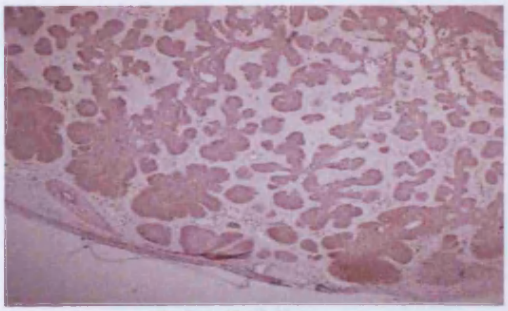
(b) appendix positive control



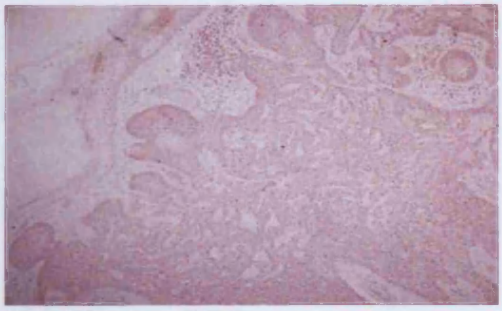
(c) nodular BCC



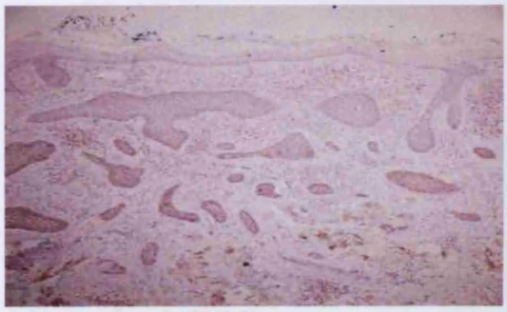
(d) superficial BCC



(e) micronodular BCC



(f) infiltrative BCC



(g) morphoeic BCC

Figure 3.24: Representative examples of Neurofilament staining in control tissues and BCC tumours classified by histological growth pattern subtype

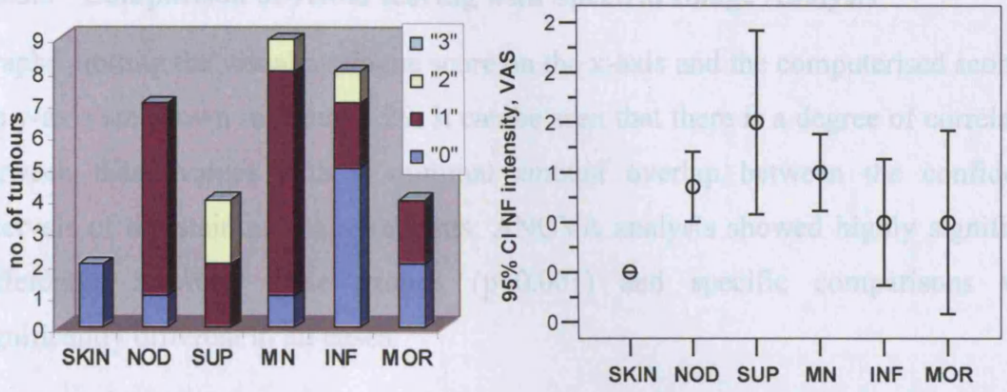
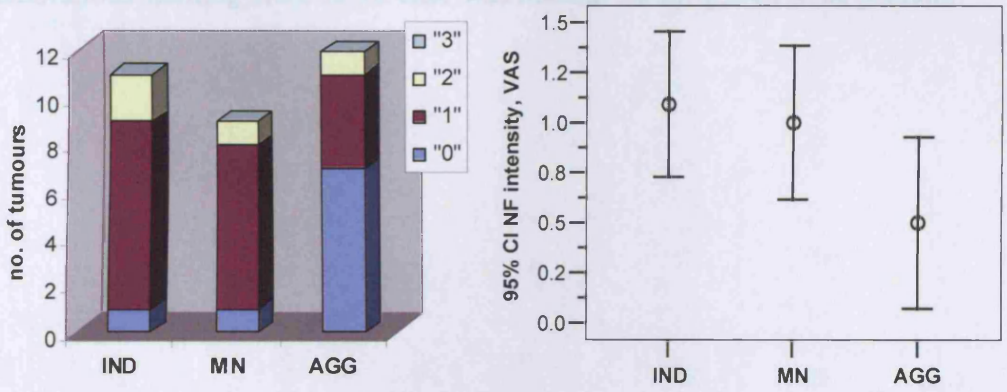


Figure 3.25: Neurofilament: means of visual analogue scoring staining values with error bars (95% CI) by tumour subtype. ANOVA analysis demonstrated significant differences between these groups (p=0.028).



Class types compared	p value.
Indolent & Micronodular	>0.05
Indolent & Aggressive	>0.05
Micronodular & Aggressive	>0.05

Figure 3.26: Neurofilament: means of staining values (as determined by visual analogue scoring) with error bars (95% CI) by tumour class. Significances of Bonferroni multiple comparisons shown.

aggressive tumours and the other groups. This contrasts with the lack of significance between the staining of these same groups when assessed by the visual method.

3.3.5.3 Comparison of visual scoring with Spectral Image Analysis

Graphs plotting the visual analogue score on the x-axis and the computerised score on the y-axis are shown in figure 3.29. It can be seen that there is a degree of correlation between these values with a minimal amount overlap between the confidence intervals of the staining measurements. ANOVA analysis showed highly significant differences between these groups ($p<0.001$) and specific comparisons were significantly different in all cases.

3.3.5.4 Intracellular localisation

Location of intracellular staining was assessed by visual examination and the results plotted on the graph shown in figure 3.30. Very little membranous staining was present in any of these tumours, with all of the staining being cytoplasmic in nature in the majority of tumours. Only in the micronodular subtype was a small element of membranous staining seen. In no case was nuclear staining seen to be present.

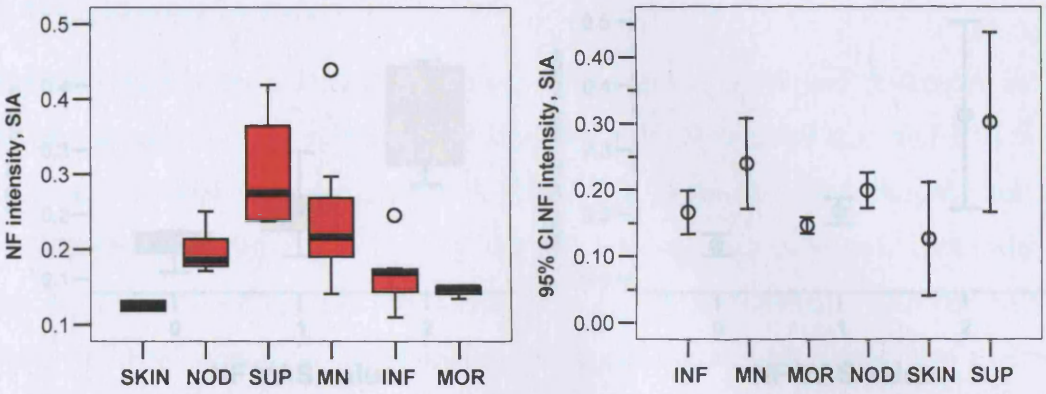
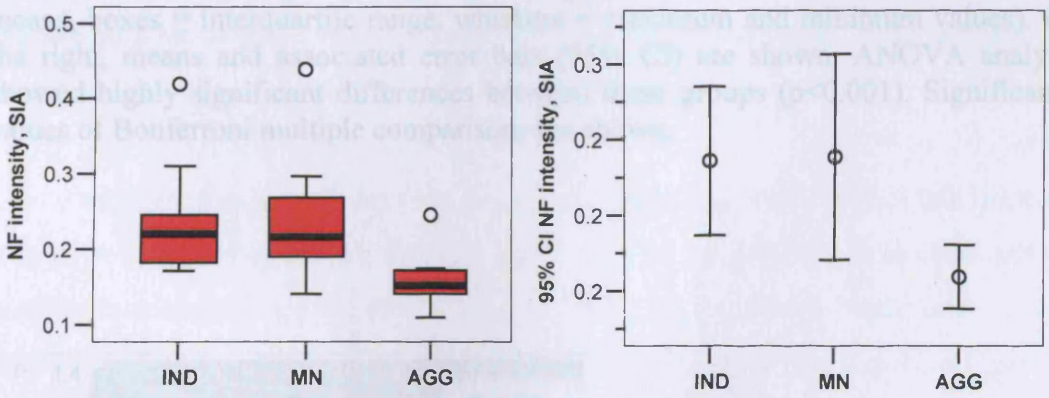
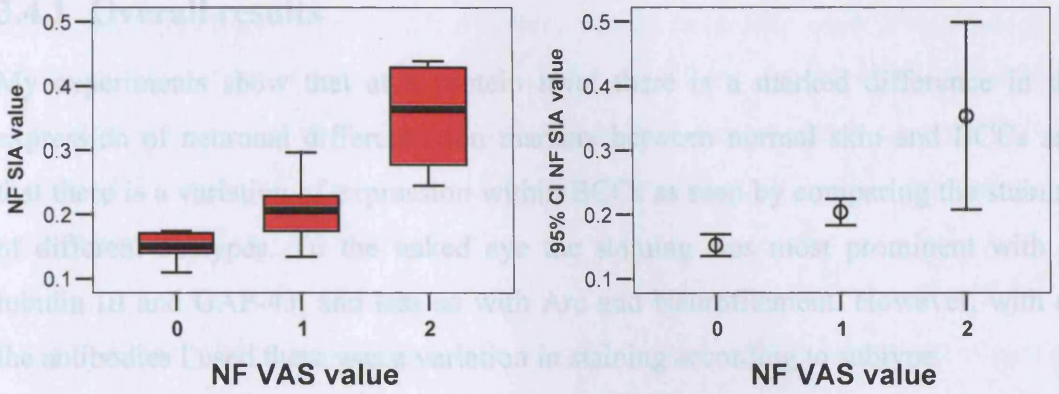


Figure 3.27: Neurofilament: means of spectral image analysis staining values with error bars (95% CI) by tumour subtype. ANOVA analysis demonstrated highly significant differences between these groups (p=0.003).



Class types compared	p value.
Indolent & Micronodular	>0.999
Indolent & Aggressive	0.030
Micronodular & Aggressive	0.034

Figure 3.28: Neurofilament: means of staining values (as determined by spectral image analysis) with error bars (95% CI) by tumour class. Significances of Bonferroni multiple comparisons shown.



Visual score groups compared	p value.
"0" staining and "1" staining	0.030
"0" staining and "2" staining	<0.001
"1" staining and "2" staining	<0.001

Figure 3.29: plots of visual analogue score data and spectral image analysis data. On the left, all absolute figures of spectral imaging data are plotted (horizontal bars = means, boxes = interquartile range, whiskers = maximum and minimum values). On the right, means and associated error bars (95% CI) are shown. ANOVA analysis showed highly significant differences between these groups ($p<0.001$). Significance values of Bonferroni multiple comparisons are shown.

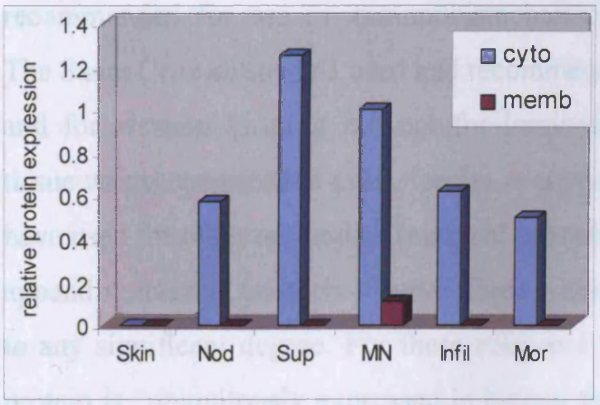


Figure 3.30: Neurofilament: Relative presence of membranous and cytoplasmic staining seen in BCCs according to subtype.

3.4 Discussion

3.4.1 Overall results

My experiments show that at a protein level there is a marked difference in the expression of neuronal differentiation markers between normal skin and BCCs and that there is a variation of expression within BCCs as seen by comparing the staining of different subtypes. To the naked eye the staining was most prominent with β-tubulin III and GAP-43, and less so with Arc and Neurofilament. However, with all the antibodies I used there was a variation in staining according to subtype.

3.4.2 Specific antibody markers used

ULK1 ("Unc51.1-like kinase 1") is the human homologue of Unc51.1, one of the earliest genes involved in neuronal differentiation in nematodes (Kuroyanagi et al., 1998). Whereas Unc51.1 is specifically detected in the nervous system of C. elegans, it has previously been reported that ULK1 is ubiquitously expressed in adult human tissues such as skeletal muscle, heart, pancreas, brain, placenta, liver, kidney, and lung.

My results however did not confirm the previous findings, and I suspect this is mainly due to the fact that the above findings were produced by genomic techniques and not immunohistochemistry. There is no commercially available antibody that is recommended for use in immunohistochemical detection of the associated protein. The Santa Cruz antibody I used had recommendation for use in immunocytochemistry and for western blotting but not for immunohistochemistry of tissue sections. No tissue was recommended even, for use as a positive control, and the routine controls I have used for other antibodies (external controls - nerve fibres with the muscle coat of appendix; internal controls – nerve fibres within BCCs and other tissues) did not stain to any significant degree. For these reasons I was unable to confirm whether ULK1 protein is "ubiquitously expressed in human tissues" as suggested by PCR, nor was I able to determine whether it is upregulated at a protein level in BCCs.

β-tubulin III is a well-known marker for cells of neuronal origin. This property was confirmed in my experiments by strong expression in the nerve fibres of positive

control appendix sections. Although it has been demonstrated in the testis in mammals (Burgoyne et al., 1988; Lewis and Cowan, 1988) it is generally thought of as being specific to neuronal tissue. In this regard it has been used as a marker of neuronal differentiation in pluripotent dermal papilla stem cells undergoing change to a neuronal phenotype (Fernandes et al., 2004). As a component of the intracellular cytoskeleton, immunostaining typically demonstrates a cytoplasmic location for the protein. This was reflected in my experiments with the notable exception of micronodular BCC tumours.

It has previously been investigated as a feature of other non-melanoma skin cancer. Scott et al investigated the expression of β-tubulin III in squamous cell carcinomata, and found that this specific isotype was upregulated in these malignant biopsies compared to normal non-tumour ski (Scott *et al.*, 1990). Additionally, it has unsurprisingly been proposed as a marker of neuroendocrine tumour development, and has been used in studies of these tumours in the gastrointestinal tract (Jirasek et al., 2002).

Its expression has not previously been recognised as a finding in basal cell carcinoma. In this study I found that it did seem to be expressed in BCCs, in many cases strongly, and that it was not expressed in normal non-tumour epidermis. It was principally expressed in the cytoplasmic compartment, and only in micronodular tumours was there any exception to this. In this case a higher degree of membranous staining was seen although this did coincide with cytoplasmic staining also.

Arc (the "activity-regulated cytoskeleton-associated protein") was discovered as a gene that was rapidly induced in active neurons in models of adult and developmental plasticity (Lyford et al., 1995) and has been described as an "immediate early gene". On the basis of this expression it is known as a marker of neuronal differentiation. It shares homology with the actin-binding protein α-spectrin, and in vitro studies suggest that it is associated with the actin cytoskeleton. Immunohistochemical studies have previously indicated that Arc protein is expressed selectively in neurons, where it is located in the cytoplasm of the cell body and in dendritic processes (Fosnaugh *et al.*, 1995). All this and its expression in rat brain following administration of cocaine

suggests that it may be involved in structural alterations underlying neuronal plasticity.

In my study Arc was one of the most highly up-regulated genes highlighted in the in-vitro gene array data analysis. The immunohistochemistry results stated above seem to imply that it is indeed expressed in human BCCs, and not in normal non-tumour skin. No significant amounts of membranous staining were seen in this case – in virtually all the cases described the staining pattern was exclusively cytoplasmic in nature. The degree of staining did not appear to be as strong as that of β-tubulin III.

Growth-associated protein (GAP-43, B50, neuromodulin) is a membranous nervous system-specific phosphoprotein which is widely considered to be involved in axonal growth, axonal regeneration and the modulation of synaptic connections (Benowitz and Routtenberg, 1997). It was initially recognized because of its greatly increased synthesis and axonal transport following axotomy (Skene and Willard, 1981; Skene, 1984). It was also shown to be synthesised at high rates during axonal development (Skene and Willard, 1981). Subsequently its expression was demonstrated in some regions of the normal adult central nervous system (Benowitz et al., 1988; Jacobson et al., 1986; Neve et al., 1987). It is interesting that high basal levels of GAP43 have been found in those areas of the adult central nervous system that are presumed to be in a functionally more plastic state than other parts of the mature brain (McGuire et al., 1988; Neve et al., 1987).

Experimental evidence of the role of GAP43 in guiding the growth of axons and modulating the formation of new connections is available. A recent study in transgenic mice showed animals which over-expressed high levels of an gene encoding GAP43 showed spontaneous formation of aberrant connections and new nerve terminals (Aigner *et al.*, 1995). Conversely, suppressing GAP43 expression has adverse effects on axon outgrowth: mice bearing a null mutation of the GAP43 gene showed defects in axonal pathfinding and most died shortly after birth, despite having what was described as a grossly normal nervous system (Strittmatter *et al.*, 1995).

As well as a central nervous system location, GAP-43 has been localized in peripheral nerve endings in the rat adrenal glands, heart, intestine and olfactory neuroepithelium

(McGuire et al., 1988; Verhaagen et al., 1989). Its presence in neuronal fibres within the skin has previously been studied, and in this case (as in my study) no staining of the normal epidermal tissues were seen (Fantini and Johansson, 1992). It is considered to be a marker for sprouting, and is usually associated with physiological events such as neuronal growth and synaptic plasticity (Gispen et al., 1991; Hoffman, 1989; Skene and Virag, 1989). The distribution of GAP-43 in the central nervous system has raised the hypothesis that throughout life GAP-43 could play a key role in the plasticity of neuronal relationships (Benowitz et al., 1988; Neve et al., 1987).

In this study it was seen to be expressed at a protein level in BCCs, but not in normal skin. Again, a variation in expression does occur amongst the various BCC subtypes, with significantly less expression in the more aggressive subtypes. Similar to β-tubulin III, a marked degree of membranous staining occurred in the micronodular subtypes in conjunction with cytoplasmic staining, whereas cytoplasmic staining occurred in isolation in all other subtypes.

Neurofilament protein expression was also seen in BCCs in this study. Neurofilaments are intermediate filaments and are one of the major components of the neuronal cytoskeleton. Three subunits constitute the basic assembly blocks of neurofilaments - these are the light (NFL protein, NEFL gene), medium (NFM protein, NEFM gene) and heavy (NFH protein, NEFH gene) subunits. In neurons the three neurofilament subunits assemble into a 10nm filamentous structure running the length of the axon, maintaining communication within the cell and having roles including intracellular signalling and maintaining the calibre of the axon. Neurofilament expression is a late marker of neuronal development in the embryo compared to β-tubulin III, which is known to be an early marker. By definition, as part of the cytoskeleton, staining is typically cytoplasmic in nature, as was seen in this study.

As with the other neuronal differentiation markers used, there was an absence of expression in normal epidermis but a presence of staining in BCCs. The staining did not appear to be as strong in these tumour sections as had been seen with GAP43, Arc or β-tubulin, but still a variation in expression did exist. No membranous staining was seen with this antibody, as all the staining seen was cytoplasmic in location.

3.4.3 Relation of molecular markers to growth pattern subtype

3.4.3.1 Neuronal differentiation markers

There is a difference in both the level of expression and cellular localisation of these neuronal differentiation markers in different histological growth pattern subtypes of BCC. This type of tumour classification has most recently been clarified by Sloane and Sexton (Sexton et al., 1990; Sloane, 1977) and it is this that has been adopted by the Royal College of Pathologists as its main classification for these tumours. This classification has a degree of relevance to outcome – it has been shown that the infiltrative subtype of tumour has the highest chance of recurrence following treatment (Jacobs et al., 1982; Rippey, 1998). The main reason, of course, for classifying tumours, is to try and correlate (histological) appearance at the time of excision with future clinical behaviour. The spectrum of aggression of these tumours is, to a degree, reflected by the variations in growth pattern that are seen, but little work on molecular biology of the tumours has managed to show any significant differences in marker expression in the different subtypes.

The reduced expression of these markers in infiltrative and morphoeic tumours could be attributed to de-differentiation as is seen in other tumours, where the phenotype of the tumour changes as it becomes more malignant (da Costa, 2001; Cumming et al., 1990). This reduced expression was not, however seen in the case of micronodular tumours, which do tend to have a more aggressive course than its nodular or superficial counterparts. It is clear that expression of these markers, for example, is not the defining feature of tumour behaviour – otherwise a much closer link would exist between the variation in aggressive behaviour and the variation in staining in these tumours. Micronodular BCC is the group that defies this link.

More evidence of differing molecular biology between micronodular tumours and other subtypes is provided by the degree of membranous staining present in these tumours in this study. This was noted with β-tubulin and GAP-43 antibodies, but not with either Arc or Neurofilament. Membranous staining was present alongside cytoplasmic staining in all cases, and in no case was nuclear staining demonstrated. I

cannot say that tumours with a micronodular type of growth pattern have an exclusive expression of β-tubulin and GAP-43 at the cell surface, but I can surmise that there is some shift in expression of these markers within the cell compared to other subtypes, both more and less aggressive in nature. This specificity of micronodular tumour intracellular localisation is a feature noted with other antibodies as discussed below (β-catenin, section 3.4.3.2) and adds to the suspicion that in many ways micronodular tumours do exhibit properties that are unique to that subtype.

3.4.3.2 β-catenin

Other molecular biology findings that correlate specific marker expression to behaviour are uncommon. One of the studies that has shown a degree of correlation demonstrated that nuclear localization of β-catenin was most prominent in infiltrative and morphoeic tumours, and least so in nodular tumours (El Bahrawy et al., 2003). β-catenin is a crucial member of the E-cadherin / catenin complex and it plays a major role in cell-cell adhesion. Its structural role includes the management of interactions between membranous E-cadherin molecules and the actin microfilament network of the cell. In addition, it is thought that β-catenin is a key player in the Wnt signalling pathway, directly mediating downstream events through transactivation of transcription factors of the lymphocyte enhancer factor / T-cell factor family. These include cell cycle regulating genes such as c-myc, and the gene encoding the matrix-metalloproteinase, matrilysin.

They discovered in immunohistochemical studies that the intracellular localisation of β-catenin was strongly associated with growth pattern subtype of the tumour. Specifically, they noted that nodular and superficial tumours lost the characteristic membranous staining of normal epidermis, and that a slightly greater degree of both cytoplasmic and nuclear staining was present. In infiltrative and morphoeic tumours it was seen that membranous staining was completely lost, and that cytoplasmic and nuclear expression was prominent, especially in the advancing edges of the tumour. Interestingly, micronodular tumours did not have this characteristic appearance, but they instead had a staining profile comparable to normal epidermis. From this work they postulated that the features of infiltrative and morphoeic BCCs may be partly due to the activity of β-catenin and that its increased expression and nuclear localisation at the advancing edges of these tumours could link this area of molecular biology to

biology to subtype. Additionally, they proposed that micronodular tumours were a separate molecular entity in this regard, since none of the features of infiltrative and morphoeic BCCs were present, yet it is known that these tumours do have an aggressive biological pattern.

3.4.3.3 Bcl-2

Bcl-2 is a 24kDa anti-apoptotic protein which was initially identified in a human B-cell lymphoma cell line. Bcl-2 expression in normal skin includes basal keratinocytes, the dermal papillae of the hair follicle, the keratinized Huxley's and Henle's layers, and the keratinized outer root-sheath cells of the isthmus and infundibulum of the hair follicle (Rodriguez-Villanueva et al., 1995). Suprabasal keratinocytes do not express Bcl-2. BCCs appear to have characteristic expression of Bcl-2: most BCCs express high levels of Bcl-2 throughout the tumour (Rodriguez-Villanueva et al., 1995; Verhaegh et al., 1995), the diffuse staining pattern is helpful in distinguishing BCC from the peripheral pattern of Tricoepitheliomas (Abdelsayed et al., 2000; Poniecka and Alexis, 1999; Smoller et al., 1994; Swanson et al., 1998) and moreover, higher levels of Bcl-2 are found in the less clinically aggressive subtypes of BCC compared to invasive subtypes (Crowson et al., 1996; Ramdial et al., 2000).

3.4.3.4 p53

Inactivating mutations in the p53 gene are one of the most frequently found defects in all tumours (Levine, 1997). The p53 gene exerts anti-proliferative effects in response to a variety of different stimuli, such as DNA damage. Current opinion is that p53 prevents cells from cycling when DNA damage has been detected, so that repair enzymes can correct the errors. Should the DNA damage be found to be irreplaceable, an apoptotic pathway is activated leading to cell death. Non-functional or mutated p53 is unable to halt the cell cycle when damage is detected, so that replication of DNA errors can occur, leading to the accumulation of activating mutations in oncogenes or loss of tumours suppressor function. Loss of functional p53 may also inhibit an apoptotic pathway and hence enhance survival of abnormal cells (Levine, 1997). Genetic mutations of p53 are typical UV-induced changes (C-T and CC-TT nucleotide changes). Despite the frequency of p53 mutations in BCC a causal role for these mutations in BCC development or progression has not been demonstrated. Patients with Li-Fraumeni syndrome are susceptible to an increased incidence of

internal malignancies, but interestingly an increase in the numbers of skin cancers has not been reported.

Rather confusingly between fifty and one hundred per cent of BCCs contain p53 mutations (Barbareschi et al., 1992; Ponten et al., 1997; Shea et al., 1992). The figures vary widely because of the different techniques used to identify p53 mutations and the importance placed on identifying mutations based on the intensity of p53 antibody immunohistochemical staining. Comparison between actual mutations (as demonstrated by genomic analysis) and immunohistochemical staining is not straightforward: Campbell et al demonstrated a lack of correlation between actual mutations and immunohistochemical staining, with an increased incidence of positive staining compared to genomically defined mutations (Campbell et al., 1993).

Data regarding the relationship of p53 staining to histopathological subtype is also mixed. Multiple studies have stated that the intensity of p53 staining does not vary between subtypes of BCC (Demirkan et al., 2000; Shea et al., 1992), whereas de Rosa et al demonstrated that p53 staining was increased in aggressive BCC subtypes (De Rosa et al., 1993).

3.4.3.5 Ki67

The growth fraction of a tumour, i.e. the proportion of the cells of that tumour committed to the cell cycle at any particular time, may be easily assessed by Ki-67 antibodies, which identify an antigen expressed in G_1, S and G_2 phases of cycling cells. This has been used extensively to attempt to correlate cellular activity with clinical tumour behaviour. However, from the small number of studies conducted on specific Ki67 expression in different BCC subtypes, it is difficult to come to a definite conclusion.

Baum et al could determine no difference between subtypes of BCC when staining for Ki67 (Baum et al., 1993) with a huge variation of staining patterns seen within tumours, and within growth pattern subtypes. However, Horlock et al showed an increased rate of Ki67 expression in infiltrative, morphoeic and superficial BCC subtypes (Horlock *et al.*, 1997).

3.4.4 Summary

In this chapter I have described how four markers of neuronal differentiation are expressed at a protein level in Basal Cell Carcinoma, as demonstrated by immunohistochemistry. This contrasts with the expected absence of expression in normal skin (epidermis and dermis, except for internal positive control nerve fibres). The level of expression differs according to histopathological subtype for each antibody, with the degree of difference being more significant when measured by an automated computerised system than by visual analogue scoring. Additionally, a difference in intracellular localisation exists when comparing BCC subtypes, with β-tubulin III and GAP-43 staining having an increased membranous component in micronodular tumours.

These results add to the existing data regarding differential antigen expression in BCC subtypes. This has been described for other markers with known intra-cellular functions such as β-catenin, p53, Bcl-2, and Ki67. The wider relevance of the addition to this list of antigens normally associated with tissues of neuronal phenotype will be discussed at further length in chapter 7.

Chapter 4: Investigation of Neuronal Markers in BCC by PCR

4.1 Introduction

Investigation of changes in BCC gene expression and sequences has mainly focussed on the major targets of hedgehog signalling, as outlined in section 1.9.2. Methods used have included in-situ hybridization and PCR methods following mRNA extraction by tissue homogenisation, needle microdissection and laser capture microdissection (LCM). The last method is particularly attractive for my purposes, as it theoretically enables the investigator to specifically select areas of tumours for genomic examination. This is not so necessary for well circumscribed tumours such as nodular BCCs, but would be of great use for disparate tumours such as infiltrative and morphoeic subtypes. To assess whether the genes corresponding to the previously examined proteins (β-tubulin III, GAP-43, Arc and neurofilament) are differentially expressed and associated with growth pattern subtype, specific isolation of both circumscribed and disparate tumour islands is required. Laser capture microdissection was therefore used in this study (as well as needle microdissection and tissue homogenisation) to examine the presence and variation of gene expression in different subtypes of BCC.

Previous published reports of LCM use in BCC has been limited to a small number of studies such as that published by Backvall, Asplund and Micke (Asplund *et al.*, 2005; Backvall *et al.*, 2005; Micke *et al.*, 2004). However, this technique is rapidly becoming part of the mainstream armamentarium in molecular diagnostics; a medline search revealed a total of 178 publications on the use of this technique in the last two years alone (2004 and 2005). These include applications in a wide variety of conditions including other cancers, infectious disease, degenerative conditions and normal hair biology.

My assumption was that RNA extracted by LCM would show more specific changes in BCC gene expression than either the standard method of homogenising whole

biopsies or needle microdissection. I have compared all three methods using the same biopsy samples to determine whether this assumption is correct.

4.2 Methods

As described in section 2.2.2.1, biopsies of tumour samples were taken from patients following ethical study approval and individual patient consent. These samples were limited in size so as not to affect the histological examination (and the surgical margins) of the rest of the specimens. All specimens were immediately snap frozen in liquid nitrogen, stored at -80°C and then embedded in embedding medium. Each biopsy was visually graded by size ("small" to "very large") and H&e slides of each biopsy were used to quantify the grade of biopsy quality (a scale of 0-10 as to how well the biopsy represented the fully excised BCC).

LCM and subsequent RNA extraction was performed at separate times to the RNA extraction following needle microdissection and whole biopsy processing. Some RNA derived from LCM samples was processed using a RNA-amplification kit ("RiboAmp", Arcturus). This is designed to amplify RNA from nanogram/μl concentrations (as are typically produced with LCM) to microgram/μl concentrations for use in downstream applications such as gene array analysis and real-time PCR. As described below, however, this amplification did not occur in this case, and so all further RNA was reverse transcribed in the standard manner and the resulting cDNA was used in PCR experiments to assess gene expression. 2% agarose gels were loaded with cDNA from less aggressive tumours in the left-hand lanes, and from more aggressive tumours in the right-hand lanes.

4.3 Results

4.3.1 Patients and biopsies

Between 26.4.04 and 7.6.04 a total of 57 patients were examined prior to surgery for lesions considered suspicious of being BCCs. Of these, 42 patients (25 males, 17 females) had lesions suitable for biopsy and punch biopsies were taken from a total of 54 lesions. The thirteen patients who did not undergo biopsy had lesions that were either very small (in which any biopsy would have affected surgical margins) or were

not likely to be BCCs. All patients gave written consent for their tissue to be used for research.

Of these 54 lesions, formal histological analysis of the definitively excised lesion confirmed that 39 were BCCs (11 nodular, six superficial, five micronodular, 17 infiltrative, no morphoeic specimens) and the remaining biopsies included those from actinic keratoses (6), squamous cell carcinomas (6), Bowen's disease (1), a tricoepithelioma (1) and a dermatofibroma (1).

Of the 39 BCC biopsies, examination of the H&e slides prepared from that biopsy showed the characteristics described in table 4.1:

Size	n =		quality (score / 10)	n =
Small	15		no tumour in biopsy (0)	8
Medium	19		poor quality (1-3)	8
Large	3		medium quality (4-7)	16
Very large	2		high quality (8-10)	7

Table 4.1: Sizes and quality of BCC punch biopsy specimens

On the basis of this 22 BCC specimens and two normal skin specimens were chosen as candidates for laser capture microdissection. As is described below, nine of these were also subjected to needle microdissection and whole biopsy processing, along with two control normal skin samples.

4.3.2 Laser Capture Microdissection

Of the 22 tumour biopsies that were used for LCM, a total of nine were excluded from further use due to either very poor architecture being preserved during processing, making accurate capturing of tumour cells impossible (five samples), or due to essentially no RNA yield in the extracted samples (four samples, see table 4.3).

Of the 13 remaining BCC RNA samples, four were used in a trial of RNA amplification (see section 4.3.2.5 below). The remaining nine suitable BCC RNA samples and two skin samples were used (without amplification) for further downstream processing.

An attempt was also made to prepare samples in which both peripheral and central populations of cells could be taken from the same tumour, with the aim of comparing gene expression in the leading edges of tumours to that in the central portion (figure 4.2). With the quality of samples being used, this was only realistically possible in one of the tumours (BCC 44) and the peripheral sample consistently had no intact RNA in the final extract. No comparison was therefore possible in this study between tissue derived from the periphery of tumour islands and the central portion.

Aspects which affect the reliability and product of this technique are discussed below.

4.3.2.1 Biopsy orientation, size and content

The biopsies used were harvested using punch biopsies ranging from 2 to 4mm in diameter. It was of course essential that these small tissue blocks were embedded in the correct orientation to ensure that a representative cross section of epidermis, dermis and subdermis was presented for sectioning. In most cases this was not difficult, but in a small number of cases the lack of defined epidermis over an ulcerated BCC and the small nature of the biopsy meant that the biopsy was initially incorrectly oriented: this was corrected by melting of the embedding material and re-orienting the block. The proportion of each biopsy that consisted of defined tumour ranged from 0 to 100%. As long as good quality tumour was present in great enough quantities for meaningful LCM it was included in this study.

4.3.2.2 Section thickness

One can imagine that the process of laser capture microdissection ensures that the selected cells adhere to the plastic of the harvesting "cap". This is analogous to a carpet of cells being lifted from the tissue section. Because it relies on surface cell adherence the optimal thickness of section is approximately 5-7μm: thinner than this and less cells are captured per laser "shot"; thicker than this and residual cells remain on the section slide, obscuring what has been captured and what has not.

4.3.2.3 Use of trypsin to improve capture selectivity

During the "Histogene" dehydration and staining steps, I found that a two minute step of immersion in trypsin greatly improved the ability to selectively remove epidermal and tumour cell populations (as suggested by Nita Agar, personal communication).

Longer than this (5 minutes) produced friable sections in which cells were non-specifically ripped off the slide, whereas less than this (none, 20 seconds and 1 minute) did not produce any discernable difference. This additional step did not appreciably affect the RNA extracted.

4.3.2.4 Timing of LCM

From the point of sectioning the tissue block to immersing the captured cells in lysis buffer (as part of the "Picopure" kit) a large number of steps are required. This takes time (especially when a number of samples are being processed) and RNA degradation can be a major problem in this setting. At all stages preservation of RNA was attempted by keeping blocks and sections on dry ice. When sampling nine BCCs at the one sitting it was interesting to note that the strength of housekeeping β-actin PCR bands appeared to decrease in the later samples processed by LCM (which were also the right-hand lanes of the agarose gels). This reduced PCR yield may correlate with the increased time delay between sectioning and cell capture in these cases, as seen in Figure 4.1. However, as can be seen in Table 4.3 this reduced PCR yield does not correlate with RNA yield or quality of RNA as determined by A_{260}/A_{280} ratio.

The moisture content of the sections was critical for successful cell capture. If any moisture was present cells would not adhere to the cap upon firing of the laser. Keeping the dehydrated sections cold (on ice) and dry (using dessicant) produced the most reliable conditions for good cell capture.

4.3.2.5 RNA amplification

The RiboAmp kit produced by Arcturus, the manufacturers of the LCM microscope and all associated products, is designed to amplify small quantities of RNA such as that produced by LCM. This is supposedly especially useful when using LCM RNA for gene array analysis (such as the gene chips produced by Affymetrix, Santa Clara, USA), in which microgram quantities of RNA are required. Given the low overall RNA concentrations in my BCC LCM samples, I attempted to use this kit on four LCM BCC RNA samples, control RNA, and RNA derived from cell culture experiments to assess the effect on RNA quantity. Results of RNA amplification are shown in table 4.2 below.

Biopsy number	Diagnosis	Original RNA		Amplified RNA	
		Concentration ng/µl	A_{260}/A_{280} ratio	Concentration ng/µl	A_{260}/A_{280} ratio
9	BCC	49.44	2.03	1.53	1.74
23	BCC	26.45	2.45	4.2	2.06
29	BCC	81.31	1.98	4.31	1.52
53	BCC	45.4	2.43	4.47	2.21
Control RNA		359.98	1.96	1604.8	2.33
Primary keratinocyte RNA		467.03	1.96	1440.9	2.36

Table 4.2: RNA concentrations and purity (A_{260}/A_{280} values) before and after use of the Arcturus "RiboAmp" RNA amplification kit.

The RNA amplification kit was effective at increasing the concentration of RNA in the samples with the higher starting concentrations (i.e. the RNA that does not need amplification to produce quality cDNA). Even in these samples the purity appears to have deteriorated, with A_{260}/A_{280} values further from 2.0. No amplification was seen using RNA with lower concentrations (the RNA that I would ideally have wished to amplify). This step was therefore abandoned, and further reverse transcription used only the raw RNA extracted following LCM, needle microdissection or biopsy homogenisation.

4.3.2.6 Final sample results

Examples of cell capture achieved by LCM are illustrated in figures 4.2 - 4.4.

With all these experimental variables taken into account, the final RNA concentrations for the 17 laser-captured samples are stated in table 4.3 below. The nine samples with greater than 10ng/µl RNA and absorbance curves suggestive of RNA presence were also used for needle microdissection and whole tissue homogenisation. Because of the low concentration of the RNA in these samples, all available RNA was used for reverse transcription in each case.

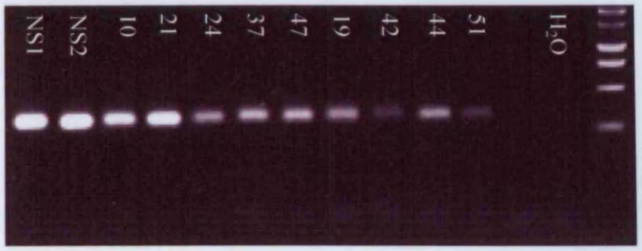


Figure 4.1: Diminishing expression of β-actin with increased length of time from sample preparation to capture of cells (NS1 and NS2 with the shortest interval, BCC 44 and BCC 51 with the longest interval).

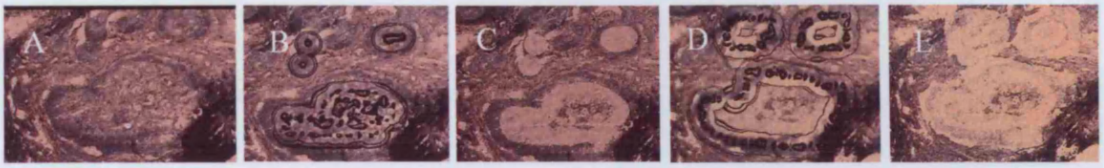


Figure 4.2: Peripheral and central biopsies of BCC tumour island; A: before capture; B: first laser-selected area; C: central biopsy removed; D: peripheral biopsy area selected; E: peripheral biopsy removed.

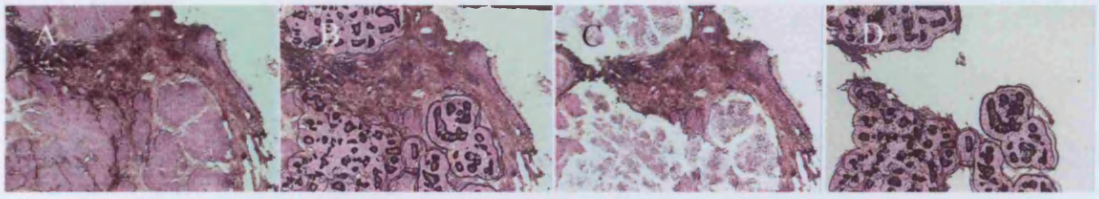


Figure 4.3: Laser capture of cells from a nodular BCC; A: pre-firing of the laser; B: areas where laser has melted the plastic of the cap overlying tumour cells; C: after removal of the cap; D: isolated cells on the undersurface of the cap.

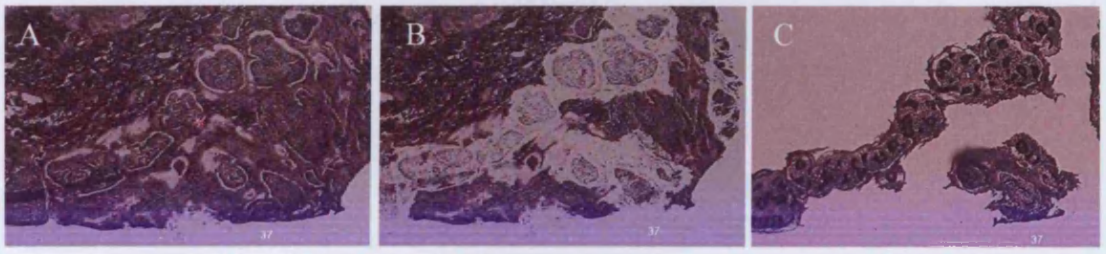


Figure 4.4: Laser capture of cells from a micronodular BCC

Biopsy number	gender	Diagnosis	quality	size	Laser Capture Microdissection RNA		
					Concentration (ng/ul)	A260/ A280 ratio	RNA used onwards
6	F	Nodular BCC	6	M	5.30	2.67	No
9	M	Micronodular BCC	5	M	49.44	2.03	Amplification
10	M	Nodular BCC	7	L	15.60	2.15	RT-PCR
12	F	Superficial BCC	6	M	1.57	1.35	No
19	M	Infiltrative BCC	6	S	52.44	1.99	RT-PCR
21	F	Superficial BCC	4	M	27.32	2.31	RT-PCR
23	M	Nodular BCC	6	M	26.45	2.45	Amplification
24	M	Nodular BCC	8	M	67.24	1.89	RT-PCR
25	M	Infiltrative BCC	8	S	1.98	1.40	No
28	M	Nodular BCC	7	S	4.34	1.68	No
29	F	Nodular BCC	8	M	81.31	1.98	Amplification
37	F	Nodular BCC	8	S	18.70	1.78	RT-PCR
42	M	Micronodular BCC	7	S	19.13	2.00	RT-PCR
44	M	Infiltrative BCC	8	M	44.35	1.40	RT-PCR
47	F	Nodular BCC	6	S	36.94	1.45	RT-PCR
51	F	Infiltrative BCC	9	M	38.47	2.14	RT-PCR
53	F	Infiltrative BCC	5	L	45.40	2.43	Amplification
NS1	F	SKIN	9	9	31.72	1.38	RT-PCR
NS2	M	SKIN	9	6	42.43	1.76	RT-PCR

Table 4.3: Features of RNA product from seventeen BCC biopsies (6 to 53) and two normal skin (NS) samples. Quality and size descriptions are as described in table 4.1.

4.3.3 Needle microdissection and whole tissue homogenisation

Many LCM trials were performed on the blocks of tissue before definitive LCM samples were finally prepared. When the remaining tissue was finally sectioned for needle microdissection and homogenisation, only a small amount of tumour tissue was left in some cases. Nonetheless, needle microdissection (NMD) was performed on all cases prior to whole tissue homogenisation. RNA was extracted from all these samples prior to reverse transcription and PCR. RNA concentrations and total amounts in the extracts from all three techniques are cited in the table below (Table 4.4).

As can be seen, the RNA quantities gleaned from the tissue was greatest following gross tissue homogenisation and least following laser capture microdissection. Extracts from needle microdissection produced intermediate amounts. Approximately three times as much RNA was produced by needle microdissection as by laser

microdissection (2mg vs 0.7mg), whereas this was still only about a quarter of the amount produced following tissue homogenisation (approximately 8mg).

Biopsy	Laser Microdissection			Needle Microdissection			Tissue homogenisation		
	RNA conc. ng/ µl	A_{260}/ A_{280} ratio	x20 µl volume (µg)	RNA conc. ng/ µl	A_{260}/ A_{280} ratio	x40 µl volume (µg)	RNA conc. ng/ µl	A_{260}/ A_{280} ratio	x40 µl volume (µg)
10	15.60	2.15	312.0	96.3	1.71	3852	20.7	1.36	828
19	52.44	1.99	1048.8	79.9	1.91	3196	23.1	1.89	924
21	27.32	2.31	546.4	41.1	1.90	1644	161.2	1.95	6448
24	67.24	1.89	1344.8	55.1	1.89	2204	237.1	1.9	9484
37	18.70	1.78	374.0	41.6	1.73	1664	336.8	1.9	13472
42	19.13	2.00	382.6	57.2	1.83	2288	261.2	1.95	10448
44	44.35	1.40	887.0	24.1	1.62	964	157.5	1.83	6300
47	36.94	1.45	738.8	47.7	1.86	1908	288.4	1.86	11536
51	38.47	2.14	769.4	36.5	1.90	1460	117.8	1.94	4712
NS1	31.72	1.38	634.4	50.6	1.78	824	412.6	1.84	16504
NS2	42.43	1.76	848.60						
Average	**35.8**		**717.0**	**50.0**		**2000.5**	**201.6**		**8065.8**

Table 4.4: Concentrations (RNA conc.), quality and total amount of RNA extracted from nine BCC (10 to 51) and two normal skin (NS) samples via laser capture microdissection, needle microdissection and tissue homogenisation.

The data in this table shows that the total amount of RNA used for reverse transcription did not significantly differ between the BCCs classified as indolent and those classified as aggressive: statistical comparisons as determined by unpaired t-tests are summarised in table 4.5 below ($p>0.05$ in all cases).

	Laser Microdissection	Needle Microdissection	Tissue homogenisation
Average indolent BCC RNA quantity	663.2	2254.4	8353.6
Average aggressive BCC RNA quantity	771.95	1977	5596
p value	0.335155	0.337686	0.197841

Table 4.5: Statistical comparison of total amount of RNA extracted from indolent and aggressive BCCs by laser capture microdissection, needle microdissection and tissue homogenisation in this study.

4.3.4 Gene expression in BCCs & control tissues

4.3.4.1.1 β-actin

β-actin was expressed in all samples using all three methods as shown in figure 4.5.

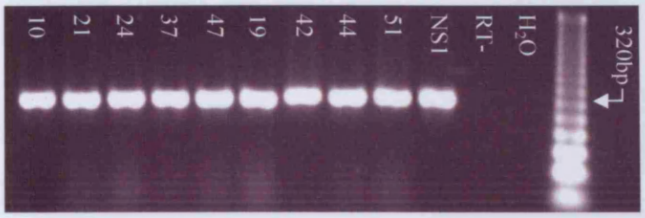


Figure 4.5a: whole tissue homogenisation samples

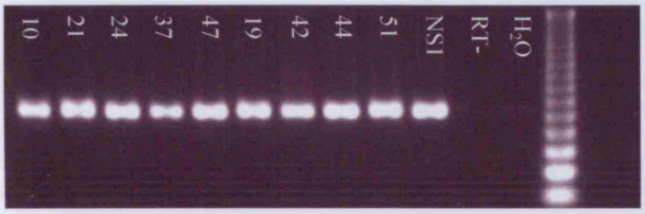


Figure 4.5b: Needle microdissection samples

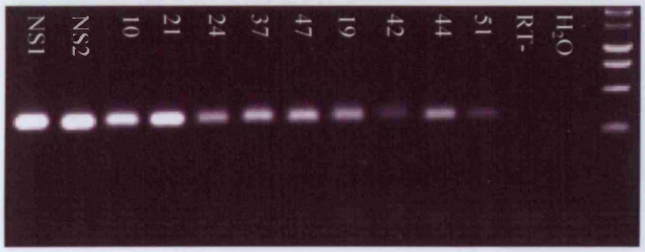


Figure 4.5c: Laser capture microdissection samples

Figure 4.5: β-actin expression in nine BCCs, two control skin samples and negative controls.

The intensity of these bands reflects the expression of β-actin genes in the tissue from which the sample was prepared, but because of differing quantities of starting RNA for each reverse transcription reaction I cannot say that they truly represent the expression per unit amount of tissue. As a result, these gels really only inform us that β-actin is expressed, but not to what degree.

The primers used do not span an intron and so any genomic DNA contamination of the RNA sample would not be picked up by another (higher) band on the gel. For this reason the RT- lane was required to check that contamination is not present.

The strongest bands are clearly seen in the samples prepared by whole tissue homogenisation. Given that the greatest bulk of tissue is generated this way this is not surprising. A tailing off of gene expression is apparent in the laser capture samples – as previously discussed this may be due to RNA degradation due to a prolonged interval between preparation steps.

4.3.4.1.2 Gli1

Gli1 was expressed in all BCC samples using all three methods, except in BCC 42 when LCM was used. These results are shown in figure 4.6. Only an extremely faint band is seen in the normal skin and RT- lanes for the homogenised tissue samples. On the whole *Gli1* was not expressed in the control skin samples, nor in the technical controls.

In the case of *Gli1*, the primers used do span an intron of the gene, and so a band at a higher level on the gel would indicate genomic DNA contamination of the sample. Neither higher genomic DNA bands nor strongly positive RT- bands are present, showing the relative purity of the mRNA from which the cDNA is created.

Given that each cDNA sample was created using different quantities of RNA, it is again hard to draw conclusions about the relative degrees of *Gli1* gene expression present in each tumour. However the mere presence of *Gli1* in BCCs but not in

skin shows that these genes are upregulated in these BCCs. This fits with data derived from genomic examination of BCCs discussed previously in chapter 1.

4.3.4.1.3 Gli2

Gli2 was expressed in most of the BCC samples using all three methods as shown in figure 4.7. *Gli2* was weakly expressed in the control skin samples when prepared by homogenisation or by needle microdissection, but was not expressed in the laser capture skin samples.

The overall levels of expression are much greater in the samples prepared by tissue homogenisation than by either of the other two methods. Even in the laser microdissection samples, however (Fig 4.7c), there does seem to be a good correlation between *Gli2* expression and BCC diagnosis.

4.3.4.1.4 β-tubulin III

Bands corresponding to *β-tubulin III* expression were only faintly seen in the BCC samples, and not in the skin samples or negative controls, as shown in figure 4.8. In all three gels it can be seen that any gene expression in the BCCs occurs mainly in the earlier lanes, i.e. in the more indolent BCCs. The aggressive and micronodular BCCs did not tend to express *β-tubulin III* except for three very faint bands (sample 42, figures 4.8a; samples 19 and 44, figure 4.8b).

4.3.4.1.5 Arc

These results are illustrated in figure 4.9. *Arc* expression was seen in the majority of BCCs prepared by tissue homogenisation, with the most intense bands in the samples from nodular BCCs 24 and 47. Similarly, widespread expression was seen in the BCCs prepared by needle microdissection, with most of the positive bands occurring in the more indolent tumour samples (gel lanes 10 through to 47). Laser capture cDNA did not produce any strongly positive bands on PCR with these primers. Although any weakly positive bands on this gel are indistinct and not well defined (Fig. 4.9c), some of these intensities may correlate with marginally elevated gene expression levels in these samples. Up-regulation of *Arc* was not seen in the negative control lanes or the normal skin samples.

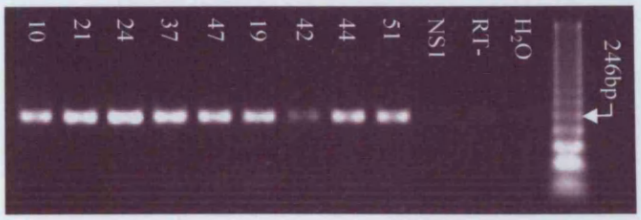


Figure 4.6a: whole tissue homogenisation samples

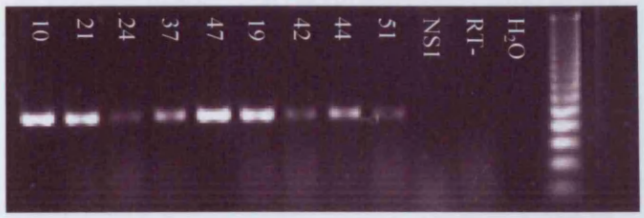


Figure 4.6b: Needle microdissection samples

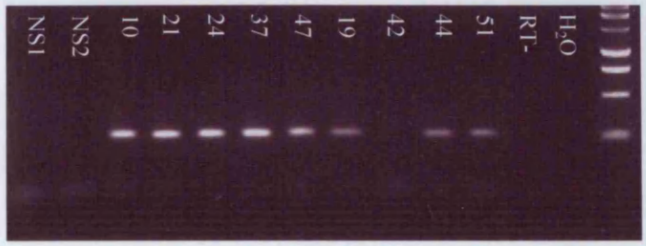


Figure 4.6c: Laser capture microdissection samples

Figure 4.6: *Gli1* expression in nine BCCs, two control skin samples and negative controls.

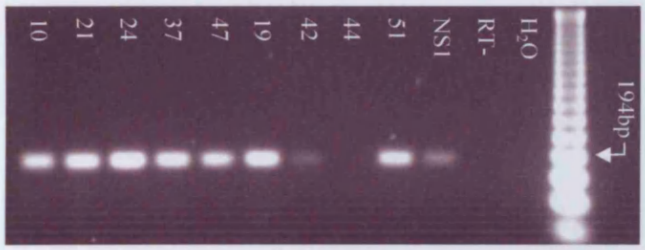


Figure 4.7a: whole tissue homogenisation samples

Figure 4.7b: Needle microdissection samples

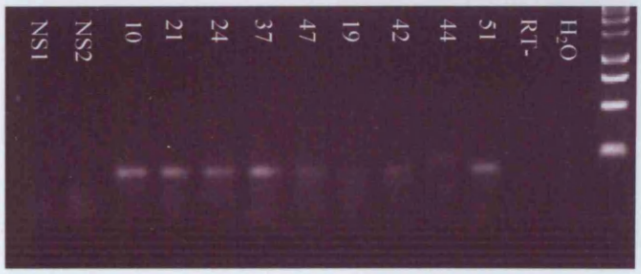


Figure 4.7c: Laser capture microdissection samples

Figure 4.7: *Gli2* expression in nine BCCs, two control skin samples and negative controls.

Figure 4.8a: whole tissue homogenisation samples

Figure 4.8b: Needle microdissection samples

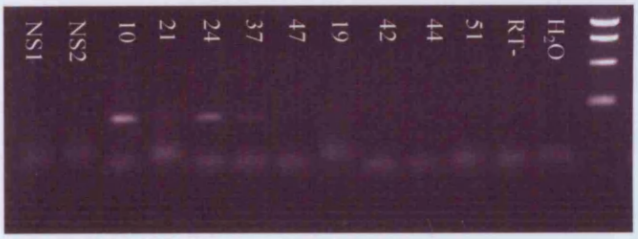


Figure 4.8c: Laser capture microdissection samples

Figure 4.8: *β-tubulin III* expression in nine BCCs, two control skin samples and negative controls.

Figure 4.9a: whole tissue homogenisation samples

Figure 4.9b: Needle microdissection samples

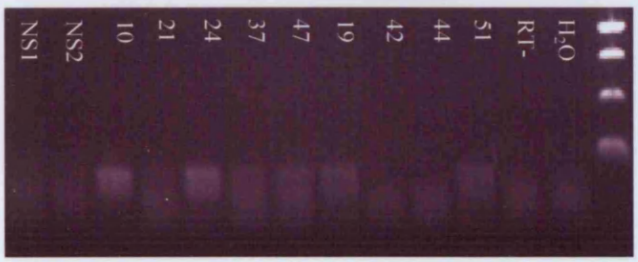


Figure 4.9c: Laser capture microdissection samples

Figure 4.9: *Arc* expression in nine BCCs, two control skin samples and negative controls.

4.3.4.1.6 GAP43

GAP-43 was seen to be strongly expressed in three of the BCCs when prepared by tissue homogenisation (BCCs 21, 24 and 19), and moderately strongly expressed in two of the other BCCs (10 and 44) when prepared by needle microdissection. No positive bands were seen in the laser capture microdissection material at all. In none of the gels did skin or negative control lanes show any gene expression positivity.

In these cases there was no predilection for elevated expression in one type of BCC versus another (e.g indolent vs aggressive). The small number of tumours that were positive is rather inconsistent, such that few definite conclusions may be drawn about *GAP-43* expression in different BCC subtypes, other than the fact that there does appear to be a degree of expression in BCCs and not in normal skin.

4.3.4.1.7 NF

Only faint expression of the neurofilament gene was seen in some of the BCCs prepared by tissue homogenisation. These weak bands were equally divided between the indolent and the aggressive tumours. No expression was seen at all in the needle microdissected specimens, and four very faint bands were again equally distributed between indolent and aggressive tumours.

Figure 4.10a: whole tissue homogenisation samples

Figure 4.10b: Needle microdissection samples

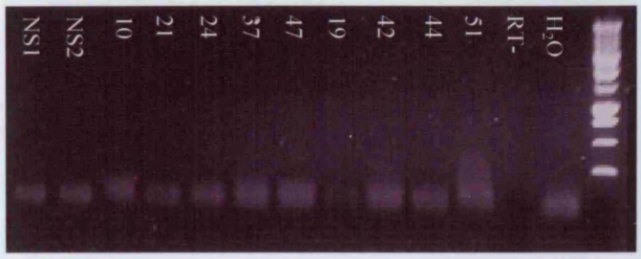


Figure 4.10c: Laser capture microdissection samples

Figure 4.10: *GAP-43* expression in nine BCCs, two control skin samples and negative controls.

Figure 4.11a: whole tissue homogenisation samples

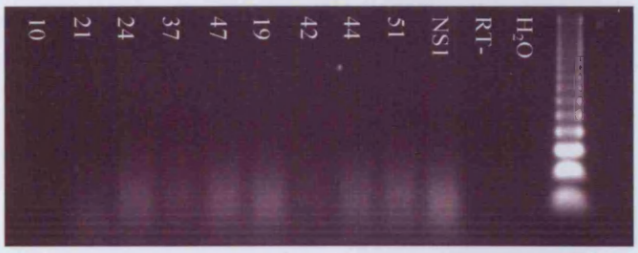


Figure 4.11b: Needle microdissection samples

Figure 4.11c: Laser capture microdissection samples

Figure 4.11: *Neurofilament* expression in nine BCCs, two control skin samples and negative controls.

4.4 Discussion

The examination of gene expression in BCCs in this chapter has been limited to visualisation of PCR products on agarose gels. This has provided a great deal of information regarding the technical aspects of RNA extraction used in this study and the expression of neuronal differentiation markers in Basal Cell carcinoma.

4.4.1 Patients and samples

The examination of gene expression required that fresh frozen biopsies be harvested and kept cold so that mRNA stability would not be affected. For this purpose, 57 patients were assessed as to whether their potential BCC would be suitable for biopsy, and from these patients a total of 54 lesions were biopsied, 39 of which turned out be BCCs. The spread of subtypes present in these BCCs (11 nodular, 6 superficial, 5 micronodular, 17 infiltrative) was typical for the plastic surgery unit in which they were taken: this spread has a slight bias towards aggressive tumours compared to previously published overall subtype statistics (Emmett, 1990; Jacobs et al., 1982; Sexton et al., 1990; Sloane, 1977).

The limitation of size of biopsy was a relative problem, but this was essential for integrity of the surgical excision margins of the tumours. The biopsies were graded by size and quality (see table 4.1) and a number of these biopsies were excluded from further use because of a small amount or absence of tumour in the biopsy. It is possible that larger biopsies would facilitate better quality and larger amounts of RNA extraction, but the permission of the histopathologists to perform this in this setting was not favourable for anything more than a punch biopsy, even in the larger tumour cases.

The selection of what tumours were eventually used in this study rested on the success of laser capture microdissection (LCM). As well as investigating the expression of neuronal differentiation markers in these tumours, I was interested to see whether LCM was significantly better than more traditional techniques in showing gene expression. For that reason the same tumours were used, restricting the numbers of BCCs for comparison to nine.

4.4.2 Technical aspects

Laser Capture microdissection is a complex technique that requires a great deal of time and experience to use effectively. Its potential benefits are that it can selectively capture cells that are visualised under the dissecting microscope, that cell populations can be specifically targeted and that surrounding tissue (accounting for "background noise" gene expression) can be excluded. In the more modern developed systems, much of the process is automated, increasing the speed of cell capture, and enabling automatic recognition of the cell areas to be targeted ("Veritas", Arcturus, Mountain View, USA).

The system that was available to me, however, was a previous model which was not automated, and was housed in a different building to where the rest of my tissue processing occurred. Following cutting and dehydration of the frozen sections, the processing time for each sample could take up to fifteen minutes. All this of course meant that the first sample processed in a batch had a delay interval during which it is possible that RNA quality was degrading. This is not reflected in the RNA concentrations of the resulting samples (see table 4.3), but stronger housekeeping β-actin bands are seen in the specimens processed quickly (see Fig. 4.1).

Needle microdissection and tissue homogenisation were performed in a routine manner, which produced higher concentrations and total amounts of RNA (as measured by spectrophotometry). This is not in itself surprising, as it is much more feasible to harvest a greater number of cells by these techniques. Because, however, the concentrations of RNA in these solutions were still fairly low (average RNA concentration in the tissue homogenisation samples was 0.20μg/μl) the full amount of RNA was used to create maximal strength cDNA in every case.

Therefore, direct comparison of the intensity of each PCR product band is not likely to absolutely reflect the level of expression of that gene per unit volume, mass, or cell number. However, various trends can be seen and are commented on in the following sections.

4.4.3 Baseline β-actin and Gli1/2 expression

β-actin was chosen as a housekeeping gene because of its ubiquitous expression in cells of epithelial origin. This was clearly demonstrated in all three gels showing β-actin expression in all skin and BCC samples. The bands are all very strong in the samples prepared by tissue homogenisation and relatively strong in those prepared by needle microdissection, reflecting the starting RNA concentrations in these samples. β-actin was also expressed in all skin and BCC LCM samples although to a lesser degree than with the other two methods.

No significant variation exists between the intensities of the PCR bands in the first two gels (LCM and NMD) and the very strong signal implies that either a lot of β-actin is expressed equally in all these samples, or that the PCR reactions have all reached the point of saturation. In this case, no difference in intensities would be seen, masking any relative difference between the samples. The relative decrease of β-actin expression from left to right in the LCM gel is not explained by the measured starting RNA concentrations. Whether the time delay in processing the later samples has accounted for this variation in expression, or whether it is merely a feature of the cell populations captured cannot be commented upon here. A more formal trial of delay in processing versus RNA extraction and housekeeping gene expression would be required to ascertain this.

Gli1 and *Gli2* were both expressed in BCCs (although not all BCCs in all cases) but not in normal skin or the technical negative controls. This correlates with data confirming Gli gene expression in these tumours (Bonifas et al., 2001; Kallassy et al., 1997; Regl et al., 2002; Reifenberger et al., 1998; Unden et al., 1997). Recent data from mouse models show that *Gli1* and *Gli2* may be responsible for different stages of tumour induction and maintenance (Hutchin et al., 2005). Even when comparing the samples prepared by tissue homogenisation and needle microdissection, there does appear to be a slightly greater degree of gene expression (for both *Gli1* and *Gli2*) in the left hand BCC lanes. This may reflect slightly decreased *Gli1* and *Gli2* expression in the more aggressive tumours.

4.4.4 Neuronal differentiation marker expression

Direct comparison of gene expression in RNA extracted from the same biopsies by different methods indicated some general trends. It appears that all the neuronal differentiation markers which were examined in chapter 3 are expressed to some degree at a genomic level in BCCs, but in general positive PCR bands were not seen when examining normal skin (the only exception to this was a very weak band with GAP-43 primers in NMD tissue).

In BCCs, gene expression results can be examined by comparing the overall expression in BCCs to that in normal skin, by comparing the expression in indolent (left hand lanes) BCCs to that in aggressive BCCs (right hand lanes), and by comparing the techniques used. More specific comparisons than these are not likely to be truly representative of gene expression in each tumour and more detailed analyses including quantitative PCR are required.

β-tubulin III was weakly expressed in BCCs, and predominantly the earlier (left hand lanes) tumour samples. BCC 10 had especially strong expression compared to the others, and this was seen in both tissue homogenisation and needle microdissection samples. Overall, expression became weaker in successive gels, but this notwithstanding, there was still some expression noted in the earlier tumours (i.e. the more indolent tumours) processed with laser capture microdissection.

Arc was moderately expressed in many of the BCC samples but again, not in normal skin. In the NMD samples the expression does appear to be more up-regulated in the earlier lanes corresponding to the more indolent tumours, but this variation is not so strongly seen in tissue homogenisation samples. The laser capture microdissection samples did not clearly express any PCR product and this contrasts markedly with the other more robust methods of RNA extraction.

GAP-43 showed some unexpected results in that a small number of bands (three) were particularly strong whereas others were completely negative in the homogenisation samples. The presence of these bands was distributed fairly evenly between indolent and aggressive tumours, but no particular comment can be made as to their

significance due to the small numbers involved. It is of course possible that within these biopsies some other material (such as that arising from a nerve fibre) was present, but this seems unlikely in the face of three such tumours showing this pattern and such bands not being picked up by the other neuronal differentiation marker primers.

Very little evidence of neurofilament expression was seen in these samples, with only a very faint band present in four of the samples processed by tissue homogenisation. No positivity was seen in the samples processed by either needle microdissection or laser capture microdissection. Of the four weakly expressed bands, no difference was noted between indolent and more aggressive tumours.

Overall it can be seen that *β-tubulin III*, *Arc* and *GAP-43* genes do seem to be up-regulated in BCCs compared to normal skin, and that this is most easily demonstrated by the techniques of either whole tissue homogenisation or needle microdissection. Laser capture microdissection, on the whole, was sensitive enough to detect *β-actin* and *Gli1*/*Gli2* expression, but it does not appear to be sensitive enough to demonstrate the expression of the four neuronal markers used.

It is hard to say whether my assumption that the higher ratio of tumour sample to background non-tumour tissue in laser capture tissue would result in a more specific gene expression profile was upheld in this study. Additionally, the sensitivity of the technique may or may not be less with this technique, but without absolute comparison to starting biopsy weights or cell numbers dissected, or relative comparison to β-actin expression, this measurement is not meaningful. In both cases, the true estimation of sensitivity and specificity relies on the knowledge of what are true and false positive and negative results. The only information available on this subject is presented above, and further work would be required to perform truly accurate comparisons of particular gene expression between tumours and between subtypes.

Chapter 5: Effects of Gli1 and Gli2 over-expression in human keratinocytes on expression of neuronal markers

5.1 Introduction

The expression of β-tubulin III, GAP-43, Arc and neurofilament has been previously demonstrated in this thesis at a protein and genomic level in BCC material. In the introduction to this thesis I have described how hedgehog signalling is crucial to the development of BCCs, and that irrespective of the site of the upstream genetic mutation, up-regulation of the Gli transcription factor genes and proteins seems to be fairly consistent.

Transgenic experiments in mice have shown us that Gli proteins may have differing roles in both the early stages of tumour development and the later stages of tumour persistence and survival. *Gli1* and *Gli2* appear to be crucial for general development, but only *Gli2* is essential for development of follicular skin appendages (Mill et al., 2003; Park et al., 2000). Clearly the initiation of BCC formation and persistence of the tumour relies on both of the molecules to an extent.

Also discussed in the introduction to this thesis is the limited amount of knowledge of what happens downstream of these Gli proteins. Current knowledge of Wnt/β-catenin, FOX proteins and cyclins has given us only a few insights into the specificity of *Gli1* and *Gli2* downstream molecular signalling. Regl et al have previously used a keratinocyte culture model to examine the interaction of *Gli1* and *Gli2* and have also examined the expression of both of these transcription factors in human BCCs (Regl et al., 2002). They demonstrated a degree of inter-dependence between the two genes, suggesting that the expression of these genes were mutually controlled.

I have already shown that markers of neuronal differentiation are expressed in human basal cell carcinoma, but it could not be demonstrated clearly with such tissue what the interaction between *Gli1* and *Gli2* is (even though expression of both genes in BCCs was demonstrated by PCR methods).

In this chapter I describe how over-expression of *Gli1* affects *Gli2* and vice versa, and how their over-expression affects the four neuronal marker genes identified in this thesis.

The reason for using this system of retroviral transfection is that it allows the controlled over-expression of target genes and proteins, in this case *Gli1* and *Gli2*. Such genetic manipulation would also be available using transgenic animal models, but the high proportion of very significant defects (at both a general and anatomically specific level) makes this a very challenging technique. Retroviral transfection of keratinocytes in culture is a robust and relatively cost-effective technique which can lead to reproducible results in a relatively short period of time.

Therefore, the aim of this chapter is to investigate over-expression of *Gli1* and *Gli2* in human keratinocytes to determine whether over-expression of these proteins up-regulates the expression of neuronal markers identified in previous chapters of this thesis.

5.2 Materials and methods

As discussed in section 2.3.3, populations of primary keratinocytes were retrovirally transfected to over-express *EGFP* alone, *EGFP-Gli1* or *EGFP-Gli2* and were subsequently grown for 72-96h. With each transfection experiment this resulted in three sets of cells in which the gene expression of *Gli* transcription factors and neuronal differentiation markers could be compared. Each cell population was harvested and mRNA was extracted using a spin-column RNA extraction kit. This was subjected to reverse transcription and real-time quantitative PCR (qPCR) using primers for Gli1, Gli2, β-tubulin III, GAP-43, Arc and neurofilament. RNA from SH-SY5Y neuroblastoma cells was used to synthesise positive control cDNA. Samples were analysed in triplicate. For each sample of each primer / cell group combination a cycle-to-threshold figure was recorded (as "c(t)"). This data was analysed in a relative quantitative fashion, with target gene expression compared in all cases to baseline β-actin expression. The relative difference between the c(t) values for each transfected cell group and the control group was calculated, accounting for β-actin expression (recorded as "Δc(t)"). Statistical analysis of these Δc(t) values was performed using the Statistical Package for Social Sciences (SPSS, Chicago, USA). Unpaired t-tests

were used to compare the expression of the Gli genes and the neuronal marker genes in the three cell groups. Standard PCR was also performed and the PCR products run on a 1.5% agarose gel.

5.3 Results

5.3.1 Retroviral over-expression of *Gli* in keratinocytes

The appearance of keratinocytes in culture is shown in figure 5.1. Messenger RNA was extracted from cultured keratinocytes and quantitative and qualitative properties were measured using a spectrophotometer; these results are shown in table 5.1 below.

	transfection 1			**transfection 2**	
	Concentration (μg/μl)	A_{260}:A_{280} ratio		concentration (μg/μl)	A_{260}:A_{280} ratio
KCs	4.75	1.91		1.06	1.93
Gli-1KCs	2.61	1.96		0.34	1.88
Gli2-KCs	4.16	1.92		0.49	1.96
SH-SY5Y	2.62	1.9		-	

Table 5.1: RNA samples extracted from transfected keratinocytes and positive control SH-SY5Y cells (KCs = *EGFP*-transfected keratinocytes; Gli1-KCs = *EGFP-Gli1* transfected keratinocytes; Gli2-KCs = *EGFP-Gli2* transfected keratinocytes)

5.3.2 Typical real-time qPCR graphs

Graphs such as those illustrated in figure 5.2 are typical representations of the exponential rise in PCR product. It can be seen that the earlier the take-off of gene product, the earlier this is likely to hit a pre-determined threshold. These examples are illustrative of one recording for each cell sample (although in reality the values were recorded in triplicate). In figure 5.2, which depicts the rise of *Gli1* gene PCR product, it can be seen that the *Gli1*-transfected cells (green) hit threshold earlier than the *Gli2*-tranfected cells (blue).

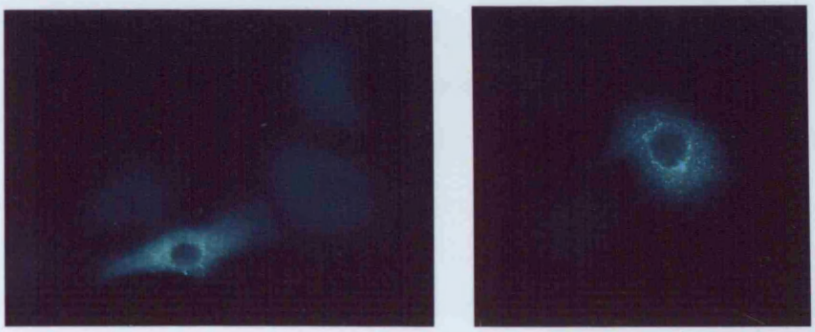


Figure 5.1: Fluoroscopic appearance of *Gli*-transfected keratinocytes in culture

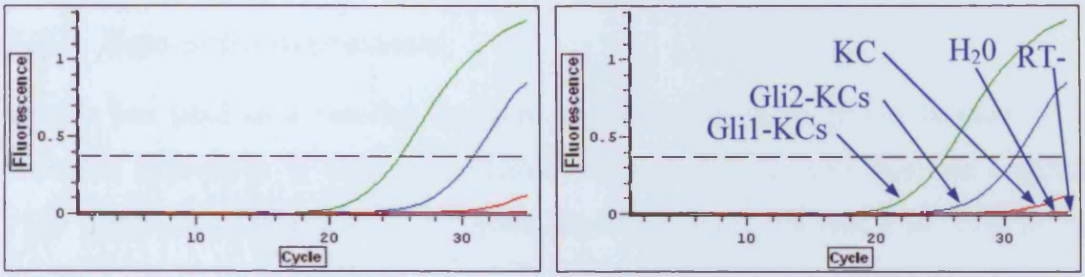


Figure 5.2: Typical graphical representation of a real-time PCR experiment in this case assessing the *Gli1* expression in various cell samples. The right-hand copy of the graph has the cell samples labelled in blue for each curve

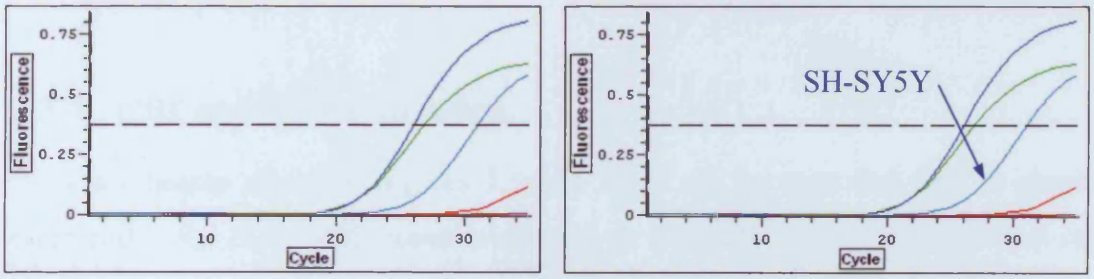


Figure 5.3: Real-time PCR curves demonstrating Arc expression in the same cell cDNA samples as in figure 5.2 (the same colours apply to the same samples). A neuronal-phenotype cell line (SH-SY5Y) has been used as a positive control in this case.

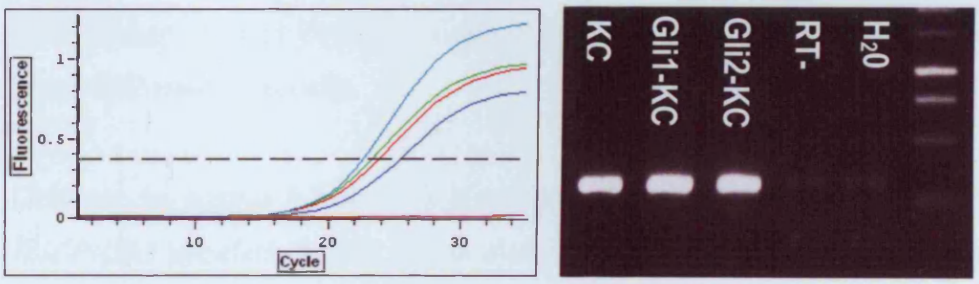
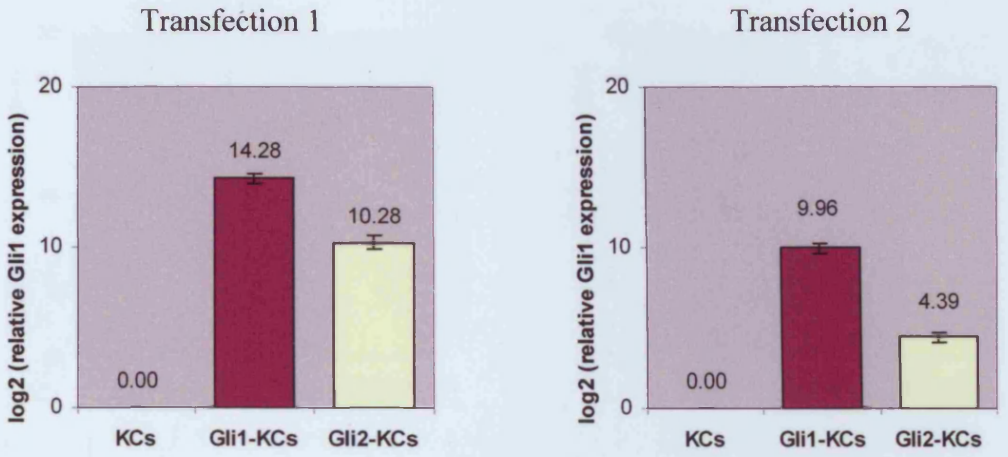


Figure 5.4: Expression of *β-actin* in all cell samples, and not in technical negative controls shown in both real-time PCR graphical format and by agarose gel electrophoresis

Expression of Gli1 in retrovirally transduced cells



	Gli1-KCs vs KCs	Gli2-KCs vs KCs
Δc(t) 1	14.22	10.86
Δc(t) 2	14.83	9.51
Δc(t) 3	13.78	10.48
Δc(t) mean	**14.28**	**10.28**
SE	**0.31**	**0.41**

	Gli1-KCs vs KCs	Gli2-KCs vs KCs
Δc(t) 1	10.28	3.94
Δc(t) 2	10.31	4.93
Δc(t) 3	9.28	4.29
Δc(t) mean	**9.96**	**4.39**
SE	**0.35**	**0.30**

Figure 5.5: Number of cycles-to-threshold for *Gli1* expression in *Gli1*- and *Gli2*-transfected cells relative to normal keratinocytes (Δc(t)). The results of two separate transfection experiments are shown. SE = Standard Error

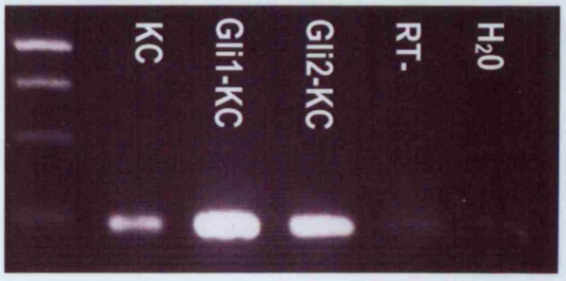
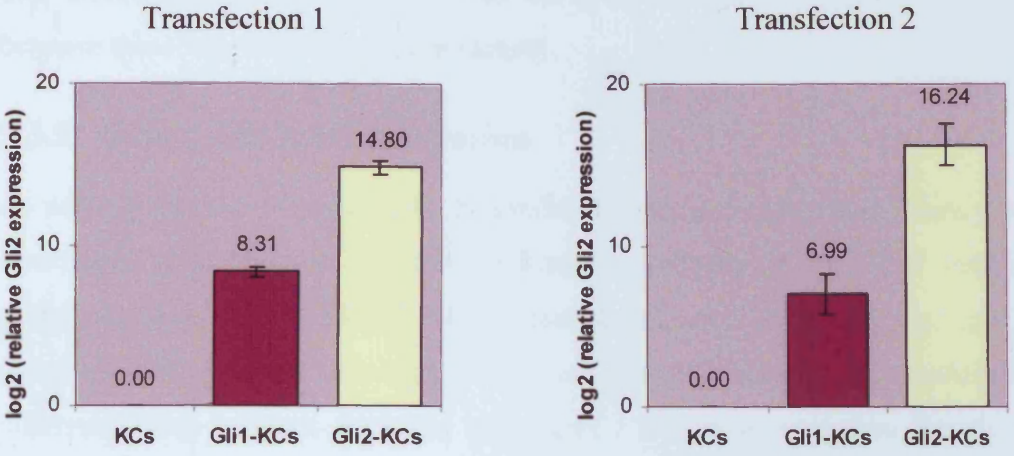


Figure 5.6: PCR products following amplification with *Gli1* primers run on an agarose gel: expression of *Gli1* in transfected cell groups is seen

Expression of Gli2 in retrovirally transduced cells



	Gli1-KCs vs KCs	Gli2-KCs vs KCs
Δc(t) 1	7.70	15.23
Δc(t) 2	8.71	15.22
Δc(t) 3	8.52	13.96
Δc(t) mean	**8.31**	**14.80**
SE	**0.32**	**0.43**

	Gli1-KCs vs KCs	Gli2-KCs vs KCs
Δc(t) 1	9.20	17.24
Δc(t) 2	6.94	17.81
Δc(t) 3	4.85	13.67
Δc(t) mean	**6.99**	**16.24**
SE	**1.28**	**1.32**

Figure 5.7: Number of cycles-to-threshold for *Gli2* expression in *Gli1*- and *Gli2*-transfected cells relative to normal keratinocytes (Δc(t)). The results of two separate transfection experiments are shown. SE = Standard Error

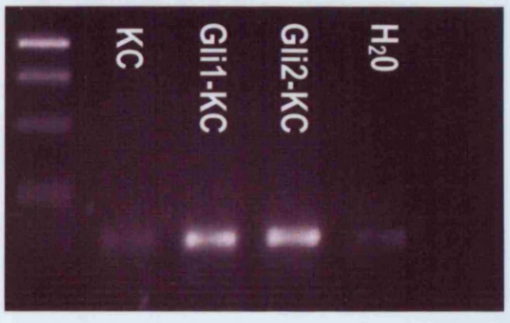


Figure 5.8: PCR products following amplification with *Gli2* primers run on an agarose gel: some expression of *Gli2* in transfected cell groups is seen

In combination with the previous *Gli1* expression data it appears that *Gli1* and *Gli2* both affect the expression of the other i.e. there is a positive feedback mechanism between these two *Gli* transcription factors.

5.3.5 Beta-tubulin III expression

As seen in figures 5.9 and 5.10, *β-tubulin III* was expressed more highly in Gli-transfected cells than in normal keratinocyte controls. In the first transfection experiment the *Gli*-transfected cells reached threshold between two and three (relative) cycles earlier than the control cells, and in the second experiment the differential was between four and six cycles. In neither case was the difference between *Gli2* and *Gli1* samples statistically significant, but in both cases there was a statistically significant difference between these samples and control keratinocytes (section 5.3.3).

5.3.6 Arc expression

Arc was expressed to a much greater degree in both *Gli1*- and *Gli2*-transfected cells than in normal keratinocyte controls, as seen in figures 5.11 and 5.12. There was no significant difference in the number of cycles to threshold between the *Gli1*-cell samples and the *Gli2* cell samples in the first experiment. In the second transfection *Gli1*-transfected cells expressed *Arc* more highly than *Gli2* cells, as reflected by the significantly earlier rise to threshold (on average over fifteen cycles earlier, data not shown).

5.3.7 Neurofilament expression

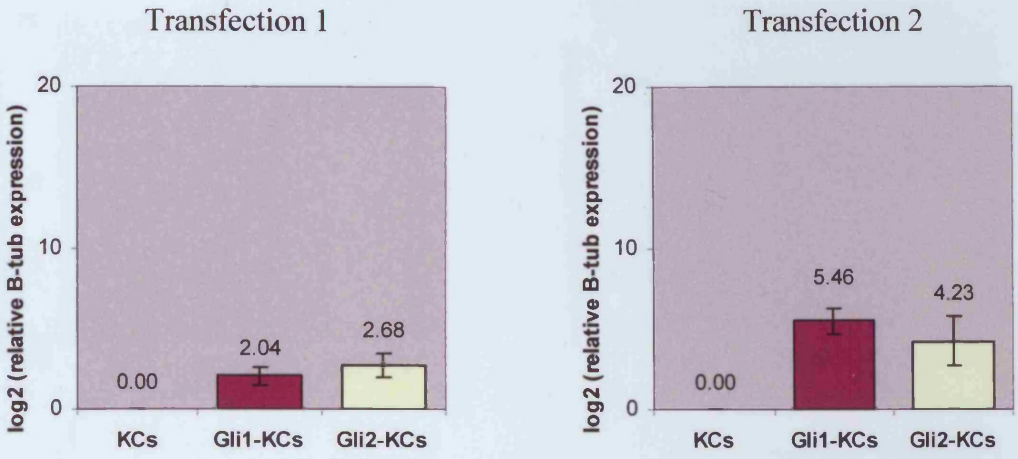
A reduction in neurofilament expression was seen in both *Gli*-transfected cell groups compared to controls, as shown in figures 5.13 and 5.14. Only data from the first transfection experiment is shown – no rise to threshold was seen in any of the cell samples in the second experiment and as such no major conclusion can be drawn, other than the suggestion that the smaller RNA concentrations present in the extracts from the second experiment were not sufficient to see the changes occurring in the first transfection.

Baseline expression was present in keratinocytes, and a decrease in average relative neurofilament expression was seen in both *Gli1*-and *Gli2*-transfected cells.

5.3.8 GAP-43 expression

In neither of the transfection experiments performed was *GAP-43* expressed at detectable levels in keratinocytes or *Gli*-transfected cells. This resulted in no cycle-to-threshold value being recorded for any sample when using real-time PCR, and no gene amplification bands present on standard PCR, as shown in figure 5.15. The only positive value obtained in the real-time PCR experiments was with the use of neuronal phenotype positive control SH-SY5Y cell RNA (data not shown) which demonstrated that the primers were indeed working.

Expression of β-tubulin III in retrovirally transduced cells



	Gli1-KCs vs KCs	Gli2-KCs vs KCs
Δc(t) 1	0.95	2.56
Δc(t) 2	2.90	3.99
Δc(t) 3	2.27	1.50
Δc(t) mean	**2.04**	**2.68**
SE	**0.58**	**0.73**

	Gli1-KCs vs KCs	Gli2-KCs vs KCs
Δc(t) 1	5.05	1.70
Δc(t) 2	4.40	3.91
Δc(t) 3	6.94	7.06
Δc(t) mean	**5.46**	**4.23**
SE	**0.78**	**1.58**

Figure 5.9: Number of cycles-to-threshold for β-tubulin III expression in *Gli1*- and *Gli2*-transfected cells relative to normal keratinocytes (Δc(t)). The results of two separate transfection experiments are shown. SE = Standard Error

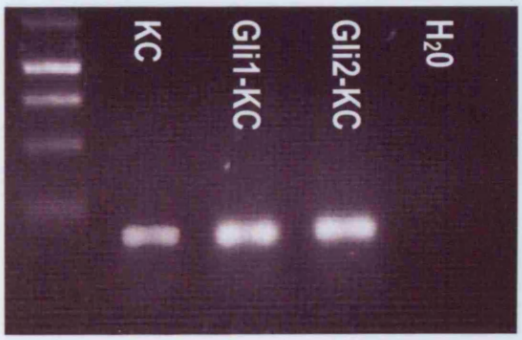
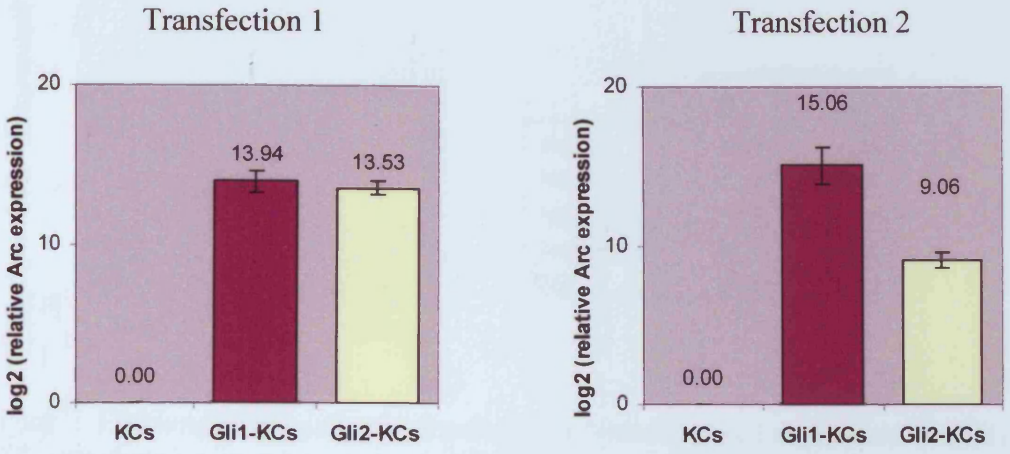


Figure 5.10: PCR products following amplification with β-tubulin III primers run on an agarose gel: expression of β-tubulin III in transfected cell groups is seen

Expression of *Arc* in retrovirally transduced cells



	Gli1-KCs vs KCs	Gli2-KCs vs KCs
Δc(t) 1	12.69	13.63
Δc(t) 2	14.75	14.22
Δc(t) 3	14.37	12.76
Δc(t) mean	**13.94**	**13.53**
SE	**0.65**	**0.43**

	Gli1-KCs vs KCs	Gli2-KCs vs KCs
Δc(t) 1	16.85	9.60
Δc(t) 2	15.44	8.02
Δc(t) 3	12.88	9.58
Δc(t) mean	**15.06**	**9.06**
SE	**1.18**	**0.53**

Figure 5.11: Number of cycles-to-threshold for *Arc* expression in *Gli1*- and *Gli2*-transfected cells relative to normal keratinocytes (Δc(t)). The results of two separate transfection experiments are shown. SE = Standard Error

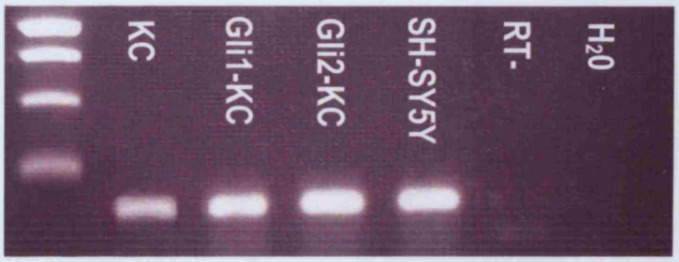
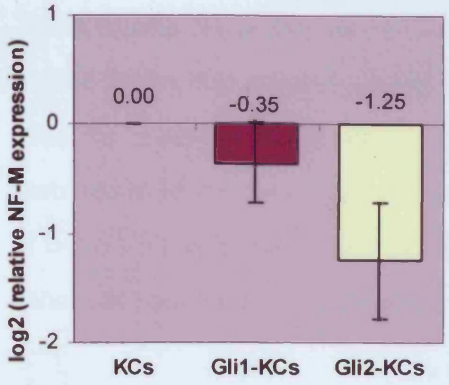


Figure 5.12: PCR products following amplification with *Arc* primers run on an agarose gel: expression of *Arc* in transfected cell groups is seen. SH-SY5Y cell cDNA was used as a positive control.

Expression of Neurofilament in retrovirally transduced cells



	Gli1-KCs vs KCs	Gli2-KCs vs KCs
Δc(t) 1	-0.75	-0.34
Δc(t) 2	-0.68	-1.24
Δc(t) 3	0.38	-2.17
Δc(t) mean	**-0.35**	**-1.25**
SE	**0.37**	**0.54**

Figure 5.13: Number of cycles-to-threshold for Neurofilament expression in *Gli1*- and *Gli2*-transfected cells relative to normal keratinocytes. The results of one transfection experiment are shown.

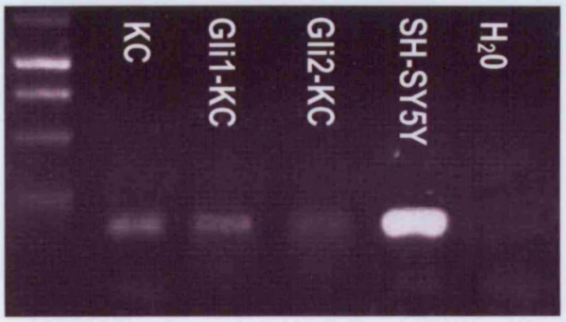


Figure 5.14: PCR products following amplification with Neurofilament primers run on an agarose gel: expression of Neurofilament in transfected cell groups is seen. SH-SY5Y cell cDNA was used as a positive control.

Expression of *GAP-43* in retrovirally transduced cells

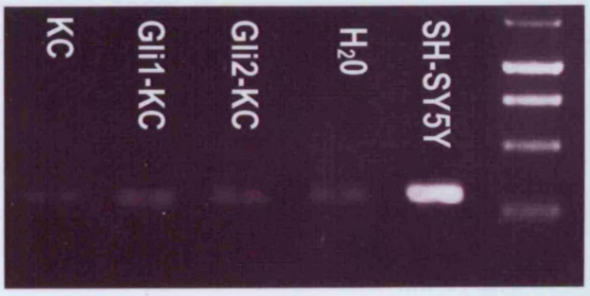


Figure 5.15: PCR products following amplification with *GAP-43* primers run on an agarose gel: expression of *GAP-43* in transfected cell groups is seen. SH-SY5Y cell cDNA was used as a positive control.

5.4 Discussion

These results show that the expression of some of the genes previously examined are indeed under the control of Gli transcription factors. However, others are not, and must be assumed to be under the control of other cell signalling pathways. The association of the target genes with a neuronal phenotype is clearly shown by the use of SH-SY5Y cell cDNA as a positive control, which expressed strongly all four target genes (as seen in the agarose gels depicted above).

β-actin was expressed fairly evenly in all the cell types examined. Since it is a gene that is intimately associated with epithelial cell function, this is to be expected. The similar levels of expression, however, correlate with the creation of these cDNA samples with the same quantity (5μg) of RNA in each case. Given that my calculations of gene expression have taken into account the variation in baseline expression of this housekeeping gene, only minor adjustments had to be made to the raw target gene c(t) values to derive relative target gene c(t) values.

Gli1 and *Gli2* were both most strongly up-regulated in the cells that were transduced to over-express those genes. This is not surprising. However, the mutual interaction between the two Gli transcription factors shows that there must be a degree of downstream inter-dependence between the two genes and their products. *Gli1*-transfected cells express *Gli2* significantly greater than controls and *Gli2* transfected cells express *Gli1* significantly greater than controls. This suggests that these genes can regulate each other's expression, and as such that there is a positive feedback mechanism occurring here.

Regl et al have extensively investigated the interactions between *Gli1* and *Gli2* in human keratinocytes and in human BCCs (Gispen et al., 1991; Regl et al., 2002; Regl et al., 2004). They have concluded that both *Gli1* and *Gli2* are expressed in BCCs and that there is a positive feedback mechanism present whereby each gene drives the expression of the other. Using gene expression time course experiments they demonstrated that *Gli1* is a direct target of *Gli2*, whereas the stimulation of *Gli2* by *Gli1* is likely to be indirect. My data concurs with these results, in as much as an

increase in *Gli1* expression was seen (compared to baseline keratinocyte levels) in the presence of *Gli2* transfection, and vice versa.

β-tubulin III and Arc were strongly expressed in those cells that over-expressed *Gli1* and *Gli2*. Both of these genes appear to be at least partly under the control of *Gli* signalling, in that they were not so strongly expressed in keratinocytes that did not have up-regulated *Gli* expression.

GAP-43 and neurofilament did not appear to be under the control of *Gli* signalling. In the case of expression of neurofilament there were marginal small decreases recorded in cells transfected with *Gli1* and *Gli2*. In the first transfection experiment only the *Gli2*-transduced cells had a significantly later rise to threshold implying a decreased gene expression. However, none of these results were replicated in the following transfection, and as such it seems that I cannot definitely say that neurofilament expression is affected by *Gli*. GAP-43 was not recorded as rising in any of the real-time PCR experiments. Similarly, no strong bands are seen in the standard PCR reaction as depicted on the agarose gel.

Overall in this set of keratinocyte transfection experiments it appears that *Gli1* and *Gli2* drive the expression of each other, and that both of them partly control the expression of Arc and β-tubulin III. They do not, however, affect the expression of neurofilament or GAP-43, which must be under alternative control pathways.

The finding that these four genes are not under similar transcription factor control but are all expressed as part of a neuronal phenotype (as in the SY-SY5Y cells) suggests that there multiple pathways that lead to specific cell differentiation features. In the case of these genes, their association with and supposed specificity to neuronal cells may be partly driven by *Gli*-mediated cell processes, but there may be other mechanisms responsible as well.

Although I have identified Arc and β-tubulin III as downstream effectors of *Gli* in keratinocytes, the expression of GAP-43 and neurofilament in BCCs shows that this is not a complete model system, and that the cell signalling associated with BCC development is complex and must be subject to influences other than mere Gli up-

regulation. Alternatively, Gli expression may be the major driving force in development of a tumour phenotype (much evidence for this has been presented in section 1.2) but other specific downstream factors that are present in human *in vivo* BCCs but not *in vítro* keratinocytes are responsible for transmitting the *Gli* signal and promoting a malignant phenotype. *Gli2* itself has been suggested by Regl et al as having direct oncogenic effects on genes which promote cell cycling (such as *E2F1*, *CCND1*, *CDC2* and *CDC45L*) while repressing genes associated with epidermal differentiation (Regl et al., 2004). As yet neither β-tubulin III nor Arc (which from these studies appear to be under the control of *Gli* signalling) have been associated with the development of a malignant phenotype in any setting. Given that all the evidence regarding their function concerns the development and repair of neuronal cells (Gispen et al., 1991; Hoffman, 1989; Lyford et al., 1995; Skene and Virag, 1989; Fernandes et al., 2004) it is highly likely that these cell signalling pathways are part of the wider network of *Gli* signalling and not direct mechanisms enacting cell transformation.

Chapter 6: Immunohistochemical analysis of neuronal markers in recurrent tumours

6.1 Introduction

The aim of this chapter was to determine the relevance of neuronal differentiation marker expression to biological behaviour. The principal aim of any treatment modality for basal cell carcinoma is tumour eradication, and the only end point that is clinically relevant is prevention of tumour recurrence.

It is known that there is a correlation between histological growth pattern subtype and the aggression that a tumour displays, but this is by no means a set rule. Although it is known that infiltrative and morphoeic tumours have a higher likelihood of incomplete resection, and a higher chance of recurrence following treatment, it is also true that micronodular tumours have higher rates of these problems than "normal". What is "normal" is exactly the problem here – a biological variation does exist and this is not entirely accounted for by histological growth pattern subtypes.

In this section I examined a number of tumour sections that represented a variety of recurrent and non-recurrent tumour types. The expression of markers of neuronal differentiation markers was quantified in these sections and correlated with clinical behaviour.

6.2 Methods

As described in chapter 2, data mining techniques were used to build a database of potentially recurrent tumours from the archives of Mount Vernon and Watford General Hospitals. Cross-checking of the patients' notes and computer records was then performed to exclude those tumours that were not definitely recurrences in the same site of the original tumour and for which both original tumour blocks and recurrent tumour blocks were not available.

From the tumours that remained, two main groups were created - tumours that had been completely excised with a margin of at least one millimetre (as determined by

routine multiple-slice histological assessment) and those that were excised with a margin that was either positive for tumour tissue or very close to tumour (less than 0.2 mm). Examples of the histological appearances of such tumours are shown in figures 6.1 and 6.2. These tumours were then classified according to factors that are known to affect recurrence including age, body site, gender, surgical excision margin and histological growth pattern subtype class ("indolent" = nodular and superficial subtypes, "aggressive" = infiltrative and morphoeic subtypes, micronodular tumours staying as a separate group).

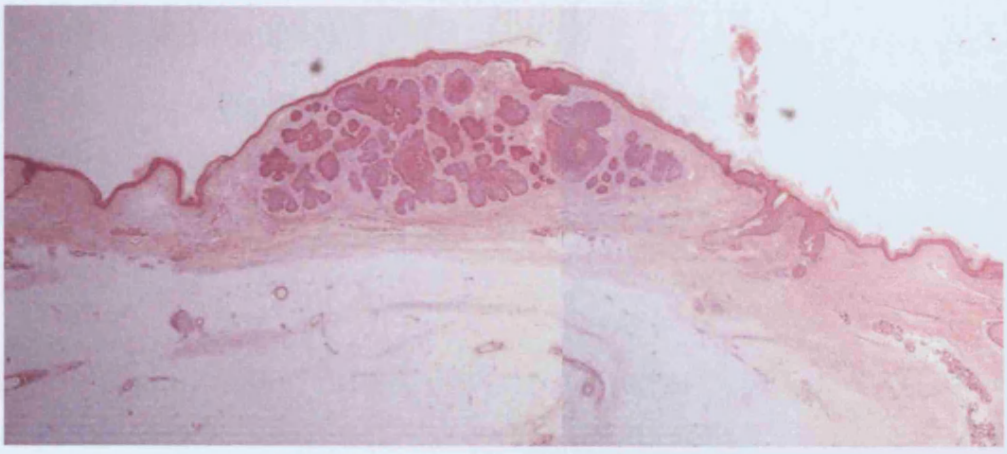
For both of the groups of original tumours that went on to recur (groups CO and IO), a matched set of tumours was then developed where the BCC did *not* go on to recur (groups CN and IN). These groups were matched by the five factors listed above to the groups where tumours did recur. The final six groups of tumours are described in table 6.1.

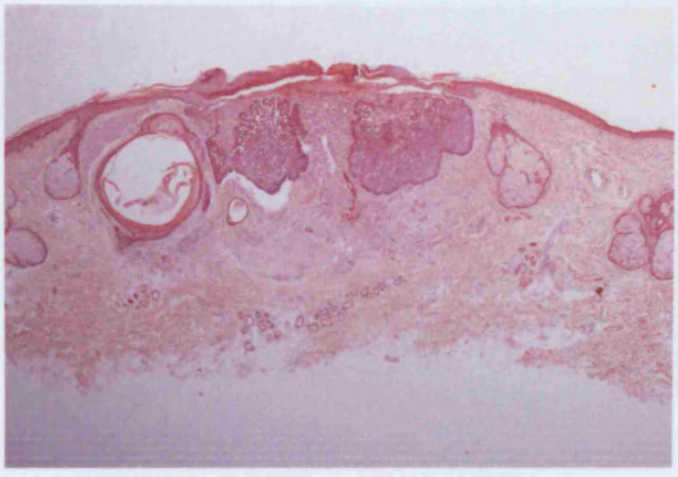
	Original tumour which goes on to recur	Recurrent tumour	Original tumour which does Not go on to recur
Complete excision	"CO"	"CR"	"CN"
Incomplete excision	"IO"	"IR"	"IN"

Table 6.1: groups of recurrent or non-recurrent BCCs studied in this chapter.

Sections of these tumours were stained for β-tubulin III, GAP-43 and Arc, and the intensity of tumour staining was analysed by both visual analogue scoring and spectral image analysis. In all cases graphs were prepared to illustrate the staining results. Bar charts were used to illustrate the frequency of visual analogue scoring staining classifications and the mean values and associated 95% confidence limits were plotted by group. Data derived from Spectral Image Analysis was presented as a boxplot, where the horizontal bar represents the mean value, the upper and lower box limits represent the upper and lower quartile ranges and the whiskers represent the maximum and minimum values (excluding outliers, shown as separate marks). This data was also then shown as mean values and associated 95% confidence limits for each group being compared.

(a)

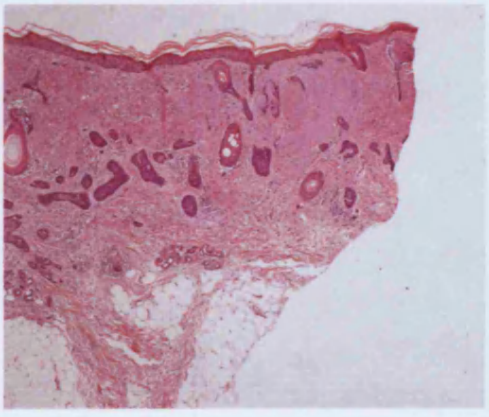




(b)

Figure 6.1: examples of the histological appearance of completely excised BCC tumours

(a)



(b)

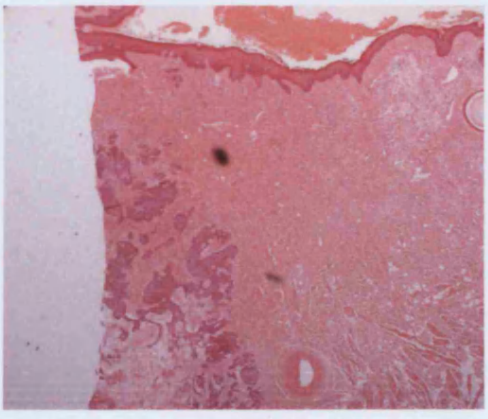


Figure 6.2: examples of the histological appearance of incompletely excised BCC tumours

6.3 Results

6.3.1 Tumours included in this study

8,444 histological reports including the code "Carcinoma, Basal Cell" were obtained from the Mount Vernon pathology archives for a five year period (1996-2001). Word searching in Microsoft Word generated an initial list of 71 tumours that were considered to be possible recurrent tumours.

Further review of patient pathology records and notes resulted in the exclusion of 40 cases. The reasons for this are shown in table 6.2.

Exclusion criterion	Number excluded
New tumour in different site to original	12
No tumour in second specimen	9
Patient notes not available	1
Excision of "recurrence" actually an elective wide local excision	7
Original or recurrent tumour tissue block not available	7
Use of radiotherapy or other treatments at this tumour site	4
Total cases excluded	**40**

Table 6.2: Causes of exclusion for tumours initially considered for this study

6.3.1.1 Original recurrent and non-recurrent cases

The histopathology sections of the original tumours that gave rise to these remaining 31 recurrent cases were reviewed. The closest surgical excision margin (lateral or deep) of the original tumour was measured and classified as shown in table 6.3.

Closest surgical excision margin	Group	Number of cases
Greater than 1mm	CO	13
0.2mm to 1mm	-	5
Less than 0.2mm / positive margin	IO	13

Table 6.3: Closest surgical excision margins in 31 tumours, stratified into three categories

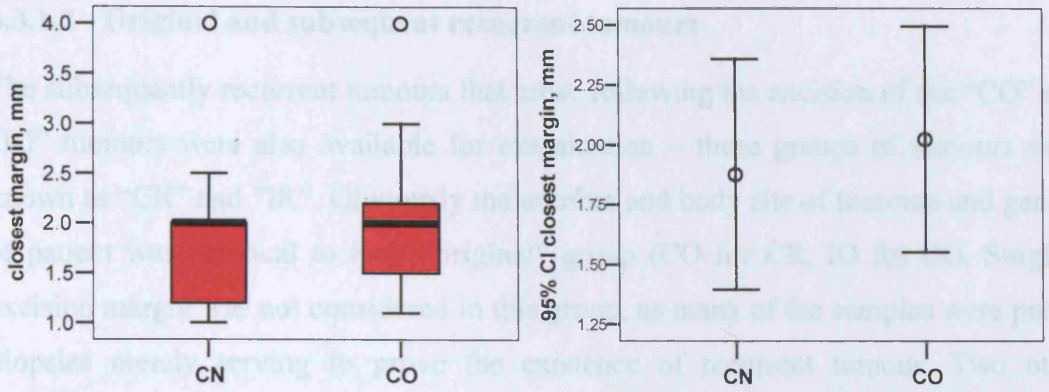
The tumours used were those where the closest surgical excision margin (lateral or deep) of the original tumour was either greater than 1mm or less than 0.2mm. For each of these original tumours a subsequent recurrent tumour was available for examination.

The original tumour cases were classified by factors known to significantly affect the likelihood of tumour recurrence and the tumours comprising the matching groups were selected on the basis of this information (groups CN and IN). These factors and their distributions are listed in table 6.4.

	Completely excised BCCs that went on to recur (group CO)	**Completely excised BCCs that did not recur (group CN)**	**Incompletely excised BCCs that went on to recur (group IO)**	**Incompletely excised BCCs that did not recur (group IN)**
GENDER				
Male	4	4	6	6
Female	9	9	7	6
HISTOLOGY				
Nodular / Superficial	4	4	8	6
Micronodular	1	1	0	0
Infiltrative / Morphoeic	8	8	5	6
SITE				
Head / Neck	10	10	12	12
Upper limb	3	3	0	0
Lower limb	0	0	0	0
Trunk	0	0	1	0
AGE				
Average age (yrs)	70.4	70.1	71.0	68.5
AVERAGE SURGICAL EXCISION MARGIN				
Lateral margin (mm)	2.1	2.3	<0.1	0.1
Deep margin (mm)	2.9	2.3	1.9	1.3
Closest margin (mm)	2.0	1.9	<0.1	<0.1

Table 6.4: Characteristics of four groups of primary BCCs – completely excised tumours which went on to recur (group CO), completely excised BCCs that did not recur (group CN), incompletely excised BCCs that went on to recur (group IO) and incompletely excised BCCs that did not recur (group IN).

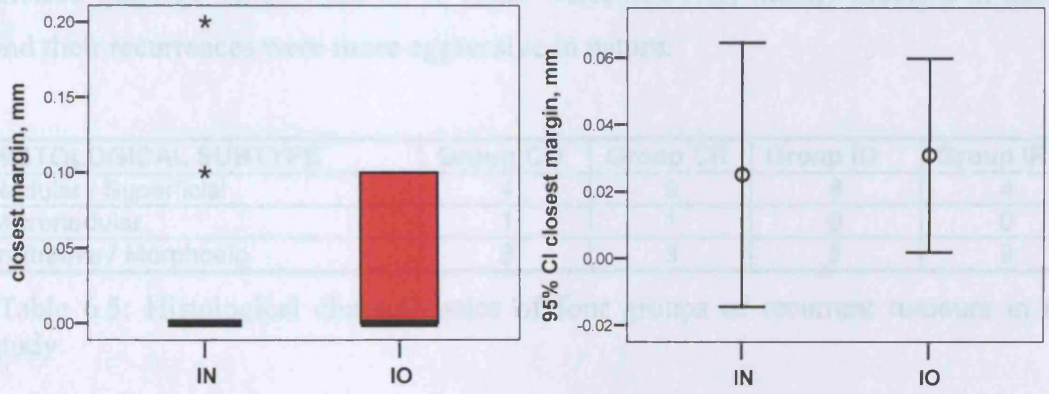
Mean surgical excision margins with associated confidence limits for these groups of tumours are shown in figures 6.3 and 6.4. It can be seen from these results that there was no significant difference between the surgical excision margins of the cases used. When analysing the means and confidence limits of these distributions using an unpaired t-test, p-values of 0.64 (CN vs CO) and 0.80 (IN vs IO) reflected the similarity of these variables in these groups.



Tumour group	Mean margin	SD
CN	1.88	0.80
CO	2.02	0.78

Statistical test	p value
Unpaired t-test	0.64

Figure 6.3: Distribution of closest surgical margin for completely excised tumours that did not recur (CN) and those that did recur (CO).



Tumour group	Mean margin	SD
IN	0.03	0.06
IO	0.03	0.05

Statistical test	P value
Unpaired t-test	0.80

Figure 6.4: Distribution of closest surgical margin for incompletely excised tumours that did not recur (IN) and those that did recur (IO).

6.3.1.2 Original and subsequent recurrent tumours

The subsequently recurrent tumours that arose following the excision of the "CO" and "IO" tumours were also available for examination – these groups of tumours were known as "CR" and "IR". Obviously the number and body site of tumours and gender of patient was identical to their "original" group (CO for CR, IO for IR). Surgical excision margin was not considered in this group, as many of the samples were punch biopsies merely serving to prove the existence of recurrent tumour. Two other important tumour characteristics (histopathological subtype and time interval to recurrence) are described below.

Histopathological subtype

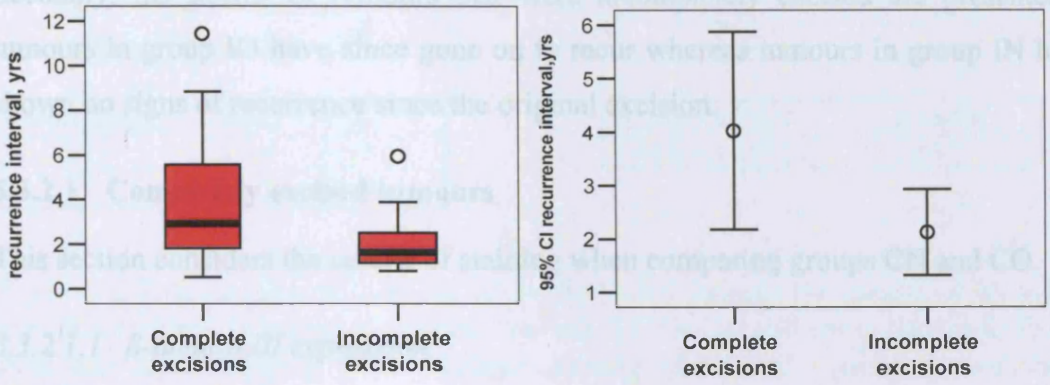
It can be seen from the results in table 6.5 that the majority of the completely excised tumours which went on to recur were of a subytpe that is known to behave aggressively (infiltrative / morphoeic). The recurrences associated with these were more typically classified as indolent tumours (nodular, superficial). The incompletely excised tumours which went on to recur were, however, mainly indolent in nature, and their recurrences were more aggressive in nature.

HISTOLOGICAL SUBTYPE	Group CO	Group CR	Group IO	Group IR
Nodular / Superficial	4	9	8	4
Micronodular	1	1	0	0
Infiltrative / Morphoeic	8	3	5	9

Table 6.5: Histological characteristics of four groups of recurrent tumours in this study

Time interval to recurrence

The results in figure 6.5 demonstrate that tumours that had been completely excised had a greater time interval to recurrence than was the case for incompletely excised BCCs. This difference is only marginally insignificant with a p-value of 0.052.



Tumour group	Mean	SD
CR	4.0	3.33
IR	2.1	1.45

Statistical test	p value
Unpaired t-test	0.052

Figure 6.5: Distribution of time interval between original and subsequent recurrent BCC tumours for each patient included in this study.

6.3.2 Immunohistochemistry: recurrent & non-recurrent tumours

This section of results compares the differences in immunohistochemical staining results between four groups of tumours. Firstly, the results of two groups of tumours that were completely excised are presented – those that have been shown to recur (group CO) compared to those that have been shown to not recur (group CN).

Secondly, the results of tumours that were incompletely excised are presented – tumours in group IO have since gone on to recur whereas tumours in group IN have shown no signs of recurrence since the original excision.

6.3.2.1 Completely excised tumours

This section considers the results of staining when comparing groups CN and CO.

6.3.2.1.1 β-tubulin III expression

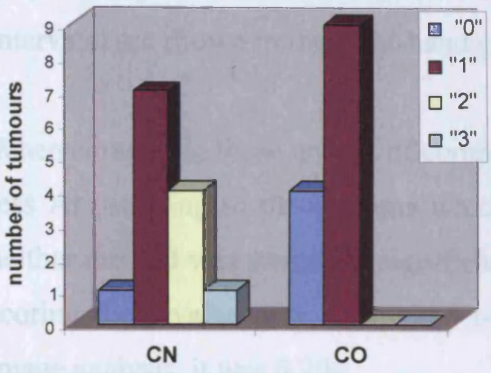
The absolute visual analogue score values of the staining of these groups of tumours were plotted by tumour group, and then the mean and 95% confidence intervals were also plotted by group. These results are shown in figure 6.6.

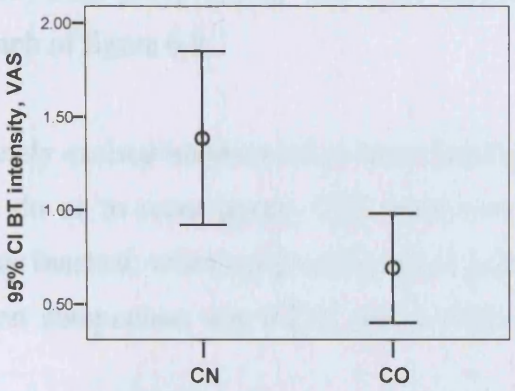
Spectral Image Analysis staining values formed a continuum and these results were plotted as a boxplot as shown along with the mean values for each group (along with 95% confidence intervals) in figure 6.7.

The results of staining in these groups show clearly that with both visual analogue scoring and spectral imaging analysis there is a significantly reduced intensity of staining for β-tubulin III in BCC tumours that go to recur when compared with those that do not. The significance of these differences is great enough such that the p-value of the unpaired t-test comparison is less than 0.001 when assessed with spectral image analysis and in the region of 0.01 with visual analogue scoring.

6.3.2.1.2 Arc expression

The absolute visual analogue score values of the staining of these groups of tumours are shown along with the mean values and 95% confidence intervals, also plotted by group. These results are shown in figure 6.8.

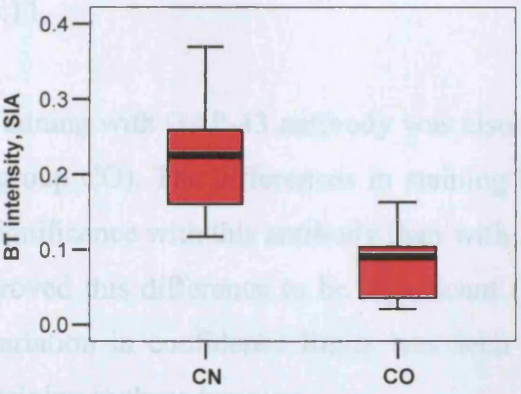


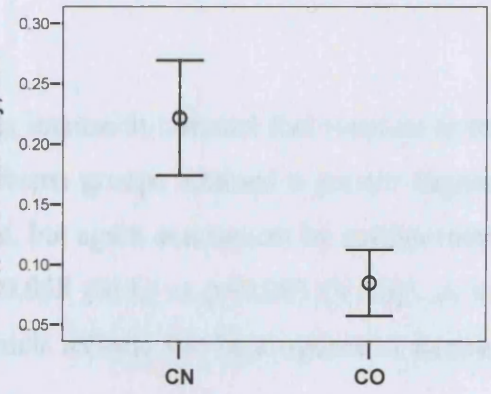


Tumour group	Mean	SD
CN	1.38	0.77
CO	0.69	0.48

Statistical test	p value
Unpaired t-test	0.011

Figure 6.6: Comparison of intensity of β-tubulin III staining (as measured by visual analogue scoring) between completely excised BCCs that did recur (group CO) and those that did not (group CN). Average staining values and significance values of statistical tests are given.





Tumour group	Mean	SD
CN	0.222	0.079
CO	0.085	0.045

Statistical test	p value
Unpaired t-test	<0.001

Figure 6.7: Comparison of intensity of β-tubulin III staining (as measured by spectral image analysis) between completely excised BCCs that did recur (group CO) and those that did not (group CN). Average staining values and significance values of statistical tests are given.

Spectral Image Analysis values were plotted as a boxplot as shown in the left-hand graph in figure 6.9. The mean values for each group (along with 95% confidence intervals) are shown in the right-hand graph of figure 6.9.

When comparing these groups of completely excised tumours it is evident that there is less Arc staining in the tumours which do on to recur (group CO). However, with neither method was statistical significance reached: when assessed by visual analogue scoring the p-value of the unpaired t-test comparison was 0.218, and with spectral image analysis, it was 0.204.

6.3.2.1.3 GAP-43 expression

Visual analogue score values of the staining of these groups of tumours are shown on the graph in figure 6.10, along with the mean values and 95% confidence intervals plotted by group.

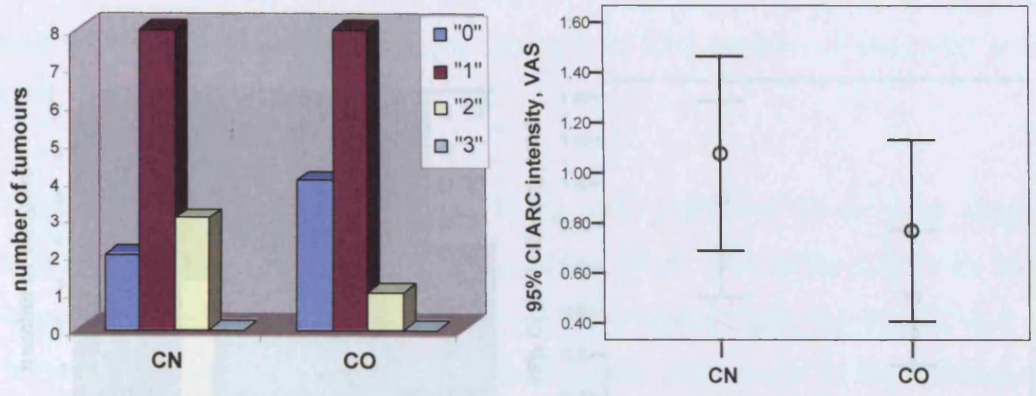
Spectral Image Analysis staining values were plotted as a boxplot as shown along with the mean values for each group (along with 95% confidence intervals) in figure 6.11.

Staining with GAP-43 antibody was also less intense in tumours that went on to recur (group CO). The differences in staining between groups attained a greater degree of significance with this antibody than with Arc, but again assessment by neither method proved this difference to be significant (p=0.058 (SIA) vs p=0.083 (VAS)). A wide variation in confidence limits was seen which reflects the heterogeneous nature of staining in these tumours.

6.3.2.2 Incompletely excised tumours

6.3.2.2.1 β-tubulin III expression

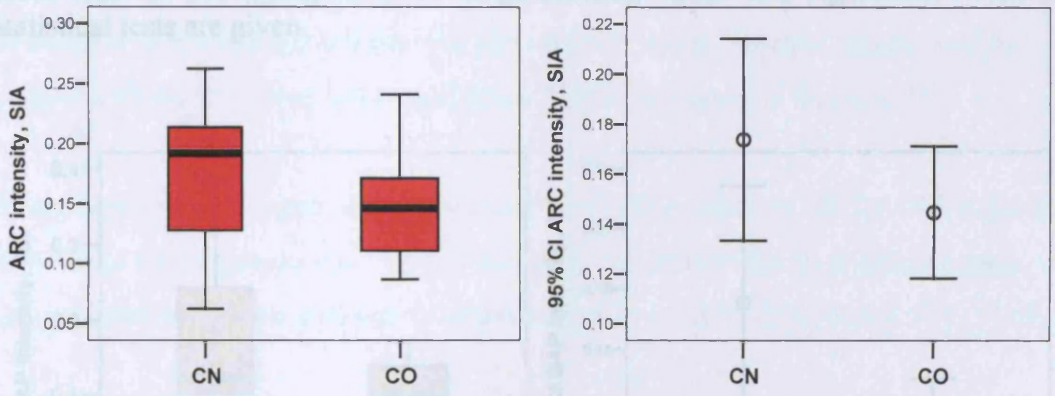
Absolute visual analogue score values of the staining of these tumours were plotted by tumour group, and then the mean and 95% confidence intervals were also plotted by group. These results are shown in figure 6.12.



Tumour group	Mean	SD
CN	1.08	0.64
CO	0.77	0.60

Statistical test	p value
Unpaired t-test	0.218

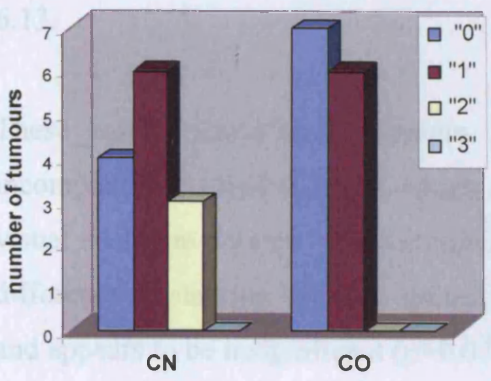
Figure 6.8: Comparison of intensity of Arc staining (as measured by visual analogue scoring) between completely excised BCCs that did recur (group CO) and those that did not (group CN). Average staining values are given.

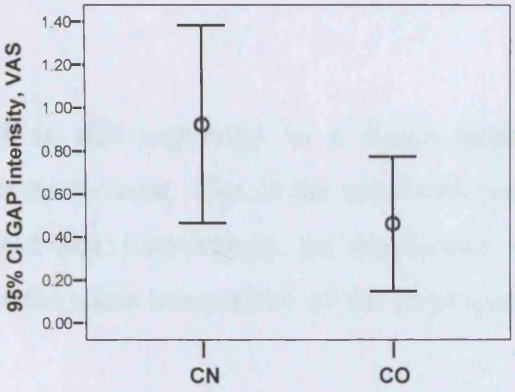


Tumour group	Mean	SD
CN	0.174	0.067
CO	0.145	0.044

Statistical test	p value
Unpaired t-test	0.204

Figure 6.9: Comparison of intensity of Arc staining (as measured by spectral image analysis) between completely excised BCCs that did recur (group CO) and those that did not (group CN). Average staining values and significance values of statistical tests are given.

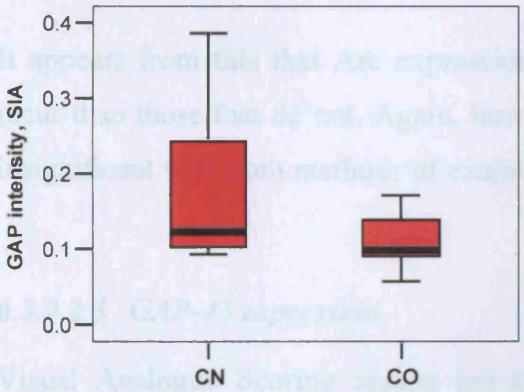


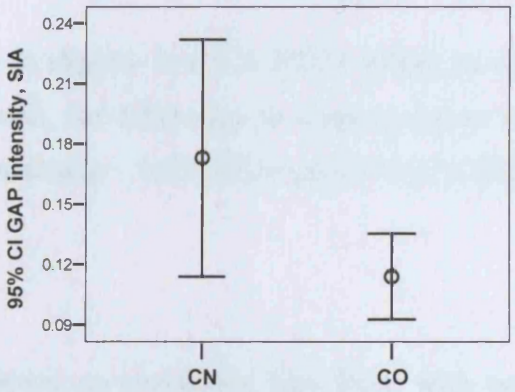


Tumour group	Mean	SD
CN	0.92	0.76
CO	0.46	0.52

Statistical test	p value
Unpaired t-test	0.083

Figure 6.10: Comparison of intensity of GAP-43 staining (as measured by visual analogue scoring) between completely excised BCCs that did recur (group CO) and those that did not (group CN). Average staining values and significance values of statistical tests are given.





Tumour group	Mean	SD
CN	0.173	0.098
CO	0.114	0.035

Statistical test	p value
Unpaired t-test	0.058

Figure 6.11: Comparison of intensity of GAP-43 staining (as measured by spectral image analysis) between completely excised BCCs that did recur (group CO) and those that did not (group CN). Average staining values and significance values of statistical tests are given.

Computer-derived absolute staining values were plotted as a boxplot as shown along with the mean values for each group (along with 95% confidence intervals) in figure 6.13.

These results show that β-tubulin III is still expressed to a lesser degree in incompletely excised tumours which go on to recur. This is the case with both the visual analogue data and the computerised data. Interestingly, the significance of the difference in staining between groups is the same irrespective of the technique used and appears to be insignificant (p=0.07).

6.3.2.2.2 Arc expression

Visual Analogue staining intensity scores were plotted as previously with means and confidence limits on the right-hand graph below. These results are shown in figure 6.14.

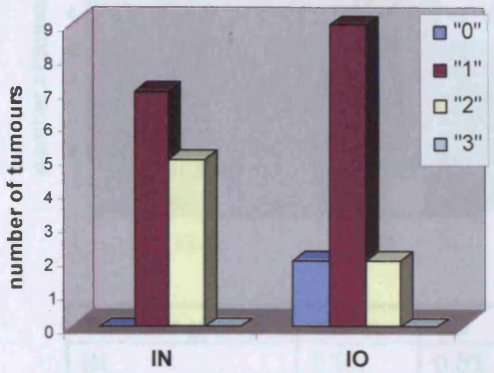
A boxplot of the staining results was generated from the Spectral Image Analysis data and the mean values with confidence limits are shown in figure 6. 15.

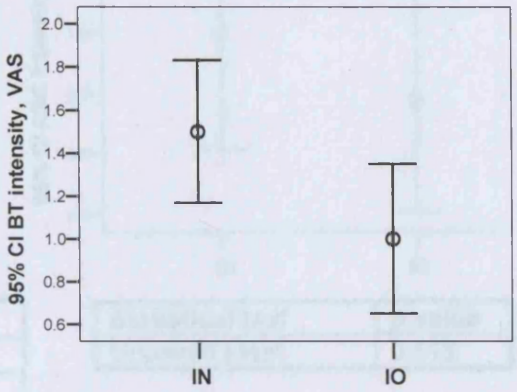
It appears from this that Arc expression is slightly lower in BCCs which go on to recur than those that do not. Again, however, the differences in staining appear to be insignificant with both methods of examination (p = 0.271 (SIA) and 0.518 (VAS)).

6.3.2.2.3 GAP-43 expression

Visual Analogue Scoring results are shown as previously described, with actual values and mean values with confidence limits plotted by group. These results are shown in figure 6.16.

Similarly, actual Spectral Image Analysis values of staining intensity were plotted as a boxplot and these results compared by considering the mean values and associated confidence limits. These results are shown in figure 6.17.

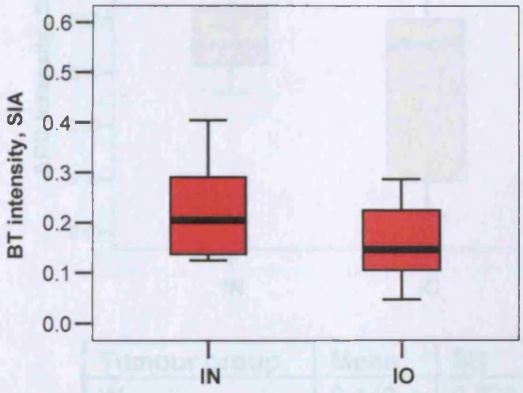


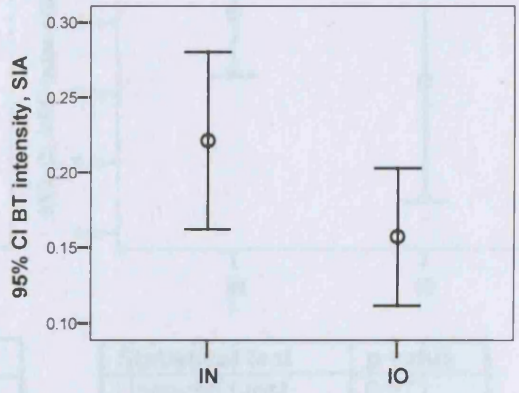


Tumour group	Mean	SD
IN	1.42	0.51
IO	1.00	0.58

Statistical test	p value
Unpaired t-test	0.070

Figure 6.12: Comparison of intensity of β-tubulin III staining (as measured by visual analogue scoring) between incompletely excised BCCs that did recur (group IO) and those that did not (group IN). Average staining values and significance values of statistical tests are given.

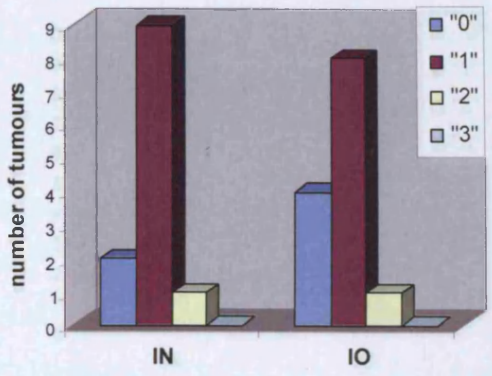


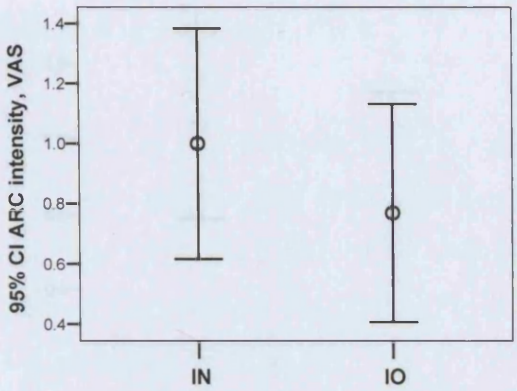


Tumour group	Mean	SD
IN	0.221	0.093
IO	0.157	0.075

Statistical test	p value
Unpaired t-test	0.070

Figure 6.13: Comparison of intensity of β-tubulin III staining (as measured by spectral image analysis) between incompletely excised BCCs that did recur (group IO) and those that did not (group IN). Average staining values and significance values of statistical tests are given.

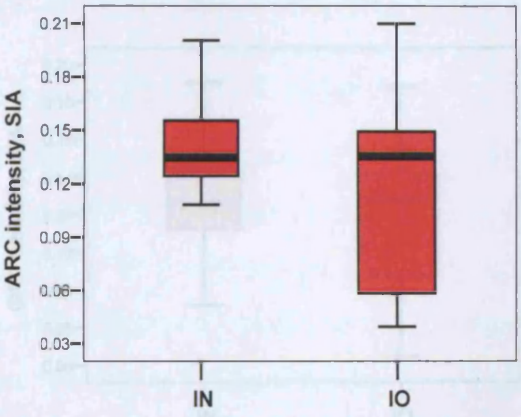


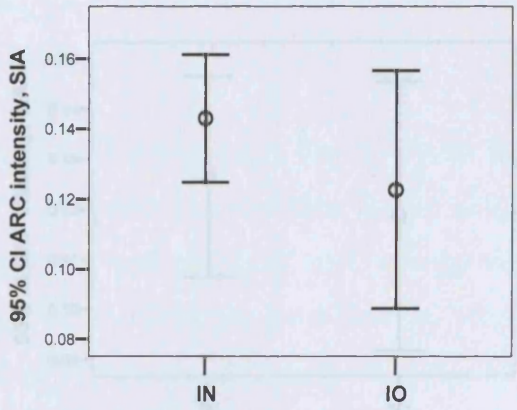


Tumour group	Mean	SD
IN	0.92	0.51
IO	0.77	0.60

Statistical test	p value
Unpaired t-test	0.518

Figure 6.14: Comparison of intensity of Arc staining (as measured by visual analogue scoring) between incompletely excised BCCs that did recur (group IO) and those that did not (group IN). Average staining values and significance values of statistical tests are given.

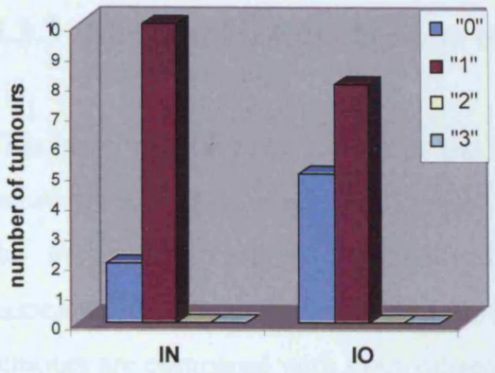


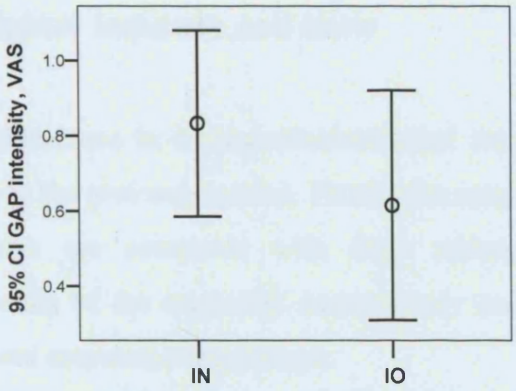


Tumour group	Mean	SD
IN	0.143	0.029
IO	0.123	0.056

Statistical test	p value
Unpaired t-test	0.271

Figure 6.15: Comparison of intensity of Arc staining (as measured by spectral image analysis) between incompletely excised BCCs that did recur (group IO) and those that did not (group IN). Average staining values and significance values of statistical tests are given.

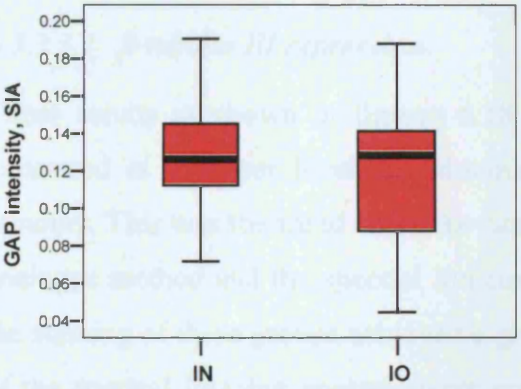


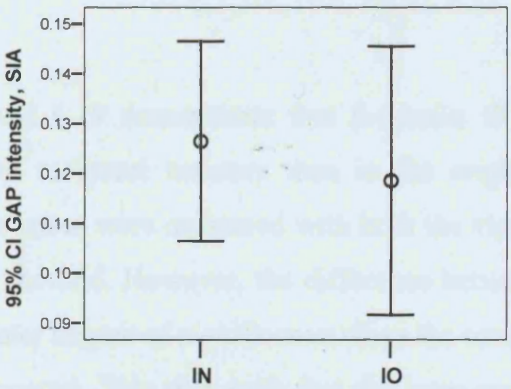


Tumour group	Mean	SD
IN	0.83	0.39
IO	0.62	0.51

Statistical test	p value
Unpaired t-test	0.243

Figure 6.16: Comparison of intensity of GAP-43 staining (as measured by visual analogue scoring) between incompletely excised BCCs that did recur (group IO) and those that did not (group IN). Average staining values and significance values of statistical tests are given.





Tumour group	Mean	SD
IN	0.126	0.032
IO	0.119	0.045

Statistical test	p value
Unpaired t-test	0.617

Figure 6.17: Comparison of intensity of GAP-43 staining (as measured by spectral image analysis) between incompletely excised BCCs that did recur (group IO) and those that did not (group IN). Average staining values and significance values of statistical tests are given.

6.3.3 Immunohistochemistry: original tumours and their recurrences

This section of results compares the differences in immunohistochemical staining between four different groups of tumours to the previous section. Firstly, the results of the original completely excised tumours are compared with their subsequent associated recurrences. Secondly, the results of the originally incompletely excised tumours are compared with their subsequent associated recurrences.

6.3.3.1 Completely excised tumours

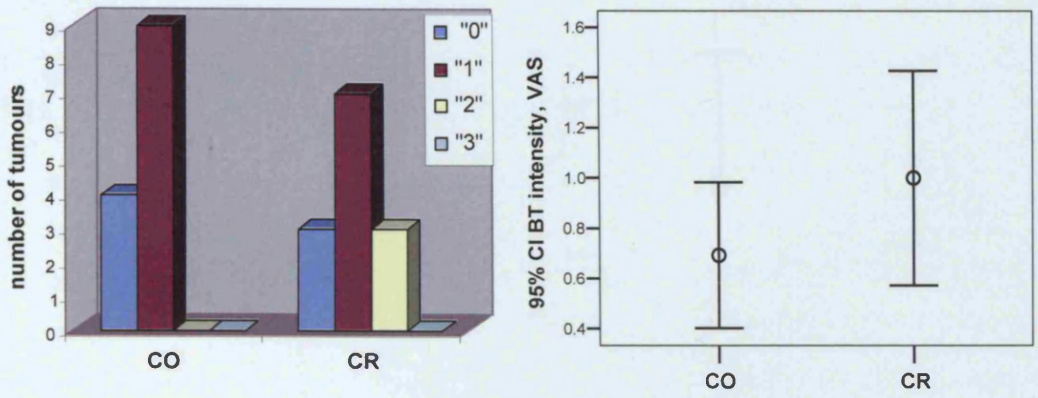
Results of BCC tumours in both group CO and group CR were plotted by group. Absolute values are shown in the left-hand graphs: Visual analogue score data consists of integral measurements, whereas spectral imaging analysis data is continuous. Therefore in these sections the visual data is presented as bar charts, whereas the computerised data is presented using boxplots with mean, interquartile and extreme values as described previously.

6.3.3.1.1 β-tubulin III expression

These results as shown in figures 6.18 and 6.19 demonstrate that β-tubulin III is expressed at a higher level in subsequent recurrent tumours than in the original tumours. This was the trend when the same cases were measured with both the visual analogue method and the spectral imaging method. However, the difference between the staining of these groups achieved a greater degree of significance when the results of the spectral imaging analysis were compared. This was such that the inter-group difference in the visually-recorded data groups was not significant, with a p-value of 0.165 (paired t-test), whereas the difference in the computerised data groups did achieve statistical significance ($p= 0.04$, paired t-test).

6.3.3.1.2 Arc expression

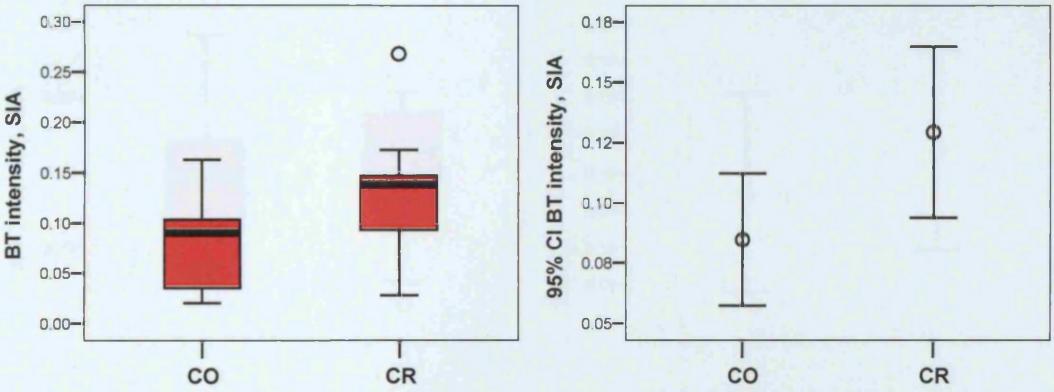
These results (shown in figures 6.20 and 6.21) demonstrate that Arc is also expressed at a slightly higher level in subsequent recurrent tumours than in the original tumours. As with β-tubulin III expression, this was the trend when the same cases were measured with both the visual analogue method and the spectral imaging method. However in this case the difference between the staining of these groups was not



Tumour group	Mean	SD
CO	0.69	0.48
CR	1.00	0.71

Statistical test	p value
Paired t-test	0.165

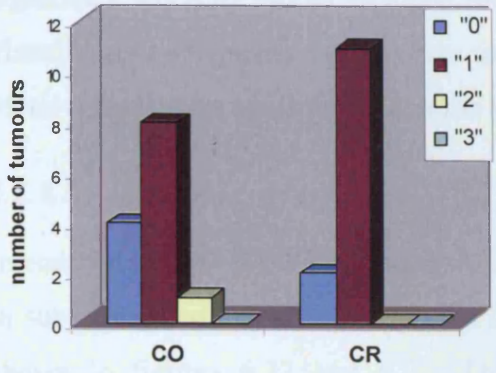
Figure 6.18: Comparison of intensity of β-tubulin III staining (as measured by visual analogue scoring) between original BCCs (completely excised, group CO) and their subsequent recurrences (group CR). Average staining values and significance values of statistical tests are given.

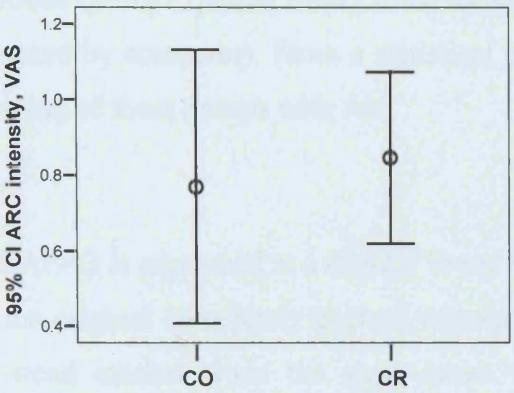


Tumour group	Mean	SD
CO	0.085	0.045
CR	0.129	0.059

Statistical test	p value
Paired t-test	0.039

Figure 6.19: Comparison of intensity of β-tubulin III staining (as measured by spectral image analysis) between original BCCs (completely excised, group CO) and their subsequent recurrences (group CR). Average staining values and significance values of statistical tests are given.

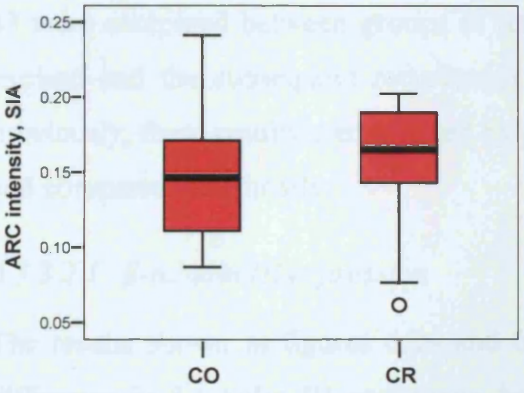


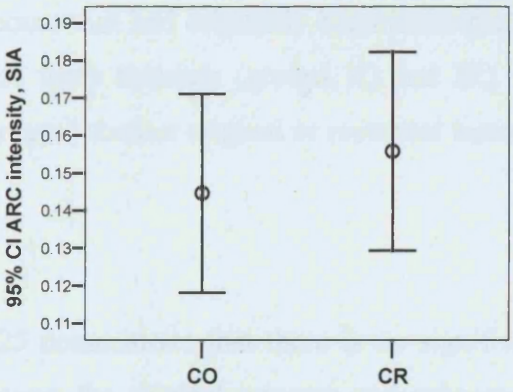


Tumour group	Mean	SD
CO	0.77	0.60
CR	0.85	0.38

Statistical test	p value
Paired t-test	0.673

Figure 6.20: Comparison of intensity of Arc staining (as measured by visual analogue scoring) between original BCCs (completely excised, group CO) and their subsequent recurrences (group CR). Average staining values and significance values of statistical tests are given.





Tumour group	Mean	SD
CO	0.145	0.044
CR	0.156	0.044

Statistical test	p value
Paired t-test	0.478

Figure 6.21: Comparison of intensity of Arc staining (as measured by spectral image analysis) between original BCCs (completely excised, group CO) and their subsequent recurrences (group CR). Average staining values and significance values of statistical tests are given.

significant when measured by either technique (p=0.67 (paired t-test) when measured visually, p=0.48 (paired t-test) when measured by computer). From a statistical point of view, there was no difference in the staining of these groups with Arc.

6.3.3.1.3 GAP-43 expression

In contrast to both β-tubulin III and Arc, GAP-43 is expressed at a slightly lower level in subsequent recurrent tumours than in the original completely excised tumours, as shown in figures 6.22 and 6.23. This trend existed when the same cases were measured with both the visual analogue method and the spectral imaging method. However, the difference between the staining of these groups was not significant when measured by either technique (p=0.17 (paired t-test) when measured visually, p=0.25 (paired t-test) when measured by computer). From a statistical point of view, there was no difference in GAP-43 staining between these groups.

6.3.3.2 Incompletely excised tumours

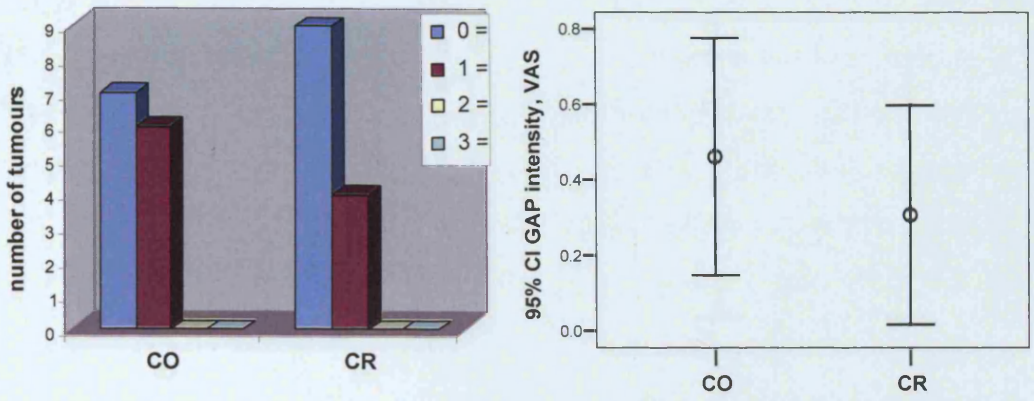
In this set of results the immunohistochemical staining of β-tubulin III, Arc and GAP-43 were compared between groups of tumours that had originally been incompletely excised and the subsequent recurrences of these tumours (groups IO and IR). As previously, these results were plotted by group (whether original or recurrent tumour) and compared statistically.

6.3.3.2.1 β-tubulin III expression

The results shown in figures 6.24 and 6.25 demonstrate that there is no significant difference in β-tubulin III expression between the original tumours and subsequent recurrent tumours. When this staining was assessed by visual analysis there was no difference in the mean staining intensity of these tumours at all. Therefore no statistical analysis has been performed on these results, as it is obvious that there is no difference. When using spectral imaging analysis only a very slight increase in staining was seen in the recurrent tumour group. However, this difference was not significant (p=0.95, paired t-test).

6.3.3.2.2 Arc expression

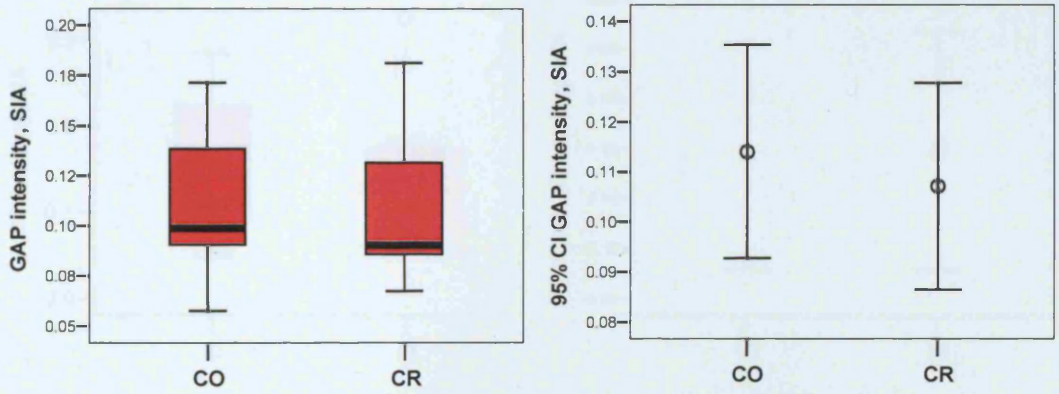
Similar to the previous results, there is no significant difference in Arc expression between the original tumours and subsequent recurrent tumours, as shown in figures



Tumour group	Mean	SD
CO	0.46	0.52
CR	0.31	0.48

Statistical test	p value
Paired t-test	0.165
Wilcoxon test	0.157

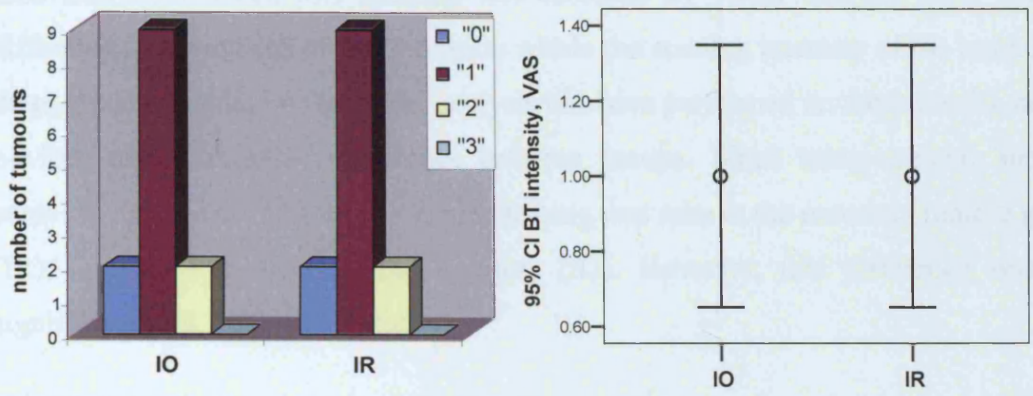
Figure 6.22: Comparison of intensity of GAP-43 staining (as measured by visual analogue scoring) between original BCCs (completely excised, group CO) and their subsequent recurrences (group CR). Average staining values and significance values of statistical tests are given.



Tumour group	Mean	SD
CO	0.114	0.035
CR	0.107	0.034

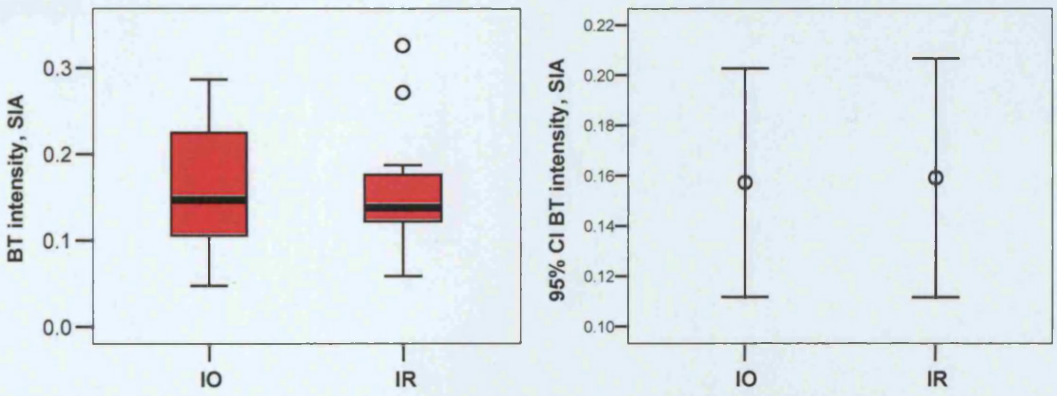
Statistical test	p value
Paired t-test	0.254

Figure 6.23: Comparison of intensity of GAP-43 staining (as measured by spectral image analysis) between original BCCs (completely excised, group CO) and their subsequent recurrences (group CR). Average staining values and significance values of statistical tests are given.



Tumour group	Mean	SD
IO	1.00	0.58
IR	1.00	0.58

Figure 6.24: Comparison of intensity of β-tubulin III staining (as measured by visual analogue scoring) between original BCCs (incompletely excised, group IO) and their subsequent recurrences (group IR). Average staining values are given.



Tumour group	Mean	SD
IO	0.157	0.075
IR	0.159	0.072

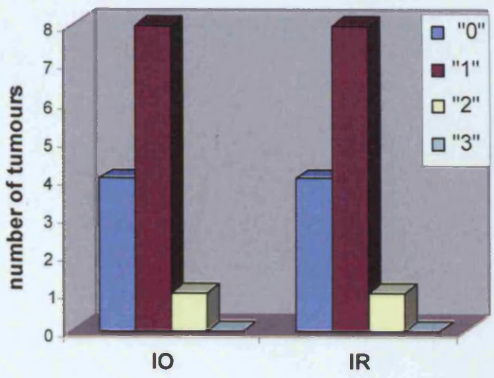
Statistical test	p value
Paired t-test	0.953

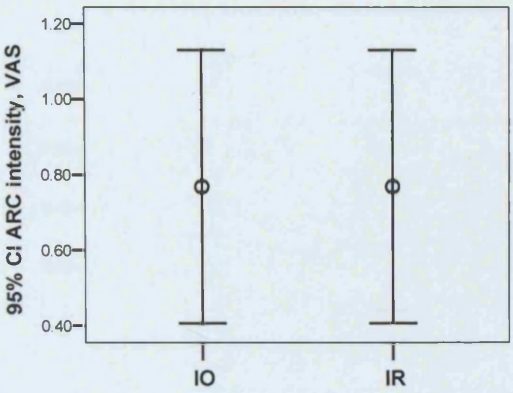
Figure 6.25: Comparison of intensity of β-tubulin III staining (as measured by spectral image analysis) between original BCCs (incompletely excised, group IO) and their subsequent recurrences (group IR). Average staining values and significance values of statistical tests are given.

6.26 and 6.27. When this staining was assessed by visual analysis there was no difference in the means of and variation within the staining intensity of the tumours in these groups. Again, no statistical analysis has been performed on these results, as it is obvious that there is no difference between groups. When using spectral imaging analysis, only a very slight increase in staining was seen in the recurrent tumour group (IR), compared to the original tumours (IO). However, this difference was not significant (p=0.72, paired t-test).

6.3.3.2.3 GAP-43 expression

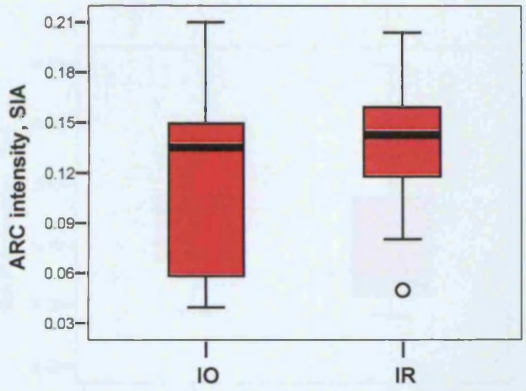
GAP-43 expression was seen to be decreased in the tumours of the subsequent recurrence group, as seen in figures 6.28 and 6.29. This was the case when the results were assessed by both visual analogue scoring and spectral image analysis. However, in neither case was a statistically significant difference found (p=0.44 (paired t-test) when measured visually, p=0.33 (paired t-test) when measured by computer). From a statistical point of view, there was no difference in GAP-43 staining between these groups.

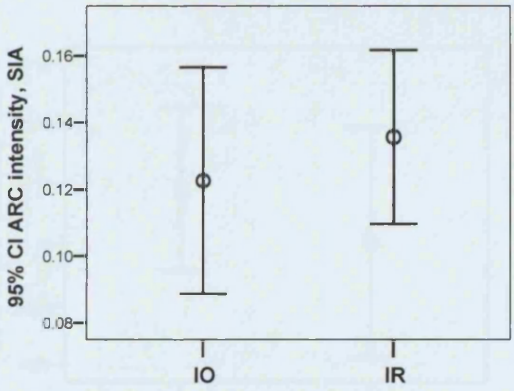




Tumour group	Mean	SD
IO	0.83	0.58
IR	0.83	0.58

Figure 6.26: Comparison of intensity of Arc staining (as measured by visual analogue scoring) between original BCCs (incompletely excised, group IO) and their subsequent recurrences (group IR). Average staining values are given.

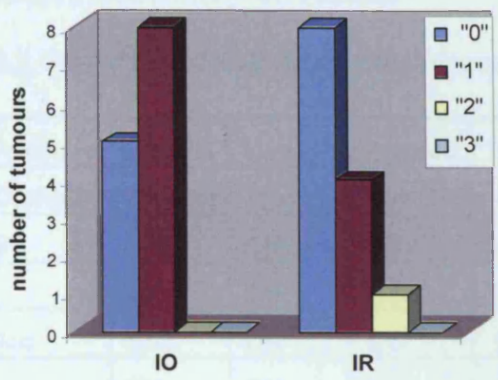


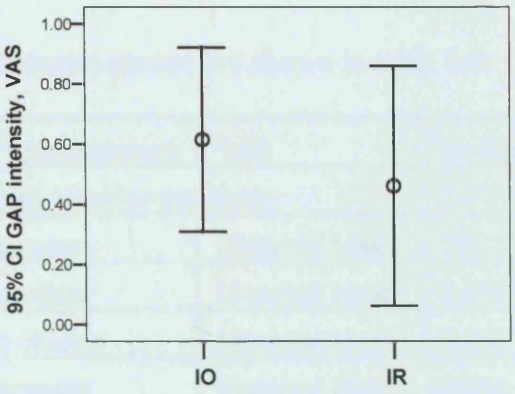


Tumour group	Mean	SD
IO	0.130	0.053
IR	0.136	0.041

Statistical test	p value
Paired t-test	0.721

Figure 6.27: Comparison of intensity of Arc staining (as measured by spectral image analysis) between original BCCs (incompletely excised, group IO) and their subsequent recurrences (group IR). Average staining values and significance values of statistical tests are given.

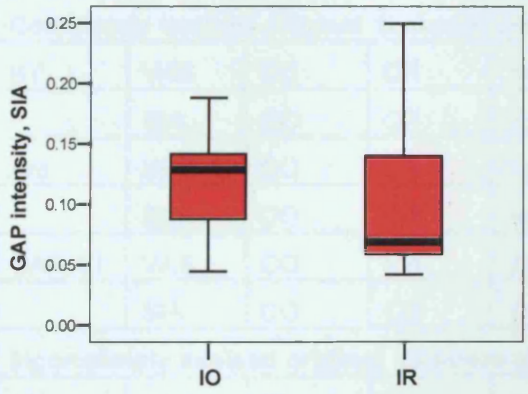


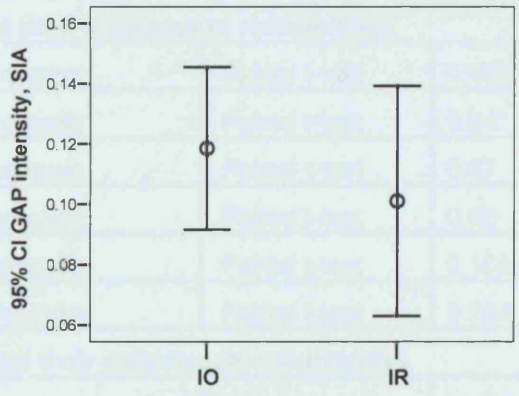


Tumour group	Mean	SD
IO	0.62	0.51
IR	0.46	0.66

Statistical test	p value
Paired t-test	0.436

Figure 6.28: Comparison of intensity of GAP-43 staining (as measured by visual analogue scoring) between original BCCs (incompletely excised, group IO) and their subsequent recurrences (group IR). Average staining values and significance values of statistical tests are given.





Tumour group	Mean	SD
IO	0.119	0.045
IR	0.098	0.059

Statistical test	p value
Paired t-test	0.326

Figure 6.29: Comparison of intensity of GAP-43 staining (as measured by spectral image analysis) between original BCCs (incompletely excised, group IO) and their subsequent recurrences (group IR). Average staining values and significance values of statistical tests are given.

6.3.4 Summary of results

All the results of statistical comparisons between groups are shown in table 6.6.

Antibody	Method	Groups compared		2nd group change	Test	p-value
Completely excised tumours which go on to recur or not recur						
BT	VAS	CN	CO	Decrease	Unpaired t-test	0.011*
	SIA	CN	CO	Decrease	Unpaired t-test	0.001***
Arc	VAS	CN	CO	No change	Unpaired t-test	0.218
	SIA	CN	CO	Decrease	Unpaired t-test	0.204
GAP-43	VAS	CN	CO	Decrease	Unpaired t-test	0.083
	SIA	CN	CO	Decrease	Unpaired t-test	0.058
Incompletely excised tumours which go on to recur or not recur						
BT	VAS	IN	IO	Decrease	Unpaired t-test	0.070
	SIA	IN	IO	Decrease	Unpaired t-test	0.070
Arc	VAS	IN	IO	Decrease	Unpaired t-test	0.518
	SIA	IN	IO	Decrease	Unpaired t-test	0.271
GAP-43	VAS	IN	IO	Decrease	Unpaired t-test	0.243
	SIA	IN	IO	Decrease	Unpaired t-test	0.617
Completely excised original tumours and their subsequent recurrences						
BT	VAS	CO	CR	Increase	Paired t-test	0.165
	SIA	CO	CR	Increase	Paired t-test	0.04*
Arc	VAS	CO	CR	Increase	Paired t-test	0.67
	SIA	CO	CR	Increase	Paired t-test	0.48
GAP-43	VAS	CO	CR	Decrease	Paired t-test	0.165
	SIA	CO	CR	Decrease	Paired t-test	0.254
Incompletely excised original tumours and their subsequent recurrences						
BT	VAS	IO	IR	No change	Paired t-test	(1.0)
	SIA	IO	IR	Increase	Paired t-test	0.95
Arc	VAS	IO	IR	No change	Paired t-test	(1.0)
	SIA	IO	IR	Increase	Paired t-test	0.72
GAP-43	VAS	IO	IR	Decrease	Paired t-test	0.43
	SIA	IO	IR	Decrease	Paired t-test	0.32

Table 6.6: Summary of differences in immunohistochemical staining by group with associated statistical significances of differences in staining. BT = β-tubulin III, VAS = visual analogue scoring, SIA = Spectral Image Analysis (* = p-values < 0.05, ** = p-values <0.01, *** = p-values <0.001).

6.4 Discussion

6.4.1.1 Factors affecting recurrence of Basal Cell Carcinoma

This study of tumours that had arisen in the sites of previously surgically excised BCCs was undertaken to assess whether the expression of neuronal differentiation markers had any predictive value for tumour recurrence.

It is known already that there are a number of clinical and histological features that correlate with the likelihood of tumour recurrence. These have been extensively examined in a number of prior publications and include age and gender of patient, site of tumour, histopathological differences between tumours and degree of completeness of tumour excision, as cited previously in this thesis.

The question of incomplete excision and recurrence has come under the particular spotlight recently. Berlin et al (2002) retrospectively reviewed 64 patients who had had incomplete BCC excision and who had been followed up for a minimum of three years. They found that size of tumour (less than 1cm in diameter), histological subtype (of nodular or superficial subtype), and location (located anywhere except the nose and ears) were all significantly related to a reduced rate of BCC tumour persistence (Berlin *et al.*, 2002). De Silva and colleagues, however, prospectively followed a cohort of patients who had BCCs incompletely excised and concluded that neither age, sex nor tumour location affected whether an incompletely excised tumour recurred or not (De Silva and Dellon, 1985).

The concept of recurrence itself in BCC is a topic that engenders much debate. Obviously if there is evidence that a tumour has been incompletely resected and that tumour may remain in the tumour bed, then re-occurrence of a BCC tumour should not be wholly surprising. In fact, as previously discussed, the recurrence rate of tumours where tissue is thought to have been left behind is not as high as might be expected. Good examples of this include the common dermatological practice of curetting BCCs and cauterising the tumour bed. In this case it is highly likely that tumour is left behind, and immediate biopsies of such BCC treatment sites have been shown to harbour remaining tumour in 33-45% of cases. However, the practice is still an accepted option because the rate of tumour recurrence is still much lower than this

(Gooding et al., 1965; Pascal et al., 1968; Rowe et al., 1989). Similarly, when discussing the management of incompletely surgically excised BCCs, I have already mentioned that the recurrence rate for these tumours is in the order of 33-39%. This of course means that the remainder of the tumours have potentially got tumour remaining in the tumour bed following excision, but this group of incompletely excised tumours does not seem to recur. This may of course be due to the shortcomings of histological analysis as previously discussed, where this may be a false positive margin result. In the case of a true positive margin, however, two major factors that cannot be accounted for include how much tumour is actually left behind following excision or curettage of the tumour, and what component a host response plays in reacting to the remaining tumour.

Rather than bracket all cases as truly "recurrent" cases, it would be reasonable to instead consider cases where remaining BCC is thought to contribute to development of a subsequent tumour as "persistence" of tumour rather than a recurrence per se. However, in the practical setting using this as a separate classification may be impractical as its use would assume that tumours described in this way fit rather more rigorous criteria (in terms of presence of residual tumour) than can often be applied.

Certainly in the case of any work that has been done on investigating the molecular biology of BCCs, no studies have been published that have used anything other than archival material based on retrospective data. This has limited these studies to immunohistochemical experiments, assessing protein expression in archival material. To take this further (to assess gene expression) in such tumours would require a vast amount of extra work. The prospect of gathering fresh samples from every BCC harvested in a department with the intention of building a frozen library of biopsies, of which only a small number will recur, is beyond even the most committed skin cancer research units.

One of the few recent papers to try and address this lack of molecular biological knowledge in specifically recurrent BCCs (as opposed to different subtypes of BCC) was published by Healy et al (Healy et al., 1995). That study selected their tumour groups in a similar manner to how I have done – seventeen tumours which had

recurred following surgical excision were examined immunohistochemically, but in this case only completely excised tumours were considered and no mention is made of their definition of "complete" excision. These were compared with seventeen similar tumours that did not recur, and these groups were matched only for age, sex and year of excision. They examined the expression of a marker of proliferation, Ki67, and also the expression of p53. They concluded that Ki67 expression was expressed at higher levels in tumours that went on to recur, and this difference was statistically significant. No statistically significant difference was noted with p53 expression.

It has been discussed that improved complete excision of BCCs reduces the risk of tumour recurrence. Improved estimation of completeness of excision is possible compared to routine histological techniques where a single slice is taken transversely through the tumour section. One can progress to taking multiple slices in multiple planes, known as "breadslicing", and this gives improved information to treating doctors and to patients following a definitive excision attempt, but even this method still only examines a small proportion of the tumour edge. The ultimate way to know whether you have completely removed all of the tumour in that area is to use Mohs' surgery. Close examination of all tumour margins at the time of excision in a plane that is parallel to the edge of the tumour means that repeated excisions are performed until all regions are clear of tumour. Hence, recurrence following this type of procedure is the lowest of all treatment modalities for BCC (Rowe et al., 1989).

However Mohs' surgery is expensive and time-consuming. Studies have been performed to compare costs and claim that there is little cost difference, but these studies have compared specific groups of tumours (BCCs of the face or ear) with specific additional diagnostic tests (including on site frozen section control) (Bialy et al., 2004; Cook and Zitelli, 1998). Mohs surgery also needs to be performed in units that regularly use it as method, limiting its availability. In this country it is traditionally reserved for tumours that are known to be high-grade or of an aggressive growth pattern, in areas where sensitive resection and reconstruction must be considered. Classic areas include the periorbital and perinasal areas, where the benefits of Mohs surgery in maximising tumour clearance while sacrificing as little normal tissue as is possible comes into its own. However, because of the practical and cost limitations in its use, the vast majority of skin cancer surgical excisions are

performed using routine surgical methods. It stands to reason that for the majority of BCCs in non-critical sites which do not need immediate histological assessment routine surgery will be cheaper and less labour intensive than Mohs' surgery. In all these cases there must always be an element of suspicion that so-called "recurrent" tumours are indeed "persistent" tumours because of relative lack of assurance that the original excision *was* definitely complete.

There is, however, some value, in considering these tumours that are routinely surgically excised, as I have. Standard surgical excision is the commonest method of surgical treatment and so this study which used archival material where excision margins had been recorded following examination of multiple slices, attempts to be representative of the majority of BCCs that are excised in this country. Questions such as those regarding the likelihood of recurrence following incomplete excision are the most clinically relevant questions that this research could hope to answer. Such incomplete margins do not exist with Mohs' surgery, and so I aimed to use my previous experimental findings to shed light on everyday problems using material that is relevant to everyday practice.

In chapter three I showed that the staining intensity of β-tubulin III, Arc and GAP-43 were related to the histological growth pattern subtypes of the tumours. Analysis of variance showed that, in terms of antibody staining, different populations of tumours were being represented. Specific comparisons of subtypes of BCCs, when grouped by predicted behaviour class (Indolent, Aggressive), showed that there were statistically significant differences in staining between these types of BCC. This however relies on the assumption that tumours with a certain histopathological appearance will fit neatly into the classification of predicted tumour behaviour. There is a good deal of evidence that the so-called more aggressive subtypes of BCC *do* behave more aggressively, but this rule is not absolute (Freeman and Duncan, 1973; Menn et al., 1971; Pascal et al., 1968; Shanoff et al., 1967). This part of my study therefore aimed to go beyond predicted clinical behaviour and attempted to determine whether expression of these markers truly does have any value in predicting if a tumour has inherent biological features which are linked to recurrence.

6.4.1.2 Groups compared and data measurement

Groups were selected to try and minimise the bias of multiple additional factors that affect recurrence. By matching the "going to recur" and "not going to recur" groups as closely as possible to each other in terms of the gender, body site, excision margin and class of histological growth pattern subtype, I attempted to highlight other potential biological differences in these groups that would affect their re-growth. Two groups of tumours that had been completely excised were compared as to whether their expression of β-tubulin III, Arc and GAP-43 were linked to whether they had recurred or not (CN vs CO). Similarly, tumours that had been incompletely excised were assessed in the same way (IN vs IO).

The original tumours were also compared with their subsequent recurrences (groups CO vs CR and IO vs IR). This was done to assess whether there was any correlation with a change in the expression of these markers and progression of the tumour. At no point were comparisons made between tumours that had been excised to different degrees (a strong factor in recurrence) – for this reason at no stage was a "C" tumour compared with an "I" tumour. Similarly, tumours in groups that differed in their demographic make-up (sex, site, age) were not compared (such as an "N" tumour compared to an "R" tumour).

The methods of Visual Analogue Scoring and Spectral Imaging Analysis were compared in chapter three. It was obvious from those results that the methods are comparable in that a near-linear distribution was shown to exist between the two techniques' results from the same tumours. This direct correlation of data has not been repeated in this chapter. I do however feel that spectral imaging analysis with its ability to specifically delineate staining intensities has benefits when considering the more subtle differences between groups such as these. When examining the comparisons between groups, in no case was there a reduction in staining when measured by visual analogue scoring and an increase as measured by the computer method (or vice versa).

The cases in which no difference was shown by visual scoring but where a change was present when assessed by spectral image analysis (comparisons IO vs IR for Arc

and for β-tubulin III staining) had very close results when assessed by computer, as reflected by the respective p-values for these comparisons (0.72 and 0.95). Given the near-infinite number of values that the spectral imaging system can return, it is unlikely that absolutely identical values would be returned for two groups of tumours with such heterogeneity of staining. A high value of the p-number is as close as I can get to reassuring ourselves that this is the case.

6.4.1.3 Number of cases involved in this study

Of 8444 histological reports classified as consisting of Basal Cell Carcinoma from two centres, a total of thirteen were selected as being recurrent tumours which developed following complete excision of the original BCC tumours. Similarly thirteen BCCs were discovered that had been originally incompletely removed and which subsequently recurred.

If considered to represent all the recurrences in this large number of tumours, these figures represent a very low rate of recurrence (a total of 26/8444 = 0.3%). However this cannot be considered to be the case. Exclusion criteria ensured that only tumours with defined excision margins and for which paraffin-embedded tumour blocks were available were included. Tumours for which radiotherapy had been used as a treatment were also excluded. Additionally, there was no provision in this population for tumours that may have recurred following excision but which were either ignored by the patients, treated elsewhere, or in whom the patients died before re-excision. This is essential information in any true study of BCC recurrence incidence; that was not the purpose of this part of the study and as such these groups of tumours cannot be considered to be representative of all the recurrent tumours.

From a follow up point of view the important groups in this study were those tumours that did not recur following BCC excision (groups CN and IN). In this case it was essential to ensure that the patients had lived long enough following the original surgery, that they had not suffered a recurrence and that adequate follow up data was available to determine this. Part of this involved checking also that no further skin cancer treatment had been sought for that area of the patient's body, so that other inventions would not act as a confounding factor. From this large number of reports initially available, groups (IN and CN) with similar numbers of tumours to the

comparison groups (IO and CO) were generated. The high number of patients excluded due to lack of adequate follow up, recurrence or short survival follow up testifies to the difficulties with performing comparative research using archival material such as this.

6.4.1.4 Immunohistochemistry of tumours that recur and those that do not recur

Antibody	Comparison	p-value as per VAS	p-value as per SIA
β-tubulin III	CN vs CO	0.011*	0.001***
Arc	CN vs CO	0.218	0.204
GAP-43	CN vs CO	0.083	0.058
β-tubulin III	IN vs IO	0.070	0.070
Arc	IN vs IO	0.518	0.271
GAP-43	IN vs IO	0.243	0.617

Table 6.7: Comparison of p-values representing differences in tumour staining between groups of tumours that do go on to recur and those that do not as measured by either spectral imaging analysis (SIA) or visual analogue scoring (VAS) using three antibodies (* = p-values < 0.05, ** = p-values <0.01, *** = p-values <0.001).

The comparisons of antibody staining in the various tumour groups showed a variety of differences, as shown in table 6.7. The main feature that was evident was that overall there appeared to be a consistently lower intensity of staining present in tumours that subsequently went on to recur following excision when compared to those that did not.

This was most marked in the groups where original excision had been complete, with radial and deep surgical margins of at least 1 millimetre. In the case of β-tubulin III expression this difference was highly significant with p-values less than 0.05. Such a strong degree of correlation with staining and recurrence was not present using other antibodies. GAP-43 staining was only marginally insignificant when assessed by computer SIA scoring ($p = 0.058$) but was less significant when staining was measured by visual methods ($p = 0.083$). Arc staining was markedly insignificant with mean values and confidence limits that overlapped greatly.

In cases where the tumour had originally been incompletely excised no significant differences in staining were present. Differences in staining with β-tubulin III were

greater than those with other antibodies in these experiments, but still statistical significance was not reached ($p = 0.07$ with both assessment methods). With both Arc and GAP-43 large overlaps of confidence limits and mean staining intensities that were not very different resulted in insignificant differences between the groups as reflected by the high p-values shown.

The generally lower level of staining seen in the tumours that did recur correlates in an interesting way with the results from chapter three. I showed that the expression of these markers was related to histopathological subtype, and that tumours which are typically *thought* to behave aggressively expressed these markers to a lesser degree than those that tend to behave indolently. In this chapter I have shown that there is indeed a reduced level of staining in tumours that *are* more aggressive, as inferred by their recurring rather than not recurring.

When the differences in staining between similarly excised groups are compared, they imply that there is less difference between the incompletely excised groups (IO vs IN) than there is between the completely excised groups (CO vs CN). In other words, tumours in the IO group are likely to stain similarly to tumours in the IN group, whereas tumours in the CO and CN groups are likely to stain differently.

If one considers these markers of neuronal differentiation as a marker of biological similarity, one sees that IO tumours are more "like" IN tumours, i.e. whether they recur or not is less a feature of the fact that they are biologically different, and it is highly likely that the degree to which they have been incompletely excised has a major effect on the subsequent outcome. CO tumours, however, do appear to behave differently in staining terms to the CR tumours, and although these groups were matched by other factors known to affect recurrence they appear to behave differently in both biological terms and in staining terms.

The IO and IN groups were also matched by site, sex, age and subtype of tumour. The lack of difference in staining between them did not correlate with the lack of biological difference – after all, one group recurred and one did not (as big a biological difference as can be measured in these cases). Yet no statistically significant difference was noted using any of these antibodies. The main factor (even

accounting for the matching of other variables) linking these cases was that they were indeed incompletely excised. It is assumed from this therefore that tumour is left behind following the incomplete excision, and the two major factors affecting recurrence at this point would be the actual amount of tumour left behind (it makes sense that a lot of tumour is more likely to recur as a clinically detectable BCC than a very small amount) and the host response to that remaining tumour. These variables can in no way be accounted for in this type of study – with the decision having been taken to "watch and wait" it is of course unknown how much tumour lurked behind, or what degree of host response is occurring in the period following the surgery. These factors may play a significant factor in the fact that many incompletely excised tumours do not recur.

One additional property of the staining of these tumours that is clear is that there is a great degree of heterogeneous staining in all groups examined. This reflects the fact that not only is there a variation of staining seen within tumour types (as discussed in chapter 3) but also that these groups are made up of tumours of different subtypes. Therefore a degree of heterogeneity would be expected and indeed this is reflected in the confidence intervals that are shown for each group with each antibody.

6.4.1.5 Immunohistochemistry of primary tumours and their subsequent recurrences

The tumours in the groups compared in this section were linked by the fact that in each case they were paired recurrences i.e. one tumour in the CO group had given rise to one of the tumours in the CR group. However, although age, gender and site differences did not exist (because each patient gave rise to a pair of tumours, one in each group) there were marked differences in the histological subtypes present in each group. Interestingly the majority of the completely excised original tumours which went on to recur were of a typically aggressive subytpe (8/13) whereas the recurrences associated with these were mainly classified as indolent tumour subtypes (9/13). The incompletely excised tumours which went on to recur were, however, mainly indolent in nature (8/13), and their recurrences were more aggressive in nature (9/13).

It is also interesting to note that the mean time to recurrence was markedly less for the tumours that were incompletely excised compared to those that were completely excised (CO→CR mean 4.0 years, IO→IR mean 2.1 years). The difference between them was not statistically significant, but only marginally so (p-value = 0.052). It makes sense that if tumours are going to recur, those with a higher starting amount of tumour bulk will grow to be clinically detectable BCCs in a shorter period of time.

Antibody	Comparison	p-value as per VAS	p-value as per SIA
β-tubulin III	CO vs CR	0.165	0.04*
Arc	CO vs CR	0.67	0.48
GAP-43	CO vs CR	0.165	0.254
β-tubulin III	IO vs IR	(1.0)	0.96
Arc	IO vs IR	(1.0)	0.72
GAP-43	IO vs IR	0.43	0.32

Table 6.8: Comparison of p-values representing differences in tumour staining between groups of original tumours and their recurrences as measured by either spectral imaging analysis (SIA) or visual analogue scoring (VAS) using three antibodies (* = p-values < 0.05, ** = p-values <0.01, *** = p-values <0.001).

The immunohistochemistry results show that on the whole there was very little difference in staining intensity between original BCC tumours and their subsequent recurrences, as illustrated by the summary of these results shown in table 6.8. The only experimental combination that produced a significant difference was the comparison of completely excised tumours and their subsequent recurrences using the β-tubulin III antibody. This comparison yielded a significant difference where the staining of original tumours was significantly less than that of the matched subsequent recurrent tumours. All the other decreases seen with β-tubulin III and Arc were not statistically significant. Only in the case of GAP-43 was there seen to be a general increasing trend of staining in subsequent recurrent tumours. However these differences were also not statistically significant.

When comparing the original tumours and their subsequent recurrences one factor is constant: every tumour in these groups is either a recurrence, or will lead to one. The biological behaviour of these tumours is a fixed point of reference (unless one considers the timing of those recurrences, but that is not a debate I will address here).

What is of interest is that the differences between the comparison groups obviously vary and when the significance of the differences between groups is examined an interesting concept arises. There are, on average, more significant differences in staining between the completely excised tumour groups (i.e. CO vs CR, p-values 0.165, 0.67, 0.165 (VAS) and 0.04, 0.48, 0.254 (SIA)) than there are between the incompletely excised tumour groups (i.e. IO vs IR, p-values 1.0, 1.0, 0.43 (VAS) and 0.96, 0.72, 0.32 (SIA)). This is illustrated graphically in figure 6.30.

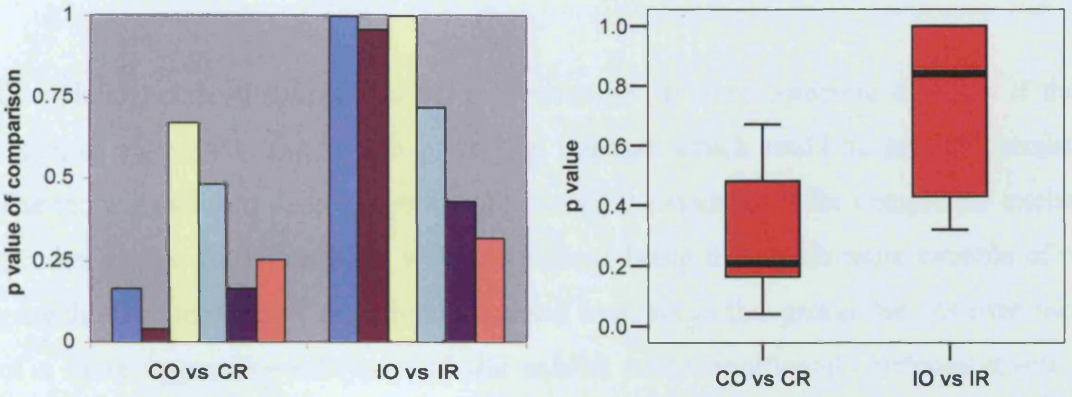


Figure 6.30: Bar chart and boxplot of p-values arising from comparisons of staining intensity in completely and incompletely excised BCCs and their subsequent recurrences. For each group six p-values are shown – two values (spectral imaging and visual analogue data) from each of the three antibodies.

It can be seen from these figures that the recurrent tumours which develop following the complete excision of a BCC are *more different* in staining to their original source tumours (i.e. lower p-values of statistical comparison), than recurrent tumours which arise following an incomplete excision are to their source tumours (i.e. higher p-values). The highest p-values in the IO/IR comparison come from visual analogue comparisons that were absolutely identical (β-tubulin III and Arc, p-values considered to be 1.0) and from the spectral imaging analysis of β-tubulin III staining (p-value = 0.96). This reflects the similarity between the staining of incompletely excised tumours and how that contrasts with the staining differences that exist between completely excised BCCs.

If one assumes (again) that the change in expression of these proteins is indeed a form of marker for altered biological behaviour, then one could postulate that there is *more*

biological difference between the completely excised original tumours and their recurrences than in the equivalent incompletely excised tumours. Or put another way, the recurrences that arise from incompletely excised tumours are more "like" their original tumours. This could of course be explained by the fact that if tumour resides in the excision site, then any recurrent tumour is likely to be a *persistence* rather than a *recurrence* per se. So actually in this category I am comparing "recurrent" tumours and "persistent" tumours, and demonstrate that although they both "recur" there are fundamental differences in their biological relationships with their subsequent recurrent tumours.

Completely excised tumours have to "overcome" a more complete excision if they recur, as opposed to incompletely excised tumours which could be said to "persist". The more significant differences noted between the staining of the completely excised tumour groups could correlate with the tumour being that much more capable of re-growth. The majority of completely excised tumours in this group that recurred *were* of a more aggressive subtype and *did* exhibit more significantly reduced levels of neuronal differentiation marker expression than those that did not.

Chapter 7: General Discussion

7.1 Clinico-pathological background to BCC

As has been discussed in the preceding chapters of this thesis, there is a wide variety of tumour phenotype included within the diagnosis Basal Cell Carcinoma. Historically, it is known that these tumours were recognised as common, and that the treatment of them was often best achieved by excisional methods.

Today, many historical observations still hold true, but a great deal of scientific refinement has been applied to the knowledge of this disease. The main developments have come with identification of both environmental and genetic predispositions to skin cancer development. In particular, exposure to ultraviolet radiation and the subsequent mutation of the patched tumour suppressor gene is of key importance to the development of BCC. Any other predisposing condition such as immunosuppression (pharmacological or otherwise) or inherited traits (Gorlin's syndrome or Xeroderma pigmentosum) which increase the likelihood of genetic abnormalities occurring or being preserved will have an additive effect on the normal causes for BCC development.

BCC is very common, and although a huge amount of data has been published on the clinical, histological and genetic aspects of the disease, there are difficulties when it comes to relating these changes to outcome. This is, of course, the most clinically relevant part of research; although better understanding of disease biology increases the chances of improving treatment, this is only possible if strong correlations can be made between treatment and outcome. Unlike more severe skin cancers such as melanoma (where outcome is definite: local, nodal or distant recurrence potentially leading to death), the problem with BCC is that outcome is a difficult term to define exactly. Local recurrence is the only end point of importance here – regional and distant metastasis is only a problem in exceptional cases. The frequency of the disease, however, still makes BCC a considerable healthcare issue (and expense) and it is this that drives the search for more effective treatment modalities.

Of course, eradication of disease and prevention of recurrence is the goal of any treatment, but in the case of BCC defining both the extent of treatment (specifically the extent of tumour mass excision or ablation) and the extent of success (do tumours recur, or do they merely persist following re-growth of residual tumour, or are they new tumours that happen to be in the same site as previous ones?) is challenging. To define the absolute rate of recurrence ten years follow-up has been used as the best indicator of disease recurrence, although of recurrences over ten years, 80% will occur in the first five (Rowe et al., 1989). For common tumours which already present a significant healthcare cost burden, the number of institutions which are able or financially willing to carry on such intense follow-up is small, and for this reason much of the data regarding tumour recurrence in this country is small. One of the more prolific researchers in this field is R.W. Griffiths, based in Sheffield, whose unit has a commitment to optimal surgical treatment and long term follow-up of BCCs. After many years of research and a number of quality publications on the subject, his conclusion is that BCCs which are conventionally excised (as proven by "breadslicing" of the histological specimens) with clearly defined excision margins have a low recurrence rate, in the order of 1.3% over 5 years (Griffiths et al., 2005). This compares favourably with other published data.

However, in the majority of cases, the treatment of BCC will remain surgical until improved treatments with equally effective or better eradication rates, less side-effects or lower costs are defined. Many such treatments are still being trialed; these include immunomodulators such as Imiquimod, retinoids such as tazarotene and intralesional interferon α-2b. These have mostly been used on either superficial BCCs or tumours that are likely to behave indolently such as nodular tumours. However, none of these therapies specifically target the changes in cell biology that define BCC, but instead they rely mostly on non-specific tissue ablation and subsequent healing of the area by the body's innate wound repair mechanisms. Treatment with specific hedgehog-signalling antagonists has been trialed in mice but this has not been attempted in humans due to the likely risk of side effects following administration (Taipale et al., 2000; Athar et al., 2004).

Therefore, in the majority of cases complete surgical excision is still the accepted gold standard. This is best achieved by Mohs surgery, but its expense and time

consumption makes it unsuitable for routine use. For this reason, the search for ways to improve the efficacy of routine surgical excision and histological reporting is most likely to have benefits to all BCC patients. The recent evidence showing that discrete tumours (such as clearly visually defined nodular and superficial cases) should be excised with a 3-4mm margin, whereas indiscrete tumours (such as those likely to be infiltrative in nature) should be excised with greater margin is currently the most important consideration in routine surgery (Telfer et al., 1999). However, there will always be cases where tumours are incompletely excised, and the management of these is not clear cut. Even Griffiths states that in a unit where concerted efforts are made to re-excise such tumour beds, there are some that slip through the net and this proportion will be much greater in units where such an aggressive policy is not followed. In these cases, additional markers that may assist in predicting outcome would be particularly useful in affecting what are sometimes difficult surgical decisions.

7.2 Molecular Biology advances in BCC

The molecular biology of BCC took a great leap forward in the 1990's following the discovery that *patched* mutations are the key alterations in Gorlin's syndrome, and that these mutations are also found in sporadic BCCs. Since that time a vast amount of other work has progressed knowledge about this cell signalling pathway, and alterations or mutations in expression of many of the main components in this pathway (*hedgehog*, *ptch1* and *ptch2*, *smoothened*, *suppressor of fused*, *Gli1* and *Gli2*) have been discovered in BCC. The action of Gli proteins as transcription factors has been established but only a limited amount is known about what happens downstream of its effect at the nucleus. Significant downstream effectors of hedgehog network signalling in BCC include the Wnt signalling pathway, other transcription factors such as FOXM1 and FOXE1, and the cyclins intimately involved in the control of the cell cycle.

Some attempt has been made to explain why the variation in BCC growth patterns and clinical aggression exists. Changes in the expression of cyclins, Bcl-2, Ki-67and p53 have all been noted in different subtypes of BCCs and it is significant that all these factors are involved in the control of the cell cycle and apoptosis. Higher expression of the anti-apoptotic protein Bcl-2 has been noted in less aggressive tumours which

correlates with decreased rates of apoptosis in these tumours. Lower levels of expression of both cyclins and Ki67 (all of which are associated with control of the cell cycle and cell turnover) have been noted in indolent tumours compared to aggressive ones. p53 expression in BCCs has received much attention but the difficulties in interpretation of the results (including the question of whether immunohistochemical positivity correlates with mutant p53 expression) has not resulted in any clear consensus.

Part of the difficulty in the investigation of the molecular biology of BCC has been that a robust cell culture model has not been available (unlike that which is routinely used in the investigation of malignant melanoma). Various attempts have been made to grow BCCs in vitro but as mentioned in section 1.11.2, few of these have resulted in successful growth in the laboratory. The perceived wisdom on this subject is that with current techniques, culture of BCCs in the laboratory is not a reliable technique, and as such is not used in the study of BCC molecular biology.

I attempted to circumvent this by creating a series of retrovirally transfected primary keratinocytes which over-express the Gli transcription factors. My hypothesis was that these cells would (at least partially) mimic BCC cell signalling and that analysis of gene expression in these cells (performed initially by Prof. F. Aberger's group in Salzburg using gene array technology) would lead to new discoveries regarding the mechanisms of Gli signalling in BCCs. The finding that molecules such as β-tubulin III and Arc are expressed in these cells led me to investigate the expression of these markers and others known to be associated with neuronal differentiation.

7.3 BCCs express markers of neuronal differentiation

7.3.1 Protein expression

In chapter 3 I have presented evidence that four molecules which are normally associated with a neuronal cell phenotype (β-tubulin III, Arc, GAP-43 and neurofilament) are expressed at a protein level in BCCs. Assessed by both visual analogue scoring and spectral image analysis, there appears to be a significant difference between the expression of these markers in normal non-tumour epidermis and in BCC tumour islands. Additionally, there appears to be a reduction in the

expression of these markers in tumours that possess infiltrative or morpheic growth patterns compared to either micronodular tumours or more typically indolent tumours with nodular or superficial growth patterns. This reduction was most marked in the cases of β-tubulin III and GAP-43. In all cases the statistical significance of these differences was greater when the immunohistochemical staining was assessed by spectral image analysis. A particular example is that of neurofilament, where only a limited difference was shown by visual analogue scoring ($p > 0.05$) but a greater degree of difference was noted when SIA was used ($p < 0.05$).

The intracellular localisation of each protein was also looked at by visual inspection. It was shown that micronodular tumours have a particular tendency to express β-tubulin III and GAP-43 in a membranous fashion, whereas other tumours tend to show mainly cytoplasmic expression. This adds weight to the concept that micronodular tumours have specific biological features that are not always shared by other tumour subtypes. This was first suggested on the basis of histological appearances by Lang and Maize (Lang, Jr. and Maize, 1986) and subsequently differences in intracellular localisation of β-catenin specific to this subtype have been shown by El-Bahrawy et al (El Bahrawy et al., 2003). All this data suggests that micronodular tumours have specific biological changes that result in not only a separate physical growth pattern, but also a unique expression profile compared to other subtypes.

7.3.2 Genetic expression

The results presented in chapter 4 show that different methods of tissue processing for RNA extraction provide different total quantities of RNA, and that laser capture microdissection (LCM) provides the smallest quantities. This is ideally a trade off where the increased specificity of cell capture is offset by the small RNA yield. The limitations of the technique resulted in nine BCCs being used to compare gene expression following LCM, needle microdissection (NMD) and whole biopsy homogenisation (WHT). The strength of housekeeping β-actin bands were most intense in the bands from samples obtained from WHT which fits with the higher average RNA yield (see table 4.4).

The expression of Gli 1 and Gli2 was variable in the BCC specimens, but notably bands were absent in the normal skin samples as well as the technical controls. No great difference was noted in the strength of the bands when comparing indolent and more aggressive BCCs.

The expression of the four sets of primers designed to amplify *β-tubulin III*, *Arc*, *GAP-43* and *Neurofilament* genes was also variable. Although β-tubulin III and GAP-43 antibodies stained most strongly in BCCs, this was not always consistent with the level of gene expression. Interestingly, a number of the *GAP-43* WHT PCR product bands were very intense, suggesting a great degree of *GAP-43* gene expression in these samples. The same tumours that were processed using needle microdissection did not show such intensity, and a different tumour showed a high level of gene expression in this case. *Neurofilament* expression was generally weak, although there did seem to be a small amount of PCR product present in four of the WHT samples and up to four of the LCM samples – no bands were seen in the NMD samples. The variability of expression with these two primer sets is difficult to draw meaningful conclusions from in terms of expression amongst the different types of BCC analysed (i.e. less aggressive tumours to the left, more aggressive tumours to the right.

The expression of *β-tubulin III* and *Arc*, however, is more interesting. For both primers using all three methods of RNA extraction, PCR product shows up in many (but not all) of the BCCs, but not normal skin and not in the technical negative controls. In addition, there is a slight preponderance of PCR product band intensity in the left-hand lanes, i.e. in the less aggressive BCCs. Less band intensity is seen in the more aggressive tumours although the difference is subtle. This correlates with the reduced expression of the corresponding proteins in the wax-embedded BCC specimens investigated with immunohistochemistry as described in chapter 3. Such a reduction of marker gene expression in more aggressive tumours has been discussed in terms of increased "de-differentiation" (da Costa, 2001) and it is highly likely that the BCCs with growth patterns that predispose to more aggressive behaviour are showing this phenomenon.

This concept of tumour de-differentiation, however, implies that a tumour is regressing to a state where such fine control over genetic expression and therefore

phenotype has diminished. It is hard to imagine that this is the case in tumours which exhibit growth patterns so very different from normal epidermis, as is the case with infiltrative and morphoeic tumours. However, if one of the purposes of tight regulation of gene expression and cell turnover includes ordered regulation of the microarchitecture of tumours, it is possible that loss of this control could result in a much more haphazard growth pattern arrangement, as demonstrated in infiltrative and morphoeic BCCs.

Exactly what effect these molecules have on the growth of BCCs is not clear from these studies. Certainly in all these cases there does seem to be a correlation between the nature of the BCC (indolent or aggressive) and the likely degree of gene or protein expression. This, however, is not absolute (as can be seen from the overlap of confidence limits in chapter 3, or the variability of PCR product band intensity in chapter 4). It may be that the expression of these genes does not have a direct role in the development of a growth pattern per se, but may be more strongly associated with the degree of differentiation (or de-differentiation) present in tumours. Irrespective of this, the fact that their expression *does* correlate with the histopathological growth patterns that are likely to be associated with an aggressive tumour course, makes them likely to be of interest in the search for biomarkers that predict for the likelihood of tumour recurrence following treatment.

7.4 Gli1 and Gli2

The results that are presented in chapter five provide evidence that both *Gli1* and *Gli2* appear to have regulatory effects on the expression of each other and on the expression of the markers described previously. Regl et al have shown that there appears to be a positive feedback mechanism whereby expression of *Gli1* and *Gli2* is positively regulated by each other (Regl et al., 2002) and these findings are confirmed in my experiments.

Real-time relative PCR quantification was used to assess how many cycles PCR reaction mixes took to reach a threshold level of PCR product. This was possible using starting cDNA which was produced using the same quantity of transfected cell mRNA in each case. The relative gene expression was therefore comparable in each

case, given that the same amount of cDNA (originating from the same quantity of RNA) was present in each PCR reaction.

Gli1 transfected cells showed a significant rise in *Gli2* expression over control cells, and likewise *Gli2* transfected cells showed a significant increase in *Gli1* expression. This trend was repeated in both cell transfection experiments performed.

The expression of the markers of neuronal differentiation varied greatly. *β-tubulin III* was moderately up-regulated by both *Gli1* and *Gli2* with these reactions in the first transfection experiment reaching threshold on average 2.04 and 2.68 cycles earlier respectively (and 5.46 and 4.23 cycles earlier respectively in the second experiment). If one assumes that the growth of PCR product is exponential this corresponds to a 4-fold and 44-fold ($2^{2.04}$ and $2^{5.46}$) relative increase in the gene expression for *β-tubulin III* in the *Gli1*-transfected cells over two experiments. Similarly, this corresponds to a 6.4-fold and 18.7-fold relative increase due to *Gli2* over two experiments.

Arc was massively upregulated by both *Gli1* and *Gli2*. *Arc* PCR reactions using *Gli*-transfected keratinocyte cDNA from the first retroviral transfection reached threshold over 13 cycles earlier than cDNA from control keratinocytes, corresponding to more than a 8,000-fold (2^{13}) upregulation of gene expression. This upregulation in *Gli1*-transfected cells was even stronger in the second cell transfection experiment, but not quite as massive in the *Gli2*-transfected cells.

The huge up-regulation of *Arc* by *Gli1* and *Gli2* shows that Arc is clearly strongly under the control of *Gli* signalling. In comparison to *β-tubulin III*, *Arc* is clearly more sensitive to *Gli* expression. It is interesting to note that in chapter four I showed that *Arc* expression was, on average, slightly stronger than *β-tubulin III* expression in cDNA derived from BCCs: it is therefore possible that in terms of *β-tubulin III* and *Arc* gene expression, my retrovirally transfected keratinocytes do act as a model for BCC.

In the first transfection experiment neurofilament expression in *Gli1*- and *Gli2*-transfected keratinocytes was seemingly decreased in *Gli*-transfected keratinocytes compared to control cells. In the second transfection no signal was recorded in any

group. Although most of the results from this second transfection occur later than those from the first, the lack of signal from this second experiment implies that neurofilament expression is either absent or very low in all the cell groups examined. Certainly expression of neurofilament at all in keratinocytes is unexpected and in the cases where a result was recorded, threshold was only reached in over thirty cycles in every case. This paucity (and possible down-regulation) of gene expression correlates only partially with the results from chapter four, in which a very small amount of neurofilament up-regulation was seen in BCC specimens. However, the intensity of the neurofilament PCR product bands was very weak, and with no signal registered in the normal skin samples, it is impossible to say whether any down-regulation occurred in these BCC samples.

GAP-43 was not expressed in any of the keratinocyte samples from either transfection and only very faint bands were seen following a standard PCR reaction. No comparative conclusion can be drawn from this other than noticing that if there is any *GAP-43* gene expression present, it is very low in all groups. This contrasts with the PCR results from BCC cDNA in chapter four, where most of the samples were negative for *GAP-43*, but a small number of samples (two WTH and two NMD samples) did show very strong expression. On this basis, the transfected keratinocytes do not appear to be an ideal model system for *GAP-43* expression in BCCs.

All these findings in transfected keratinocytes have been compared with PCR results from BCCs and in some cases (such as *β-tubulin III* and *Arc*) they compare favourably. However, not all these findings correlate well with the immunohistochemical findings presented in chapter three. In that case, *β-tubulin III* and *GAP-43* proteins were expressed strongly in the more indolent tumours, with markedly decreased expression in more aggressive tumours. Arc protein expression was less intense than either of these, and neurofilament was the weakest antibody of all. However, in all cases significant differences were shown between the expression of these proteins in aggressive and non-aggressive BCCs.

It is possible that the keratinocyte model as it stands reflects to some degree the expression of *β-tubulin III* and *Arc* in BCCs, but less comprehensively *GAP-43* and *neurofilament*. Also, the variation in gene expression seen in the BCC samples (with

tumour aggression an important factor) makes it difficult to compare transfected keratinocyte results to BCCs *in general*. The most that can be concluded from this is that in some cases the comparative expression between normal keratinocytes and transfected keratinocytes (whether *Gli1* or *Gli2*) does appear to highlight differences in gene expression, as is the case in the BCC PCR results.

When comparing the BCC PCR results and the immunohistochemistry results there is certainly evidence to suggest that there is a variation in expression of these markers at both gene and protein levels. The association of marker expression with tumour aggression, however, is not so strong at the gene level as it appears to be at the protein level, and it is likely that post-translational modification accounts for this.

7.5 Association with tumour biology

The results presented in chapter six show that there are differences in immunohistochemical staining between tumours that go on to recur and those that do not. The material used for this study was archival material for which follow-up data was available for at least eight years, and because of this I can be as sure as is possible that these cases definitely represent cases where cases have truly recurred or have not. The groups that were examined were matched for broad anatomical site, gender, age, excision margin (if complete) and histopathological subtype class (i.e. typically indolent or typically aggressive). The only major differences between the groups was their recurrence status, and so it was possible to conclude that the differences in staining for the antibodies previously used in chapter 3 would be due to intrinsic differences in the biology of the tumours that are associated with recurrence.

Few previous studies have attempted to make such correlations, a recent example being that by Healy et al who demonstrated that there were significant differences in Ki-67 expression (but not p53 expression) between tumours that go on to recur and those that do not. The results outlined in chapter six demonstrate that there are indeed some differences between these tumours, but few of these are significant. The only marker that was significantly reduced in recurrent tumours was β-tubulin III, which was markedly reduced in tumours that recurred following complete excision ($p=0.011$ by visual analogue scoring, and $p<0.001$ by spectral image analysis). The difference between groups of tumours that were incompletely excised were not significant in any

case, although again β-tubulin III came the closest to this with a p-value of 0.07. In the case of each antibody the significance of the difference between the incompletely excised groups of tumours was less than the completely excised groups.

The main conclusion to draw from this is that completely excised BCCs that recur differ more greatly in one aspect of their molecular biology (their expression of β-tubulin III) from their non-recurring counterparts than the incompletely excised recurring BCCs do from their non-recurring counterparts. The completely excised tumours that do recur are significantly different to the BCCs that do not in both their clinical course and their expression of β-tubulin III. None of the other markers, however, showed significant differences in staining patterns according to clinical recurrence status.

The most pressing clinical question that this relates to is of course the management of incompletely excised BCCs. As has been discussed, there is a historical precedent for basing the decision of whether to re-excise the scar and tumour bed on histopathological, age and location grounds. Unfortunately none of the antibodies used in this study are expressed at significantly different levels in incompletely excised tumours which do or do not recur. Therefore, none of them have a future as an immunohistochemical marker of likely future recurrence. Although a prospective trial of β-tubulin III expression in completely excised tumours could be set up, it is unlikely that a patient with a histologically completely removed BCC would be offered revisional surgery, whatever the expression of the marker. For these reasons, the future of biomarkers in incompletely excised BCCs will not feature any of the markers I have investigated here.

7.6 Conclusions

7.6.1 BCC histogenesis

There has been much debate about the histogenesis of Basal Cell Carcinoma as discussed in section 1.1. The greatest quantity of recent data proves that the outer root sheath area (ORS) of the hair follicle (the "bulge") is the most likely source of BCCs, although this may not be absolute. Such data includes

1) BCCs resemble tricoepithelioma, a benign hair follicle tumour (Walsh and Ackerman, 1990).
2) BCCs have a similar immunohistochemical profile to the hair follicle ORS (Asada et al., 1993; Kruger et al., 1999).
3) BCCs are generated more frequently following carcinogen addition in hair follicle anagen (Miller et al., 1993b).
4) BCCs seldomly occur on non-hairy skin (Kore-eda et al., 1998).
5) B-CAM is expressed in suprabasal cell layers, the ORS of the hair follicle and in BCCs (Bernemann et al., 2000) but not in normal skin.

The role of stem cells in BCC development (whether they reside in the ORS of the hair follicle or elsewhere) is as yet uncertain. Stem cell biology is progressing rapidly in all fields of cancer biology and there has, as yet, been no definite clarification of a site harbouring stem cells that may become BCCs. It is thought that stem cells may have a significant role to play in both normal development and in neoplasia, and increasingly the evidence for links between these two states is becoming stronger. Not only are stem cells (with unlimited replicative potential, low proliferation rates and several possible fates) thought to be responsible for tumours upon acquisition of genetic mutations (Reya *et al.*, 2001), but it appears that the reverse may also be true: teratocarcinoma cells can contribute to normal development if placed in early mouse embryos (Mintz and Fleischman, 1981). This demonstrates that under certain conditions tumour cells may be "de-programmed" to become normal and totipotent. Ruiz i Altaba, who has published extensively on the subject, has concluded that "tumours may be considered as organs that normally do not develop although the potential of their development exists all the time". He goes on to suggest that "this potential could reside in stem cells, which are present in many if not all adult tissues, and that the need for the body to remain morphologically plastic and therefore evolutionarily fit, by retaining stem cells in the adult has a price – tumourigenesis" (Altaba et al., 2002).

This concept is relevant to the link between epidermal and neurological tissues: the stem cells that give rise to skin are the same as those that give rise to the neurological system. The epidermis and the neurological system both derive from ectoderm: at 18 days of development a cranial area of ectoderm in the embryo is influenced by the

underlying prechordal and notochordal mesodermal plate and transdifferentiates to form neurectoderm (Larsen W.J., 1998). The role of the hair follicle here is that the epithelial linings of the follicle (including the outer root hair sheath) are ectodermal, ie. arising from the same original stem cells that the neurological system did.

A number of pieces of evidence outside of pure embryology add weight to the idea that epidermal and neurological tissue could share an ability to express similar genes, and this may help to explain the findings of this thesis:

1) Gli transcription factors were originally isolated in glioma brain cancer tissue, hence their name, but yet are critical for development of Basal Cell Carcinomas of the skin.
2) It is now known that hedgehog signalling has critical effects on development in general and on development of neurological malignancies such as medulloblastomas: both BCCs and medulloblastomas are key features of Gorlin's syndrome.
3) As cancers are a result of loss of cell proliferation and differentiation control, then it is quite possible that genes that are repressed in differentiated adult tissues may be re-expressed during the de-differentiation process that occurs in neoplasia (da Costa, 2001).
4) In a keratinocyte model system, *Gli* drives the expression of *Arc* strongly and *β-tubulin III* moderately. Both of these genes are expressed in BCCs, but are associated with neuronal development and repair following injury.

Although the expression of markers of neuronal expression in BCCs was a surprising finding to me originally, it is not so unlikely given the lines of argument stated above. What is however confusing, is that it seems that the expression of these markers is strongest in the most indolent tumours and as the tumours become more aggressive the expression of the "unmasked" markers is lost. There may be a peak of repressed gene expression at the point of initial loss of normal epidermal cell function (i.e. upon development of an indolent tumour) and that a progression to an aggressive tumour results in loss of the normal growth control (hence disorganized growth pattern of infiltrative tumours) and loss of this specificity of gene expression (hence reduced expression of these neuronal differentiation markers).

7.6.2 Gli-mediated cell signalling

It appears from the results of this thesis that another downstream target of *Gli* signalling in BCC has been identified. Whether *Arc* and *β-tubulin III* are direct targets, or are more indirectly linked to *Gli* expression is not clear from my studies. The strengths of the retroviral keratinocyte transfection system include the ability to over-express specific *Gli* transcription factors and determine the relative gene expression resulting from this. From my studies examining the expression of these markers of neuronal differentiation it appears that there is a great deal of overlap between the transcriptional control of *Gli1* and *Gli2*, and that as well as both having an effect on the expression of *Arc* and *β-tubulin III* they have positive feedback effects on each other.

The drawbacks of this putative model system include the fact that it is an isolated cell culture system and, as has been shown by the difficulties in culturing BCC cells in the laboratory, there is a degree of location-specificity in BCCs. This has been previously described as the stromal dependency of the tumours, and this interaction between tumour cells and their immediate environment may have strong bearing on the tumour cell phenotype. This has not, as yet, been replicated in the laboratory (whether for BCC cells or cultured retrovirally transfected keratinocytes) and advances in this field may improve upon this. This crucial difference may account for the differences between the model system findings and the results in BCCs.

7.7 Further developments of this research

This research has focussed on the expression of markers of neuronal development in Basal Cell Carcinoma and in a putative keratinocyte model system. Further work that would improve upon the results presented could include a number of alterations to the work already performed.

The control tissues used in this work were mainly normal non-tumour skin distant to the site of the BCCs examined and normal non-Gli-transfected keratinocytes. It would be of great interest to examine hair follicles from the same patients and from control non-tumour patients to see if these markers that are expressed in BCCs are expressed

in the outer root sheath of the follicle. This would add weight to the arguments in favour of the hair follicle histogenesis of BCCs.

Examination of the gene array data that led to the discovery of neuronal markers being up-regulated in *Gli1*-transfected cells could lead to other molecules being considered. No other markers that were known to be associated with a neuronal phenotype were noticed in the array performed by our collaborators (Prof. F. Aberger et al, Salzburg), but the sequence of investigation in this thesis could be repeated for other markers.

It would have been ideal to use larger biopsies of tissue, and in combination with better Laser Capture Microdissection abilities, obtain more uniform and concentrated RNA extracts from each tumour by each method. In the setting of more concentrated RNA (as was the case with the cultured cell model extracts) a more meaningful comparison could be made using real-time quantitative (or relatively quantitative) PCR. Unfortunately the amount of tissue I was able to harvest from the vast majority of BCCs was limited to a small biopsy, and following a number of trials of laser capture microdissection, this left a smaller amount of tissue for needle microdissection and whole tissue homogenisation than I would have wished for. Were the series of steps required for this thesis to be repeated I would choose to try and only take samples for larger tumours, thus enabling a larger biopsy to be harvested without affecting the surgical examination margins. This would then allow me to generate greater quantities of RNA and compare the results between tumours and methods more effectively.

The technique of Laser Capture Microdissection is a difficult one to master. The equipment for it is mainly marketed by Arcturus (Mountain View, California, USA) and since the production of the "PixCell II" (which was available to me), two successive generations of machines have been produced ("AutoPix" and "Veritas"). The main advantages of the new machines are that they have greater degrees of automation and therefore the speed of cell capture is much increased. This may have the advantage of decreasing the chance of RNA degradation during the crucial period between section dehydration and immersion of the cells in lysis buffer. To get the most out of an expensive system such as this (the new machines cost in excess of

£120,000 in 2006), I feel that it is a technique that is best reserved for use by those who have a great deal of experience in molecular biology and who are used to extracting RNA from very small samples.

7.8 Summary

In summary, I have characterised the expression of neuronal markers of differentiation in Basal Cell Carcinoma and reported that they are expressed in BCC whereas this is not the case in normal epidermis. Also, this expression is reduced in tumours with a more aggressive phenotype. In a retroviral keratinocyte model, expression of *β-tubulin III* and *Arc* appear to be more strongly controlled by Gli signalling than *GAP-43* or *neurofilament*. Of these, only β-tubulin is expressed at different (lower) levels in tumours that do recur following complete surgical excision compared to those that do not. No significant differences in expression of these markers correlated with recurrence following incomplete excision. Further work will be required to ascertain the sequence of downstream effects of *Gli* signalling and how these interact with genes associated with both neuronal development and tumour aggression.

Chapter 8: Appendices

		------β-Tubulin III---			--------GAP-43---------			------NF-M+H---			------Arc----------		
		Int	localisation		Int	localisation		Int	localisation		Int	Localisation	
			Cy	Me		Cy	Me		Cy	Me		Cy	Me
SKIN1		-			-			-			-		
SKIN2		-			-			-			-		
BCC1	Nod	++	++	-	+++	+++	-	-			++	++	-
BCC2	Nod	++	+	+	++	+	+	+	+	-	++	++	-
BCC3	Nod	+++	++	+	+	+	-	-			-		
BCC4	Nod	++	+	+	++	+	+	+	+	-	+	+	-
BCC5	Nod	+	+	-	+++	++	-	+	+	-	++	++	-
BCC6	Nod	++	+	+	++	+	+	+	+	-	+	+	-
BCC7	Nod	-			++	++	-	-			-		
BCC8	Sup	++	++	-	-			+	+	-	+	+	-
BCC9	Sup	+++	++	++	+	+	-	-			+	+	-
BCC10	Sup	+	-	+	+			++	++	-	++	++	-
BCC11	Sup	+++	+	+++	++	+	+	++	++	-	+	+	-
BCC12	MN	+++	+	+++	+++	-	+++	+	+	-	++	++	-
BCC13	MN	+++	+	+++	+++	+	+++	++	++	+	++	++	-
BCC14	MN	++	-	+++	++	+	++	+	+	-	+	+	-
BCC15	MN	++	-	++	++	+	+	+	+	-	+	+	-
BCC16	MN	++	-	++	+	+	-	+	+	-	++	++	-
BCC17	MN	++	+	++	++	+	-	+	+	-	+	+	-
BCC18	MN	++	+	+	+	+	+	-			+	+	-
BCC19	MN	++	+	+	+++	+	+++	+	+	-	+	+	-
BCC20	MN	++	+	+	+	+	-	+	+	-	-		
BCC21	Inf	+	+	-	+	+	-	++	++	-	+	+	-
BCC22	Inf	+	+	+	+	+	-	++	++	-	+	+	-
BCC23	Inf	-			+	+	+	+	+	-	-		
BCC24	Inf	-			-			-			-		
BCC25	Inf	++	+	+	+	+	-	-			-		
BCC26	Inf	-			-			-			-		
BCC27	Inf	-			+	+	-	-			-		
BCC28	Inf	+	+	+	-			-			+	+	-
BCC29	Mor	+	+	+	-			-			-		
BCC30	Mor	-			-			+	+	-	-		
BCC31	Mor	-			-			+	+	-	-		
BCC32	Mor	+	+	-	-			-			+	+	-

Table 8.1: Intensity of tumour immunohistochemistry staining and cellular localisation in two normal skin samples and 32 BCCs as determined by visual analogue scoring. Nod = Nodular; Sup = superficial; MN = Micronodular; Inf = Infiltrative; Mor = morphoeic. Int = intensity of tumour staining; Cy = cytoplasmic staining, Me = membranous staining. "-" = no staining; "+" = weak staining; "++" = moderate staining, "+++" = intense staining.

	Type	β-tubulin III intensity			GAP-43 intensity			ARC intensity			NF intensity		
SKIN1		0.055	0.054	0.045	0.150	0.156	0.153	0.141	0.155	0.151	0.129	0.141	0.126
SKIN2		0.033	0.038	0.044	0.137	0.122	0.121	0.166	0.158	0.171	0.128	0.113	0.115
BCC1	Nod	0.336	0.430	0.433	0.390	0.403	0.339	0.293	0.266	0.280	0.236	0.214	0.171
BCC2	Nod	0.195	0.213	0.203	0.451	0.505	0.437	0.140	0.136	0.124	0.168	0.179	0.166
BCC3	Nod	0.289	0.288	0.329	0.352	0.418	0.423	0.247	0.277	0.287	0.212	0.219	0.230
BCC4	Nod	0.348	0.411	0.393	0.334	0.364	0.353	0.234	0.291	0.285	0.176	0.183	0.177
BCC5	Nod	0.130	0.515	0.526	0.241	0.246	0.280	0.203	0.181	0.180	0.232	0.238	0.281
BCC6	Nod	0.312	0.191	0.147	0.388	0.388	0.463	0.232	0.263	0.236	0.200	0.184	0.173
BCC7	Nod	0.245	0.302	0.300	0.541	0.573	0.560	0.350	0.329	0.311	0.207	0.166	0.160
BCC8	Sup	0.177	0.192	0.214	0.173	0.194	0.283	0.342	0.414	0.348	0.226	0.247	0.239
BCC9	Sup	0.388	0.414	0.411	0.195	0.197	0.243	0.204	0.202	0.186	0.254	0.235	0.232
BCC10	Sup	0.282	0.235	0.289	0.397	0.386	0.479	0.375	0.380	0.368	0.289	0.329	0.313
BCC11	Sup	0.701	0.603	0.681	0.640	0.645	0.612	0.468	0.484	0.505	0.430	0.432	0.395
BCC12	MN	0.233	0.317	0.288	0.284	0.263	0.206	0.209	0.212	0.207	0.171	0.160	0.168
BCC13	MN	0.710	0.676	0.432	0.552	0.597	0.562	0.419	0.417	0.397	0.238	0.200	0.211
BCC14	MN	0.541	0.493	0.484	0.402	0.374	0.390	0.174	0.165	0.185	0.481	0.433	0.402
BCC15	MN	0.179	0.225	0.270	0.324	0.294	0.321	0.265	0.268	0.246	0.216	0.219	0.209
BCC16	MN	0.159	0.129	0.111	0.401	0.492	0.441	0.232	0.215	0.225	0.210	0.198	0.163
BCC17	MN	0.337	0.329	0.428	0.217	0.234	0.206	0.466	0.639	0.489	0.261	0.278	0.266
BCC18	MN	0.244	0.309	0.281	0.364	0.346	0.333	0.398	0.353	0.383	0.316	0.312	0.262
BCC19	MN	0.233	0.254	0.338	0.396	0.391	0.414	0.294	0.267	0.254	0.141	0.136	0.145
BCC20	MN	0.352	0.369	0.460	0.489	0.441	0.487	0.298	0.258	0.277	0.225	0.217	0.209
BCC21	Inf	0.093	0.125	0.129	0.147	0.147	0.147	0.193	0.162	0.191	0.161	0.176	0.181
BCC22	Inf	0.049	0.045	0.061	0.173	0.201	0.182	0.170	0.153	0.191	0.254	0.242	0.239
BCC23	Inf	0.073	0.077	0.087	0.242	0.223	0.219	0.184	0.183	0.181	0.171	0.173	0.170
BCC24	Inf	0.038	0.041	0.031	0.196	0.220	0.181	0.148	0.162	0.168	0.147	0.164	0.148
BCC25	Inf	0.075	0.089	0.073	0.112	0.108	0.113	0.142	0.160	0.134	0.162	0.172	0.172
BCC26	Inf	0.068	0.088	0.058	0.162	0.161	0.158	0.171	0.175	0.180	0.178	0.175	0.171
BCC27	Inf	0.043	0.055	0.063	0.129	0.144	0.136	0.142	0.152	0.161	0.119	0.144	0.138
BCC28	Inf	0.033	0.032	0.032	0.152	0.151	0.141	0.139	0.152	0.150	0.108	0.104	0.117
BCC29	Mor	0.066	0.073	0.095	0.195	0.176	0.186	0.144	0.157	0.155	0.137	0.151	0.166
BCC30	Mor	0.120	0.137	0.160	0.121	0.124	0.112	0.138	0.136	0.149	0.140	0.131	0.133
BCC31	Mor	0.233	0.238	0.248	0.124	0.118	0.119	0.162	0.176	0.162	0.147	0.129	0.169
BCC32	Mor	0.159	0.171	0.154	0.119	0.125	0.131	0.216	0.199	0.189	0.161	0.151	0.135

Table 8.2: Intensity of tumour staining in two normal skin samples and 32 BCCs as determined by spectral image analysis: Nod = Nodular; Sup = superficial; MN = Micronodular; Inf = Infiltrative; Mor = morphoeic. Three Spectral Image Intensity readings are given for each tumour.

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