

**The Cell Biology Of Basal Cell Carcinoma.
Relationship To Histology And Clinical Outcome.**

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Abstract

Basal cell carcinomas presents with extremely diverse clinical and histological appearances and behaviour. Currently there is little understanding of the biological processes that determine these variations. In an attempt to understand these differences, this thesis evaluated some aspects of the cell biology of BCC both a prospective series and in archival specimens. A variety of measurements were assessed in combination with patient factors (age, presentation etc.) and medical factors (type and adequacy of treatment).

The cell kinetics of BCC was studied *in vivo* following administration of bromodeoxyuridine, which was analysed by flow cytometry. The growth fraction (Ki-67 immunohistochemistry) and the contribution of cell loss to the overall tumour kinetics were studied by evaluating apoptosis (morphologically) and the bcl-2, bax and p53 protein expression, using immunohistochemistry, in both the prospective and archival specimens (including non recurrent, recurrent and horrifying BCCs).

It was apparent that BCCs are highly proliferative tumours with a median Ts of 7.6 hours (range 5.0-14.6) , Tpot 2.8 days (range 4.0-18.3 days), LI 14%, and Gf 32%. Cell production rates were related to the histological growth pattern with infiltrative and morpheic tumours having a higher Gf than the nodular tumours ($p<0.01$) and a shorter Tc and Tpot. Cell proliferation was not related to differentiation status.

The median apoptotic index was 1% (range 1%-5%) and in the absence of apoptotic rate measurements, it was difficult to equate the contribution of apoptosis to the paradox of the slow clinical growth of BCCs. However, the concept of a high apoptotic rate was not supported by bcl-2 and bax protein expression. 88% of BCCs expressed bcl-2 and 23% expressed bax. The relationship between p53 expression and apoptosis was unclear since there was no correlation of p53 with bax, bcl-2 or apoptosis. The apoptotic parameters displayed some relationship to the histological growth patterns. The infiltrative and morpheic tumours exhibited the least apoptosis and least bcl-2 expression ($p=0.02$), but p53 did not correlate with tumour histology.

The contribution of biological factors in determining outcome (the development of recurrence or a horrifying tumour) in BCC are limited because patient factors (late presentation) and treatment factors are dominant. Incomplete excision was associated with recurrence and the development of a horrifying tumour when compared to non recurrent tumours ($p<0.01$). Primary radiotherapy was also associated with the development of a horrifying tumour ($p<0.01$).

A novel treatment modality, the optomechanically flash scanned carbon dioxide laser, was evaluated to assess its ability to completely ablate BCCs. Complete ablation was associated with ablation depth ($p<0.01$) and tumour type ($p=0.01$). Superficial BCCs were most suitable for this modality but required lasering to the middle dermis or deeper for complete eradication.

Identification of problem BCCs at an early stage still requires further research but this thesis highlights the need for further improvement in surgical treatment.

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Abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic Acid
FACS	Fluorescence activated cell sorter
PBS	Phosphate buffered saline
LI	Labelling index
LI(im)	LI measured by immunohistochemistry
LI(flow cytometry)	LI measured by flow cytometry
Ts	S phase duration
Tpot	Potential doubling time (days)
Tpot(im)	Tpot calculated from LI measured by immunohistochemistry
Tpot (flow cytometry)	Tpot calculated from LI measured by flow cytometry
Tc	Cell cycle time
GF	Growth fraction
IL	Interleukin
CDK	Cyclin dependent kinase

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Chapter 1

Section 1. Clinicopathological aspects of basal cell carcinoma.

1.1 History

1.1.1 Clinical definition

Centuries before the true definition of basal cell carcinoma, there were many fitting descriptions. Early physicians noted that whereas complete removal resulted in cure, incomplete removal resulted in recurrence (Bennett 1974; Crouch 1983). This thesis emphasises the impressions of these early observers.

Hippocrates, in the 5th century, founded a school of medical thought based on accurate observation and recording of signs, symptoms and progress of diseases. He did not define skin cancer, but made some interesting observations that may be the easiest descriptions of skin cancer and its treatment in his book of Alphorisms (Lloyd 1978). *“Ulcers lasting a year or longer cause underlying bone to be eaten away and the resulting scars depressed..”*

Celcus (30BC-AD50) may have been describing BCC in “De Re Medicina” when he defined skin cancer more accurately. *“A carcinoma... This disease occurs mostly in the upper parts of the body, in the region of the face, nose, ears, lips...There is a fixed irregular swelling, sometimes there is also numbness. Around it are tortuous veins.”*

This relates to the most common locations of BCC and the telangiectasia which are a common feature (Spencer 1935).

In the dark ages the term Noli-Me Tangere (touch me not) evolved for slowly spreading lesions of the skin. In the absence of an accurate diagnosis this term included BCC, Syphilis, Lupus Vulgaris, and other skin cancers. Noli-Me Tangere, as a phrase, arose due to the experience of successive recurrences following treatment and their apparent deterioration following caustics. Four centuries later, Jaques Daviel, a French surgeon, encouraged surgery, as the treatment of choice for certain varieties of Noli-Me Tangere. He gave the first account of cure by surgery of 10 patients with a long history of skin cancer, with no metastasis who were most probably suffering from BCCs (Daviel 1755).

Arthur Jacob, an Irish ophthalmologist (Figures 1.1 and 1.2), is often accredited for the earliest accurate report of BCC in the Dublin Hospital reports when he meticulously described: “ *an ulcer of peculiar character which I have observed to attack the eyelids, extend into the eyeball orbit and face. The characteristic features of this disease are the extraordinary slowness of progress, and peculiar condition at the edges and surface of the ulcer, the comparatively inconsiderable suffering produced by it, its incurable nature unless by extirpation, and its not contaminating the neighbouring lymphatic glands.*” He described accurately the surface features. “*The edges are elevated, smooth and glossy, with a serpentine outline and are occasionally formed into a range of small tubercles or elevations...The part within the edges in some places a perfectly smooth, vascular secreting surface, having veins of considerable size ramifying over it: which veins give way, causing slight haemorrhage..*” He concluded that the disease was not to be confounded with genuine carcinoma or with lupus or Noli-Me-Tangerie. In his conclusion he appealed for other surgeons to publish their experiences with this disease (Jacob 1827). Subsequently BCCs were often called Jacobean ulcers.

In the next 20 years there were a large number of descriptions of BCC including Mackenzie’s description of “eating cancer of the face” (Mackenzie 1830) and Lebert who is said to have been the first to use the term Rodent Ulcer (Lebert 1851). Rodent ulcer was finally acknowledged as a clinical entity following Hutchinsons analysis of 42 cases which was concluded by “14 Aphorisms respecting rodent ulcer”(Hutchinson 1860).



Figure 1.1. Arthur Jacob



Figure 1.2. The ulcer of peculiar character illustrated by Jacob



Figure 1.3. Jacques Daviel

1.1.2 Histological Definition

The first microscopic description was probably by Hutchinson in 1860. (Hutchinson 1860) He commented on the dense fibrous stroma found around some BCCs: *“Sections of the indurated edge of this ulcer do not exhibit the cell structures met with epithelial or schirrhous cancer but only those of organising fibrous tissue.”*

Thiersch, in 1865 recognised that there were different subtypes of this tumour and describe a superficial and a deep variety (Thiersch 1865). The cell of origin of BCC was disputed: Moore, Hulke and Warren believed it to be a variety of epithelioma; Thiersch, a cancer of sebaceous glands; Thin a carcinoma of sweat Glands; Sangster and Hume a carcinoma of hair follicles (Thiersch 1865; Moore 1867; Hulke 1871; Sangster 1882; Hume 1884). The histological definition of this tumour as BCC was by Krompecher (1903) when he proposed its derivation from the basal layer of the epidermis and used the term Basalzellenkrebs (Krompecher 1903). This definition opened the debate of the cell of origin of basal cell carcinoma which still continues 90 years later (Section 1.2.2).

1.1.3 Historical Treatments

A vast array of treatment options have been described for BCCs. Evidence for the use of cautery for skin diseases is found in the Edwin Smith Papyrus which is thought to have been compiled in 2500BC. Edwin Smith, an American Egyptologist bought 2 papyri from an arab named Mustapha Aga. Smith deciphered the papyri and found that 1 patient, with an ulcer on the breast was treated with a fire stick consisting of a hollow block of wood inside which a stick is rotated to produce heat. When the fire stick was glowing it was applied to the tumour as a form of cautery (Breasted 1930). Hippocrates also commented on the treatment of skin cancer by drugs, surgery and cautery (Lloyd 1978). *“What drugs will not cure the knife will; What the knife will not cure , the cautery will; What cautery will not cure must be considered incurable.”*

Celsus also discussed of treatment options and prognosis: *“Some have used caustic medicaments, some the cautery and some excision with a scalpel”*. He stated that only in the first stages was removal possible whilst in the latter stages carcinoma was irritated by treatment (Spencer 1935). These writings put on record that skin cancers were recognised and that a variety of treatment modalities were applied to remove them but some were incurable.

Caustics were a popular early treatment, even described by Celsus. In 1844, E.W. Tuson, a surgeon to the Middlesex hospital stated that *“the use of a paste made with Chloride of Zinc and flour as an external application in an ulcerated cancer is well known”* (Tuson 1844). Arsenical compounds were also very popular, The best known was Plunkett’s powder whose recipe was kept secret until published by the Plunkett family in 1760. Early seventeenth century treatments involved peculiar remedies including: *“Agrimony (agrifolia Eupatoria) The leaves and seeds are outwardly applied, being stamped with old swine’s grease helpeth old sores, cancers and invertebrate ulcers”* (Culpeper 1952).

Daviel (Figure 1.3), as previously described, was the first to recommend complete surgical excision of certain Noli-Me-Tangere and to recommend surgical margins although this was not widely practised for years. *“The seat of the cancers of the eyelids, nose and neighbouring parts are absolutely in the periosteum, perichondrium and fat”* and that *“There can be no hopes of cure without taking off these membranes, the fat or even parts of the very cartilages” that are contaminated.*” He also discussed the inadequacies of some treatments: *“Some are content to treat them with palliatives such as frog spawn water, and other such ingredients as serve only to amuse the patient, while others more bold, touch them with liquid caustics, or the lapis infernalis, from which they have no better success than the application of water”*(Daviel 1755).

In 1806 P.J.L. Bouchet pointed out the importance of early treatment of Noli-Me-Tangerie but warned of the dangers of neglect. *“When left untreated they always*

progress but the patients life may be spared for a long time as generalised cancerous involvement does not occur till late”(Bouchet 1806).

However surgery did not become popular until renaissance of plastic surgery which began in 1816 with Carpue’s account of forehead rhinoplasty (Carpue 1816). Spencer Wells described an advancement flap for a BCC in the medical and Times Gazette in 1854 (Spencer Wells 1854). Hutchinson used a forehead flap split into 2 parts for a BCC of the upper and lower lids in 1860 (Hutchinson 1860). Reverdin introduced free skin grafting in 1869 for repairing defects caused by ablating skin cancers (Reverdin 1870). Finally Wolfe described full thickness grafts in 1879 (Wolfe 1875). Moh combined surgery with zinc sulphate, as a fixative in the technique of chemosurgery in 1956 (Mohs 1956).

X rays were first used to treat a BCC in 1899 (Stenbeck 1900). Sequeira then established radiotherapy as a treatment of choice in 1901 in cases in which complete removal by the knife was impractical (Sequeira 1901). It was not until 20 years later that radionecrosis was recognised as a complication. Subsequently a variety of increasingly sophisticated techniques have been described including chemotherapy (retinoids and 5 fluorouracil) and laser treatments.

In this thesis the importance of early complete excision is emphasised and the use of a new “fire stick”- The carbon dioxide laser is also evaluated.

1.2 Definition of basal cell carcinoma

BCC is defined by the World Health Organisation Committee on the Histological Typing of Skin Tumours (Ten-Seldam and Helwig 1974) as a locally invasive, slowly spreading tumour arising in the epidermis or hair follicle and in which in particular the peripheral cells usually stimulate the basal cells of the epidermis. Although other terms such as basaloma or epithelioma have been used the term carcinoma seems justified due to the locally invasive behaviour (Jacobs, Rippey et al. 1982).

1.3 Histogenesis of BCC

Despite a multitude of studies investigating the cell of origin of BCC, the histogenesis has not unequivocally been determined. Virtually all dermal structures have, at some point in time been proposed as a potential source.

Epidermal origins were first implicated by Krompecher in his accurate histopathological study of BCC (Krompecher 1903). He regarded these tumours as carcinomas of the basal cells of the epidermis and attempted to classify them into 4 groups. Subsequently there has been considerable debate. Support for the epidermal origin outlined in Krompecher's thesis came from Montgomery, Willis and Teloh and Wheelock (Montgomery 1935; Willis 1945; Teloh and Wheelock 1949; Lever 1954). Kyrle explained the failure of the cells to keratinize on the basis that the basal layer has 2 functions. Firstly to differentiate into pavement epithelium and secondly to act as a matrix for sebaceous gland formation. He concluded that tumours from the cells with the first task were SCCs while tumours destined for the second task became BCCs (Kyrle 1915).

However many other theories have been proposed including the adnexal structures as the prime source of this tumour. Mallory considered the hair matrix, sebaceous and

sweat glands because he demonstrated fine epithelial fibres identical to those found in embryonic hair follicles (Mallory 1910). Haythorn supported Krompechers classification but opposed the epidermal origin theory. He concluded that they arose from the hair matrix due to the observation of perfectly formed hair shafts within the tumours and silver staining techniques revealed a basement membrane similar to that of a hair follicle. He expressed the belief that sebaceous glands but not sweat gland could also form BCCs. Haythorne questioned Krompechers belief that the cells of origin were from embryonal basal cells (and therefore did not differentiate into large prickle cells) because he considered that the tumour should be highly malignant if that were the case. To resolve this point he derived them from the pilosebaceous apparatus (Haythorn 1931). Nicholas and Favre also favoured this view (Lever 1949). Kraitz believed that only basal cells with a potential to develop glandular cells could transform into BCC (Lever 1954). Foot redirected the origin back to the dermal adnexal structures and suggested that the culprit was from the distorted primordia of dermal adnexae rather than from ordinary basal cells (Foot 1947). The tumours could then imitate the embryonal development of any of the adnexal structures and suggested the term adnexal carcinoma (first described by Haythorn).

An argument has also existed as to whether there may be more than 1 single origin. Warren assumed a multicentric origin, the tumours having several distinct points of growth from the overlying skin and occasionally from appendages and concluded that this was of no clinical significance (Warren 1872). Masden argued against a multicentric origin by pointing out that patchy tumour death from a unicentric source gives the appearance of many points of origin (Madsen 1942). Zackheim contended these opinions by reporting that the BCCs arose from multiple closely placed foci that fuse to form a single structure (Zackheim 1963). The origins of the foci were epidermal or the follicular root sheath. Subsequent computer 3D reconstruction now supports a unicentric origin (Lang, McKelvey et al. 1987).

The opinion that BCC was not a carcinoma, but a nevoid tumour of embryonal cells was first expressed by Adamson and introduced a novel theory of the histogenesis of BCC. It was proposed that BCCs were nevoid tumours originating from latent embryonal foci of pilosebaceous follicles or sweat ducts aroused from their dormant

state at a later period of life (Lever 1954). Wallace and Halpert also suggested that they were benign tumours of the hair matrix or anlage and therefore proposed the term Trichoma (Wallace and Halpert 1950). Glasunow was convinced that the distribution of the lesions corresponded so closely to the position of the embryonal facial fissures or folds as to prove the tumours of embryonal origin and called them facial fissure carcinoid (Lever 1954). Lever believes that BCC are not carcinomas because they do not metastasise. He also considered that it was unlikely that these tumours arose from any single dermal appendage because the majority exhibit differentiation towards a variety of structures simultaneously. Furthermore, the basal cell, as the cell of origin, seemed unlikely because firstly, of the absence of intercellular bridges in BCC, secondly that there is essentially little difference between squamous cells and basal cells apart from development and therefore unlikely that 2 different cancers can result due to malignant transformation of these cells. Lever therefore regarded that BCC as the least differentiated form of adnexal tumours and considered them nevoid tumours of embryonal primary epithelial germ cells which lie dormant until the onset of neoplasia (Lever 1954). Pinkus considered that the origin was from a different primitive cell, the immature pluripotential cells that form continuously throughout life and have the potential to form any of the dermal structures (Pinkus 1959).

Recent keratin studies have not completely resolved this issue. The keratin phenotype of BCC is in keeping with a follicular origin (lower outer root sheath) (Asada, Schaart et al. 1993) but also compatible with an interfollicular stem cell differentiating towards follicular structures (Markey, Lane et al. 1992). Electron microscopy data is also inconclusive but supported an epidermal origin (Reidbord, Wechsler et al. 1971).

Current theories that postulate that primary epithelial germ cells, as is the case with linear unilateral basal cell nevus or, in adults, a progenitor stem cell, result in most BCCs. These are slow cycling cells with great proliferative potential that proliferate via transit amplifying cells (Figure 1.4). Limited evidence suggests that these are situated in discrete locations such as the base of the rete ridges, bottom of the hair follicle or the bulge of the outer root sheath. These cells have not yet been fully characterised and therefore their relationship to BCC is unclear.

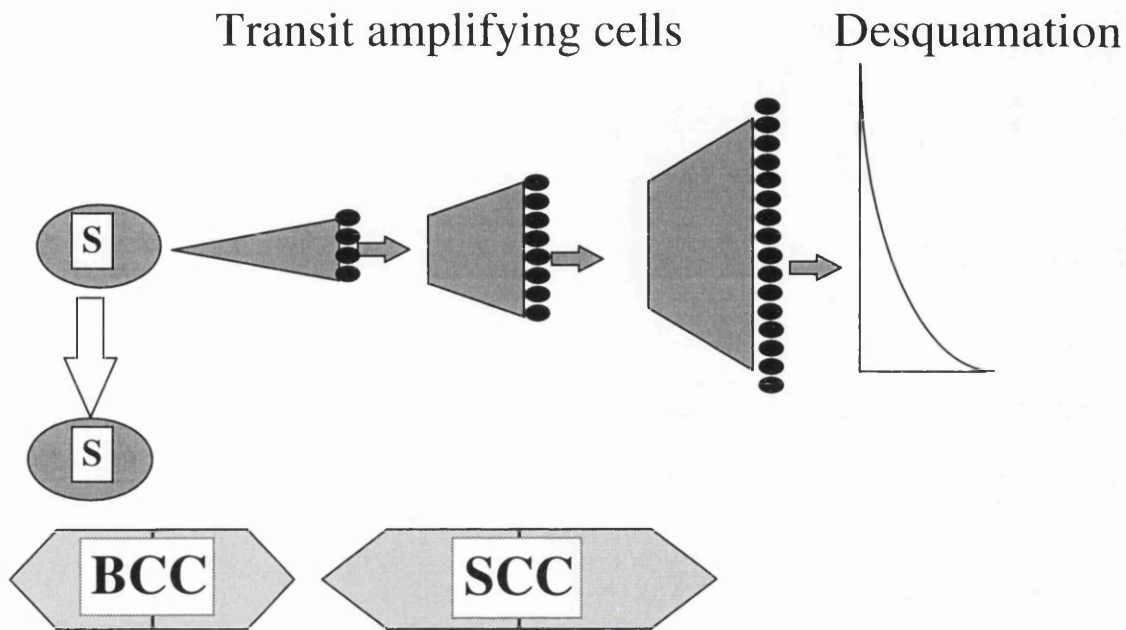


Figure 1.4. BCC may originate from a pluripotent stem cell or an early transient amplifying cell.

1.4 Incidence

The true incidence and prevalence figures for BCC in Great Britain are unknown since the Office for Population and Census surveys do not collect the figures for BCC. The term non melanoma skin cancer (NMSC) is used and refers to both BCC and Squamous cell Carcinoma (SCC) of the skin. The figures for NMSC are also not fully reported and are therefore inaccurate. BCC is considered the most common cancer that human beings acquire in a lifetime (Miller 1991). There are approximately 30,000 - 40,000 new cases per year in the U.K. In the USA there were about 1,200,000 new cases in 1995. The average incidence in the USA is 101 new lesions per 100,000 white persons. In the world (excluding the Soviet Union) there were 2.7 million cases in 1985 (Goldberg 1996). On a par with melanoma, the incidence of BCC has risen by

238% in 14 years (Ko, Walton et al. 1994). This increase has been attributed to 2% decrease in ozone, from chlorofluorocarbons, increasing the penetration of UVB through the atmosphere. The ratio of BCC:SCC:Malignant Melanoma is 32:4:1. The mortality of BCC is unknown.

1.5 Environmental risk factors

Epidemiological evidence suggests that sun exposure is the principal etiological agent for BCC. This is because:

- a) Annual incidence rates of BCC increase with increasing proximity to the equator. The annual incidence of BCC in Hawaii is 692 per 10,000 persons and in Iceland the figure is 10 per 100,000 (Stone, Elpern et al. 1986). World War 11 veterans posted in the Pacific had a greater number of BCCs than those posted in Europe. (Ramani and Bennett 1993) and it is recognised that the UV dosage per unit time is about 200% greater in the Pacific than in Europe (Fears 1983). Latitudinal variation is not only related to ultraviolet level but also due to behavioural differences (clothing habits and recreational exposure) due to weather and temperature.

- b) Non melanoma skin cancer is more common in outdoor workers (Marks, Jolley et al. 1989). As a result it has been considered that chronic sun exposure patterns determine the development of BCC. However migrant studies suggest that, like melanoma, heavy sunlight exposure in childhood contributes to the risk in adulthood. Whether or not there is childhood sensitivity of skin or whether a very large amount of the total dose is received at that age is not clear (Marks 1995). Some studies contradicted conventional wisdom and find no correlation between cumulative sun exposure and BCC (Vitasa, Taylor et al. 1990) (Gallagher, Ma et al. 1990). In 1 a history of severe sunburn in childhood and increased recreational exposure between the ages of 0-19 was a significant risk factor. Chronic occupational exposure was, however, associated with an increased risk of SCC (Gallagher, Ma et al. 1990).

c) The majority of tumours occur on sun exposed sites such as the head and neck (85%) with 30% occurring on the nose alone (Miller 1991). Some recent studies suggest that this trend may be changing with up to 40% of BCC occurring on other sites, with an increasing proportion on the upper trunk of younger people which may be due to altered clothing and sunbathing habits (Marks 1995).

Ultraviolet sources other than sun, such as PUVA treatment for psoriasis has also been associated with BCC (Reshad, Challoner et al. 1984). Other environmental agents include ionising radiation. This may be following therapies such as radiotherapy for malignancies or scalp irradiation for ringworm in childhood. Ionising radiation from atomic warfare has also resulted in the development of BCC (Albert, McGhee et al. 1984; Allison 1984; Ron, Modan et al. 1991; Sadamori, Mine et al. 1991). Arsenic is also an important etiological agent. This has been present in asthma remedies such as Bells mixture and also an important ingredient of Plunketts powder, previously described as a remedy for BCC.

1.6 The Biological effects of UV radiation

The putative carcinogen in sunlight is UV-B (280-320nm). UV radiation of a shorter wavelength of 200-280nm (UV C) is largely filtered by the atmosphere. The longer wavelength UVA (320-400nm) has less deleterious effects on human skin. UV-B has a multitude of actions that may produce or encourage malignant change and tumour progression. At shorter wavelengths the damage occurs due to absorption of UV by critical molecules such as the nucleic acids. At longer wavelengths indirect damage occurs due to the production of active intermediates from other chromophores such as the hydroxyl radical and the superoxide anion. The chromophores include porphyrins, quinones and flavins. Direct and indirect mechanisms effect damage on DNA, mitochondria, and membranes. Therefore defence of UV damage is by way of UV protection, DNA repair or by scavenging or antioxidant pathways.

1.6.1 DNA Damage

The peak absorption of DNA is dictated by the component nucleic acids but is approximately 260nm. Longer wavelengths do not cause direct DNA damage but can damage proteins. The mutation caused by UV-B has a characteristic signature (Figure 1.5). Two thirds of the substitutions are C-T substitutions and about 10% of these changes occur at 2 adjacent cytosine moieties. The UV light breaks chemical bonds between the pyrimidine bases. New bonds then form between the cytosines that disrupts their 3D structure forming a pyrimidine dimer. If DNA repair is not achieved then these changes can alter DNA integrity. During replication the 2 cytosines pair with adenines instead of guanines. During the next replication the adenines then pair with thymidine. Thus CC-TT substitutions take place.

Other non pyrimidine dimer damage can also occur. 6,4 photoproducts, purine photo products, single strand breaks and DNA-protein cross links have all been demonstrated. This may result in cell death or sublethal damage. If these changes effect important genes such as p53, patched or ras then malignant transformation may occur. The p53 mutation acts as an initiation step since cells do not apoptose when exposed to UVr and it also acts as a promoter since cell cycle arrest and DNA repair is not accomplished resulting in the accumulation of additional DNA damage.

1.6.2 Membrane damage

UV A and B can cause lipid peroxidation (the oxidation of unsaturated fatty acids to hydroperoxides) and this can cause membrane damage, altering permeability and transport systems of fibroblasts and keratinocytes.

1.6.3 Protein damage

There is evidence that some proteins are particularly sensitive to radiation in the solar UV range. These include aromatic amino acids in antioxidant enzymes, catalase and peroxidase. Repair enzymes are also sensitive to UVA radiation and this may play a role in mutagenesis.

1.6.4 Cytokine modulation

UV can lead to a strong enhancement in the release of several cytokines including IL-1,3,6 and TNF. These cytokines may modulate both the local and systemic immune response.

1.6.5 Alteration of immune function

It is believed that surveillance of somatic tissues for malignantly transformed cells is an important function of the immune system and is supported by increased number and increased aggressiveness of mutations in immunosuppressed populations.

Experimental evidence has supported immune alterations as mechanism for cancer development. Mice exposed to UVr develop skin cancers and also show reduced splenic antigen presenting cells (Noonan, Kripke et al. 1981; Kripke and Applegate 1990; Streilein 1993). UVr induced tumours are also rejected from identical unexposed mice. Unexposed mice also fail to reject UVr induced tumours and loose hapten induced contact hypersensitivity if they are given the lymphocytes from UVr exposed mice suggesting that it is a systemic rather than immune suppression. (Kripke and Morison 1986). A local immunosuppression can also be demonstrated by low dose UV exposure (Toews, Bergstresser et al. 1980).

Of particular interest is the genetic variation between strains of mice in which contact hypersensitivity has been impaired by UVr. This has resulted in the development of the terms UV susceptible and UV resistant as terms to describe the ability to lose or

maintain contact hypersensitivity after UV exposure. The susceptible phenotype is dominant and the resistant is recessive. Subsequent human studies have demonstrated the existence of similar phenotypes. In mice, these phenotypes are determined by polymorphic alleles in 2 loci, namely TNF α and Lps. Both of these loci are involved in the control of TNF α production. It has subsequently been demonstrated that TNF α modifies the immune system by impairing the function of Langerhan cells (Streilein, Taylor et al. 1994). Recent work suggests that urocanic acid, which is present in the stratum corneum may be the photoreceptor mediating the immunosuppression. UV-r isomerises urocanic acid from the trans to the cis form. This the has an immunosuppressive action by upregulating TNF α production (Streilein, Taylor et al. 1994).

As previously mentioned UV susceptible and UV resistant phenotypes occurs in humans. About 40% of Caucasians are UV susceptible. In one study, 93% of patients with NMSC and 100% of patients with melanoma were UV-r susceptible (Streilein, Taylor et al. 1994). This may be genetically determined by polymorphic loci that determine skin type and pigmentary factors but also by other loci that govern cell mediated immunity as in mice.

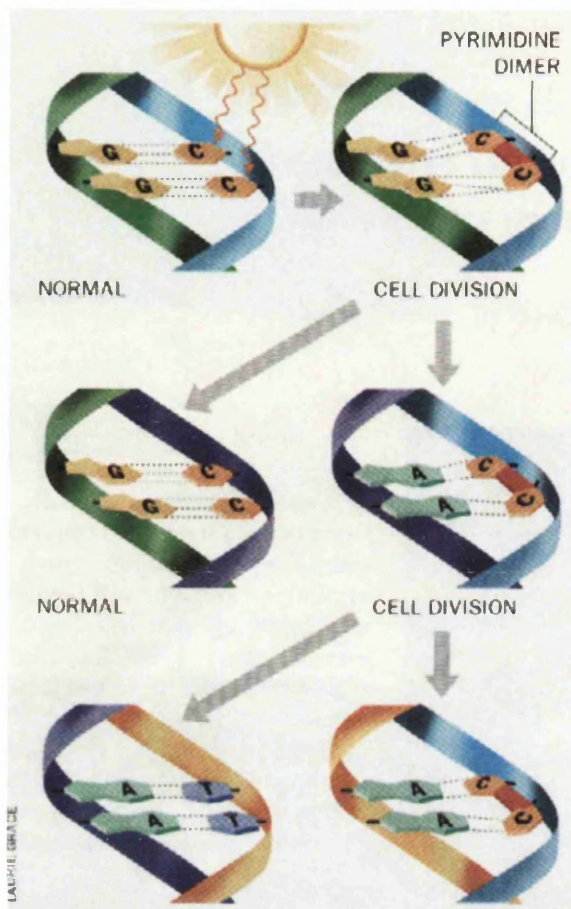


Figure 1.5. The mechanism of UV induced CC-TT substitution.

1.7 Constitutional Risk Factors

99% of persons who develop BCC are white and 95% are between the ages of 40 and 79 years (Kopf 1979; Roenigk, Ratz et al. 1986). BCC is slightly more common in males. The age specific incidence of NMSC in males is double that of females (Scotto, Kopf et al. 1974). Race and ethnic group are also important risk factors. Epidemiological studies suggest that it is the photoprotective effects of melanin that accounts for these differences. There is increased risk in whites, especially those with blue eyes, fair complexion, sunburn easily, suntan poorly, freckle with sun exposure, have red blond, or light brown hair or are of Celtic ancestry (Scots, Irish, Welsh). 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 (Gloster and Brodland 1996). The incidence of NMSC is 70 fold greater in whites than blacks (232.6 per 100,000:3.4 per 100,000) (Scotto, Kopf et al. 1974). Albino blacks develop more BCCs than normally pigmented blacks. Most BCCs in blacks are pigmented which can lead to delayed diagnosis, although it is reported that the BCCs are more aggressive. Multiple BCC is rare in blacks and usually occurs in patients with Gorlin's syndrome.

Immunosuppression (iatrogenic, malignant, or infective) increases the risk of BCC. The effect is more pronounced with SCC, therefore the ratio of SCC to BCC in transplant patients reverses from 1:4 to 4:1. Traumatic scars may also result in BCCs. They have been reported in scars (Ewing 1971), burn scars (Castillo and Goldsmith 1968; Horton, Adamson et al. 1969; Hursthouse 1969; Kantor, Berger et al. 1970; Bashir 1976; Earley 1983; White 1983; Wiener and Scher 1987), tattoos, vaccination scars, and chronic ulceration.

1.8 Defence against UV damage

1.8.1 DNA repair

Human populations display a range of inherent susceptibilities to cancers when given a common carcinogenic insult. Some people will not develop a malignant or premalignant lesion while others may develop a multitude of cancers. DNA repair mechanisms are responsible for repairing DNA damage to potential proto oncogenes or tumour suppressor genes following a carcinogenic insult. Differences in cancer susceptibility may be explained by variations in DNA repair mechanisms. This results in persistent DNA damage and oncogene activity. The importance of DNA repair as a mechanism for UVr protection is exemplified by the DNA repair disorders such as Xeroderma pigmentosum (Section 1.9).

A variety of mechanisms exist for repairing different types of DNA damage. The mismatch repair system acts on single base mismatches and small displaced loops of 4-5 base pairs. Mutations in genes in this pathway occur in patients with Hereditary non polyposis coli colonic carcinomas. However, it is the nucleotide excision repair pathway that detects and repairs ultraviolet photoproducts. A variety of genes in this pathway are mutated in Xeroderma Pigmentosum. Furthermore, it has been demonstrated that patients who develop BCCs have a reduced DNA repair capacity compared to normal subjects and also patients with a family history of BCC or actinic keratosis have reduced DNA repair capacity. DNA repair capacity has also been demonstrated to fall with age. The maximum rate of pyrimidine dimer repair in normal skin cells is reported to be 5×10^4 cells per hour, a rate that can barely cope with the damage imposed by full sunlight. Hence a 25% relative decrease in repair capacity seen with age would facilitated the accumulation of persistent DNA damage that may activate potential oncogenes (Grossman and Wei 1995).

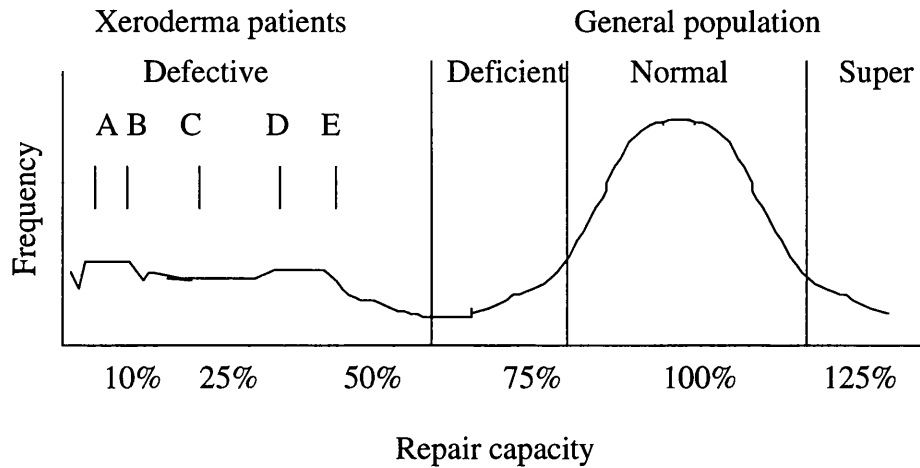


Figure 1.6. Theoretical distribution of DNA repair capacity among populations (Grossman and Wei 1995).

1.8.2 Antioxidant pathways

Antioxidants defend against reactive oxygen intermediates that arise at longer UV wavelengths. These intermediates can damage DNA, membranes and proteins. Glutathione is present in most mammalian cells in high concentrations and plays a crucial role in the protection of cells against oxidative damage. The exact mechanism of protection is not known, however, it involves in the detoxification of hydrogen peroxide or organic peroxides, the quenching of free radicals and the cycling of free radical reductases. Antioxidant vitamins including A, C and E have a multitude of antioxidant functions although the exact mechanisms require further characterisation. A variety of water soluble antioxidants are present in the plasma including glucose, pyruvate, uric acid, ascorbic acid and bilirubin.

Antioxidant proteins and enzymes include superoxide dismutase (which converts the superoxide ion to hydrogen peroxide), catalase (destroys hydrogen peroxide) and glutathione peroxidase. These enzymes are present in the skin but their exact role in UV protection is not fully elucidated at present.

1.9 Cancer Syndromes and Basal Cell Carcinoma

A group of genetic diseases exist which predispose an individual to the development of multiple primary tumours. Investigation of these diseases have provided invaluable information regarding the biological and genetic events that determine the development of cancers. There are 4 syndromes which predispose to the development of multiple BCC.

1.9.1 Naevoid Basal Cell Carcinoma Syndrome (Gorlin's syndrome)

This syndrome is a highly penetrant autosomal familial cancer syndrome in which affected individuals are predisposed the development of multiple BCC at an early age. They may develop thousands of BCCs in a lifetime. About 30-50% of patients do not have a family history suggesting that there is a high rate of spontaneous mutation. Segmental or unilateral disease can occur which is probably attributable to mutation in 1 cell of and early embryo (Gutierrez and Mora 1986). The essential components of this syndrome were described by Binkley and Johnson in 1951 and by Howell and Caro in 1959 (Binkley and Johnson 1951; Howell and Caro 1959). Gorlin and Goetz described the syndrome in 1960 (Gorlin and Goltz 1960) and subsequently their name became attached. In addition to multiple BCC can patients develop other cancers including medulloblastoma, meningioma, ovarian or cardiac fibroma. There are often skeletal abnormalities including otontogenic keratanocysts, bifid ribs, frontal bossing, polydactyly and generalised overgrowth. Intracranial calcification may occur and there is characteristic pitting in the palms and soles of the feet. Expression of the features vary both within and between families which probably reflects different mutations, modifier genes or environmental factors such as sunlight exposure.

The gene responsible for the syndrome was mapped to chromosome 9q22-31 (Farndon, Morris et al. 1994). Subsequently this region has been found to code for the human homologue of the Drosophila polarity gene, *patched* (Bale, Gailani et al.; Gailani, Bale et al.). It is considered that the *patched* gene acts a tumour suppressor gene. The inherited loss of function of 1 homologue, in the germ cell, does not result

in BCC, although it does result in developmental abnormality. The loss of the second homologue is required for carcinogenesis. Subsequent studies have shown that sporadic BCCs also have patched mutations in approximately one third of cases which suggest that patch inactivation is important in sporadic BCC also (Gailini and al 1996).

1.9.2 Xeroderma Pigmentosum

This is a rare autosomal recessive genetic disease, first described by Kaposi in 1874. Its prevalence in the USA is estimated at 1:250,000. Patients are extremely sensitive to sunlight and experience a 2000 fold increase in sun related skin cancers. There is a spectrum of disease depending on the Xeroderma pigmentosum subtype (XPA-XPG) When an infant with XP is exposed to sunlight they are uncomfortable and develop persistent erythema on all exposed sites. There may be photophobia. This is followed by freckeling and brown macular pigmentation. If the skin is not protected it will develop features suggesting accelerated ageing, then the development of actinic keratosis followed by melanoma and NMSC in childhood. Death in the second or third decade was common before the introduction of effective sunscreens. Other generalised features include bird like facies with a pinched but protuberant nose, ocular deterioration from keratitis and corneal vascularisation, central nervous system degeneration with growth retardation, cerebellar ataxia and spastic quadriplegia.

The disease is due to defective DNA repair by the nucleotide excision and repair genes. A variety of different enzymes exist in this pathway. Each disease subtype is related to a specific enzyme deficiency. It is this repair system that is responsible for the restoration of DNA structure following pyrimidine dimer formation due to sunlight. In these individuals DNA repair is normal following X-ray exposure since other DNA repair systems are invoked to deal with the double strand breaks.

1.9.3 Bazex's syndrome

This is a rare X linked syndrome that predisposes individuals to multiple BCCs at an early age. Additional features include atrophoderma resulting in “ice pick marks”, hypotrichosis and hypohidrosis. The disease has been mapped to chromosome Xq24-27 (Vabres, Lacombe et al. 1995). The relationship between BCC and abnormalities of the hair follicle is of great interest since follicular keratinocytes are considered a the cell of origin of BCC.

1.9.4 Muir-Torre Syndrome

Originally describe in 1976, this syndrome is characterised by the presence of 1 or more sebaceous neoplasms in association with 1 internal malignancy (Colonic tumours most frequently). The clinical and pathological features overlap with hereditary non-polyposis colorectal cancer syndromes. The tumours of either syndrome show the same characteristic microsatellite instability. Furthermore it has been shown that the mismatch repair gene MSH2 is mutated in both syndromes. Other tumours including BCC and SCC are found in Muir-Torre syndrome, however, it is not fully established whether they are an integral part of this condition or incidental.

1.10 Premalignant lesions of basal cell carcinoma

Unlike SCC, in BCC there are no sun induced premalignant lesions such as Actinic Keratosis or Bowen's disease. In SCC, this progression exemplifies the multistage model of carcinogenesis, namely initiation, promotion and progression. BCCs usually arise de novo, however there are 2 unusual lesions in which BCCs can develop.

1.10.1 Fibroepithelioma of pinkus

This is a tumour composed of strands of epithelial cells that are in contact with the overlying epidermis, admixed with a fibrous stroma. They usually occur on the lower back appearing as flesh coloured tags. Basal cell carcinomas are reported to develop in a variable portion of these lesions.

1.10.2 Nevus sebaceous (Borst-Jadassohn epithelioma)

This is a nevus composed of an abnormal number of skin appendages that has the propensity to develop a basal cell carcinoma in approximately 10% of cases. They are usually present at birth as a raised papular, yellow area and are most commonly found on the scalp. Histologically increased numbers of sebaceous and eccrine glands are found. The hair follicle element of the pilosebaceous unit is generally under represented. Syringocystadenoma papilliferum may also develop in these nevi.

1.11 Clinical features of basal cell carcinoma

In general, BCCs tend to present as slowly growing lesions that begin as a lump, an ulcer, bleeding or small non healing lesion. Some may appear as a crusting erythematous lesion. A multitude of classification systems have been developed. Basal cell carcinomas may be primary or recurrent. A third clinical type the horrifying tumour (also called giant, aggressive or mutilating) is a useful term and is used in this thesis. Basal cell carcinomas may also be classified according to the clinical appearance, histological manifestations or a combination of both. As a consequence of the variety of different classifications, confusion may arise resulting in inconsistencies between studies. In order to simplify the abundance of descriptive terms, it is practical to classify BCCs firstly, according to clinical and then secondly according to histological classifications. This is because the clinical appearance may not always correspond to the histological findings (See Chapter 7). In this scheme the

features of pigmentation and ulceration are not applied separately since these features can be seen on all of the subtypes (Emmett 1990).

1.11.1 Clinical classifications

The simplest clinical classification has recently been highlighted (Goldberg 1996) and divides BCC into 5 distinct groups. There is not always correlation with the clinical and histopathological findings. A similar clinical classification has been described by Emmett with subdivision according to the microscopic features (Emmett 1990).

a) Superficial basal cell carcinoma

These tumours are red, finely wrinkled and scaled with small superficial ulcerations (See Figure 1.7). They often have an ill defined or scalloped border. At close examination a pearly edge is often seen if the skin around the BCC is stretched. They may resemble subacute or chronic dermatitis. Superficial BCC tend to be situated on the trunk. Their incidence is approximately 35%. Histologically the tumour cells appear as multiple buds growing downward from the epithelium with abnormal or fibrosed skin between the tumour cells. Macroscopically this may give rise to a leading edge of tumour spreading outwards from a scarred central area. This appearance has led to the development of the term Forest Fire BCC. Debate exists as to whether the multifocal appearance is a true finding or an artefact due to the vertical sections (Lang, McKelvey et al. 1987).

b) Nodular BCC

Nodular BCCs are the commonest clinical type and found in 45% of a large series (Emmett 1990). This type presents as a pearly papule or nodule within the skin that may ulcerate centrally or bleed (Figure 1.9 and Figure 1.20). Fine vessels may run over the surface. If there is no ulceration they are soft and appear cystic. Hence the term nodulocystic BCC has evolved. The term cystic should however be used for

histological rather than macroscopic appearances only. The nodular BCC may display a variety of histological features.

c) Morpheic BCC

Morpheic BCC present as a white patch or plaque due to the fibrotic response that results (Figure 1.11). They are usually flat but close examination by stretching the skin often reveals a pearly margin. Histologically cords of tumour are seen in a dense fibrous stroma. They are not well circumscribed and therefore the tumour margins may be difficult to assess visually which can result in higher incomplete excision rates.

d) Infiltrative BCC

8% of primary BCC are infiltrating (Emmett 1990). There is no characteristic clinical appearance. They present as a red or grey scaling area. There may be some ulceration. These tumours tend to deep infiltration and may have less fibrous tissue reaction around them which also makes visualisation of the margins difficult (Figure 1.12). The dermis is less altered than other tumours which may result in later presentation. There is often a morpheic component present. Approximately 16% of other tumour subtypes may have an infiltrating component present.

e) Basosquamous (metatypical) BCC

These tumours are rarer (incidence 1.4%) and have the clinical and histological appearance of both BCC and SCC. Histologically there are features of SCC in conjunction with BCC. The question remains as to whether this type metastasise more commonly.

1.11.2 Staging

Carcinomas of the skin are staged according to a TNM classification (see Table 1). This system is utilised in some publications.

PRIMARY TUMOUR	TX	CANNOT BE ASSESSED
	T0	No evidence of primary tumour
	Tis	Carcinoma in situ
	T1	>2cm
	T2	2-5cm
	T3	>5cm
	T4	Involves extradermal structures (muscle, bone , cartilage)
Regional Nodes	Nx	Cannot be assessed
	N0	No regional nodes
	N1	Regional nodes present
Distant Metastasis	Mx	cannot be assessed
	M1	Distant metastasis

Table 1.1. The TNM classification of skin cancers.



Figure 1.7 A Superficial BCC



Figure 1.8 A forest fire BCC



Figure 1.9 A nodular BCC



Figure 1.10 A pigmented nodular BCC



Figure 1.11 A morpheic BCC



Figure 1.12 An infiltrating BCC.

1.12. Histological Features

1.12.1 General

In tandem with the huge variation in clinical appearance there is also an extensive diversity morphology. Accordingly a multitude of terms, classifications and descriptions have been applied. These have been based on the perceived cells of origin, accepted differentiation status and growth patterns. This section reviews the pitfalls and benefits of classification systems, histological features and culminates with we consider to be the optimum classification system .

Certain features are shared between most subtypes. Commonly tumours are composed of islands or nests of basaloid cells with palisading of the cells at the periphery and a haphazard arrangement of cells in the centre of the islands. The cells have a hypochromatic nucleus with poorly defined cytoplasm. Intercellular bridges usually are not seen on light microscopy. Numerous mitotic figures and a correspondingly large number of mitotic figures have been described (Kerr and Searle 1972). Although this point is examined closely in this thesis. The majority of tumour islands show some attachment to the epidermis although some tumours show deep extension even into bone or cartilage. Perineural invasion is rare (Mark 1977). The tumours are surrounded by a characteristic stroma which separates them into different patterns. The stroma contains variable amounts of acid mucopolysaccharides Laminin and collagen types 4,5and 7 are present in the basement membrane that separates the tumour cells from the stroma (Van Cauwenberge, Pierard et al. 1983; Jones, Steinman et al. 1989). Amyloid deposits are present in the stroma of about 50% of tumours (Looi 1986). The adjacent dermis shows solar elastosis in about 90% of cases. The overlying epidermis may also show the changes of solar keratosis.

A variable inflammatory infiltrate is found. It is usually composed of T cells, the majority of which are of the helper/inducer type (Habets, Tank et al. 1988; Habets, Tank et al. 1989). Natural killer cells and Langerhans cells are also present. A plasma

cell infiltrate correlates with ulceration (Emmett 1982). Regression is associated with lymphocytic infiltrate that penetrates the tumour nests and disrupts the palisading edge resulting in apoptotic tumour cells. Past regression is seen by areas of new collagen deposition within the tumours. Clinically scarring is seen in the tumours (Curson and Weedon 1979).

1.12.2 Classifications

As with the histogenesis of BCC there has been much debate and confusion regarding its classification. Krompecher was the first to classify the tumour into 4 types (Krompecher 1903).

- i) Solid pouch like down growths of the epithelium*
- ii) Epithelial masses containing cysts*
- iii) Glandular form made up of interlacing strands of epithelial cells*
- iv) Nests of epithelium formed parakeratotic pearls*

In error he included tumours of the mucous membranes (SCCs). Owen recognised that these tumours were different and excluded these from the group (Owen 1930). Darrier and Fernand and subsequently Montgomery recognised basal cells carcinomas with features of squamous cell carcinomas and named them basal squamous cell epitheliomas (Darrier and Ferrand 1922; Montgomery 1935). Foot proposed a classification based on the adnexal theory of origin and proposed 3 main types (Foot 1947). (He did not include basal squamous cell tumours). The classification is as follows:

- i) Pilar* (imitates the architecture of a hair follicle)
 - Proper
 - Primordial
 - Cylindrical or ribbon
 - Cystic
- ii) Sudiparous* (containing onion like bodies of concentrically arranged squamous cells)

Adenoid

Hydradenomatous

iii) *Basal celled* (composed of elongated fusiform cells that grow downward from the rete pegs)

Lennox and Wells criticised this type of classification because of the difficulty of placing a tumour in any 1 group due to the variety of subtypes that could be found within each tumour; therefore making the system unusable. Instead they divided tumours according to the predominance of one of the following 4 features (Lennox and Wells 1951):

i) *Palisading*

ii) *Pigmentation*

iii) *Fluid formation*

iv) *Whorls*

While this type of division is of descriptive interest there was no correlation with clinical behaviour. Lever's classification, which remains the most popular and widely used classification system, at present, has the same pitfalls as the previously described systems. i.e. That it contains no information significance and that it is difficult to fit a tumour into any one single category. Lever's classification divides BCCs into 2 groups; undifferentiated and differentiated (Lever 1949).

i) *Undifferentiated (solid)*

These are a corresponding group to Foot's Primordial type. Various sized tumour masses of cells with a peripheral palisade and central haphazard arrangement are found. There may be contact with the epidermis. There may also be variation in cell size and basophilic staining.

ii) Differentiated

Keratic. -In addition to undifferentiated areas there are parakeratotic cells with elongated nuclei and eosinophilic cytoplasm which represents some keratinization. These cells surround horn cysts. These are composed of fully keratinized cells surrounding a keratin filled space which represents attempts at hair shaft formation.

Cystic:- Cystic spaces develop due to tumour cell degeneration or stromal degeneration or sebaceous differentiation.

Adenoid:- Gland like or tubular structures are present arranged in strands or radially around stroma resulting in a lace like pattern.

Lever's classification did not neatly incorporate morpheic or superficial subtype as distinct histological variants but included them as a clinicohistopathological combination that followed the histological definitions. In this account noduloulcerative types are described as comprising any of the described histological features. Pigmented tumours are noted to contain melanin granules, melanocytes and melanophages. The morpheic variant is portrayed as having increased connective tissue proliferation in which are narrow strands of tumour cells. Superficial tumours show buds and irregular proliferations attached to the underside of the epidermis. Palisading is present. They do not penetrate deeply unless they have persisted for long periods of time. The Premalignant Fibroepithelioma and Nevoid Basal Cell Epithelioma Syndrome are also included in this classification. Lever disputed the existence of basal squamous cell carcinoma but conceded that mixed forms can arise with the development of a secondary SCC in a pre-existent BCC.

Pickering modified and simplified the previous schemes in an attempt to find a clinically useful method which would direct treatment (Pickering and Nickel 1957). He did not, however include any data to substantiate his concept. The tumours were divided according to clinicohistopathological criteria into cystic, pigmented, superficial, iceberg or submarine and sclerosing. This depended on appearance,

position and morphology. These types were in accordance to Lever's descriptions apart from the submarine type which was described as a deeply infiltrating cystic adenoid type found in the nasolabial fold.

Thackary introduced and developed a novel classification system based on their "habit of growth" or marginal characteristics (Thackary 1951). He described them as:

- i) *Group 1* Circumscribed with a well marked palisade layer and basement membrane often with evidence of differentiation
- ii) *Group 2* Intermediate between group 1 and 3.
- iii) *Group 3* Infiltrative tumours seen as streaks or columns invading connective tissue planes usually with absent basement membranes.

This was the first classification system that was based on the growth pattern. The data also demonstrated the prognostic value of this method. The infiltrative tumours with the poorest and circumscribed the best prognosis. This is the basis of most modern, clinically useful systems.

Sloane improved and expanded this system to 4 subtypes (Sloane 1977).

- i) *Nodular*:- Well circumscribed nodules with a rounded or pushing edge
- ii) *Nodular*:- With infiltrative margin
- iii) *Infiltrative*: Poorly circumscribed with an infiltrative rather than expansile growth. The tumour islands may be well separated and have spiky edges. The palisade layer is poorly formed.
 - Sclerosing:- With an extensive fibrotic stroma
 - Non sclerosing:- Only a thin rim of stroma around the tumour islands.

iv) *Multifocal*:- The tumours were apparently multifocal in the plane of section.

The infiltrative and multifocal types were demonstrated to have a higher recurrence rate. In the same year an atlas showing 26 histologically variants of BCC was

published (Wade and Ackerman 1978) and although beautifully descriptive has no bearing on prognosis and is therefore clinically inconsequential.

A further modification has been published by Emmett who divides BCC into localised and diffuse groups (Emmett 1990) (Table 1. 2).

TYPE	SUBTYPE	PERCENTAGE
LOCALISED	Papulonodular	45
	Solid	
	Cystic	
DIFFUSE	Infiltrating	8
	Multifocal	35
	Superficial	
	Multifocal superficial	
	Cicatrising (field fire)	
	In depth	
	Morpheic	8.9
	Metatypical	1.4

Table 1.2. Emmett's Classification based on analysis of 2277 BCCs (Pigmented, ulcerated or Gorlin's tumours can be any type) (Emmett 1990).

Confusion arises in the multifocal category especially in the distinction between the multifocal superficial and the superficial subtype. Indeed multifocal in depth is also confusing bearing in mind the islanded nature of most BCCs.

Jacobs, while studying aggressive tumours produced an alternative classification, again based on the growth pattern which had prognostic value (Jacobs, Rippey et al. 1982). This was based on 3 major types, each containing subtypes. (See Figure 1.13)

A recent classification system has been published (Sexton, Jones et al. 1990), also based on the growth pattern and therefore is of prognostic value. The term micronodular was introduced. This in part replaces tumours that would previously be described by Emmett as multifocal in depth and therefore allows the term superficial to remain uncomplicated. The groups are:

i) Superficial in which multiple tumour peninsula extended from the epidermis and/or adnexae and abutted or penetrated the papillary dermis.

ii) Nodular which consisted of a rounded mass of tumour cells with a well defined contour and peripheral pallisading.

iii) Micronodular in which the tumour islands tended to be smaller and more numerous than the nodular type but still retained a uniform contour.

iv) Infiltrative, was characterised by an irregular contour to the tumour islands with a tendency to form spikes. The tumour islands could be large or small usually with poorly developed peripheral pallisading.

v) Morpheic. In this subtype, the tumour islands were small and elongated with jagged ends and the stroma is sclerotic. The neoplastic tissue infiltrates as narrow strands or cords of cells.

This classification is illustrated in Figure 1.14.

1.12.3 The classification of BCC utilised in this study.

In this present a classification similar to the above (Sexton, Jones et al. 1990) was utilised because the tumours could be simply placed into a single category and it was postulated that this may carry some useful prognostic information (See Chapter 4). In addition, for completeness, the tumours were also classified according to their differentiation status (Lever). This resulted in a fully comprehensive system which was fully descriptive, useable and also with prognostic significance. We considered that this was the most optimal system that, at present, has been described in the literature.

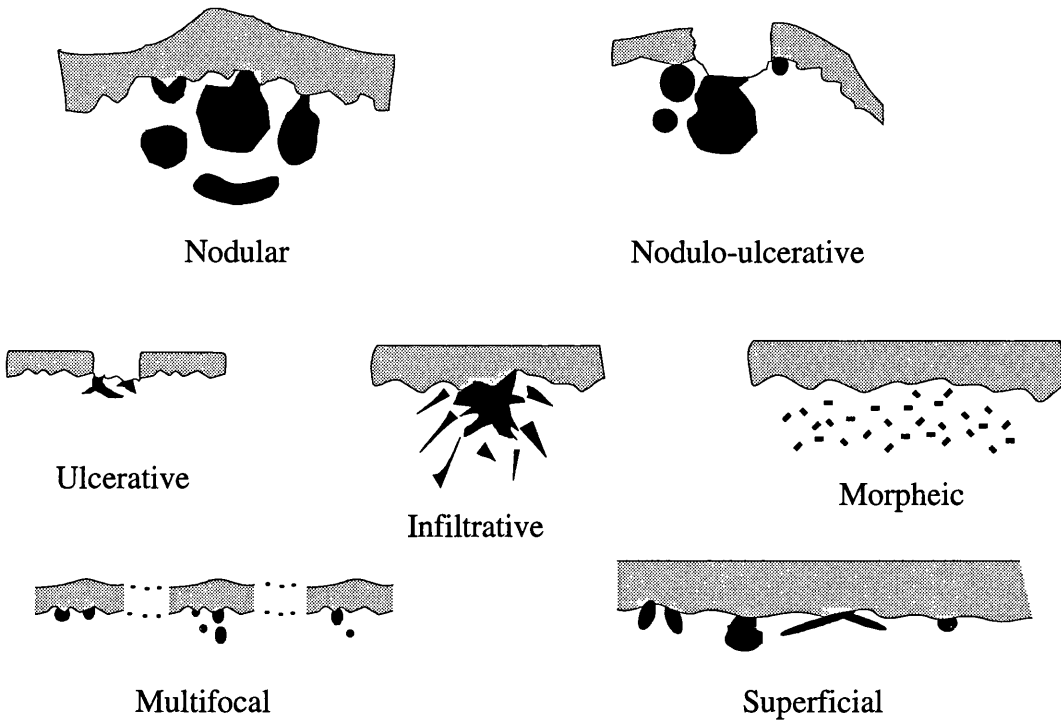


Figure 1.13. A proposed classification of growth pattern for BCC. (Jacobs, Rippey et al. 1982)

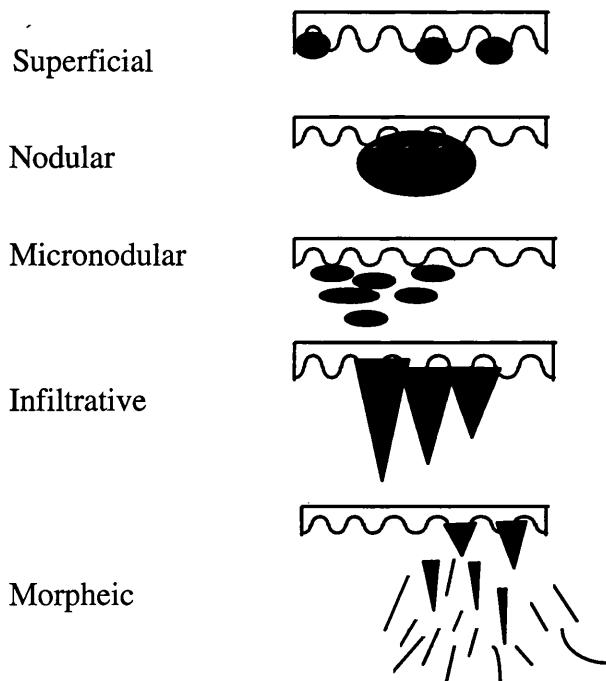


Figure 1.14. The classification of BCC utilised in this study. The figure is based on descriptive terms by Sexton (Sexton, Jones et al. 1990).

1.13 Trends and Prognostic Markers in BCC

The ability to determine prognosis is fundamental to the management of patients with malignant disease. Basal cell cancer is an unusual example because, in the absence of metastatic spread, prevention of local recurrence is of the greatest importance. Combined with the fact that 80% of these tumours occur on the head and neck, a cosmetically very sensitive area where recurrence may result in increased disfigurement adds to the final objective for cure at first attempt. Furthermore recurrent tumours tend to be of aggressive subtype which may hinder secondary treatment and hence recurrent-recurrent rates have been known to reach 40%. A small proportion of tumours that may have been cured by relatively simple methods may then require aggressive and disfiguring treatment modalities in order to achieve cure. A smaller proportion may become incurable. Prognostic markers may facilitate the choice of treatment modalities and guide treatment planning at an early stage to minimise treatment side effects and maximising cure rates. In order to study prognostic features clinical and histological characteristics of primary recurrent and aggressive tumours have been evaluated. A major difficulty in analysing recurrent and aggressive BCCs is the paucity of information regarding the type and adequacy of the original treatment. Thus the data not only reflects the tumour biology but also the treatment efficacy and the competence of medical staff. Therefore, factors regarding recurrence are discussed as generalities, followed by treatment related factors in more detail.

1.13.1 Primary basal cell carcinoma (non recurrent)

The primary BCC is a tumour that presents having received no previous treatment. A variety of general factors are important. These include:-

1.13.1.1 Age and sex

Primary BCC occurs most commonly in adults aged between 60 and 70 years. Although there is wide age range (Figure 1.15). In a small plastic surgical series a

younger age distribution was reported with 23% occurring in patients under 40 (Hauben, Zirkin et al. 1982). This may represent referral patterns rather than true incidence.

1.13.1.2 Anatomical position

Depending upon the published series, the sex distribution is approximately equal (Betti, Inselvini et al. 1995). The anatomical distribution is shown in Table 1.3. The face is the commonest site. Midfacial tumours are most common with lower face the least common.

	Primary		Recurrent	
	Kopf (3531)	Koplin (3054)	Kopf (273)	Koplin (164)
POSITION				
Scalp	4%	11%	16%	10%
forehead	15%	7.5%	14%	2.5%
Periorbital	5%	14%	8%	2.1%
Nose	30%	25.5%	18%	38%
Face	21%	-	15%	
Chin	-	2.5%		2.5%
Cheek	-	16%		18%
Lips	-	6%		2%
Ear	7%	11%	21%	4.5%
Neck	4%	7%	9%	2.5%
Trunk	10%	7%	11%	3%
Upper Limb	3%		18%	
Lower Limb	2%		6%	

Table 1.3. Anatomical distribution of BCC and percentage of recurrences reported in 2 large series.

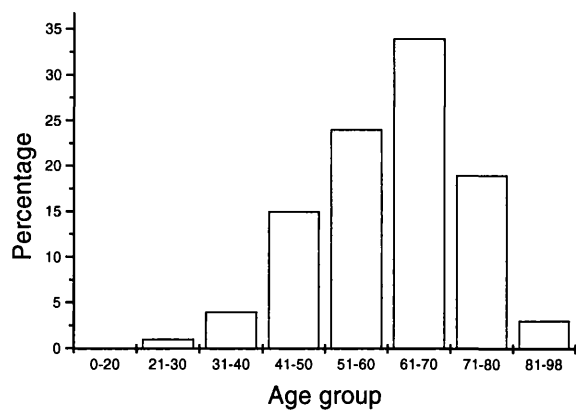


Figure 1.15. The age distribution of 2654 patients with BCC (Kopf 1979).

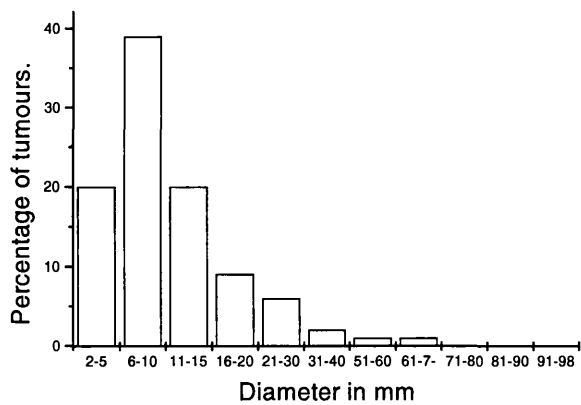
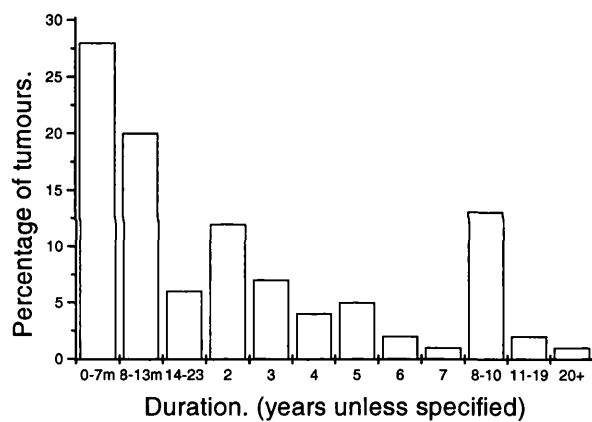


Figure 1.16. Size and duration of 3531 BCCs (Kopf 1979).

1.13.1.3 Size and duration

Over half of the patients will perceive they will have had the tumour for under 1 year although there is a wide range with some patients presenting up to 20 years after developing a BCC. 80% of patients present with a lesions of less than 15mm (Kopf 1979) (Figure 1.16.)

1.13.1.4 Histology

Variation in classification hinders a precise account of the incidences in clinical type. In general the papulonodular type (including noduloulcerative and cystic descriptions) are the most common and represent 45-60%. The superficial (or multifocal) represent approximately 8-10%. Infiltrating types are reported at 8%. Morpheic (sclerosing) are recorded between 2-9% and metatypical at 1-2 %. 1-6% of BCCs are pigmented (Kopf 1979; Hauben, Zirkin et al. 1982; Emmett 1990). There is no relationship between type and age. Nodular tumours are found most commonly on the nose (83% of nasal BCC) and less frequently on the extremities (31%) or trunk (41%) (Betti, Inselvini et al. 1995) The superficial subtype however are found most commonly on sun unexposed areas such as the trunk (42%) and extremities (44%). The morpheic subtype is reported to be equally distributed over all body sites except the anterior chest and abdomen. Inconsistencies in histological classification systems also hampers accounts of histological variants. According to the classification system previously described by Sexton the ranking for histological subtypes is as follows; nodular 21%, superficial 17%, micronodular 15%, infiltrative 7% and morpheic 1%. However a 38% of tumours in this series were of mixed pattern which prevents true estimates of the incidence of the dominant subtype. The proportions, however are in agreement with the classification system described by Emmett (See Table 1.2 in previous section). Another study reported 69% of facial BCCs as solid, 18% as adenocystic and 13% as morpheic (Hauben, Zirkin et al. 1982).

1.13.2 Recurrent BCC

A recurrent BCC is defined as the reappearance of a histologically verified BCC within or contiguous to the scar resulting from the treatment procedure. The cumulative rate of appearance of recurrences is shown in Figure 1.17. The history of recurrence is therefore over a 10 year period since about 20% occur between year 6 and 10. However 66% recur within the first 3 years.

1.13.2.1 Age and sex

Increasing age has been associated with recurrence in patients treated with curettage and electrodesiccation and not in those treated with radiotherapy or surgery (Kopf 1979). Patient sex has no influence (Kopf 1979) except in 1 series where young women were had a higher incidence than men of the same age (Robins and Albom 1975). Rigel found a significant difference for both age and sex (greater recurrence in male under 50) in post Mohs surgery recurrence (Rigel, Robins et al. 1981).

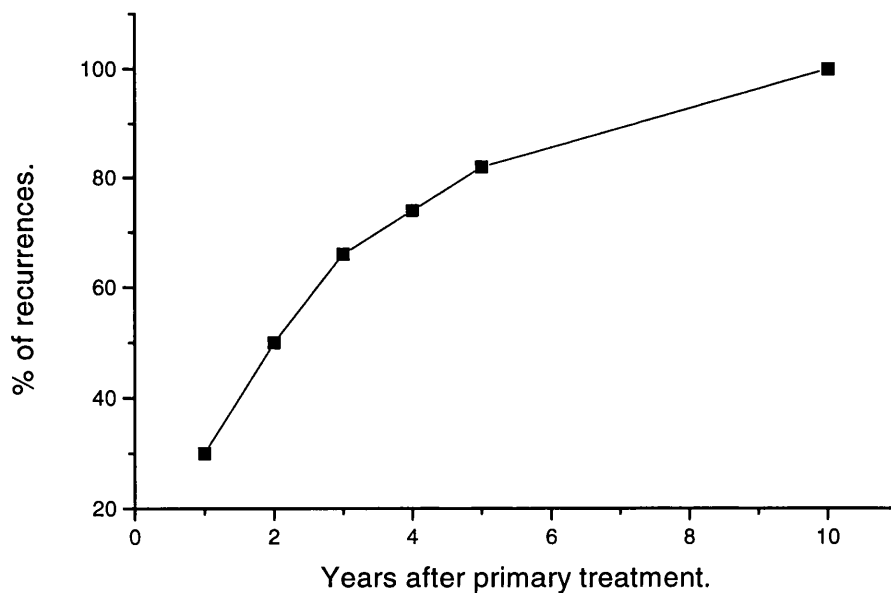


Figure 1.17. The cumulative rate of appearance of recurrent BCCs after primary treatment (Rowe, Carroll et al. 1989).

1.13.2.2 Position

There is considerable variation of recurrence rates with tumour position. One study showed that 86% of primary BCC occurred on the face but that 95% of recurrent lesions are found in this site (Koplin and Zarem 1980). The nose, periorbital area and scalp having the greatest recurrence rates (See Table 1.3). Whether this reflects alteration in the biology of the tumours in these positions or the fact conservative excisions in favour of cosmetic surgery are performed is uncertain. The finding that there is no difference in subclinical extension of tumours in these sites supports the latter option (Breuninger and Dietz 1991). Cosmetic conservatism has also been proposed as the mechanism for high incomplete excision rates of BCC on the eyelids (Rakofsky 1973).

1.13.2.3 Size and duration

In general tumour duration and size (Kopf 1979) at presentation has not been found to be related to recurrence except in 2 studies. Kopf found that these factors were important for recurrence in tumours treated with curettage and electrodesiccation (Kopf 1979), whilst Rigel found these measurements important for tumours treated with Mohs surgery (Rigel, Robins et al. 1981).

1.13.2.4 Histology

Thackary, followed by Sloane, were the first to emphasise the importance of an infiltrative or multifocal histological subtype as a factor related to recurrence. Other authors have found less association between recurrence and histology but this may reflect the classification system utilised (Pascal, Hobby et al. 1968). (Shanoff, Spira et al. 1967). This point has been emphasised by Weidenbecher who summarised that the usual classification of BCC provides no information as to the degree of aggressiveness nor adequate information as to the form of treatment required. (Waller and Weidenbecher 1977). As an example, the findings of Menn et al and Freeman and Duncan, who noted infiltrating strands of tumour in some recurrences but reported that the nodular type was the commonest histological variant in a recurrent group

(Menn, Robins et al. 1971; Freeman and Duncan 1973). One author commented “*We summarise that some of the tumours classified as nodular BCCs by of Menn et al and Freeman and Duncan had, in addition micronodular or infiltrating elements and thus in our classification would have been called nodular-infiltrating or nodular-micronodular BCCs*” (Lang and Maize 1986).

Emmett found high recurrent rates in Infiltrating tumours (34.5%). Dixon reported significantly higher recurrence rate in multifocal infiltrative and morpheic subtypes compared to nodular or noduloulcerative type (Dixon, Lee et al. 1991). Roenigk reported a higher odds ratio of morpheic and metatypical tumours in a recurrent group (Roenigk, Ratz et al. 1986) Hauben classified tumours into morpheic, solid and adenocystic groups and found the morpheic and solid tumours recurred more than the adenocystic tumours (Hauben, Zirkin et al. 1982)

Other histological criteria have also been evaluated an related to recurrence. Dellon associated the presence of irregularity of 75% of the peripheral palisade and lack of lymphocytic infiltrate with recurrence (Dellon 1985). There was no association with squamous differentiation. The irregularities in the peripheral palisade is a feature of infiltrative tumours (Sexton, Jones et al. 1990). Dixon’s findings supported these findings observing that shape of cell group, growth pattern, contour of invading edge, degree of peripheral palisade and nuclear pleomorphism were all of prognostic significance (Dixon, Lee et al. 1993). Fibrosis, cystic change and atypical squamous change were all of possible significance. Hauben, however, found no association with peripheral palisading, lymphocytic infiltrates or squamous differentiation but did find an increased recurrence rate with those tumours with high mitotic activity (>10 per 5 high power fields) and the absence of ulceration (Hauben, Zirkin et al. 1982). The report of a higher growth fraction in previously completely excised BCCs would support this finding (Healy, Angus et al. 1995) but Dixon however, found the mitotic index of no significance.

The relationship between histology, recurrence and complete excision is complex and closely interrelated. The higher recurrence rates most probably reflect the higher incomplete excision rates for the more infiltrative tumour subtypes and also the

difficulty in reporting the completeness of margins in vertical section by conventional histology.

1.13.3 Aggressive BCC

The term aggressive has not clearly been defined. Some authors refer to clinically aggressive tumours (Poole, Cochrane et al. 1975; Gormley and Hirsch 1978; Itayemi, Abioye et al. 1979; Weimar, Ceilley et al. 1979; Binstock, Stegman et al. 1981; Jacobs, Rippey et al. 1982; Leffell, Headington et al. 1991) with deep invasion, causing widespread destruction of underlying tissues (Jacobs, Rippey et al. 1982). Other terms for aggressive clinical behaviour such as mutilating (Dvoretzky, Fisher et al. 1978; Schwartz, Vickerman et al. 1979; McGurk and Edwards 1984), giant [Beck, 1983 #13616; Betti, 1997 #17090; Canterbury, 1990 #12178; Curry, 1977 #15025; Dudzinski, 1984 #13597; F, 1989 #12350; Herring, 1994 #11006; Love, 1985 #13389; McElroy, 1996 #17242; Randle, 1993 #11357; Sahl, 1994 #11058; Schwartz, 1986 #13039; Warthan, 1994 #11026 or horrifying tumours have also been used (Jackson and Adams 1973; Bianchini and Wolter 1987).

Confusion may arise because some studies apply the term aggressive for the morphological appearances of high mitotic index, absence of peripheral palisading, hyaline fibrosing stroma, and thin sheets of invading neoplastic cells with an irregular spiky pattern. rather than clinical appearance (de Rosa, Vetrani et al. 1990; De Rosa, Staibano et al. 1992; De Rosa, Staibano et al. 1993; De Rosa, Barra et al. 1994; Staibano, Boscaino et al. 1996).

1.13.3.1 Age and sex

In Jackson's series, 33 horrifying tumours were compared with 435 non horrifying tumour (Jackson and Adams 1973). He concluded that there was no means of distinguishing them from the usual BCC. The horrifying tumours did, however, occur in younger men (24% younger than 41). Leffell found a high proportion of histologically aggressive tumours among young adults (Leffell, Headington et al.

1991). Poole's case report of infiltrative BCC of the forehead, requiring a free flap, occurred initially in a 36 year old male. Binstock's analysis of 5 cases and a literature review of a further 17 found that 60% of patients were younger than 40. Randle compared 50 giant BCCs (T3) with small (1cm), T1 and T2 (2-5cm) tumours and found no age or sex difference.

1.13.3.2 Site

Jackson (Jackson and Adams 1973) found no anatomical site preference. Binstock considered that a disproportionate number of large aggressive BCCs occurred on the scalp. Leffell (Leffell, Headington et al. 1991) found histologically aggressive tumours occurred on the nose, temples and forehead.

1.13.3.3 Size and duration

In Jackson's series 17 of 33 cases did not present until 5 years after the appearance of the BCC and 9 patients waited 10 years. 27 of these cases presented with lesions greater than 3 cm. In Leffell's series the tumours ranged from 4-17cm. Vico considered these factors imperative in the development and incorporated them into a classification of aggressive BCC, namely (Vico, Fourez et al. 1995):

- i) Initial diameter greater than 1 cm.
- ii) Greater than two recurrence despite adequate treatment.
- iii) Extracutaneous extension.

1.13.3.4 Previous treatment

The role of previous treatment as an etiological factor for aggressive BCCs has been discussed in the literature and in particular, the importance of inadequate initial treatment allowing deep tumour extension or the possibility of treatment, especially radiotherapy altering tumour biology (Pierce, Klabunde et al. 1953; Jackson and Adams 1973; Randle, Roenigk et al. 1993; Vico, Fourez et al. 1995).

82% of a series of giant tumours were reported to be present for greater than 1 year and 68% had received previous treatment. 16% had been treated 3 or more times (Randle, Roenigk et al. 1993). The treatment type and adequacy was not mentioned. 16 of Jacksons horror cases had radiotherapy 11 unsuccessfully including . 3 of these cases that developed metastatic disease. He concluded however that radiotherapy did not have a role in the aetiology of the tumours and that they were biologically aggressive from the start. 15/33 horror case of the orbit and face reported by pierce had received prior radiotherapy (Pierce, Klabunde et al. 1953). Leffell suggested that inadequate treatment of histologically aggressive tumours results in recurrence and long term morbidity emphasising that there is a subgroup of potentially problem tumours in the population. Vico cited recurrence as a defining factor in BCC and suggested that inadequate excision allows deep extension of the tumours but the adequacy of initial treatment was not noted in this series (Vico, Fourez et al. 1995).

1.13.3.5 Histology

Jackson concluded that horrifying tumours were histologically indistinguishable from ordinary BCCs but provided no evidence for this (Jackson and Adams 1973). Binstock suggested that aggressive scalp BCCs were more likely to be morpheic or metatypical (Binstock, Stegman et al. 1981). Vico observed that adenoid differentiation in the presence of a sparse lymphocytic infiltrate was associated with aggressive behaviour (Vico, Fourez et al. 1995). 72% of Giant BCCs had aggressive histological pattern. (micronodular, morpheic or metatypical) (Randle, Roenigk et al. 1993).

Attempts to identify histologically aggressive BCCs with tumour markers have been applied to morphologically aggressive rather than to clinically aggressive tumours. Histologically aggressive tumours have been demonstrated to express higher AgNor counts and marked p53 immunostaining (De Rosa, Staibano et al. 1992; De Rosa, Staibano et al. 1993). Also a discontinuous basement membrane with a myelofibroblastic response with increased Vimentin , actin and desmin immunostaining compared to histologically non aggressive tumours [De Rosa, 1994 #10972. In addition clinically aggressive tumours have been demonstrated to have a

higher microvessel count due to increase angiogenesis (Staibano, Boscaino et al. 1996).

1.13.3.6 Predisposing factors

Jackson noted that some patients with horrifying BCCs were associated with unusual aetiologies including mustard gas burns (2 patients) radiodermatitis (1 patient), trivalent inorganic arsenic ingestion (1 patient) and Gorlin's syndrome (1 patient) (Jackson and Adams 1973). Immunosuppression has been associated with a higher incidence of histologically and clinically more aggressive tumours (Weimar, Ceilley et al. 1979; Oram, Orengo et al. 1995). X ray induced tumours have also been shown to behave more aggressively in terms of recurrence and bony infiltration in comparison to sunlight induced tumours (Spoor, Lindo et al. 1977). Additionally X ray induced tumours showed more anaplastic features by electron microscopy compared to other tumours (Spoor, Lindo et al. 1977).

1.13.4 Metastatic BCC

The criteria for metastatic BCC are well established. The primary tumour must originate from the skin and not the mucous membrane and the metastasis must be at a distant site and not a simple extension. The primary and the metastasis must also be of a similar histological subtype (Lattes and Kessler 1951).

Metastatic BCC is rare with a reported incidence from 0.0028% to 0.55% (Snow, Sahl et al. 1994). There is a male to female ratio of 2:1. The mean age of onset of the tumours are 48 years with the metastasis following 12 years later. The patients typically have a large recalcitrant BCC resistant to treatment. The scalp and the ear are the commonest positions, possibly due to the thin skin and large vessels. After these genitalia and trunk are the next most common sites (von Domarus and Stevens 1984; Snow, Sahl et al. 1994). Evidence suggests that there is increased frequency of metastasis with increased size and depth of invasion of the BCC. 75% of metastatic

BCCs are stage T2-T4. Snow suggested that large tumours, especially with a history of radiotherapy may metastasise (Snow, Sahl et al. 1994). The metastatic potential of multiple BCC is unclear but it has been reported for lesions on the back (Menz, Sterrett et al. 1985).

The relationship between histological subtype and metastasis is unclear. Morpheic tumours have been reported to metastasise in less than 1% of cases, but about 15 % of the tumours displayed squamous differentiation in another series (von Domarus and Stevens 1984). Metatypical BCC has also been associated with metastasis (Farmer and Helwig 1980). Perineural invasion has been associated with recurrence rather than metastasis. The route of metastasis is reported to be lymphatic or haematogenous. Facial BCCs tend to metastasise to the regional lymph nodes and organs above the diaphragm including brain, dura, oesophagus and heart (Lo, Snow et al. 1991; Snow, Sahl et al. 1994) although liver and kidney involvement has been described. (von Domarus and Stevens 1984; Howat and Levick 1987). BCCs of the trunk tend to metastasise to the regional lymph nodes or lung, however, pericardial and abdominal viscera have also been involved (Degner, Kerley et al. 1991; Snow, Sahl et al. 1994). Boney metastasis is usually observed in conjunction with other sites of metastasis especially from lesions of the eye, scalp or temple. Metastatic BCC to the skin is rare but associated with a good survival (0.3-7years.) when compared to other forms of metastasis where the survival is poor at approximately 8 months (von Domarus and Stevens 1984).

1.13.5 Risk of a New Primary BCC

A new BCC is defined an independent occurrence at a site not contiguous to the another BCC. It is not a recurrence.

The incidence of a new BCC increases with time after the first BCC. The cumulative rate is approximately 22% within the first year rising to 45% in the fifth year. The greatest risk (hazard) for developing a new BCC is in the first year (a 16% chance) and then it fall to about 10% in the next 4 years follow up (Marghoob, Kopf et al.

1993). The risk increases with increasing number of previous BCCs (Epstein 1973). A previous SCC is also a risk factor for the subsequent development of a BCC (Schreiber, Moon et al. 1990).

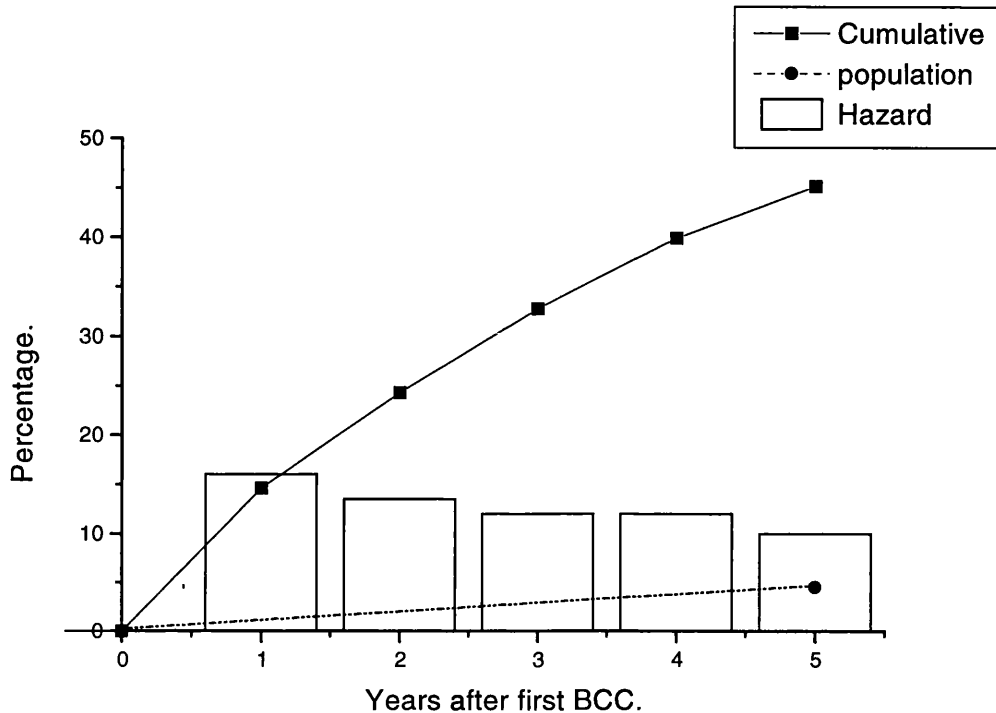


Figure 1.18. The 5 year cumulative risk increases to 45% at 5 years. This is compared to a normal population which is less than 5%. The chance of developing another BCC falls with time after the first BCC from 16% to 10% (Hazard).

1.13.6 Regression in BCC

The phenomenon of regression in BCC is recognised in the literature (Curson and Weedon 1979; Franchimont, Pierard et al. 1982; Halliday, Patel et al. 1995). Indeed Masden included it in his theory of the histogenesis of multifocal BCC (Masden 1942). Clinically regression may take the form of central scarring. Histologically it is characterised by a dense lymphocytic infiltrate at the margins of the tumour nests with prominent apoptosis (Curson and Weedon 1979). Evidence suggests that regression is immunologically mediated by a cell mediated response rather than humoral

mechanisms. There are increased numbers of CD4 T lymphocytes around actively regressing tumours and relatively few B lymphocytes (Hunt, Halliday et al. 1994). There are also greater expression of IL2R which suggests activation of lymphocytes (Hunt, Halliday et al. 1994). It has been postulated that these mechanisms may be partly responsible for the cure of some tumours after incomplete excision (Goldwyn and Kasdon 1978).

1.14 Treatment of BCC and prognostic factors

The goals of treatment for BCC are firstly, cure and secondarily, cosmesis since the majority of tumours are on the head and neck. Good local control is synonymous with cure due to the rarity of metastasis. Conservatism for cosmesis may result in recurrence and therefore increased the long term morbidity. This applies especially for tumours in the vicinity of the nose or the eyes.

A variety of treatment modalities have been described for the treatment of BCC. This, in part, may be due to the fact that the majority of BCCs are first referred to dermatologists who traditionally tended to favour non surgical or simple ablative surgical techniques rather than excision. Because many untreated BCCs are small, and therefore do not penetrate deeply into the dermis, the results of ablative treatments are acceptable and therefore in favour. A problem may arise when a tumour, inappropriate for this type of treatment is managed by such techniques, resulting in recurrence or deep infiltration. A knowledge of available therapies and the indications for each are important for the correct management of BCCs.

1.14.1 Curettage and electrodesiccation

This technique involves removal of the lesion with a curette under local anaesthesia followed by electrodesiccation of the treated area. The curette finds a plane between the soft tumour and harder dermis or stroma. It is therefore best used for “mushy” nodular tumours (Wolf and Zitelli 1987). 5 year recurrence rates range between 3 and

18% which reflects technique and patient selection. This modality is suitable for small previously untreated lesions. Size, site and treatment time span are independent risk factors for recurrence (Silverman, Kopf et al. 1991). One study found high rates of residual tumour after excision of a curettage and cautery bed especially in facial BCC compared to tumours on the trunk (Suhge d'Aubermont and Bennett 1984). Recurrent lesions should not be treated by this technique since re recurrence rates may reach 40% (Rowe, Carroll et al. 1989). The predominant criticism of this modality is the lack of histological information regarding growth pattern and excision margins. Therefore the first indication of a high risk or potentially problem tumour may be a recurrence.

1.14.2 Cryotherapy

Liquid nitrogen to destroy BCCs is reported to have a high success ranging from no recurrence (Kuflik 1980) to 12.9% (Kuflik 1978) but there is only one study with a five year follow up which reveals a 7.5% recurrence rate (Fraunfelder, Zacarian et al. 1984). In this series recurrence was higher in larger tumours (>10mm) of infiltrative subtype (18%). Patient selection, therefore, represents an important determinant of outcome for Cryotherapy. Cryotherapy has been combined with cautery as an technique for reducing recurrence post curettage recurrence (Abadir 1980). The criticism of absent histological information also applies.

1.14.3 Radiotherapy

BCC is a radiosensitive tumour and patients can be treated in an outpatient setting without the added complications of surgery. A variety of techniques are available including interstitial brachytherapy, superficial contacttherapy and conventional fractionated radiotherapy. 5 year recurrence rates range from 4.1% (Knox, Freeman et al. 1967) to 31% (Nordman and Nordman 1978). Size has been found as an independent prognostic makers for recurrence following radiotherapy with one author

reporting a 4.4% recurrence for tumours less than 1cm rising to 9.5% for larger tumours (Silverman, Kopf et al. 1992). Age, sex, site duration or time span before treatment have not been demonstrated as important prognostic factors.

The cosmetic benefit of radiotherapy diminishes with time due to depigmentation and atrophy. Radionecrosis and the induction of new malignancies are risk factors and therefore this therapeutic modality is usually restricted to older patients. 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 for surgery (Avril, Auperin et al. 1997) . In this series surgical recurrence was low (0.7%). Radiation has lost favour for the treatment of BCCs and is only used for approximately 8% of BCCs in the UK (Motley, Gould et al. 1995). Radiation may also be administered for palliation in elderly patients with large potentially incurable lesions.

1.14.4 Surgical excision

Complete excision of BCCs remains the goal of surgery. This is dependant on the skills of the surgeon and partly on the ability of the pathologist to correctly report the histological tumour margins. The surgeon must be confident enough in his/her reconstructive capabilities so as not to compromise the excision margin of the tumour. The surgeon must also comprehend the microanatomy of the BCC so that appropriate margins are excised. 5 year recurrence rates range from 0.7% (Avril, Auperin et al. 1997) to 23.4% (Hauben, Zirkin et al. 1982). One study showed that for primary BCCs anatomical location on the head and males were independent prognostic markers (Silverman, Kopf et al. 1992). In particular Anatomical location, on the nose and periorbital regions have the greatest risk for recurrence following surgery (Koplin and Zarem 1980; Emmett 1990). Histological subtypes associated with recurrence have previously been discussed. Apparent or unrecognised incomplete excision is most probably responsible for this.

1.14.5 Surgery and incomplete excision of BCC

In contrast to other cancers, such as melanoma, incomplete excision has become an important entity in BCC. This is because firstly, BCCs occur most commonly on the face so that cosmetically conservative excisions become apparent due to reconstructive uncertainty. E.g. High rates of incomplete excision have been demonstrated in the eyelids (50%) especially of females (57%) (Rakofsky 1973). Secondly non surgical specialities may treat BCCs and rely on ablative rather than excisional techniques and thirdly because publications reporting low recurrence rates for incompletely excised tumours may discourage re excision. Furthermore a question has to be raised regarding the value of a histological report of marginal clearance when only random sampling is performed of the edges and deep margins of the tumour by conventional or vertical sections. For example Lang found 4 of 10 recurrent BCCs initially treated by excision had incomplete excision margins. A reasonable explanation for the recurrences of the other 6 would be false complete reporting of the margin.

Tumour size and tumour subtype influence surgical excision margins. Epstein showed higher incomplete excision rates in larger tumours of a morpheic or infiltrating subtype (Epstein 1973) when excising tumours with a 2mm margin. Breuninger demonstrated higher incomplete excision rates for recurrent tumours compared to primary tumours and those of scirrhous (morpheic) compared to solid types (Breuninger 1984). Sexton demonstrated higher incidence of positive margins in infiltrative, morpheic and micronodular tumours (26.5%, 33.3% and 18.6%) compared to nodular and superficial tumours. (6.4% and 3.6%) The degree to which these cancers subtly extend into the surrounding tissue (subclinical extension) is probably the main determinant for incomplete excision and therefore recurrence. This exemplifies the importance of an appropriate prognostically useful classification system.

1.14.5.1 Subclinical extension of BCCs. What surgical margin is appropriate

The microanatomy of the BCC is such that the tumour extends out from the visible border of the tumour. This extension is commonly asymmetrical and the extent is dependant on factors such as tumour size and subtype. A knowledge of these factors are essential for planning of surgical excision margins.

1.14.5.2 Lateral margins

Wolf, by preoperative markings of the skin in 1mm increments, found that a 4mm margin would eliminate 98% of tumours less than 2cm. Margins of 3mm and 2mm would have eliminated the tumours in 85% and 75% of cases (Wolf and Zitelli 1987). Epstein demonstrated that a 2mm margin was adequate in 95% of his patients. However the mean size was only 8mm. The mean size in the failures was 13mm indicating that subclinical extension is related to tumour size (Epstein 1973).

TUMOUR TYPE	TUMOUR SIZE	% POSITIVE EXCISION MARGIN FOR EACH EXCISION MARGIN		
		2mm	3mm	5mm
Primary(all type)	10mm	30	16	5
Primary (morpheic)	10-20mm	48	34	18

Table 1.4. The relationship of incomplete excision for excision margins for different types of BCC (Wolf and Zitelli 1987.)

Burg studied the subclinical extension by subtracting the tumour size from the defect size in 72 BCCs treated by Mohs surgery (Burg, Hirsch et al. 1975). Subclinical extension was related to duration of tumour, location, tumour diameter, number of previous treatments, treatment type and histology (Table 1.4). It can be seen that increasing tumour size, recurrent tumours and morpheic tumours have greater subclinical extension. This factor is responsible to their higher incomplete excision

rates and correspondingly greater recurrence rates. This study has however been criticised due to the fact that the defect may expand after tumour removal resulting in falsely high measurements.

Primary or recurrent	Primary 5.5+/-4.5mm		Recurrent 8.9+/-4.8mm			
Case history	1year 4.8+/-2.3mm	2 years 6.3+/- 3.4mm	3-5 years 8.3+/-3.4mm		>5 years 8.9+/-5.0mm	
Location	Lips 4.8+/- 3.0	Nose 5.5+/- 2.5mm	Cheek 8.1+/- 5.6mm	inner canthus 8.2+/- 2.2mm	temple and scalp 9.5+/- 5.0mm	Forehead 9.7+/- 5.0mm
Number of previous treatments	1 previous 7.2+/-3.5mm		>1 previous 9.4+/-5.1mm			
Kind of previous treatments	Surgery 8.2+/-5.1mm		Xray 8.2+/-4.9mm			
Histology	Solid 6.5+/-3.8mm		Morpheic 9.3+/-5.5mm			

Table 1.5. Means and standard deviations of subclinical extension in mm of BCC (Burg, Hirsch et al. 1975).

Breuninger has subsequently confirmed these findings by measuring the tumour size and subtracting it from the safety margin of excision to calculate subclinical extension in 2016 tumours. Subclinical extension was expressed mathematically according to type, diameter and recurrence resulting in exponential functions which varied highly significantly. Unlike Burg no difference was found with tumour position [(Breuninger and Dietz 1991).

Breuninger also characterised the asymmetric lateral growth patterns and their frequencies (Breuninger and Dietz 1991). Type a) and b) are the most common for both primary and recurrent BCC (Figure 1.19).

1.14.5.3 Deep margins

Table 1.6 shows the depth of infiltration of various BCCs from a series of 1421 tumours excised by Mohs surgery (Breuninger, Flad et al. 1989). It can be seen that the depth of infiltration of recurrent and morpheic BCC is greater than primary or solid BCCs. As a consequence the incomplete excision rate and recurrence rate are greater for these subtypes.

Depth	PRIMARY		RECURRENT	
	Solid	Morpheic	Solid	Morpheic
Upper dermis	3.8%	2.2%	4.9%	1.2%
Middle dermis	24%	12.5%	30.3%	16%
Lower dermis	42%	35.4%	26.0%	40.8%
Subcutaneous tissue	26.3%	36.2%	90.2%	83%
Subtotal	91.1%	86.3%	90.2%	83.0%
Muscle	3.6%	8%	9.7%	13.2%
Perichondrium	0.3%	3.5%	0	2.6%
Periostium	0	2.2%	0	1.2%

Table 1.6. The depth of invasion of Primary and recurrent BCC of Solid and morpheic histological subtype (Breuninger, Flad et al. 1989).

The pattern of deep infiltration by BCCs is also important. Only 27% showed symmetrically central deep extension. The majority of tumours exhibited peripheral deep extension by way of single or multiple down growths. This has important implication regarding the value of conventional midline or cruciate type histological sections compared to sections taken perpendicular to the skin as in Moh's technique

(Mohs 1968) or other published methods (Breuninger 1984) since outgrowth of tumour may be missed resulting in a false complete excisional histology report.

The closeness of the surgical margin has been associated with increase risk of recurrence. Lang found that recurrent tumours had a significantly closer margin than non recurrent groups (Lang and Maize 1986). Furthermore 60% of his recurrent tumours were initially incompletely excised compared to 13.5% of a non recurrent group. Breuninger found no recurrence in his group of tumours excised with histological control of the edges (Breuninger 1984).

1.15.5.4 Uncertainties in Histological margins

The value of a histological report to determine the presence or absence of residual tumour the margins of a tumour will depend on the amount of the margin that has been examined. This holds especially true for BCC which has been demonstrated to grow asymmetrically. Traditional breadslicing of a tumours will provide excellent information regarding the tumour histology and growth pattern but will only sample less than 0.5% of the margin (Wolf and Zitelli 1987). This may be adequate for small primary nodular tumours which have the least lateral and deep subclinical extension. It is most probably inadequate for large recurrent infiltrative tumours. Lateral subclinical extension may be missed (See figure 1.21).. Conversely it may be difficult with small sections to be confident of the correct orientation of a specimen. This may result in an apparent incomplete excision in a completely excised tumour. Marking of the tumour edge with Indian ink is common practice to avoid this complication (Paterson, Davies et al. 1992).

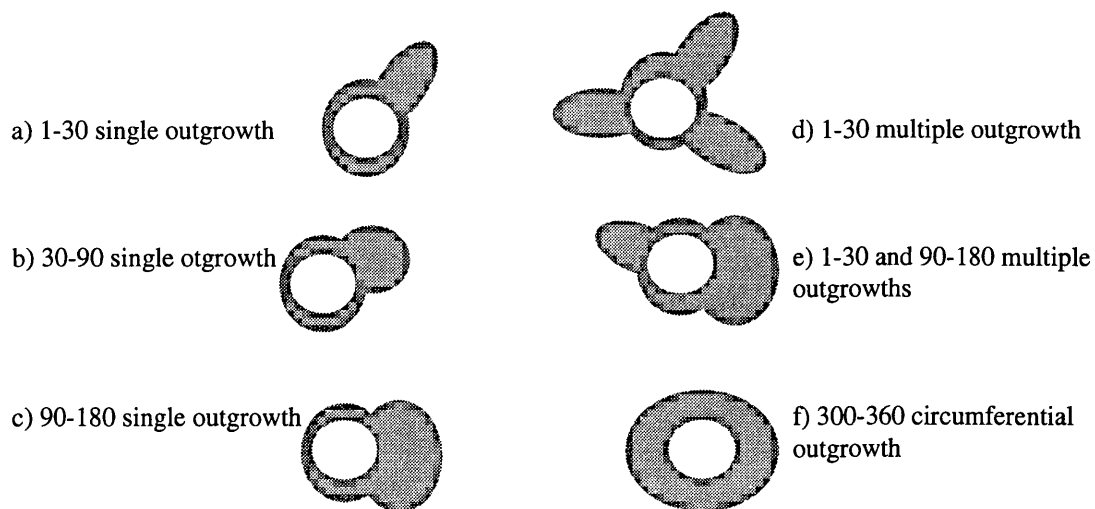


Figure 1.19. Horizontal growth patterns of BCC.

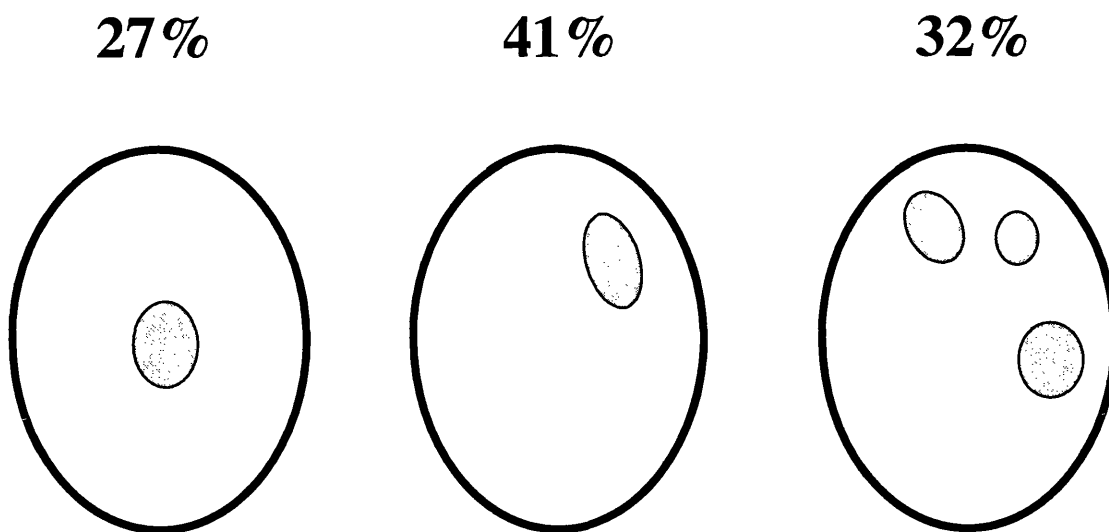


Figure 1.20. The pattern of deep extension of BCC.

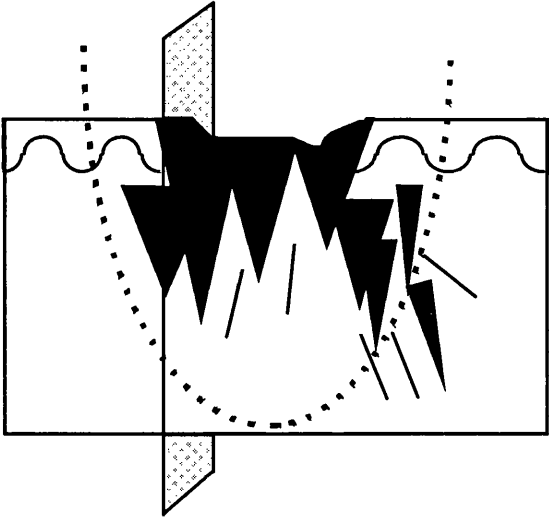
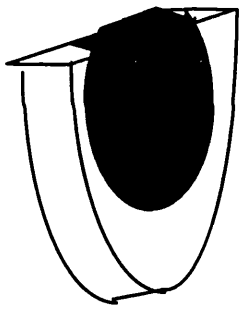


Figure 1.21. The mechanism by which an incomplete tumour margin may be missed.



Tumour surrounded by normal tissue



Line of section due to incorrect orientation
would reveal a false incomplete excision

Figure 1.22. Diagram of skin excision showing the potential for an apparent incomplete excision resulting from incorrect orientation on sectioning.

As a consequence Mohs chemosurgery developed in which the tumour is excised with successive shave type excisions. The histological sections were therefore perpendicular to the skin and therefore minimising the possibility of a false incomplete excision. Breuninger addressed this problem and compared the accuracy of a midline vertical section with horizontal sections of excised tumours. Horizontal sections were showing incomplete excisions up to 3mm deeper than that of a clear vertical section emphasising the inadequacies of midline sections (Breuninger, Flad et al. 1989).

1.14.5.5 Recurrence following incomplete excision

The question regarding the outcome of inadequately excised BCCs is complex. On the one hand there is evidence that incomplete excision and the size of excision margin is directly related to recurrence, on the other hand the literature also supports the finding that not all incompletely excised BCC recur (Pascal, Hobby et al. 1968; Dellon, DeSilva et al. 1985; Richmond and Davie 1987). Three important issues must be raised. Firstly a specimen with marginal involvement does not necessarily reflect that tumour may remain in situ. It may reflect histological abnormalities such as a poor specimen orientation (See Figure 1.22). Secondly some tumours may be well circumscribed and compact in the stroma and may “shell out” thus appearing incompletely excised when they are not, such as curettage specimens. Thirdly the history of recurrent BCC is over 10 years and therefore follow up needs to be long to be meaningful. Fourthly, studies comparing recurrence of complete versus incomplete specimens must be interpreted with care because with conventional breadslicing of specimens the true margins cannot be assured.

Pascal reported 42 incompletely excised, 17 suboptimal and 84 adequately excised BCCs in a series where they were treated with curative attempt, multifocal lesions were excluded and re excisions were excluded. The tumour edges were marked with ink to prevent poor specimen orientation at histological assessment. Follow up was 10 years. The recurrence rate was 33%, 12% and 1.2% respectively. Even though the re excisions were excluded, it was noted that residual tumour was found in 3 of 6

specimens (Pascal, Hobby et al. 1968). Gooding reported a 34.8% recurrence rate in recurrent rate in 66 inadequately excised BCCs with a 5 year follow up (Gooding, White et al. 1965). Shannoff reported a 67% recurrence in incompletely excised tumours. De Silva reported a 41% recurrence in 38 incompletely excised tumours with a 5 year follow up and noted no difference in patient age, sex or tumour location (De Silva and Dellon 1985). Dellon questioned if there was a particular subtype more prone to recurrence and applied criteria previously described in recurrent tumours (Dellon 1985). (Namely irregularities in the peripheral palisade, absence of lymphocytic response, ulceration and squamous differentiation). Tumours with >75% irregularities had a 39 fold chance of recurrence, weak or absent lymphocytic infiltrate concurred a 4 fold risk and tumour ulceration a 2.8 fold risk of recurrence. Squamous differentiation conferred no risk. Follow up was a minimum of 11 years (Dellon, DeSilva et al. 1985). Whether this is because nodular tumours which have marked peripheral palisading appearing incompletely excised because they shell out (as seen the photomicrograph in Dellon's paper) or because there is residual tumour remains to be seen. It is interesting that studies noting re excisions often remark on the small percentage of residual tumour found. Whether this is due to regression of the residue or because there was no residue remaining is unknown. Richmond studied 60 incomplete excisions including 37 recurrent tumours with a wait and see policy. Incompletely excised recurrent tumours recurred more frequently than primary tumours (43% and 30% respectively) Those that had received both radiotherapy and surgery recurred the most frequently (91%). Recurrence occurred in 4 of 4 patients with both lateral and deep margins involved. From the remainder of the data it was difficult to estimate which margins are involved and the histological subtype for the remainder of the patients. The 5 year follow up in this series was incomplete.

Question still remains as to a wait and see policy or an immediate re excision policy is most appropriate. Richmond's series demonstrated as a result of an expectant policy that 2 of 7 recurrent tumours required more than 1 procedure for cure. This rose with previously treated tumours to 3/3 (with primary radiotherapy) and 5/10 (surgery and radiotherapy previously). The patients who were initially treated by surgery were all cured with 1 procedure. None of seven patients who had immediate re excision developed a recurrence. It is my own view that a plastic surgical population of patients

will have a greater proportion of larger, aggressive growth pattern tumours located on the face and therefore recurrence after incomplete excision would be most probable. Furthermore, a low rate of incomplete excision of nodular tumours would be expected in this setting and therefore the group of tumours that appear incomplete but are, in fact, complete should be small. Immediate re excision should therefore reduce the overall morbidity.

1.14.6 Mohs Micrographic Chemosurgery.

Originally developed by Dr. Frederick Mohs in the 1930s at the university of Wisconsin, this technique involved the fixation of the neoplastic tissue, in situ, with zinc chloride paste followed by excision by slicing successive sections perpendicular to the skin. After each excision the specimen the under surface of each layer is examined section by section and residual tumour mapped so that it was removed with the next excision (Mohs 1968). The main advantage of this technique is that histological evaluation of the tumour is performed until there is no residue remaining. This increases histological accuracy and almost guarantees complete excision and minimises the removal of uninvolved tissue, therefore, reducing morbidity. This results in low recurrence rates. This technique is suitable for recurrent or problem tumours. The main disadvantage is that it is slow and time consuming. Furthermore, evaluation of bone involvement cannot be performed in the timespan of the procedure because decalcification techniques takes weeks. Microscopic control of areas with bony involvement has to be performed at a later stage. In order to accelerate the procedure the fresh tissue technique was developed whereby the tissue examined by frozen section, however the layer by layer excision and histological examination remains unchanged. Therefore, more than 1 stage can be performed at a time (Mohs 1976). 9.3% and 97% cure rates have been reported for the fixed and fresh tissue respectively. (Mohs 1976) This technique has a recurrence of 8.7 times less than that of all non Mohs modalities for primary BCCs (Rowe, Carroll et al. 1989).

11.14.7 Non Surgical Techniques

Newer non surgical techniques do not have the high cure rates of established methods however, are currently under trial. Oral retinoids may produce regression but not cure and are more effective as chemo prevention in patients with Gorlin's syndrome, Xeroderma pigmentosum or in transplant patients (Hodak, Ginzburg et al. 1987; Moon, Levine et al. 1997). Intralesional interferon alpha -2b has also been demonstrated to cure 67% of BCCs in the short term (Greenway, Cornell et al. 1986; Thestrup Pedersen, Jacobsen et al. 1990; Buechner 1991). Photodynamic therapy with either topical or systemic haematoporphyrin derivatives is also under evaluation (Tosca, Balas et al. 1996). Topical application of photosensitisers has the advantage of less systemic side effects however is only suitable for superficial tumours. Chemotherapy has also been used for large inoperable BCCs and in the rare cases of metastatic BCC. Cisplatin appears more effective than doxorubicin, bleomycin, cyclophosphamide, 5 fluorouracil and vinorelbine. Combination chemotherapy may provide some useful palliation (Goldberg 1996). Laser ablation has also been advocated since recent improvements in laser technology have resulted in small user friendly and portable lasers. Scanning and ultrapulse techniques have reduced thermal diffusion and unwanted thermal necrosis extending outward from the ablation zone thereby minimising local tissue damage, facilitating healing and reducing scarring (Chapter 7).

Publications concerning the effectiveness of the carbon dioxide laser to treat basal cell carcinoma reveal diverse results. In 1 study 50% local recurrence at 1 year was attributed to cautious use of the laser, another study demonstrated no recurrence in 370 superficial BCC with a 20 month follow-up (Adams and Price 1979; Wheeland, Bailin et al. 1987)

Prevention by education of children and parents of the carcinogenic effects of ultraviolet radiation is important. Adequate protection from clothing and sunscreens should be emphasised. However while there is evidence that sunscreens reduce actinic keratosis there is no evidence there is a reduction in BCC. Follow up of the high risk patient so that early treatment of a new BCC or of a recurrent can be implicated is essential

Chapter 1

Section 2. Cell kinetics and human tumours

1.15 Introduction

For several decades it has been suspected that rapid tumour growth was associated with a poor prognosis (Tubiana M. and Courdi A. 1989). In early studies, crude growth rates were calculated from clinical measurements of tumour size and, despite obvious limitations, it was recognised that rapidly growing tumours, especially of the breast, had a poorer prognosis (Richards G.E. 1948; Rigby-Jones P. 1963; Boyd N.F., Meakin J.W. et al. 1981). Subsequently, an objective and quantitative measurement of doubling time by sequential X-ray was introduced. However, the periods of observation contravened plans of early treatment. Despite these drawbacks several studies showed that longer clinical tumour doubling time was associated with a better prognosis (Joseph D.A., Morton D.L. et al. 1971; Galante E., Gallus G. et al. 1986). Subsequently as the knowledge of cell biology grew, it became evident that greater insight into the tumour behaviour may be derived from understating cell kinetics at a cellular level. It is now possible to measure a multitude of kinetic parameters. In tandem with the advances in kinetic measurements, vast advances in the underlying genetic mechanisms of cancer have revealed that cancer genes alter cell cycle control. Therefore knowledge of the cell cycle and cell proliferation is fundamental to understanding of tumour biology.

1.16 Cell production-the cell cycle

The initial understanding of the controls of cell division followed the description of the cell cycle in 1951 by Howard and Pelc (Howard A. and Pelc S.R. 1951). Studying

uptake of p^{32} into *Vicia faba* they noted mitosis to be preceded and followed by periods of reduced uptake. This was later correlated with a number of steps in a replicative cell cycle which have now been well characterised. The G1 phase (Gap 1) represents the start of the cycle; once stimuli are received to commence replication, the cell proceeds along a biochemical pathway which includes an initial 'start' signal. Quiescent cells may move from a G0 phase into G1, though this distinction is essentially arbitrary. Once the cell passes the 'start' point it is committed to divide and proceed to the S phase (synthesis phase). During this period the necessary metabolites for DNA and chromosomal synthesis are amassed and the genome replicated. The S phase is followed by a second period of relative inactivity, denoted as the G2 (Gap 2) phase in which the cell contains a tetraploid number of chromosomes (i.e. four sets as opposed to a normal complement of two). The cell now undergoes mitosis, producing two daughter cells, to restore the normal diploid number of chromosomes. The progeny can either re-enter the cycle at the G0/G1 phase, proceed to differentiate, or undergo apoptosis (Figure 1.23).

1.16.1 Cyclins and cyclin dependent kinases

Completion of the cell cycle requires the co-ordination of a variety of macromolecular syntheses, assemblies and movements. The chromosomes must be replicated, condensed, segregated and decondensed. The spindle poles must duplicate, separate and migrate to opposite ends of the nucleus. Co-ordination of these complex processes is thought to be achieved by a series of changes in a family of molecules termed the cyclin dependent kinases (CDKs). As the name suggests, these are a family of kinase enzymes, that require for their activity, the binding of another family of molecules termed the cyclins. The complex of cyclin bound to CDK activates the kinase activity, thus permitting the phosphorylation of critical substrates which are involved in the machinery of cell cycle control. The various members of the cyclin and CDK families are expressed at different phases during the cell cycle with different combinations being responsible for driving the cell cycle from one stage to another. According to this paradigm, cell cycle stage is determined by the constellation of

proteins activated or inactivated by phosphorylation as a result of the activity of the CDKs during that stage [reviewed by Hunter, 1994 #148].

In mammalian cells each specific CDK and cyclin are transcribed for a brief period during the cell cycle with the messenger RNA being translated and then the proteins are rapidly degraded. Thus each cyclin and CDK are present only during a specific period of the cell cycle and each is only capable of binding and thus activating a specific partner. The CDKs and cyclins known to be involved in human cell cycle control are listed in Table 1.7.

In mammalian cells, the cyclin-CDK complexes which are most closely linked to the start of G1 phase are the D type cyclins and their partner CDKs, primarily CDK4. Three D type cyclins exist with most cells expressing type 3 and types 1 or 2 depending on tissue specificity. The D type cyclins bind to and activate CDK4, and in some cells also CDK6, to drive the cell through early G1. The D type cyclins have a short half life of approximately 30 minutes and are highly inducible by growth factors and may thus act as sensors to such (Solomon 1993). This leads to the hypothesis that deregulation of D type cyclins may reduce the dependency of cell cycle progression on extracellular signals and may thus be a route for oncogenesis. This has subsequently been found to be the case, with over expression of cyclin D1 being involved in certain breast carcinomas (Lammie et al., 1991).

CYCLIN	CDK PARTNER	ROLE IN CELL CYCLE CONTROL
A	CDK2 & CDK1	Controls progression from G1-S and through S
B1	CDK1	Controls progression from G2 to mitosis
B2	CDK1	Controls progression from G2 to mitosis
D1	CDK4 & CDK6	Controls progression in early G1
D2	CDK4 & CDK6	Controls progression in early G1
D3	CDK4 & CDK6	Controls progression in early G1
E	CDK2	Controls progression from G1 to S phase

Table 1.7 Cyclins and cyclin dependent kinases (CDKS) involved in the control of the cell cycle in humans.

Similar progressive binding of the cyclin molecules and their specific CDK partners takes place throughout the cell cycle as indicated in Figure 1.23. The control of expression of many of these cyclins and CDKs is not only regulated by factors external to the cell cycle, such as growth factors, but also by a number of specific CDK inhibitors. Many such proteins exist and a Particular member of this family is p53 which has been found to be of importance in skin malignancies Due to their action as inhibitors of the cell cycle, these proteins bear the physiological characteristics of putative tumour suppressor genes. p53 acts by activating the transcription of p21 which binds CDK4 in competitive fashion and so reducing its activation by cyclin D (Serrano, Hannon et al. 1993).

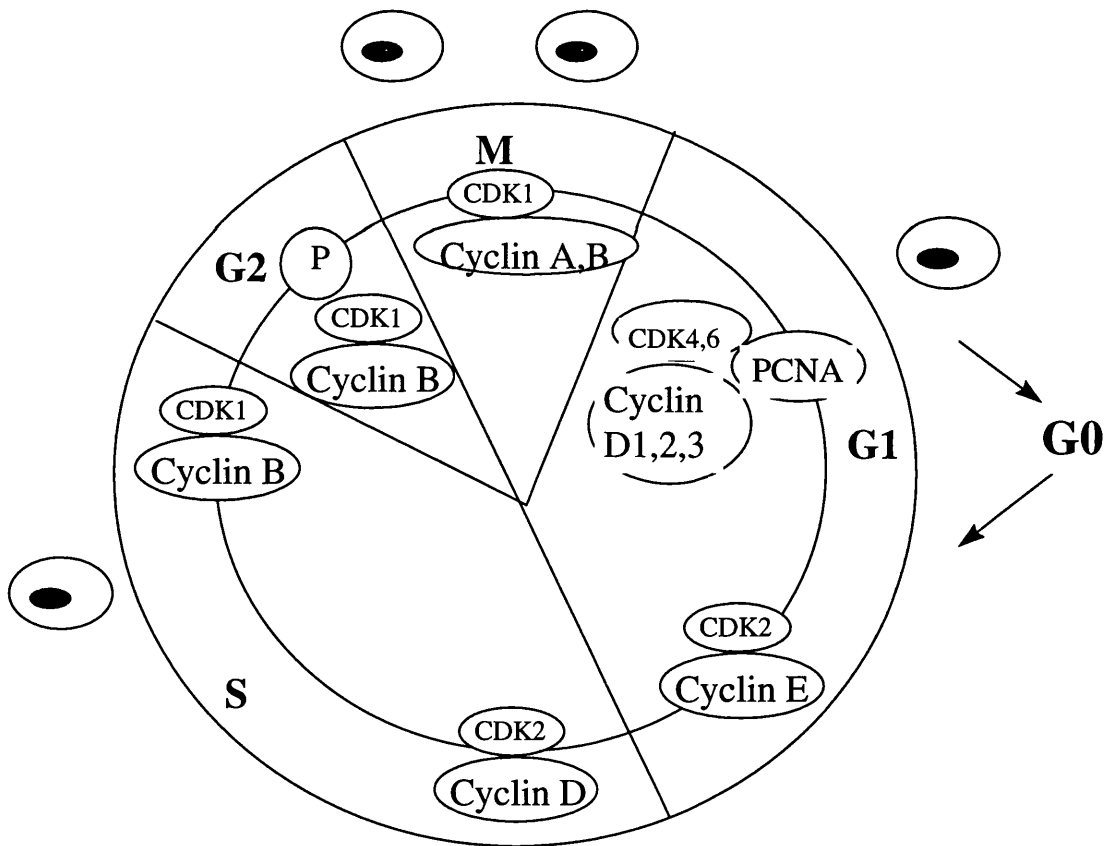


Figure 1.23. An illustration of the cell cycle with the interactions of the various Cyclin dependant kinases and cyclins.

1.17 Cell loss

Cells may be lost in a tumour due to cell death or by shedding . Shedding of cells can be either into the surrounding environment (exfoliation) or into the lymphatic or blood vessels (metastasis). Cell death may result from apoptosis or necrosis. These two processes are distinctly different pathways, although, both may be found in close proximity to each other. e.g. an event that causes necrosis may trigger apoptosis in the surrounding tissue as a result of the accumulation of cellular debris and inflammation.

1.17.1 Necrosis

Necrosis is essentially an accidental occurrence being the outcome of a severe injurious change in environment of the affected cells. e.g. a tumour might outgrow its blood supply. The ultrastructural appearances of necrosis are organelle swelling and subsequent cellular disintegration. Destruction of membrane integrity leads to release of lysosomal enzymes which accelerates disintegration. At a late stage, the nuclear chromatin disappears (karyolysis) and there is usually an inflammatory reaction in the surrounding tissue which may result in scarring.

1.17.2 Apoptosis

Programmed cell death or apoptosis is a process whereby developmental or environmental stimuli activate a genetic program to implement a specific series of events that culminate in the efficient disposal of the cell. Apoptosis is essential for normal development and deregulation of the process leads to a spectrum of defects ranging from embryonic lethality to tissue specific perturbation of postnatal development. Regulation of cell death is an essential defence against viral infection and the emergence of cancer. Too much cell death can result in impaired development and degenerative disease. Morphologically, the earliest changes are compaction of the nuclear chromatin into sharply circumscribed, uniformly dense masses that abut on the nuclear envelope and condensation of the cytoplasm (cytoplasmic boiling).

Continuation of the condensation is accompanied by convolution of the nuclear and cellular outlines and the nucleus often breaks up at this stage to produce discrete fragments. The surface protuberances (blebbing) then separate with sealing of the plasma membrane converting the cell to into a number of membrane bound apoptotic bodies of varying size in which the closely packed organelles appear intact. Some of the bodies lack a nuclear component. whereas others contain one or more nuclear fragments. In tissues, the apoptotic bodies are rapidly taken up by adjacent cells and degraded within lysosomes. There is minimal disruption of surrounding cells and no inflammation. It is estimated that the apoptotic bodies remain visible, by light microscopy, for only a few hours (Wyllie, Kerr et al. 1980; Kerr, Winterford et al. 1994).

The apoptotic pathway is complex and not fully understood. It is further explored in Section 3 on the discussion of the bcl-2 oncogene which may be important in the development of BCC.

1.18 Tumour kinetics

1.18.1 Compartmentalisation of proliferation

In normal tissue or tumour, cells can be considered to be compartmentalised according to their proliferative status. Some cells are actively proliferating and are in the cell cycle. Other cells may be out of cycle or in G0 for a variety of reasons: They may be differentiating or maturing. They may be poorly vascularised and therefore deprived of nutrients or oxygen. These cells may be able to re enter the cell cycle if the microenvironment improves. The compartment of cell loss is an important consideration in tumours. Cells may be lost due to necrosis if the nutrient deprivation is persistent. Programmed cell death or apoptosis may also be responsible for cell loss. Finally exfoliation or metastasis may also account for considerable cell loss.

1.18.2 Tissue specific control of proliferation

In normal tissues the control mechanisms that control cell production and cell loss are finely balanced either by inhibiting all cell division beyond a certain developmental stage (*static populations*) e.g. nerves and striated muscle, or by exactly balancing cell production with cell loss (*continuously renewing*) e.g. small intestine, skin and the haemopoetic system. In the latter group, there is also the capacity to vary the cell production to account for excessive loss due to injury. A third group of normal tissues exist that are normally quiescent e.g. liver, kidney, lung but which can respond to injury by a compensatory burst of proliferation (*conditionally renewing*).

In cancer the control of proliferation is altered to produce unbalanced growth in which there is a net gain in cell number. This may result from variations in cell production due to differences in the duration of the cell cycle or from differences in the proportion of cells in the cell cycle (growth fraction). Additionally, a reduction in cell loss may also contribute to a net gain. It is therefore apparent that tumour cells are not simply rapidly dividing cells evading the normal homeostatic mechanisms, but are under varying degree of control depending on the deregulation of the control systems operating in the tissue of origin.

1.19 The measurement of cell proliferation

There are several methods which can be used to assess proliferation characteristics, each having advantages and disadvantages. These can be subclassified into state and rate measurements. The former reflecting the percentage of a proliferation associated population at any particular time and the latter representing measurements in which the cell cycle transit time is calculated to allow estimation of a doubling time.

1.19.1 State measurements

1.19.1.1 *The labelling Index*

The LI is defined as the number of cells that take up a DNA precursor. The proportion of cells. Traditionally tritiated thymidine was able to estimate the number of cells actively involved in the S phase of the cell cycle and calculate the thymidine-labelling index (TLI) as a marker of proliferation (Taylor J.H., Woods P.S. et al. 1957). This was achieved by pulse labelling of cells with tritiated thymidine. The percentage of the population labelled was given to be the LI. However, Several assumptions were made. Firstly, the free availability of the label to the cells. Secondly, active uptake of the label to the cells and thirdly, efficient detection of the incorporated radioactivity.

1.19.1.2 *The Growth Fraction*

The growth fraction (Gf) is the proportion of cells in a tumour that are proliferating (in the cell cycle). Direct measurement is difficult because of the uncertainty of defining which cells are in the cell cycle. At present it is considered that that Ki 67 labelling is the closest measurement of the Gf. If Tc and Tpot are known the Gf can be calculated from:

$$Gf = \frac{\text{Log}_e 2 \times Tc}{Tpot}$$

With the advent of novel monoclonal and polyclonal antibody staining systems alternative methods to equate the Gf have been developed such as the Ki-67 (Gerdes, Schwab et al. 1993) antibody or PCNA (Miyachi K, Fritzler et al. 1987).

1.19.1.3 *Ki67 antibody staining*

The Ki67 antigen is expressed in all stages of the cell cycle except G0 (Sasaki, Murakami et al. 1987). There is some variation. In G1 it is expressed in the nucleoli, during S and G2 there is increased intensity with nucleoplasmic distribution and the peak activity is in M where the antigen is chromatin associated. Recent evidence has

demonstrated that that its expression may be an absolute requirement for cell proliferation (Duchrow, Gerdes et al. 1994). Although its function and role has not been totally elucidated, it may have a role in the nuclear matrix, condensation of chromosomes or the breakdown of the nuclear envelope prior to mitosis (Schluter, Ducrow et al. 1993). The Ki67 gene is localised on chromosome 10 and encodes a doublet protein of 345 and 395 kDa.

The best known antibody for detecting Ki 67 was the IgG1 murine monoclonal antibody derived from mice immunised with extracts from a Hodgkin's disease cell line L428 (Gerdes, Schwab et al. 1993). However this was hampered by the necessity to use frozen sections thought to be due to the fragility of the Ki67 epitope. More recently, a new series of antibodies has been developed by immunising mice using protein sequences obtained from the central repeat of the Ki67 gene of E. Coli (Key and Becker 1993). As a result the MIB 1 antibody has been developed that has been shown to recognise the Ki67 antigen in formalin fixed paraffin embedded tissue sections after antigen retrieval techniques (Cattoretti, Pileri et al. 1993).

1.19.2 Rate measurements

1.19.2.1 The Volume Doubling Time

The volume doubling time (Td) represents the time interval in which a tumour doubles in volume and this measurement is therefore subject to cell loss, the growth fraction as well as any host cells contained within the tumour mass. It can be derived from: i) Serial sacrifice of animals and weighing the tumours. ii) Sequential measurements with callipers and the use of appropriate formulae for converting linear dimensions to volume. iii) Displacement of fluid giving a volume measurement.

Most tumours reduce growth rate with increasing size and represent a Gompertzian growth curve. ie and exponential growth with an exponential slowing superimposed with increasing age. The Td for normal tissues is infinitely long since cell production

and loss are perfectly matched. The Td of tumours varies between one day in fast rodent tumours to many months in slowly growing human tumours.

1.19.2.2 The Potential Doubling Time

The potential doubling time (Tpot) is a “rate measurement” and is defined as the time within which a cell population would double its number if cell loss did not occur. Thus, if all of the cells were proliferating (GF=1) and there was no cell loss then the Tpot would equal the Tc. It is a good measure of cell proliferation of a tumour because it takes into account the Tc and the GF.

Tpot is determined from an estimate of the proportion of the cells in any phases of the active cell cycle and the duration of that phase. They are usually calculated from either the mitotic index and the duration of mitosis or more commonly from the labelling index (LI) ie the proportion of cells which take up a DNA precursor and the duration of S phase. DNA precursors traditionally included tritiated thymidine which was analysed by high resolution autoradiography but now bromodeoxyuridine is utilised more commonly and analysed by flow cytometry.

T pot is calculated from
$$\mathbf{T_{pot}=\lambda \times T_s/LI}$$

λ is applied because of the nonlinear distribution of cells in the cell cycle such that on average there are twice as many cells in the postmitotic phase G1 than in the immediately G2 premitotic phase (Steel and Lamerton 1966).

To apply this formula the duration of the appropriate phase is required. For mitosis this was obtained by strathmokinetic techniques. Spindle poisons such as colcemid are use to block mitosis and the time taken for the metaphases to double is the mitotic duration. Similarly, repeated injections of tritiated thymidine can be given to calculate phase duration. The advantage of the bromodeoxyuridine and flow cytometric method is that S-phase duration can be calculated from one biopsy using the relative movement method (Begg, McNally et al. 1985).

1.19.2.3 Cell Cycle Time

The cell cycle time (T_c) is the time interval within which one cell completes a mitotic cell cycle. i.e. from birth at mitosis to eventual splitting to two progeny at the next mitosis having passed sequentially through G1, S and G2 phases of the cell cycle. Traditionally this has been obtained with the percent labelled mitosis (PLM) method. After injection of tritiated thymidine, multiple biopsies, at time intervals, are taken and the amount of radiolabelled mitosis in each sample was determined. Cell cycle times can be calculated from the plot (Figure 1.24) Variation in phase length between cells prevent the curves from being idealised as shown and results in less distinct curves with dampening in successive cell cycles. Computer assisted analysis is, therefore, required.

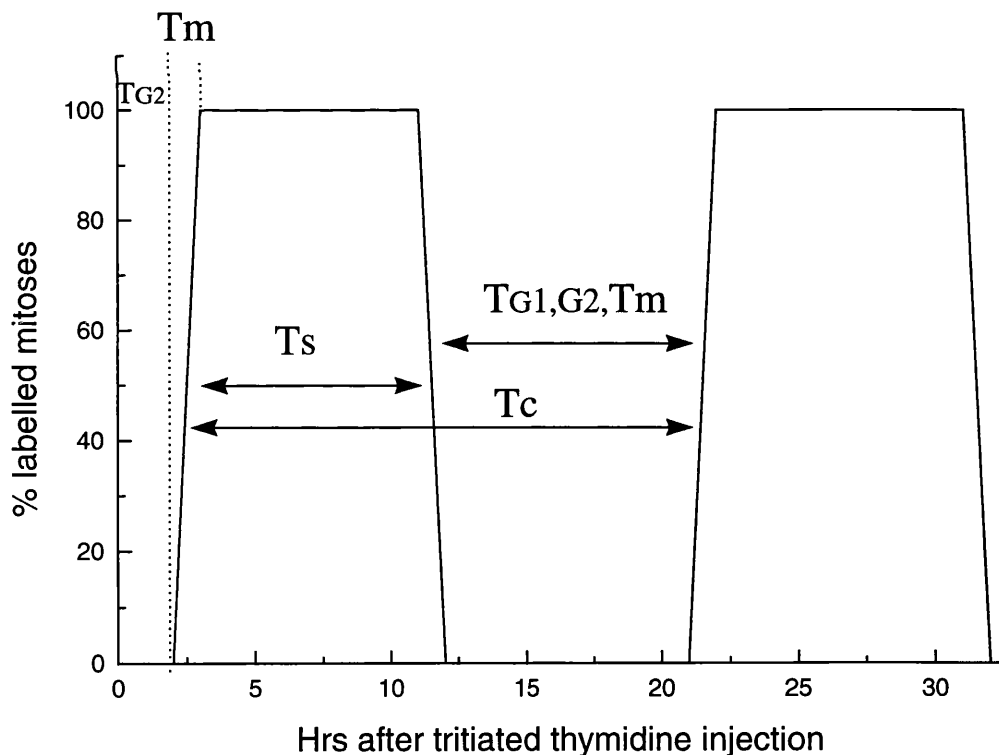


Figure 1.24 Idealised curve for the cell cycle time measurement by the PLM method.

1.20 Measurement of cell death

As discussed previously, variety of mechanisms exist for cell death. There may be discrete apoptotic cells, large areas of necrosis, dead cells may autolyse and resorb or be phagocytosed and removed. Their fate will depend on whether they contribute to cell volume or not. Cell loss factor (ϕ) can be calculated from the discrepancy between the T_{pot} and T_d (Steel and Lamerton 1966), although this formula tells nothing of the mode of cell death

$$\phi = 1 - T_{pot}/T_d$$

Apoptosis as a cause of cell death, in isolation, can be recognised, although the in-situ recognition is rather difficult (Kerr and Searle 1987). Apoptosis can be recognised by morphological criteria, however the cells undergo rapid phagocytosis and are not always readily recognisable, nevertheless from examination of thin resin embedded tissue sections, it has been estimated that the ratio of apoptotic cells to normal cells in experimental tumours ranged between 0.2% and 7.9% (Nakagawa, T et al. 1995).

The morphological changes in the nuclei that accompany apoptosis are associated with the activation of a specific endogenous endonuclease that cleaves double stranded DNA of chromatin at the internucleosomal linker DNA (Wyllie, Arends et al. 1992). On gel electrophoresis these products display a ladder pattern of multiples of 180-200 base pairs. This indicates apoptosis but is not quantitative. Consequently a DNA strand break staining method was devised for quantifying apoptotic cells in situ (TUNNEL) (Gavreili, Y. et al. 1992). This method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the 3'-hydroxyl terminal of DNA and the TdT catalysed addition of several thousand normal or modified mononucleotides to these 3'-termini which can be visualised and quantified. This method may not demonstrate many more cells than are found with conventional histology (Ansari,

Coates et al. 1993). Furthermore, evidence suggests that in situ end labelling is not entirely specific because necrotic cells are detected (Ansari, Coates et al. 1993).

1.21 Measurement of cell kinetics with flow cytometry and bromodeoxyuridine

In Vivo measurement of cell kinetic parameters has been plagued with the impracticalities such as the necessity for radioactive injections, the need for multiple biopsies and the weeks or months required for the development of autoradiographs. A method of measuring cell kinetic parameters was developed that does not require a radioactive label and can be performed on a single sample (Begg, McNally et al. 1985). The method uses 5-bromo-2-deoxyuridine (BUDR), a thymidine analogue, that is incorporated in S phase. The crucial step in this technique was the development of monoclonal antibody that recognised BUDR (Gratzner 1982). In 1985, The Gray Laboratory developed a method to calculate the T_s from a single in Vivo measurement by using double staining techniques and flow cytometric analysis. BUDR uptake could be measured as a function of DNA content allowing the calculation of T_s , LI and T_{pot} by the relative movement method (Chapter 2) This has subsequently become the method of choice in a number of centres especially for the determining prognostic measurements for radiotherapy schedules.

1.21.1 Flow cytometry

The flow cytometer is a well established powerful and versatile analytical tool that has been developed over the past 25 years (Figure 1.25). It allows the simple and rapid quantification of substances in minute quantities and has 2 main applications. Firstly, in combination with antibody staining techniques it can simultaneously assay a wide range of cellular parameters including proteins and nucleic acid content. Secondly, flow cytometry can be used as a cell sorting device to isolate and further utilise one cell type from another.

The principle that underlies the function of flow cytometry is the measurement of fluorescence derived from the specifically labelled cells or cell contents, proteins or other macromolecules. These labelled particles are delivered precisely, one by one, through a beam of laser light. Hydrodynamic focusing of the particles in a fluid system is the principle behind the precision delivery system. Fluorochrome markers, incorporated into the cell or protein target, become excited by the laser light and respond by emitting light of a specific longer wavelength. The emitted light is separated and collected by the optical system of the flow cytometer and converted into an electrical and finally a digital proportional to the amount of the original substance being measured. The flow cytometer is a powerful analytical tool because the measurement is taken for each particle and not the average of the total population.

There are three basic requirements for accurate flow cytometry.

- 1) Sample preparation is all important, a prerequisite for FCM is a good single cell suspension separated by mechanical and enzymatic methods. There is an axiom in flow cytometry, “garbage in, garbage out”.
- 2) The target particle, protein or cell usually requires a highly specific label. This is often a monoclonal antibody or dye. The antibodies are then labelled with a fluorochrome tag such as fluorescein Isothiocyanate (FITC) or phycoerythrin (PE). There has been a huge expansion of monoclonal antibodies in the last decade and therefore the number of quantifiable factors has increase dramatically. The choice of fluorochromes has also enabled 2 or 3 parameters to be assayed and therefore inter-relationships can be studied in detail. It is therefore essential that the spectra of the emitted light can be separated and does not overlap to a great degree.
- 3) Specialised fluidic systems are required for precise delivery of the particles into the laser light. The particles are orientated within a flow chamber to intersect with the laser light by hydrodynamic focusing, most commonly using laminar flow with viscous drag . The fluid at the centre of a moving channel flows faster than the fluid at the periphery. This forms a flow front in the form of a parabola. As a result of

this velocity gradient, the particles are drawn towards the centre by hydrodynamic focusing to form a stable parabola prior to the entry to the detector point.

- 4) Detection systems are required to detect the emitted light from the fluorochromes in response to laser stimulation. The emitted light is scattered prior to collection on photodetectors that convert light signals into electrical signals. In order to distinguish the output of several combined fluorochromes light is optically filtered into separate bands of differing wavelengths. This is achieved by dichroic mirrors, interface phenomena and absorption filters, which selectively deflect or allow the passage of various pulses of light, depending on their wavelength (Figure 1.26). Finally the separated signals are processed and digitally converted prior to graphic display. Analysis is facilitated by computer software programs.



Figure 1.25. A Becton Dickinson (San Jose, Calif) flow cytometer.

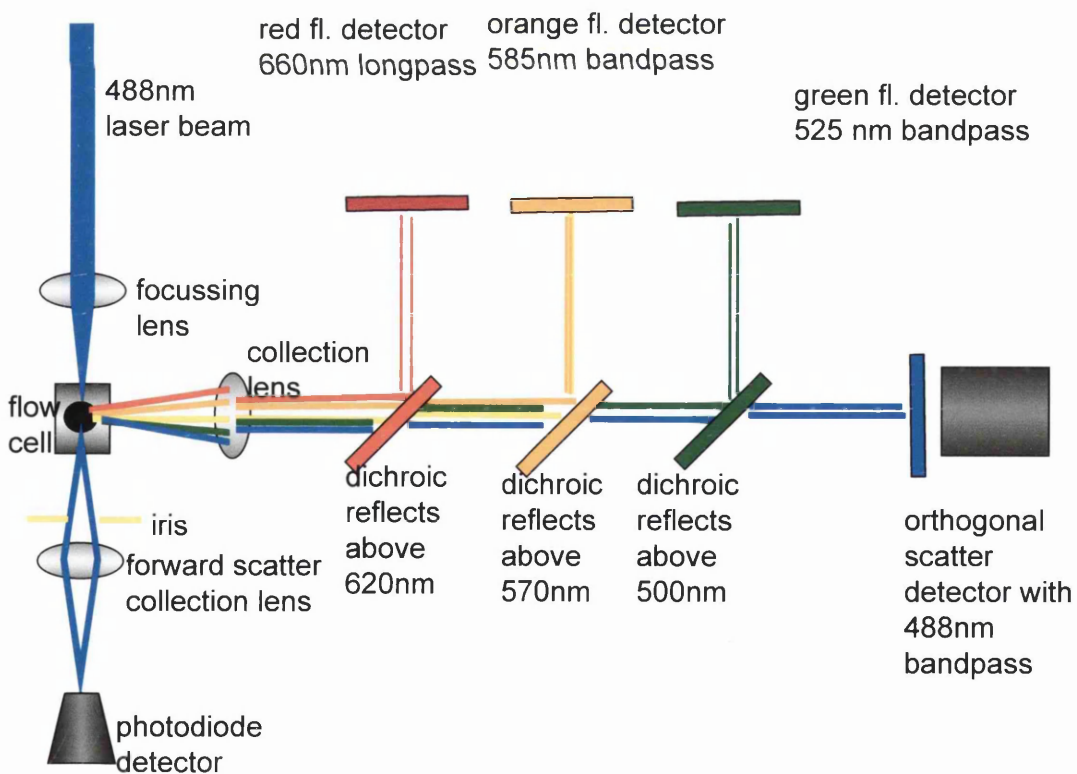
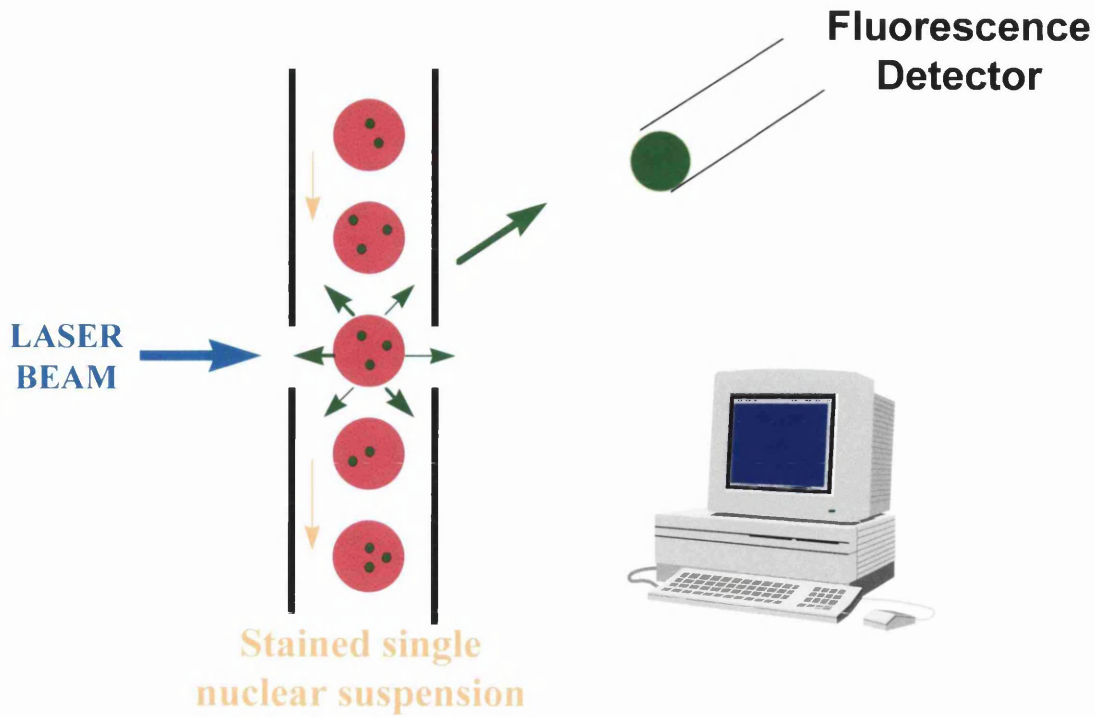


Figure 1.26. Diagrammatic illustration of the flow cytometer.

1.22 Clinical applications of cell kinetic measurements

The importance of the fine balance between cell production and cell loss in normal tissues has already been discussed. Uncoupling of this balance results in degenerative, hyperproliferative or malignant disease. In order to be able to treat or prevent cancer more effectively an understanding of the fundamental proliferative abnormalities is essential.

It is now widely accepted that the rate at which human tumours proliferate is of major significance in not only determining the development and progression of cancer but also in the response of tumours to treatments such as radio and chemotherapy (Wilson, Dische et al. 1995).

Historically measurement of TLI led to a rapid growth of interest in measuring proliferation kinetics in both normal and malignant tissues. Clinicians began to utilise data from these studies in an attempt to identify differences in the growth characteristics of malignant cells as opposed to normal tissues, with the findings of a number of these studies suggesting the following:

- i) TLI values show considerable variation, both within a group of similar tumours and between different histological types. The highest values were found in lymphomas (Malaise E.P., Chavaudra N. et al. 1973) and the lowest in adenocarcinomas (Steel G.G. 1977).
- ii) An association exists between the doubling time of tumours and the TLI (Malaise E.P., Chavaudra N. et al. 1973).
- iii) TLI is seen to be higher in more aggressive, less differentiated tumours (Tubiana M. 1986)

iv) TLI may indicate the risk of disease progression, as first observed in breast carcinoma (Tubiana M. 1986).

However the impact of these studies was disappointing firstly, thymidine labelling was lengthy involving weeks of processing. Secondly it soon became evident that cell proliferation was not the only factor determining tumour growth, Nevertheless several groups persisted with this and other methods and demonstrated the prognostic importance of the LI (Tubiana M. and Courdi A. 1989).

With the development of more sophisticated “rate measurements”, that could practically be performed on a patient basis, interest in the use of cell kinetic measurements developed especially to predict the outcome of radiotherapy. Treatment scheduling could be tailored to an individual basis based on the relevant biological characteristics as well as clinical feature (Wilson G.D. 1991).

1.23 Cell kinetics of BCCs and of keratinocytes

Clinical observation of BCC suggests that the volume doubling time of BCC is long since the tumours are considered slow growing. There is one comprehensive *in vivo* tritiated thymidine study to examine cell proliferation in BCC (Weinstein and Frost 1970). Intratumoural injection of tritiated thymidine was given to patients with multiple BCCs and sequential biopsy specimens were combined from different tumours to evaluate conglomerate kinetic information for nine tumours in a PLM method. The study did not continue long enough to calculate the T_c by the usual method. Therefore, the T_c may be falsely high because it was assumed that all of the cells were in the cell cycle. (i.e. the G_f was 1). The figure quoted therefore probably best represents the T_{pot} rather than the T_c . The values calculated for the cell cycle time was 217hrs, the S phase duration 20hrs, G2 duration 7hrs, G1 duration 188 hrs and mitosis was 1.5hrs. The labelling index from a variety of patients ranged from 5-13.2% The data however was taken from areas with uniform mitotic figures which represents the maximum DNA synthesising activity within these tumours therefore

skewing the data since it does not account for the heterogeneity of proliferation seen in BCCs. These values did not support the clinical observations of slow growth and therefore a high cell loss factor was implicated.

Other studies examining cell kinetics of transplanted tumours demonstrated higher labelling indices at the periphery rather than centrally (Grimwood, Ferris et al. 1986). This may also explain the high LI found by the previous study. Kerr and Searle suggested that high rates of shrinkage necrosis (apoptosis) resulting in the formation of councilman bodies may be responsible for the clinical and kinetic discrepancies (Kerr and Searle 1972). Subsequent studies with Ki67 immunohistochemistry have supported the high rates of cell proliferation in BCC by demonstrating growth fractions that range between 4-33% (Baum, Meurer et al. 1993).

Data concerning the kinetics of normal keratinocytes in vitro or in vivo are difficult to interpret not only due to technical considerations but also due to the due to the heterogeneity of behaviour of keratinocytes. Furthermore, the majority of studies are in vitro culture systems that are designed to maximise keratinocyte growth. The table below shows figures from a number of studies of normal skin in vivo (Leigh, Lane et al. 1993).

PHASE OF CELL CYCLE	LI (%)	MI (%)	Ts (hrs)	Tm (hrs)	Tc (hrs)	Tt (hrs)
No. of studies	37	9	16	3	10	10
Range	1.2-61.0	0.006-1.6	5.9-16.0	1-1.5	50-326	154-641
Mean	3.7	0.6	8.3	1.2	209	326

Table 1.8 Keratinocyte kinetic parameters. (From the keratinocyte handbook)

Chapter 1

Section 3 Oncogenes and cancer - The control of cell proliferation and cell loss.

1.24 Introduction

Control of proliferation, differentiation and cell loss are central to the maintenance of tissue homeostasis. Uncoupling of these processes alters the constraints on normal cellular behaviour and represents a basic step towards tumour formation. Cancer cells are readily distinguished from normal cells by their ability to proliferate unrestrained by normal regulatory mechanisms and colonise territories normally reserved for other cells (Vogelstein and Kinzler 1993). Like most cellular processes, proliferation and differentiation are under the control of specific regulatory genes which may in turn be modulated by a number of extrinsic factors. Evidence that cancer has a genetic basis is provided by the observation that many carcinogens have been shown to induce DNA damage. Cancer research has become increasingly directed towards elucidating mechanisms responsible for the regulation of proliferation and differentiation.

Particular interest centres on the role of oncogenes which represent altered expression of normal regulatory genes, referred to as proto-oncogenes. Oncogenes may be defined as genes whose deregulated expression is involved with the essential steps of initiation, promotion and progression of the malignant state. This is achieved through their involvement in proliferation, differentiation, cell loss and metastatic potential. These genes act in a dominant manner, encoding proteins whose activity promotes the malignant phenotype. In addition, a second class of gene has been recognised, whose reduced expression contributes to malignant progression, these have been termed tumour suppressor genes. Mutations of these genes usually behave in a recessive fashion. i.e. both copies require inactivation for the effects to be seen. A third class of genes called mutator genes have also received much interest lately. These genes code

for proteins which maintain genomic integrity and when mutated result in genetic instability and are important in syndromes such as Xeroderma pigmentosum.

The normal control of proliferation and differentiation involves a series of steps initiated by extrinsic growth factors which bind to cell surface receptors. The signal is then transduced across the cytoplasm and nuclear membrane. In the nucleus, these signals act by transcription regulation of genes involved in proliferation and differentiation. Proto-oncogenes and tumour suppressor genes code for proteins involved in these pathways which may escape the normal constraints, if their expression is deregulated (Vile R.G. and Hart I. 1993).

This process of malignant transformation does not occur in a single step or by the actions of one gene in isolation. Transformation is postulated to be the result of co-operation between several genes, usually from different classes (Bishop J.M 1991). This is supported by the observation that transfection of one active oncogene may lead to the immortalisation of a cell line, but that co-transfection of another is required for tumorigenicity (Vile R.G. and Hart I. 1993). A variety of classification systems have been proposed for oncogenes. One concept for the classification of oncogenes is shown in Table 1.9. Factors altered during tumorigenesis are involved in either growth and proliferation or cell loss. Therefore, proto oncogenes and their products may either act by supporting cell proliferation or by inhibiting cell loss; overexpression therefore results in a net gain in cell number. In contrast tumour suppressor genes may act by inhibiting cell proliferation or promoting cell death and when their function is lost results in a net gain of cells (Simon, G et al. 1995).

FUNCTION	ABERR- ATION	DESCRIPTION	EXAMPLE
Growth	Gain of function	Growth factor, growth factor receptor, component of cell proliferation transduction pathway	c-sis, int-2, c-erb
Suppressor eg growth	Loss of function	Factors suppressing cell growth, tumour suppressor genes residing in a growth suppression signalling pathway.	p53, ?patched
Promotes death	Loss of function	gene product executing cell death program, component of signalling pathway triggering apoptosis.	Bax, Fas
Suppresses death	Gain of function	Factors suppressing cell death	bcl-2 or bcl-x

Table 1.9 A Classification of oncogenes and gene products (Simon, G et al. 1995).

A recent concept of a gatekeeper gene has been introduced that provides a useful framework for the temporal spacing of the key early genetic events in cancer (Sidransky, 1997) The observation that mutations in critical oncogenes are nearly always found in early precursor lesions give rise to the notion that that their deregulation is essential for the initiation and development of a specific neoplasm. Once the gatekeeper gene has been deregulated, then the genetic threshold of the neoplastic process, in a given tissue, is passed which is followed by clonal expansion and the accumulation of multiple genetic events. If the other genetic hits occur in the absence of the gatekeeper alterations, then the precursor lesion or tumour may not arise (Sidransky , 1997).

1.25 The gatekeeper gene for BCC

Systematic analysis of chromosome and allelic loss reveal that BCCs differ from most other epithelial cancers in alterations are mostly confined to a single chromosome arm, the long arm of chromosome 9 (Quinn 1996). The Gorlin's gene mapped to the same area (9q22-31) suggesting that this gene was behaving like a tumour suppressor gene (Farndon, Del Mastro et al. 1992). Gailani found that loss of alleles on 9q was present irrespective of BCC subtype or clinical behaviour suggesting that the gatekeeper gene resides in this locus (Gailani, Bale et al.). The recent finding that the human homologue of the *Drosophila* patched gene has strong homology with the 9q22 locus points strongly to this being the candidate gene (Hahn, Wicking et al. 1996; Johnson, A. et al. 1996). The *patched* is mutated in both sporadic and familial BCC suggests that patched was an important developmental gene that acted as an tumour suppressor gene in the development of BCC (Galaini, Stahl-Backdahl et al. 1997). It is therefore a strong contestant for the gatekeeper gene. Epidemiological and genetic evidence points to UV induced DNA damage being the prime culprit in patched inactivation.

The exact function of patched is not entirely known. From studies in the *Drosophila* it is known that patched acts via the hedgehog signalling pathway (Ingham, Taylor et al. 1991). This group of genes can alter cell proliferation via effectors such as the Wnt and TGF β proteins (Tabata and Kornberg 1993). The function of patched is to inhibit the hedgehog signalling and therefore loss or mutation may result in increased proliferation. It is also thought that patched may effect cell adhesion . A recent finding that transgenic keratinocytes expressing sonic hedgehog genes, grown in immunodeficient mice displays histological features like BCCs supports this hypothesis. *bcl-2* expression was also increased which is a regular finding in BCC. Patched regulates its own expression and therefore no expression was found in normal skin. There is over expression of patched in BCCs suggesting that the feedback loop is not functional in patched mutations (Unden, Zaphiropoulos et al. 1997).

1.26 Other genes involved in the induction or progression of BCC

Although BCC does not have the typical induction-promotion-progression sequence of many cancers i.e. From a premalignant form to an invasive cancer followed by metastasis, a variety of other genes have been implicated that may participate in the progression of BCC.

1.26.1 p53 Tumour Suppressor Gene

The p53 gene and its protein have become the centre of intensive study since it became clear that 50% of human cancers contain loss or mutations in this gene. The gene lies on the short arm of chromosome 17 (17p13.1) and encodes a 53 kilodalton phosphoprotein protein of 393 amino acids. It was discovered whilst investigating the SV40 DNA Virus which contains an oncogene encoding an oncoprotein known as the large T antigen. Crawford and Lane found a protein complexed to the large T antigen whilst attempting to isolate it (Lane D.P. and Crawford L.V. 1979). Subsequently it became apparent that the large T antigen formed the complex to inactivate p53. This mechanism was later established to be the route of action of many tumourigenic virus including E1b and the human papilloma virus (Lane D.P. and Bnechimol S. 1990).

1.26.1.1 Structure of p53

The human p53 protein has been divided structurally and functionally into 4 domains (Levine 1997). At the N terminus the first 42 amino acids code for a transcriptional activation domain. Position 13-23 are conserved amongst many species. Amino acids in this region bind to the TATA associated factors and activate transcription. Alternatively, the transcriptional activation can be negatively regulated or suppressed by proteins such as MDM2 (Lin, Wu et al. 1995).

A sequence specific DNA binding domain resides between amino acids 102 and 292. This area is also conserved among species. This region is complexly folded into a β sheet and then formed into 2 α helical loops. When the protein is in a tetramer this region binds specific DNA sequences. More than 90% of the misense mutations reside at the sequence specific DNA binding domain. These mutations fall into 2 classes. The first type results in the defective contact of the protein and results in loss of the transcriptional activation function of p53. The second type disrupts the complex folded structure of the p53 protein which results in a protein that binds the monoclonal antibody Pab240 (Cho, Gorina et al. 1994).

The oligomerisation domain resides between amino acids 324 and 355. This region is responsible for the formation of p53 tetramers in solution.

The C terminal 26 amino acids forms a domain that regulates the ability of p53 to bind to specific DNA sequences. There is evidence to suggest that p53 requires activation before there is sequence specific DNA binding. The C terminal is probably essential for the regulation of this process (Hupp and Lane 1994).

1.26.1.2 Function of p53

The p53 protein is a transcription factor that enhances the rate of transcription of 6 or seven known genes that carry out the p53 dependent cellular functions of a cell. Normally the p53 protein is kept at a low level since it has a short half life (20 minutes). It is thought that ubiquitin mediated proteolysis plays a role. p53 may also exist in a latent inactive form (Levine 1997) (Review). Activation of p53 is by way of a variety of signals. Double stranded DNA breaks following γ irradiation or DNA repair intermediates from UV or chemical DNA damage activates p53 production. This causes post translational modification of inactive forms and also lengthens the half life of p53. It is thought that p53 and DNA damage detectoin proteins may recognise the DNA disruption. Hypoxia and reduced ribonucleoside triphosphate pools have also been implicated as triggers (Graeber, Osmanian et al. 1996).

Following activation of p53, downstream events take place by causing cell cycle arrest or by initiating apoptosis. The mechanism takes place by the transcriptional activation of specific target genes (Table 1.10). p53 can also cause transcriptional suppression of a number of genes that contain the TATA promoter sequence of nucleotides.

GENE	FUNCTION
P21,WAF1, Cip1	Inhibits CDKs and cyclins and PCNA resulting in cell cycle arrest.
MDM2	Inactivates p53 therefore forming a autoregulatory loop.
GADD45	Causes cell cycle arrest by binding PCNA and also involved in DNA repair.
Cyclin G	Unknown function
Bax	A death factor that promotes apoptosis.
IGF-BP3	Blocks the signalling of mitogenic growth factor.

Table 1.10 Genes that are transcriptionally activated by p53

1.26.1.3 Cell cycle arrest

p53 is reported to cause cell cycle arrest at G1,S and G0/G1 phases (Kastan M.B., Oninye O. et al. 1991). The function of cell cycle arrest is to allow for DNA repair before DNA replication occurs. Otherwise flawed DNA would be passed to cell progeny. A reversible G1 arrest therefore provides time for repair and the S phase block prevents immediate duplication of damage. This is mediated, in part, by transcription suppression of PCNA and c-fos. However activation of other genes is also important. p53 causes activation of p21, WAF1, Cip1 (Xiong, Hannon et al. 1993; El-Deiry, Harper et al. 1994; Pines 1994) This protein complexes with cyclins, CDK and PCNA. p21 inhibits the G1 acting cyclinE-CDK2 also the G1/S acting cyclin A-CDK2 and also cyclin D-CDK4 complexes. As a consequence the Retinoblastoma protein is not phosphorylated and therefore E2F-DP proteins are not

liberated which are a requirement for cell cycle progression. There is therefore cell cycle arrest.

Although p21 appears to be the most pleiotropic mediator of p53 dependant cell cycle arrest other genes may also be activated and play a role. CyclinG may be activated and complex with the CDK required for DNA repair. The excision repair gene ERCC3 may also be induced. The Growth arrest and damage gene GADD45 may also be induced that may interact with the essential replication factor PCNA (Smith, Chen et al. 1994).

1.26.1.4 Induction of Apoptosis

p53 activation is one pathway by which apoptosis can be induced (Yonish -Rouach E.D., Resnitsky J. et al. 1991). In this way, cells with unstable genomes, due to DNA damage or cells in an abnormal environment i.e. located in a place with limiting survival factors with activated oncogenes that commit them to cell replication are eliminated by a p53 dependant apoptotic event (Levine 1997) (Review). This is probably why most cancers select against wild type p53 function. The mechanisms of p53 activation await further clarification, however it is probable that p53 either activates the transcription of apoptotic promoting genes such as bax or that survival genes such as bcl-2 are repressed (Miyashita and Reed 1995). Figure 1.27)

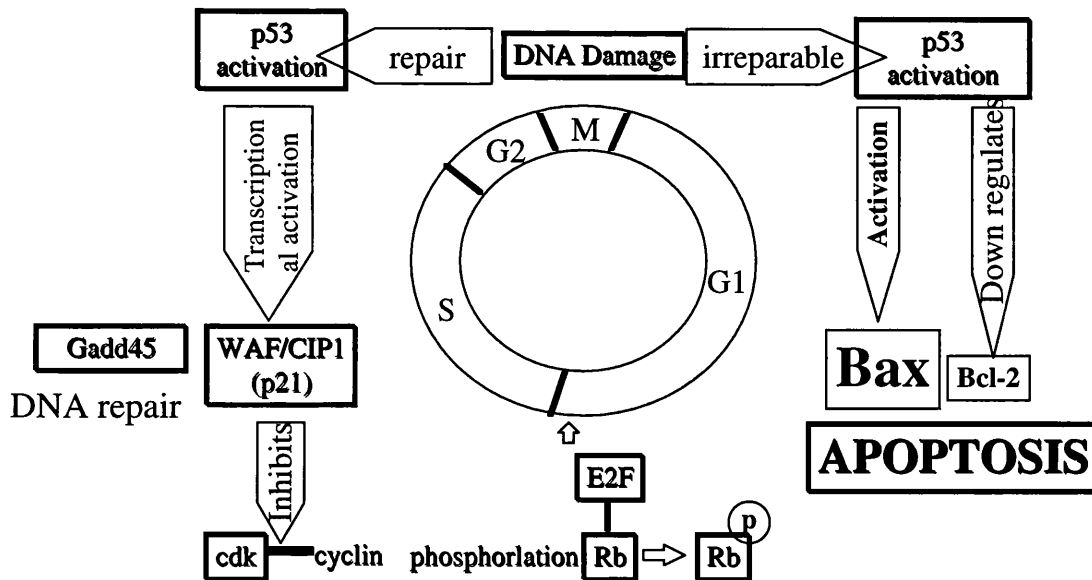


Figure 1.27 Some of the functions of p53.

1.26.1.5 p53 and cancer

P53 mutation is the most common cancer related genetic change. In addition to point mutation other abnormalities have been detected such as allelic loss, rearrangements, and deletions. Functional inactivation of p53 by viral or cellular oncoproteins is also oncogenic. Mutant forms may be inactive with the mutation causing loss of function. The loss of both alleles is required for the oncogenic effect as in the true definition of a tumour suppresser gene. However mutant p53 can bind with wild type p53 and form hetero oligomers thereby inhibiting the action of p53 by a dominant negative effect. Thirdly mutant p53 can behave as a dominant oncogenes, by upregulating other genes (Shimamura and D. 1996) (Review).

When all of the p53 mutations are grouped together they identify several codons at which exceptionally high numbers of mutations are clustered.(mutation hot spots.)

In one extensive study 98% of 280 mutations were found to occur in a 600 base pair sequence from codon 110-307 (Hollstein M., Sidransky D. et al. 1991) (Review).

p53 mutations tends to be tumour specific which most probably reflects the underlying mechanism of action of the carcinogen (Hollstein M., Sidransky D. et al.

1991) (Review). For example G:T mutation occurs commonly in the third base pair of the codon 249 in patients with hepatocellular carcinoma in area where there are high rates of chronic hepatitis B and dietary exposure to aflatoxin β 1. The specificity of this mutation may reflect a sequence which is particularly refractory to DNA repair. Similarly in squamous cell carcinoma of the skin, 20% of p53 mutations may be as a result of UV induced gene injury as indicated by tandem mutations in which 2 cytosine bases are replaced by thymidine residues as a result of pyrimidine dimer production (Rees 1994) (Review).

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 premalignant 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 p53 mutations may be a late event and associated with tumour progression. Most colorectal tumours arise via a series of genetic changes firstly in the APC gene then in the ras gene followed by DCC and finally p53 mutations (Levine 1997) (Review).

The association of p53 alteration and tumour progression has resulted in the development of p53 evaluation as a prognostic marker. Overexpression of p53 is an independent marker for poor outcome in lung ,gastric and node negative breast cancer (Harris C. and M. 1993). (Review).

1.26.1.6 p53 mutations and BCC

The genetic events that determine the development of BCC compared to SCC are contrasting. In SCC the progression of normal skin to a premalignant actinic keratosis is accompanied with a UV-induced p53 mutation (Campbell, Quinn et al. 1993). This then prevents apoptosis of sunburn cells allowing clonal expansion and further mutations to form SCC. p53 mutations may be found in up to 90% of SCCs (Rees 1994) (Review). Epidemiological evidence of cumulative sun exposure also supports this progression. In BCC, however, the association of sun exposure is weaker and there is no recognisable premalignant stage. p53 mutations are found in BCC although

are present in about 40-56% of cases (Gailani, Leffell et al. 1996). It is less common than allelic loss found at the Gorlin's gene locus, suggesting that p53 mutation occurs later in the sequence. Furthermore sun-induced p53 mutations were independent to allelic loss in the Gorlin's locus suggesting that sun may not have been the prime carcinogen for patched inactivation. Mutation of p53 is therefore associated with tumour progression and may therefore act as a prognostic marker for aggressive tumours. Further work has revealed that that only one p53 allele was inactivated which in direct contrast to other epithelial cancers where both alleles are inactivated (van der Riet, Karp et al. 1994). It is possible that in BCC the mutated p53 acts as a dominant proto oncogene or by the dominant negative mechanism by binding wild type p53. It may be that more aggressive tumour progress by loosing the second p53 allele.

1.26.1.7 p53 immunohistochemistry.

Although p53 is widely expressed, the level of protein is such that, under standard immunohistochemical conditions it is difficult to detect in normal skin (Campbell, Quinn et al. 1993). Mutant p53 results in a stable protein with a longer half life which results in accumulation. There was therefore enthusiasm for using p53 immunohistochemistry as a method for screening for p53 mutation. Subsequently weakness have been found in this technique. Firstly deletions or nonsense mutations that result in truncated proteins may not contain the correct epitope and there not stain. Secondly there may be dissociation between the intensity of the immunostaining and the presence of a mutation. False positive results may result from normal accumulation of wild type p53 as a response to DNA damage. False positive results have also been found where there is no evidence of mutation. (Campbell, Quinn et al. 1993). Conversely false negative results have also been found when there is a mutation present on DNA sequencing (Campbell, Quinn et al. 1993). Patients with Li Fraumeni syndrome are immunohistochemically negative for p53. Positive or negative immunostaining cannot therefore necessarily be taken to imply the presence or absence of a mutation.

1.26.2 bcl-2 and the control of apoptosis

Cell death is regulated by a fine balance of genes and gene products that are either survival factors or specific death factors. Thus activation or removal of either of these factors can initiate or prevent apoptosis. Apoptosis can be divided into 3 different phases: Initiation, effector and degradation. The initiation phase depends on the type of apoptosis inducing stimulus. The effector and degradation phases are common to all apoptotic processes (Kroemer 1997) (Review).

The bcl-2 gene family encode over a dozen proteins encoded by most species and are the most relevant classes of apoptosis regulatory genes products acting on the effector stage of apoptosis. Expression of some such a bcl-2 and bcl-x1 suppress apoptosis (survival) others such as Bak and Bax promote apoptosis (death). bcl-2 was the founder member of the family and was first identified as part of the most common translocation (14:18) in human B cell follicular lymphoma (M.L., S.D et al. 1986). bcl-2 is widely expressed during embryonal development but in the adult is confined to stem cells and long lived cells such as resting B lymphocytes and peripheral sensory neurones (Hockenbery, Zutter et al. 1991). Targeted expression of bcl-2 to lymphoid tissue in transgenic mice leads to and increased number of mature resting B cells and potentates their life span. Affected T cells have increased resistance to the cytotoxic effects of radiation and glucocorticoids. bcl-2 knockout transgenic mice develop a low incidence of malignant lymphoma. Co-expression with c myc with bcl-2 gives a markedly enhanced incidence of tumours probably due to the ability of bcl-2 to block c-myc induced apoptosis (Harrington, Fanidi et al. 1994) (Review).

bcl-2 is a member of a large family of genes with multiple representatives in mammals, nematodes and virus. The proteins of the bcl-2 family are localised predominantly on the outer mitochondrial membrane. In addition bcl-2 is also found on the nuclear membrane and also in the endoplasmic reticulum. It is thought that localisation to the mitochondria is essential for function. The gene has 4 conserved regions called bcl-2 homology region1, 2, 3 and 4. (BH1-4) (Kroemer 1997).

The death agonist/antagonist rheostat is determined by the ratio of death antagonists (bcl-2, bcl-x1, bcl-w) to agonists (bax, bak, bcl-xs, bad). The life/death rheostat is mediated in part by the competitive dimerization between pairs of agonists and antagonists. BH1 and 2 are required for the death antagonists such as bcl-2 and bcl-x1 to heterodimerize with bax and repress cell death. However BH3 is required for the death agonists such as bax or bak to form heterodimers with bcl-2 or Bcl-x1 to promote apoptosis. Regulation of apoptosis is not only by the relative concentrations of the agonists and antagonists but also by post translational modification. Proteases may cleave and therefore inactivate bcl-2. Eg HIV protease cleaves bcl-2 which may explain the death of HIV lymphocytes. Phosphorylation of serine residues also modifies the activity of the bcl-2 family. Phosphorylation may be controlled by growth factor receptors and kinase cascades thereby forming a signal transduction cascade.

The exact mechanism by which the bcl-2 family control apoptosis is unknown. However it is thought that the bcl-2 proteins interact with the mitochondrial permeability transition pores. This disrupts the mitochondrial membrane which marks the point of no return of the apoptotic pathway.

1.26.2.1 bcl-2 in cancer and BCC

As previously stated excessive growth of a neoplasm may result from uncontrolled cell proliferation or limited cell death. Most knowledge concerning oncogenic events concentrates on mechanisms of increased proliferation and there is less information regarding defective cell death in neoplasms. Diminished cell death has however been implicated a primary event in two third of follicular and a quarter of high grade B cell lymphomas due to the translocation of the bcl-2 oncogene into juxtaposition with the immunoglobulin heavy chain. This results in high expression of bcl-2 thus conferring a survival advantage to the neoplastic cells. A similar mechanism has been postulated for squamous cell carcinoma of the lung of which a third show bcl-2 positively.

Basal cell carcinoma has also been shown to positive immunohistochemical staining for bcl-2 in the majority of cases. It is supportive of the theories of the basal layer of origin of BCC since only the basal layer of normal skin expresses bcl-2 and cutaneous SCC tends to be immunonegative for bcl-2 (Cerroni and Kerl 1994; Nakagawa, Yamamura et al. 1994; Morales Ducret, van de Rijn et al. 1995; Verhaegh, Sanders et al. 1995; Rossen, Haerslev et al. 1997; Wikonkal, Berg et al. 1997). This finding however does not support kinetic data regarding the clinically slow growth rate of BCC despite the high growth fraction and rapid cell cycle time with high rates of apoptosis.

1.27 Experimental Aims

The aims of this series of experiments were to:

1. Study the cell biology of BCC by evaluating cell proliferation by state and rate measurements. State measurements included the LI (BURD) and GF (Ki-67). Rate measurements involved the *in vivo* administration of BURD with followed by analysis with flow cytometry to calculating the Tpot and Tc (Chapters 3 and 5).
2. Study the cell biology of BCC evaluating cell loss by quantifying apoptosis by morphological methods. The importance of the contribution of the bcl-2, bax and p53 oncogenes in the regulation of cell loss was investigated by immunohistochemical methods. (Chapters 3 and 6).
3. Evaluate the contribution of the cell production and cell loss to the histological characteristics of BCCs (growth pattern and differentiation status). A novel classification system was developed to facilitate this (Chapters 3-6).
4. Evaluate the contribution of the morphological and biological factors to the prognosis in BCC. Cell production and cell loss was analysed in primary, recurrent and horrifying BCC. The importance of the biological factors in determining outcome, in relation to medical and patient factors, was also explored. (Chapters 4-6)
5. Assess the efficacy of a novel treatment modality, the optomechanically flash scanned carbon dioxide laser was evaluated in Chapter 7.

Chapter 2

Materials and Methods

2.1 Introduction

The prime objective of this study was to investigate the underlying cell biology of BCC by studying the cell kinetic parameters and some of the oncoproteins that control cell production and cell loss. Having developed an insight into these mechanisms we then proposed to establish whether these measurements correlated with the histological and clinical subgroups found in this tumour. Tumours were therefore divided into defined histological and clinical groups. In the third chapter precise quantitative cell cycle measurements were evaluated from a small cohort of patients by *in vivo* administration of bromodeoxyuridine analysed by flow cytometry. Further examination of these specimens by immunohistochemistry was used to quantify and identify distributions of proteins within these tumours and establish any relationship between each other and clinicopathological features. In the fourth, fifth and sixth chapters, the relationship of some of these measurements was correlated with histopathology and clinical outcome in a large cohort of non recurrent, recurrent and horrifying BCC. These chapters were aimed at improving the understanding of the precise control of cell proliferation and cell loss in BCC and its relationship with clinical or histological criteria. In the seventh chapter the assessment of a novel treatment of BCC (the optomechanically flash scanned carbon dioxide laser) was assessed in a heterogeneous patient population.

2.2 Patient Material

2.2.1 Bromodeoxyuridine study

Whilst BrdUrd is free of acute toxicity, it carries a very small risk of mutagenicity, teratogenicity and may become cytotoxic in higher doses (Goz B. 1978). Therefore administration of BrdUrd to patients required Mount Vernon Hospital Ethical Committee approval. Patients less than 65 years of age were excluded. Consultant and informed patient consent was also required. As BrdUrd was administered 4-6 hours prior to tumour biopsy it was most practical to include patients who attended as in patients only. Crystalline BrdUrd (CRC Drug Formulation Unit, University of Strathclyde, Glasgow) was made up as a fresh solution (200mg in 20ml of 0.9% normal saline) immediately before each administration as it degrades within a short time in the aqueous form. The bolus was administered intravenously over a 2 minute, period 4-6 hours prior to surgery. The time between administration and excision of the tumour was recorded. Following tumour removal a wedge of tissue was removed so as not to interfere with the examination of the tumour margins by the pathologist. The sample allocated for analysis was the placed in 70% ethanol and stored at 4°C until analysis.

2.2.2 Archival material

3 clinical groups were evaluated.

Primary (non recurrent) BCC. These patients were selected from 1991 and 1992 and therefore 5 year follow up data was known from the clinical notes. The paraffin blocks were identified from the Mount Vernon histopathology archives.

Recurrent basal cell carcinomas. These patients were identified from several sources. Firstly from reports encountered while studying the primary patients of 1991 and 1992. Secondly from patients attending mount Vernon during the first 6 months of

this study. Thirdly, from patients encountered in the BrdUrd study. Attempts were made to obtain the first and successive biopsy blocks from the Mount Vernon archives and from other hospitals if necessary.

Horrifying basal cell carcinomas. From the preceding chapter it can be seen that a variety of nomenclatures exists for clinically or histologically problem tumours. Of the multitude of terms, horrifying as a clinical description is defined the most clearly as being **large** (greater than 3 cm) **destructive** (invading vital structures beyond the surrounding skin and subcutaneous fat e.g. cartilage, bone or brain), **locally uncontrollable** (recurrence despite 4 or more adequate attempts at eradication) or **metastatic**. In this present study Jackson's criteria were modified since 3cm was considered too small and would not exclude many superficial basal cell carcinomas. We defined horrifying tumours as:

1. **Large.-** Greater than 5 cm. All tumours had to meet this criteria and include 1 of the following:
2. **Destructive.-** Invading vital structures beyond the surrounding skin and subcutaneous fat e.g. cartilage, bone or brain
3. **Locally uncontrollable.-** Recurrence despite 4 or more adequate attempts at eradication
4. **Metastatic.-** Growth of tumour in tissues not in direct continuity with the primary site.

The tumours were identified by personal recollection from each of the Mount Vernon consultants. A further cohort of horrifying tumours was obtained in collaboration with the Craniofacial Unit. (N.W) at Charing Cross. Attempts to obtain the first biopsy and all subsequent samples was made.

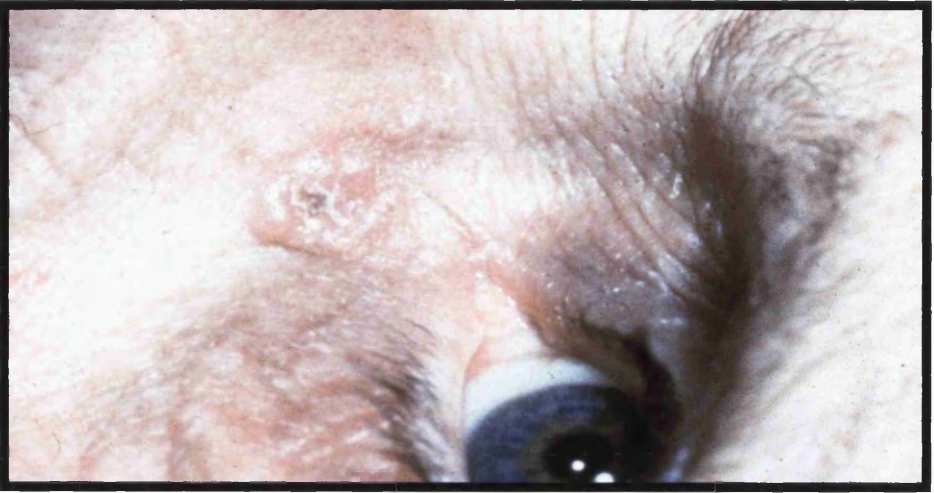


Figure 2.1 Primary BCC (left). Recurrent BCC in radiotherapy site (centre). Late presenting horrid BCC (right)

2.3 Clinical data

In order to evaluate the clinical significance of oncoprotein expression and proliferative parameters the following clinical data was sought from each patient's records.

1. Age
2. Sex
3. Time that the BCC has been present.
4. Date of operation
5. Site of tumour
 - Head and Neck
 - Limbs
 - Trunk
6. Clinical subtype of tumour
 - Primary
 - Recurrent
 - Horrifying
7. Treatment type
 - Surgery
 - Radiotherapy
 - Other
8. Adequacy of surgical margin
9. Date of recurrences
10. Length of follow up

2.4 Histopathological Data

Four micron H & E sections from all tumours studied in this project were examined by myself and a Consultant Histopathologist. (PR). Where possible, sections of tumours excised at Hospitals outside Mount Vernon, were also reviewed to obtain the required histological data. The classification of BCC is complex. We chose to classify the tumours according to the growth pattern similar to that described by Sexton

(Sexton, Jones et al. 1990). This being that this type of classification is clinically usable and can separate the tumours into discrete subgroups which show some relationship with clinical outcome. We also combined this classification with a description of the degree of differentiation according to a simplified Lever type classification (Lever 1971). This provided a comprehensive system that is a complete description and may provide further prognostic information. The following data was therefore collected.

1. Conformation of diagnosis

2. Growth pattern. (Figure 1.14 and figure 2.2)

a.) Superficial in which multiple tumour peninsula extended from the epidermis and/or adnexae and abutted or penetrated the papillary dermis.

b.) Nodular which consisted of a rounded mass of tumour cells with a well defined contour and peripheral pallisading.

c.) Micronodular in which the tumour islands tended to be smaller and more numerous than the nodular type but still retained a uniform contour.

d.) Infiltrative, was characterised by an irregular contour to the tumour islands with a tendency to form spikes. The tumour islands could be large or small usually with poorly developed peripheral pallisading.

e.) Morpheic. In this subtype, the tumour islands were small and elongated with jagged ends and the stroma is sclerotic. The neoplastic tissue infiltrates as narrow strands or cords of cells.

3. The most predominant differentiation subtype.

a.) Undifferentiated (Solid). The tumour masses show no differentiation with the peripheral layer of nuclei arranged in a pallisade arrangement and the central nuclei in a haphazard fashion.

b.) Adenoid. There are formations suggesting tubular or gland like structures that results in a lace like appearance. Some tumours may contain cells with a secretory appearance.

c.) *Cystic*. There are cystic spaces at the centre of the tumour masses that may result from degeneration of tumour cells, degeneration of stromal components or by sebaceous differentiation.

d.) *Degree of squamous metaplasia*. Keratinization may be found in BCCs. This may be partial and result in the formation of parakeratotic whorls or bands or it may be complete and result in the formation of horn cysts. Some BCCs may appear as an intermediary between BCC or SCC since they may have an oesinophilic tint due to partial keratinization and also contain prickles cells.

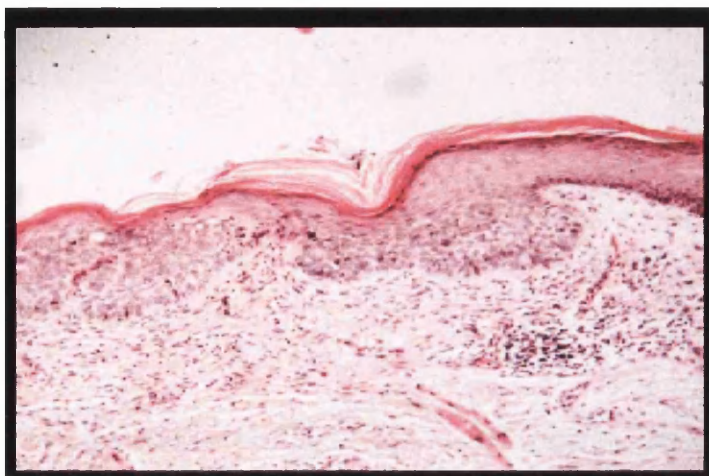
4. *Apoptosis* was identified by the characteristic nuclear features on haematoxylin and eosin staining such as crescents of collapsed chromatin along the nuclear envelope and fragmentation of the nucleus into spheres in single cells. (See figure 2.2) The spontaneous apoptosis was quantified in this study by counting the number of apoptotic bodies in 10 high power fields (x400) and expressing this number as a fraction of the total cell number. This was in contrast to the confluent apoptosis that may be found secondary to necrosis that was not accounted for in this study.

5. *Mitosis* was recognised by the characteristic nuclear condensation seen across the cell in metaphase. The mitotic index was calculated in a similar manner to the apoptotic index.

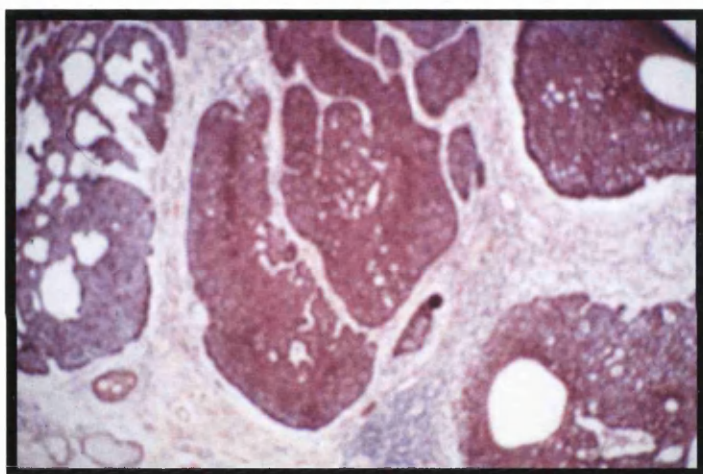
6. *Necrotic cells* were identified by the histological appearance of cytoplasmic and organelle destruction and loss of membrane integrity usually appearing in confluent areas. This was almost always associated with an inflammatory response. Necrosis was assessed semi-quantitatively and scored as none, +, ++, or +++ depending on the extent.

7. *Ulceration* was recorded if there was discontinuity of the epithelium over a BCC.

a.



b.



c.

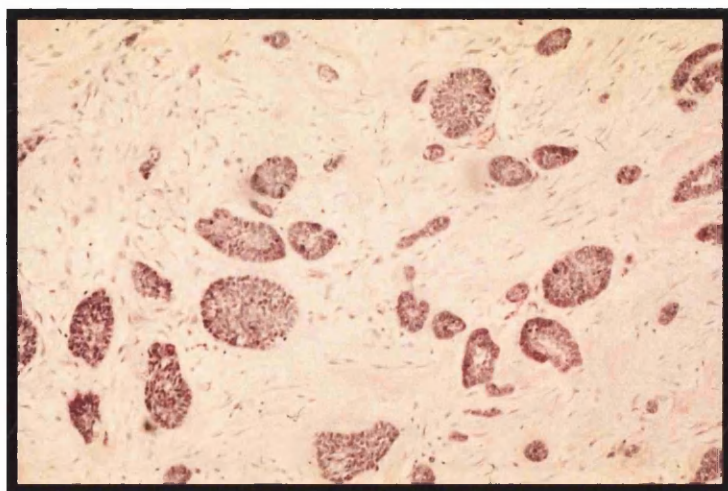
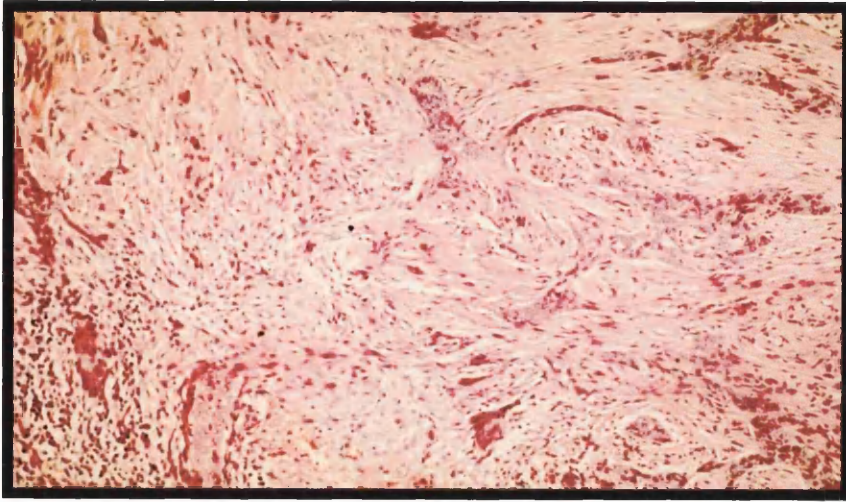
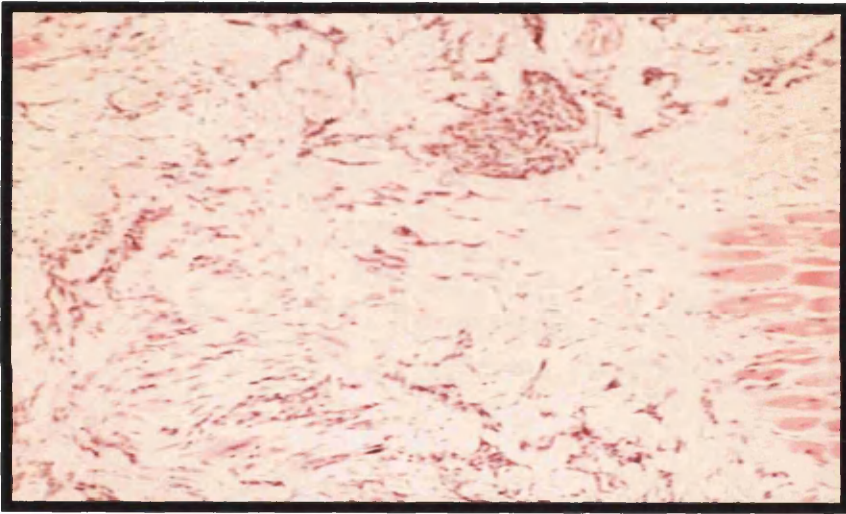


Figure 2.2. a.) Superficial BCC. b.) Nodular BCC. c.) Micronodular BCC.

d.



e.



f.

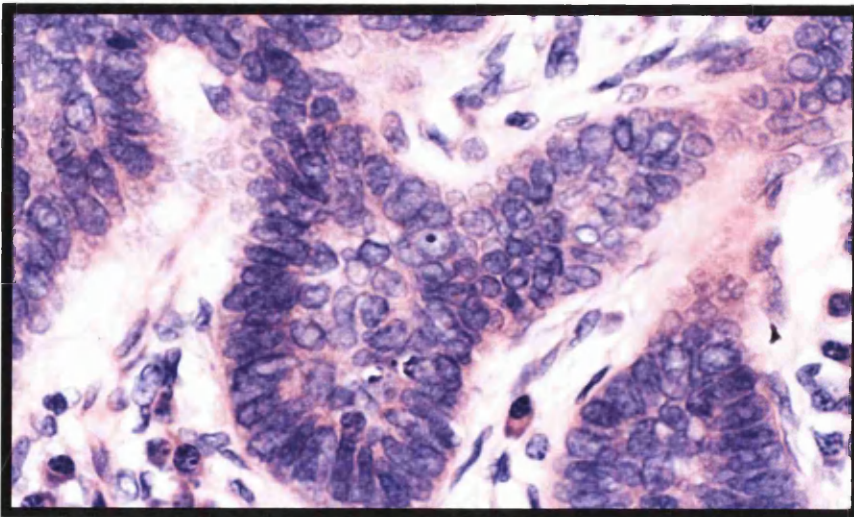


Figure 2.2. d.) Infiltrative BCC. e.) Morpheic BCC. f.) Apoptotic bodies seen in a BCC.

2.5 Tissue fixation

Tissues samples were preserved by one of two methods.

- 1) Formalin fixed: specimens were fixed in 10% formal saline for at least 24 hours prior to embedding in paraffin using standard procedures. Sections for histological analysis were subsequently stained with haematoxylin and oesin. (H&E) again using standard procedures.
- 2) Ethanol-fixed tissue: Fresh samples were directly fixed in 70% ethanol to allow prolonged storage at 4°C. Specimens were routinely preserved using this method for BrdUrd analysis.

2.6 Flow Cytometry

2.6.1 Sample preparation and BrdUrd staining of nuclei

A 3mm³ segment of ethanol fixed tumour was minced and the nucleus extracted by enzymatic digestion in 10ml of 0.4% pepsin in 0.1M HCL (Sigma Chemical Co.) in a 25ml universal tube. The mixture was the constantly agitated, in a warm room at 37°C for 45-55 min on a rotary wheel apparatus and then filtered through a 35µm nylon mesh. The suspension was centrifuged at 2000rpm for 5 minutes and the nuclear pellet resuspended in 2.5ml of 2M HCL for 12 min at room temperature. This step is required to partially denature the double stranded DNA to allow access of the BrdUrd monoclonal antibody to be incorporated without excessive disruption of the DNA helical structure which may interfere with the stoichiometry of propidium iodide staining. The sample was then washed twice in PBS, prior to the addition of a 1:25 dilution of rat anti-BrdUrd antibody (Hybridoma Unit, Institute Of Cancer Research, Sutton, Surrey, UK) in PBS containing 0.5% Tween-20 (Sigma Chemical Co.) and

0.5% normal goat serum (NGS) (Sigma Chemical Co.). The suspensions were incubated at 1 hour at room temperature then after washing in PBS, the second antibody was added. This comprised of a 1:25 solution of goat anti-rat IgG-FITC conjugate (Sigma Chemical Co.) in the PBS/Tween/NGS solution. The suspension was incubated at room temperature for a further 30 minutes then washed in PBS and resuspended in 2ml of PBS containing 20 μ l of 1mg/ml propidium iodide solution to label DNA.

2.6.2 Description of the FACScan

The FACScan system is an automated cell analyser developed for both research and clinical applications. It consists of a bench top sensor module coupled with a computer module which controls both acquisition and analysis of data (Figure 1.25 and 1.26). Cells or nuclei enter the flow chamber in single file and are irradiated by a 15mW, 488nm air-cooled argon-ion laser. Dichroic mirrors spectrally filter emitted light, separating and deflecting longer wavelengths whilst transmitting shorter wavelengths. Two laser light scatter signals are collected termed forward and side scatter (Figure 1.26). The former gives information on cell size whilst the latter represents the signal generated as a result of the structure and nature of the cell. The emitted wavelengths are reflected and transmitted one of three photomultiplier detectors, FL1, FL2 and FL3, following which the electronic signal is digitised and processed by the computer.

At 530nm the FL1 detector is optimised for FITC detection whilst the FL3 detector transmits wavelengths in excess of 650nm, suitable for detection of red light emitted by propidium iodide. The FL2 channel detects intermediate wavelengths (585nm), emitted by phycoerythrin in the red/orange band (this channel was not used in the present investigations). Dual parameter collection of data on FL1 and FL3 allows bivariate dot plots to be formulated, recording expression of FITC-labelled data on the FL1 channel against DNA content on the FL3 channel. Doublet-discrimination can also be performed on the flow cytometer prior to recording data, a region was set around the nuclear profile, using the FL3 area and width, to exclude any residual doublets, aggregates and cellular debris, to acquire a single nuclei suspension.

2.6.3 Data Analysis

Data was analysed using a computer acquisition/analysis programme, Lysys II (Becton Dickinson, San Jose Calif). Events were quantified by the imposition of computer generated windows (CGW) to define specific regions on the histogram or two-dimensional dot plot. Regions can be set around populations to omit extraneous interference from debris or from populations of cells whose data is not required, as shown in Figures 2.3 and 2.4. These regions are retained and superimposed on the control sample, to allow comparison of the number of nuclei and fluorescence in identical regions.

2.6.4 Calculation of the kinetic parameters from BrdUrd labelling

2.6.4.1 Time between injection and biopsy.

The time interval determines how far the BrdUrd labelled cells have moved around the cell cycle after drug administration. BrdUrd has a short half life in vivo and only labels cells involved in DNA synthesis at the time of injection, the starting point is therefore the S phase. The starting point is the S phase which is where the labelling occurs. The position of the labelled cells is calculated from their DNA content measured by propidium iodide staining. Therefore the time interval between injection and biopsy should be long enough to detect movement, but insufficient for all of the labelled cells to have divided. An ideal time interval would be half the S phase duration which should produce a profile of adequate redistribution. A number of studies have shown a considerable variation in Ts between groups of tumours. (Wilson G.D. 1993) The median Ts of the oral tumours was 10 hours while that of bronchial tumours is about 15hours. In this study we used a biopsy interval of 4-6 hours to account for these variations.

2.6.4.2 Processing the tumour sample

In early studies, whole cells were separated and studied by means of enzyme cocktails. This had the disadvantages of low cell yields and the need for immediate post

operative processing. Schutte modified this method by fixing the sample in 70% ethanol to allow prolonged storage at 4°C (Schutte B., Reynders M.M. et al. 1987).

Pepsin has the advantages of standardising procedures and reducing the variations of enzyme cocktails. Furthermore, the yields are larger than other methods thereby reducing the sample size required; this is an important factor in BCC since many samples are small. In analysing nuclei rather than whole cells, the complications of non specific cytoplasmic staining are reduced, although the ability to analyse other potentially useful cytoplasmic antigens is lost.

2.6.5 Data analysis.

Figure 2.3 and 2.4 show examples of data from a BCCs labelled by BrdUrd. Both show a diploid DNA content. The upper panel shows the dot plots of FL3-A (DNA content) on the x axis and the FL1-H (BrdUrd uptake) on the y axis. The BrdUrd labelled cells have been denoted in green by setting a region around those cells with positive staining. The histograms have been created from this dot plot. The middle panel shows the total DNA content in which markers 1 and 2 have been set around the G₁ and G₂ populations respectively. These are required for the calculation of the relative movement to estimate T_s. In the lower panel, a DNA histogram has been created for the DNA labelled cells only. This histogram generates the total number of BrdUrd labelled cells and marker 3 calculates those which have divided and become G₁ cells and this must be corrected for the estimate of the true LI. Marker 4 defines the cohort of BrdUrd labelled cells still progressing through S and G₂. The mean DNA content of these cells is required to measure the relative movement in conjunction with the mean DNA content of G₁ (marker 1) and G₂ (marker 2)

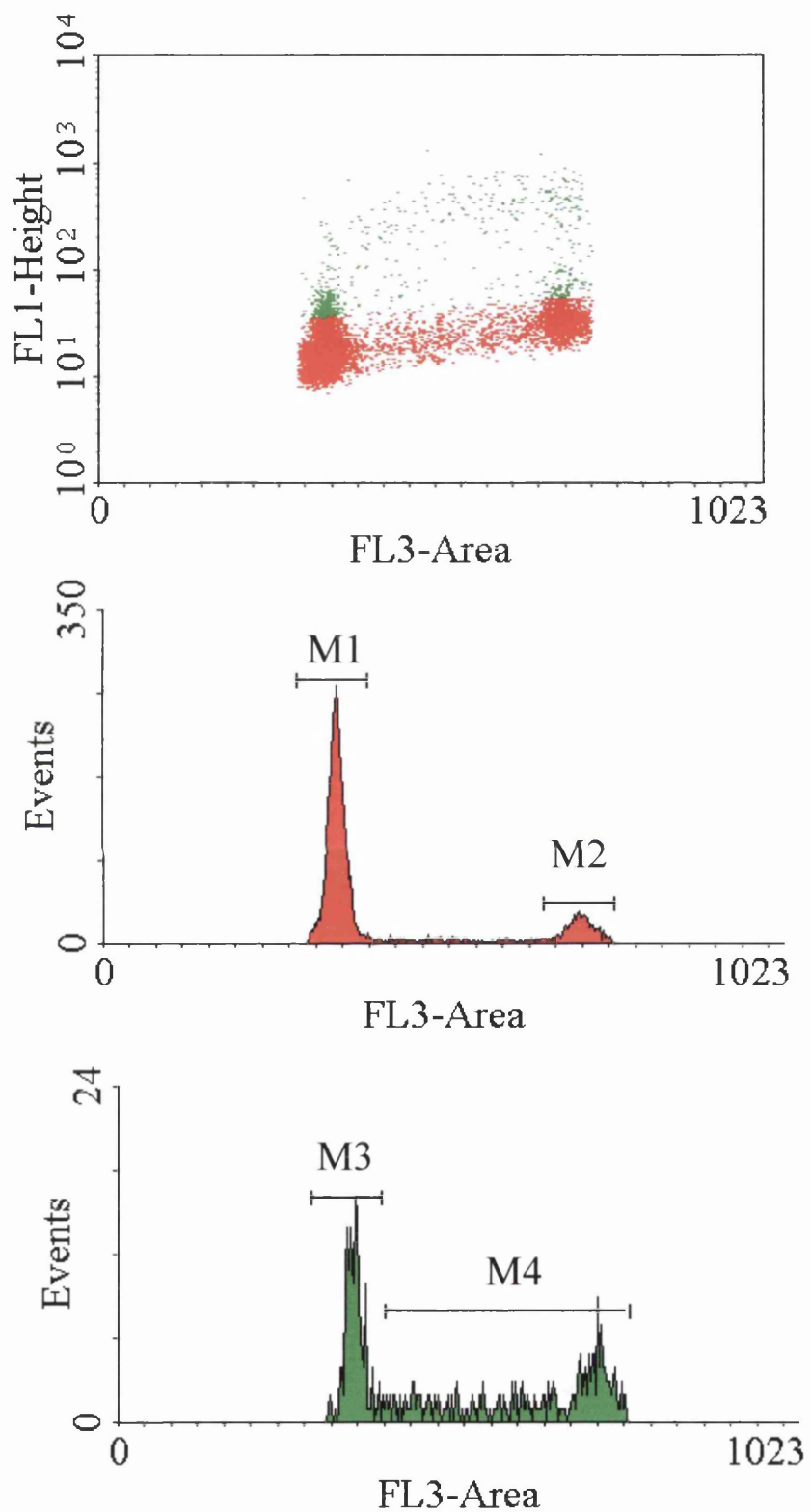


Figure 2.3. Data obtained from the FACS analysis of a BCC labelled with BrdUrd. (See text for details.)

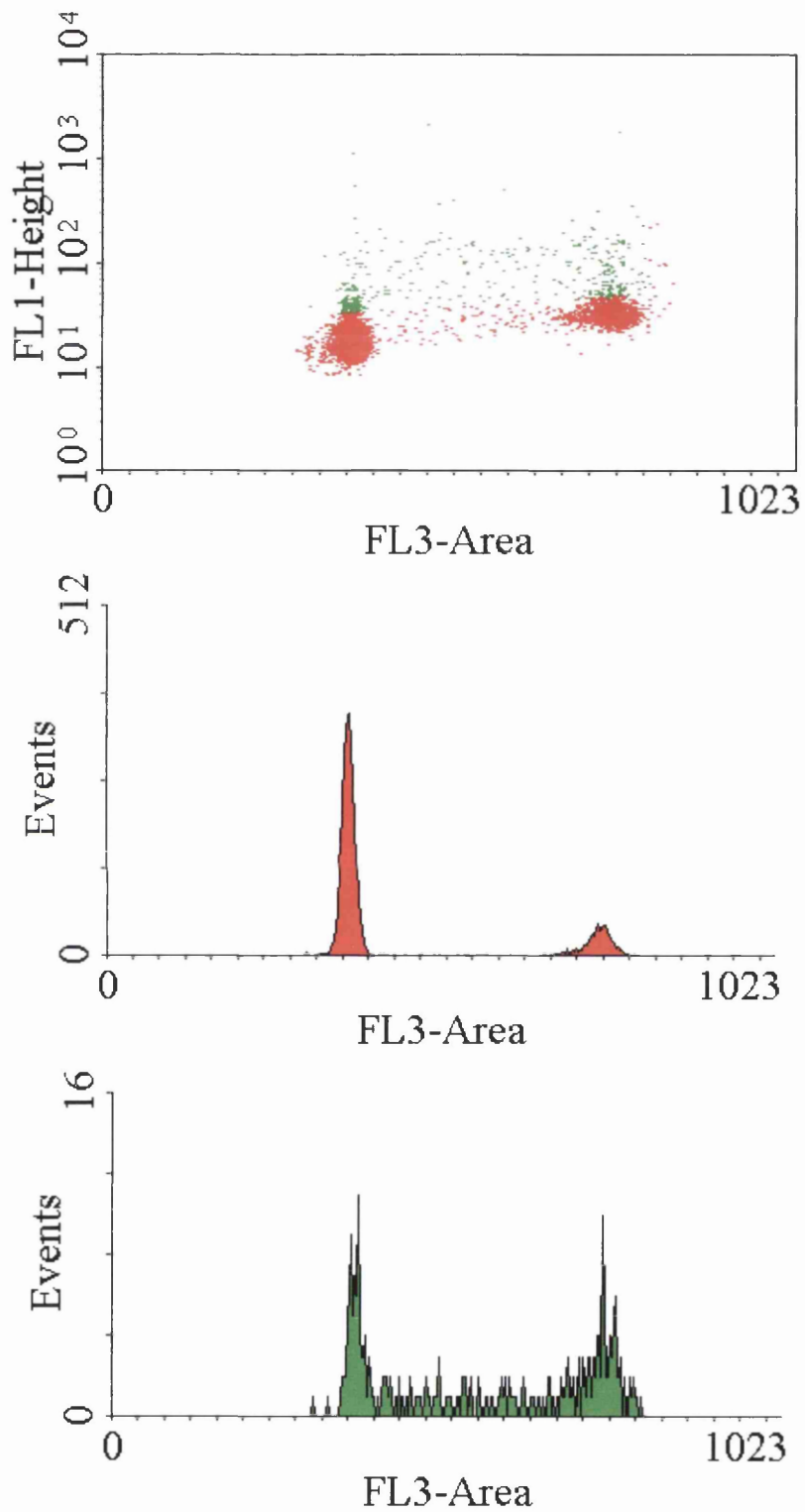


Figure 2.4. Data obtained from the FACSscan analysis of a BCC labelled with BrdUrd. (See text for details.)

2.6.5.1 The labelling index

The labelling index (LI) is defined as the percentage of cells exhibiting significant BrdUrd uptake, making a simple correction for those cells which underwent division in the period between injection and biopsy. The LI is calculated from the analysis of the distribution of BrdUrd labelled cells as a percentage of the total cell number.

$$LI = \frac{\text{Total BrdUrd labelled cell} - 0.5(\text{G1 BrdUrd labelled cells})}{\text{Total cells} - 0.5(\text{G1 BrdUrd labelled cells})}$$

Therefore:

$$LI = \frac{\text{Total BrdUrd labelled cell} - 0.5(M1)}{\text{Total cells} - 0.5(M1)}$$

The calculation includes half of the labelled G1 cells because of division and sharing of BrdUrd between daughter cells.

In aneuploid tumours the LI can be calculated for the tumour population alone by setting the regions around the cells with aneuploid DNA content only. In diploid tumours calculation of the LI includes all of the cells in the specimen including connective tissue and stromal cells. Because BCC is diploid we also calculated the LI by immunohistochemical analysis so that only the tumour nodules were included.

2.6.5.2 The duration of S phase

Begg proposed the principle of relative movement to calculate the Ts from a single biopsy (Begg AC., McNally N.J. et al. 1985). This is dependant on 2 assumptions. Firstly that there is uniform distribution of labelling throughout the S phase such that the mean DNA content of the BrdUrd labelled population gives a value of mid S. The term relative movement describes the movement of the cohort of labelled cells relative to G1 and G2. In order to measure RM the mean DNA content of the G1 and G2 populations are calculated as described. The RM at time 0 is calculated by subtracting the mean DNA content of the G1 cells from the DNA content of the

BrdUrd labelled cells and dividing by the G1 DNA content subtracted from the G2 DNA content.

$$RM = \frac{FL(\text{BrdUrd}) - FL(G1)}{FL(G2) - FL(G1)}$$

Therefore:

$$RM = \frac{FL(M4) - FL(M1)}{FL(M2) - FL(M1)}$$

FL denotes the mean fluorescence values of the populations of the cells. The relative movement at time 0 is considered to be midway between G1 and G2 and is therefore 0.5. As labelled cells progress through S phase towards G2 the value will reach 1.0 when all of the undivided BrdUrd labelled cohort of cells are in G2. Secondly, it is assumed that the cohort of labelled cells progress through S phase in a linear fashion. Thus when the $RM = 1$, the labelled cells in G2 represent the cells in the early part of S phase at time 0. Therefore the progression of cells with a relative movement from 0.5 to 1 defines the T_s . Therefore;

$$T_s = \frac{1.0 - 0.5x}{RM - 0.5} \quad \text{Where } t \text{ is the injection - biopsy duration.}$$

2.6.5.3 The potential doubling time

The T_{pot} can be calculated from the above parameters by the following formula:

$$T_{pot} = \lambda \frac{T_s}{LI}$$

Where λ is a correction factor to account for the nonlinear age distribution of the tumour population due to cell division. This varies between 0.693 and 1.38, but experimental observations suggest a value of 0.8. (Wilson 1992)

2.6.5.3 The cell cycle time

The cell cycle time can be calculated knowing the Growth and the T_{pot} assuming there is a growth fraction and that cell loss is random with respect to age or proliferative status. Where the growth constant would be:

$$b = \frac{\log_e 2}{T_{pot}}$$

$$T_{pot} = \frac{\log_e 2}{\log_e \alpha}$$

Where α = the number of proliferating daughter cells produced at each cell division.

Thus:

$$GF = \alpha - 1$$

$$\text{Therefore } T_c = T_{pot} \times \frac{\log_e(GF+1)}{\log_e 2}$$

$$\text{ie } T_c = T_{pot} \times \frac{\text{all new proliferating cells}}{\text{all new cells}}$$

2.7 Immunohistochemistry

Immunohistochemistry (IHC) has become an established routine histological technique for the identification of cell constituents including many oncoproteins. It has the advantages of being relatively simple to perform and interpret. It also has the advantage of being able to evaluate the spatial location of antigens within a tumour structure or cell. At present it does not have the speed and quantitative power of flow cytometry although computer aided image analysis is narrowing this gap. The advantages and disadvantages of each technique make them complimentary to each other. Developments in the variety of monoclonal and polyclonal antibodies available have facilitated the ability of IHC to detect an enormous diversity of cellular

constituents. In tandem there have been considerable development of the secondary immunoenzymatic detection systems suitable for light microscopy.

The majority of routine histology laboratories use neutral buffered formalin as the fixative of choice for tissue reservation: this may not be the preferred method for preservation of some tissue antigens. There is therefore growing demand for antibodies that can be used on formalin fixed paraffin embedded material. Many antigens are more reliably or exclusively demonstrable in fresh frozen sections.

Reliable IHC is dependant on selecting the correct fixative that provides maximum preservation and of tissue morphology and minimal loss of antigenicity. Inconsistencies noted in aldehyde fixed material are largely due to antigenic sites being "hidden" by cross linked proteins. Unmasking of hidden epitopes has been achieved by proteolytic digestion prior to immunostaining. Shi described an alternative technique in 1991(Shi S.R., Key M.E. et al. 1991). This involved the microwaving of tissue sections in the presence of heavy metals solutions. More recently citrate buffer has replaced the toxic heavy metals to unmask hidden antigens (Cattoretti G, S et al. 1993). Subsequently it has been demonstrated that pressure cooked paraffin sections can also be used. Although the exact mechanism by which heat mediated antigen retrieval works, it is thought that proteolysis and alteration of protein tertiary structure are probably the critical factors.

2.7.1 Staining procedure.

2.7.1.1 Microwave pre-treatment.

Sections were first dewaxed in xylene and rehydrated through decreasing concentrations of alcohol (100%, 90% and 70%) For some antibodies (See table 1) endogenous peroxidase activity was blocked using a solution of 3% hydrogen peroxide in methanol for 30 minutes at room temperature. The slides were then washed well in water before immersion in 200ml of 10 mM citric acid adjusted to pH 6 with 2N NaOH. The sections were then microwaved (800W Goldstar) on high power for a number of 4 minute cycles (See table 1.1). Between each cycle the volume

was topped up to the original volume. The slides were removed from the microwave and left to stand for 10 minutes at room temperature before being washed well in running water.

2.7.1.2 Primary antibody staining

After microwave pre-treatment the slides were removed from the running water and rinsed in Tris buffer saline (TBS) pH 7.6. Some antibodies require nonspecific binding to be blocked with normal swine serum diluted 1:5 for 10 minutes. This was tipped off. The primary antibody was then added at the correct dilution with TBS and incubated at 4°C overnight. The primary antibody was then washed off 3 times in TBS (1 minute for each wash.)

Primary	Manuf acturer	Type	Clone	Block endogenous peroxidase	Number of microwave cycles	Block non specific binding	Primary dilution
Ki-67	DAKO	rabbit polyclonal	MIB-1	Yes	4	Yes	1:200
BrdUrd	DAKO	Mouse IgG monoclonal	Bu20a	Yes	3	No	1:30
Bcl-2	DAKO	Mouse IgG monoclonal	124	No	2	No	1:40
Bax	Immunotech	Mouse IgG monoclonal	4F11	No	4	No	prediluted
P53	DAKO	Mouse IgG monoclonal	D-07	No	3	No	1:60

Table 2.1 showing the characteristics of each primary antibody utilised in this study.

2.7.1.3 Secondary antibodies

The appropriate biotinylated secondary antibody at the correct dilution. Biotinylated rabbit antimouse antibody (Dako) was used at a dilution of 1:300 (in TBS) for all of

the monoclonal antibodies and incubated for 1 hour at room temperature. Biotinylated swine anti rabbit (Dako) at a dilution of 1:350 (in TBS) was used for Ki-67 staining. The secondary antibody was washed off with 3 TBS washes (1 minute each).

2.7.1.4 Detection systems and chromogens..

This was achieved using Dako Avidin Biotin Complexed with horseradish peroxidase (ABC)1:100 in TBS was added and incubated for 1 hour at room temperature This binds to the biotinylated secondary antibody localising the peroxidase which acts as a chromagen when in the presence of an electron donor (substrate) and hydrogen peroxide. A peroxidase- substrate complex forms. The substrate becomes oxidised as it catalyses the reaction of hydrogen peroxide to water. In this study the substrate was 3-Diaminobenzidinetetrahydrochloride (DAB). On oxidation a brown insoluble product forms which can be visualised. (See figure 2.5)

Following addition of ABC the slides were then washed twice in TBS and once in tris buffer (TB). Visualisation was performed by the addition of Diaminobenzidine (7.5 mg in 10ml of TB with 3 drops of 1% hydrogen peroxide added.) for 10 minutes. The slides were then rinsed in TB and washed well in running tap water prior to counterstaining in haematoxylin for 15 seconds. They were blued in running water for 5 minutes in tap water and the dehydrated in graded alcohol and mounted in DPX.

2.7.2 Controls

Positive and negative controls were performed for each tumour to ensure that each run had been performed correctly. For Ki-67, BrdUrd and Bcl-2 this consisted of normal tonsil. For p53 this was colonic carcinoma, for Bax breast tissue was used as recommended by the manufacturers.

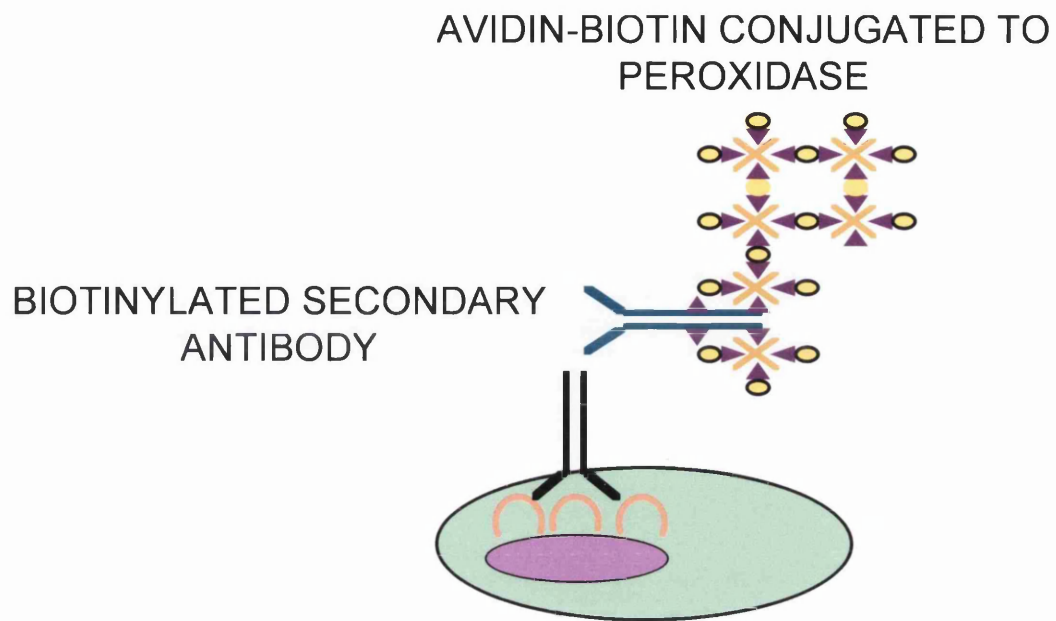


Figure 2.5 The immunohistochemistry technique.

2.7.3 Solutions

2.7.3.1 *Tris Buffer*

2500ml 0.1M Tris (BDH10315)

2000ml 0.1M Hydrochloric acid (BDH28507)

Adjust to Ph7.4-7.6

2.7.3.2 *Tris Buffer Saline*

500ml Tris buffer

4500ml distilled water

40.5g sodium chloride

Adjust pH to 7.6

2.8 Quantification

Slides were visualised with a Zeis Axioscope microscope and quantified with the aid of a TV monitor connected via a camera to the microscope. Nuclear staining with Ki67 or BrdUrd was counted with the aid of a grid on the TV monitor. Therefore, on positive cells in tumour island could be counted and proliferating and non proliferating cells in the stroma could be excluded. Positive cells were expressed as a fraction of all of the tumour cells in each high power field. (x400). At least ten high power fields were counted per tumour. The spatial localisation of these cells within the tumour islands was also recorded.

Cytoplasmic staining with Bcl-2, Bax was scored according to intensity (none, weak, intermediate and strong) and also to the percentage of the tumours that were stained. (None, 0-25%, 26-50%,51-75%, 76-100%). P53 nuclear staining was also stained according to the percentage of the tumour that was positive.

2.9 Statistical method

All data was stored on an P.C. in Excel charts (Microsoft). Data was manipulated using the JMP statistics data base. (SAS Institute Inc., Henley, Berkshire, UK) The normality of distribution of a data set was analysed by the Shapiro-Wilk test. Paired data sets showing a normal distribution were compared using the Student's '*t*' test. If more than 2 groups were compared then the anova test was utilised. Non parametric data was compared either with the Wilcoxon rank sum test or the Kruscal - Wallace test (if greater than 2 groups). Ordinal data groups were analysed by Chi square analysis. Variation in data, such as the degree of proliferation between tumours was compared by evaluating the coefficient of variance

Correlations between data sets was compared with Spearman rank order correlation because most data sets were non parametric. Further analysis of 2 measurements by different methods such as the LI in chapter 3 was by Bland and Altman analysis. This allows assessment of the 'limits of agreement' between values as opposed to correlation.

Chapter 3

Investigation of cell proliferation and cell loss in basal cell carcinoma.

3.1 Introduction

The underlying biological factors that determine the growth characteristics of BCC are not fully characterised and very few studies address this issue. Tumour growth is determined by the balance of cell proliferation and cell loss. Early work by Weinstein and Frost suggested that there were high rates of cell production with a cell cycle time was 217 hours (Weinstein and Frost 1970) (Chapter 1). However constraints determined by the technology and techniques at that time may have restricted the accuracy of the results since cell loss was not accounted for and the growth fraction was assumed to be 1. Furthermore the labelling index may be unduly high since only those areas of uniform mitotic figures were counted which does not account for the heterogeneity of proliferation. The PLM data was derived from multiple tumours and not individual lesions and this might reduce the reliability of the measured parameters. The first aim of this chapter was to reassess the cell kinetics of BCC with newer, more powerful techniques utilising bromodeoxyuridine labelling analysed by flow cytometry to assess cell production in individual tumours.

High rates of cell loss have been attributed as an explanation for the slow growth of BCC (Kerr and Searle 1972). Attempts to quantify this have suggested that apoptotic rates in BCC are higher than melanoma but less than SCC (Mooney, Ruis Peris et al. 1995; Wikonkal, Berg et al. 1997). The relationship and relative contribution of these values to the growth of BCC is unknown. The second aim of this study was to attempt to quantify apoptosis and also examine other methods of cell loss such as necrosis and desquamation.

The control of apoptosis is complex but a central role is played by the bcl-2 family of oncoproteins. These in turn are controlled by upstream mechanisms including the function of tumour suppressor genes such as p53. p53 induces apoptosis following DNA damage. The third aim of this chapter was to evaluate these oncoproteins and to assess their role in determining apoptosis or cell survival.

3.2 Materials and methods

Patients attending Mount Vernon Hospital for excision of BCC were included in the study. It was convenient to use In Patients rather than Day Case Patients because of the requirement of a time interval between administration of BrdUrd and biopsy removal. The patients were required to be older than 65 years old and informed patient and consultant consent was necessary for the ethical committee approval. The patients were injected with a 200mg bolus of BrdUrd 4 -6 hours prior to surgery. Following tumour excision, a small segment was fixed in 70% ethanol and the remainder fixed in formalin and sent for standard histological evaluation. The paraffin block was then retrieved and the H+E specimen re evaluated. The tumours were classified according their growth pattern and differentiation. Apoptotic bodies were also counted and necrosis assessed semi-quantitatively. 5µm sections were cut for immunohistochemical analysis of Ki-67, BrdUrd,P53, bcl-2, bax. Analysis of the ethanol fixed section was performed as previously described. The study could not be continued because of the failure of the Licence of BrdUrd to be renewed by the CRC drug formulation unit, University of Strathclyde, Glasgow.

3.3 Results

3.3.1 Patients and tumours

31 patients were given BrdUrd. Results are available in 19 patients with a total of 24 tumours. Sample from 11 patients were not suitable. 2 patients had squamous cell carcinomas, 2 patients actinic keratosis, in 1 patient the biopsy revealed chronic inflammation only, and in 4 patients the labelling was too poor for analysis. The Immunohistochemical data was unavailable in 1 due to failure of the section to remain on the slide during antigen retrieval. There were 13 males and 6 females. The mean age was 80.5 years. (Median 78 years range 67-92). 17 tumours were primary and 7 were recurrent. The size ranged from 5-50mm (mean 19mm, median 20mm). The mean size of the primary and the recurrent tumours was 17 mm and was 25mm respectively (This was not statistically significant). Female patients had a greater proportion of the recurrent tumours compared to the males. (5/8 vs 2/14.) This was statistically significant. (Chi square $p=0.01$) The position of the tumours were: Upper face 6, middle face 4, lower face 1, ear 3, Scalp 2, trunk 3, leg 4.

The histological features are as follows.

GROWTH PATTERN	DIFFERENTIATION			
	Undifferentiated	Adenoid	Squamous metaplasia	Cystic
Nodular	2	8	2	0
Infiltrative	4	0	2	0
Micronodular	4	0	0	0
Superficial	1	0	0	1

Table 3.1. The histological types of BCC.

3.3.2 Flow cytometry

3.3.2.1 DNA Ploidy

Analysis of the DNA revealed that all of the BCCs were diploid with no additional peaks indicating an aneuploid population.

3.3.2.2 Cell production. State and rate measurements

The LI could not be measured by selecting the tumour cells according to their DNA content in the FACs analysis since all of the BCCs were diploid.. The LI therefore was derived from tumour and non tumour populations. The LI ranged from 0.01-0.08 (mean 0.03, median 0.03) and did not follow a normal distribution. (Shapiro-Wilk $p < 0.05$) The distribution is shown in Figure 3.1.

The duration of the S phase ranged from 5.0-14.6 hours (Mean 8.1, median 7.6 hours) and did not exhibit a normal distribution (Shapiro-Wilk $p < 0.05$) (Figure 3.2).

As a consequence of the considerable variation in LI and Ts there is large range in Tpot values (range 3.4 days-40.8 days) (Figure 3.3). It can be seen from Figure 3.1 that the greatest variability was seen within the LI and Tpot as evidenced by the C.V.

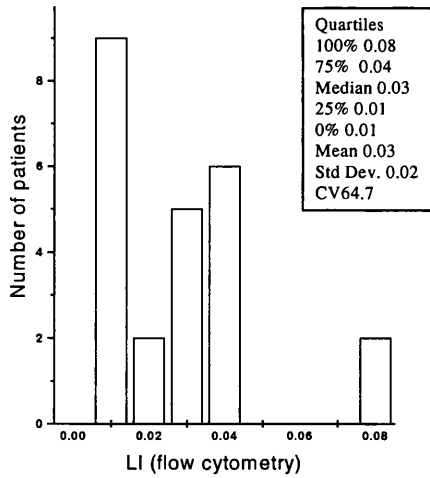


Figure 3.1. The distribution of LI (Flow cytometry)

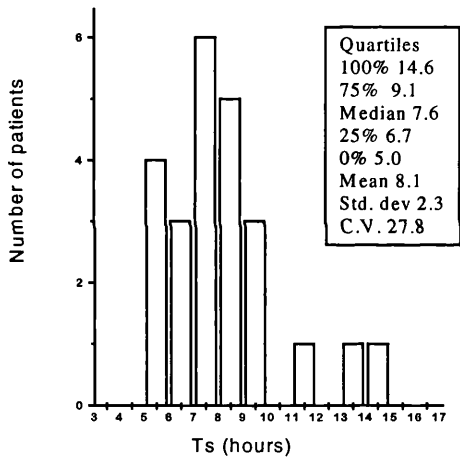


Figure 3.2. The distribution of the Ts.

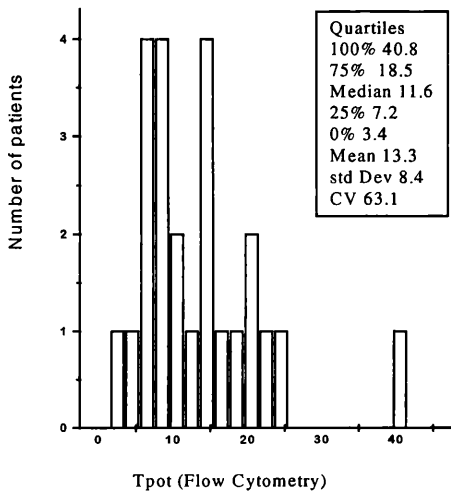


Figure 3.3. The distribution of Tpot.

3.3.2.3 Inter relationships between the flow cytometry derived data

An inverse relationship between the Tpot and the LI is seen in Figure 3.4 and is described by an exponential decay curve. At high LI the Tpot is short and at a low LI the Tpot is long, although the LI has less influence on the Tpot at lower LI.

As can From Figure 3.5 there is less correlation between the Ts and Tpot. Although at LI less than 0.02 the Tpot and Ts are related. (Figure 3.6).

The LI was not related to the Ts in this study. (Spearman rank order correlation $p=0.2$) (Figure 3.7).

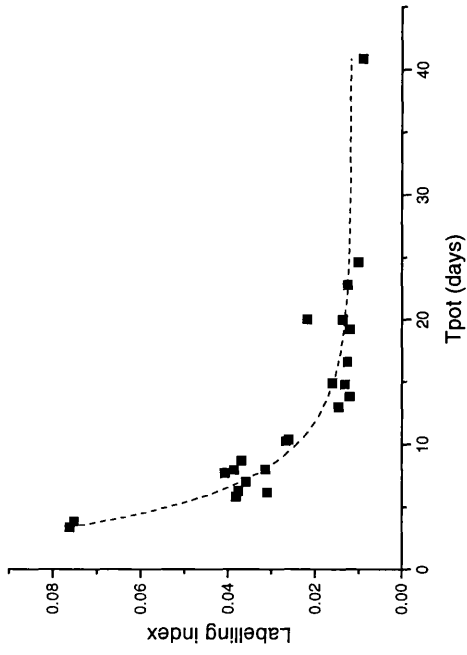


Figure 3.4. The Inverse relationship between the Tpot and LI (flow cytometry).

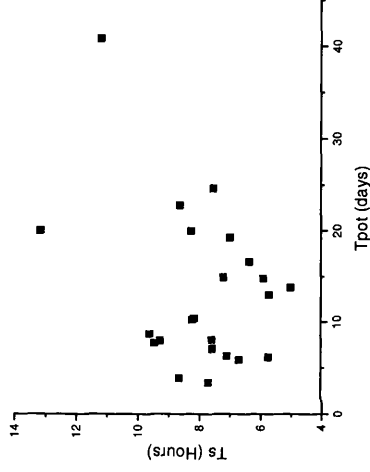


Figure 3.5. The relationship between the Ts and Tpot.

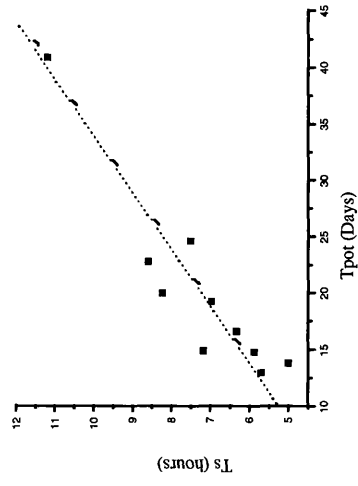


Figure 3.6. The relationship between the Tpot and Ts at low LI (Less than 0.02)

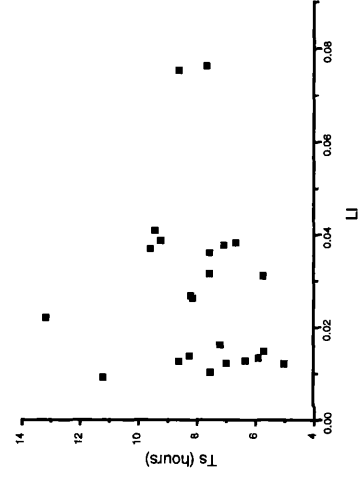


Figure 3.7 demonstrating no relationship with the Ts to LI.

3.3.3 Immunohistochemistry

3.3.3.1 Cell proliferation. State and rate measurements.

The LI was also measured by immunohistochemistry (LI (im)) and the positive tumours counted on a TV monitor so that only the tumour population of cells was included. This was in contrast to the technique of measurement by flow cytometry. The range of LI is demonstrated in Figure 3.8.

Immunohistochemical staining of BrdUrd incorporation was carried out on histological sections. Analysis of the percentage positively stained cells could be limited to the tumour population based on morphological criteria. The range of LI's determined with this method is demonstrated in Figure 3.8. Using this LI and the flow cytometry derived T_s , a T_{pot} was calculated for the tumour cell population only. This was based on the assumption that the T_s reflects the rate of DNA synthesis in the tumour cells as these are most likely to be undergoing DNA synthesis and cellular proliferation. The distribution of the T_{pot} is shown in Figure 3.9. It is clear that both the LI(im) and $T_{pot}(im)$ indicate a much more rapid proliferative group of tumours than with flow cytometry analysis alone. The median LI rose from 0.03 to 0.14 and the T_{pot} decreased from 11.6 to 2.8 days. This data suggest that the contribution of host cells within BCC is significant, and leads to a major underestimation of the tumour associated LI. In many tumours. The direct comparison of the LI is shown in Figure 3.10a and b. The upper panel shows the absolute values As can be seen from Figure 3.10 the LI, measured by flow cytometry was not related to the LI measured by immunohistochemistry (Spearman rank order correlation $p=0.2$) The lower panel shows a Bland Altman analysis in which the flow cytometry derived LI has been subtracted from the LI(im) and plotted as a function of the average value. It can be seen that the LI (im) was always higher than the LI (flow cytometry) due to the contribution of stroma within the neoplastic cells. The mean difference in labelling index was 0.10. It was also an interesting finding that the difference in the LI (im) and LI (flow) increased as the mean of both LI increased.

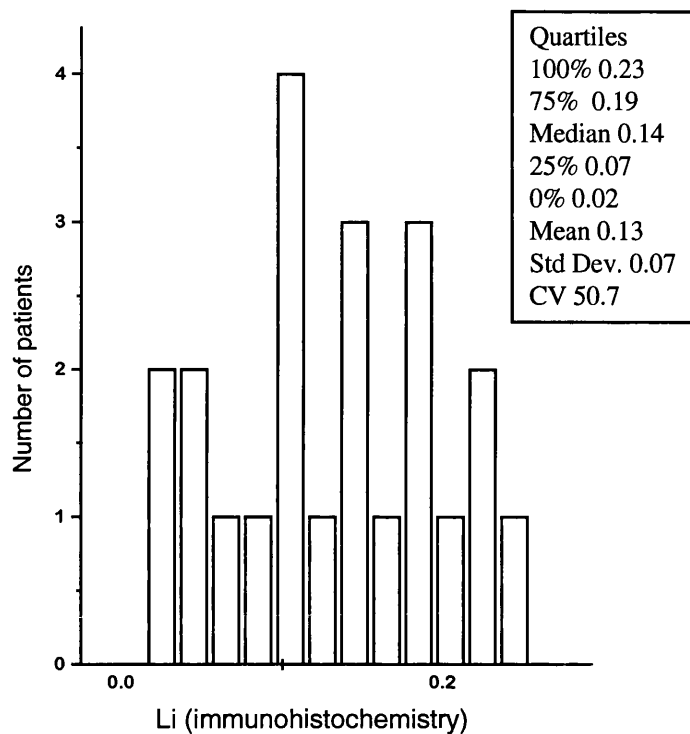


Figure 3.8. The distribution of LI (im).

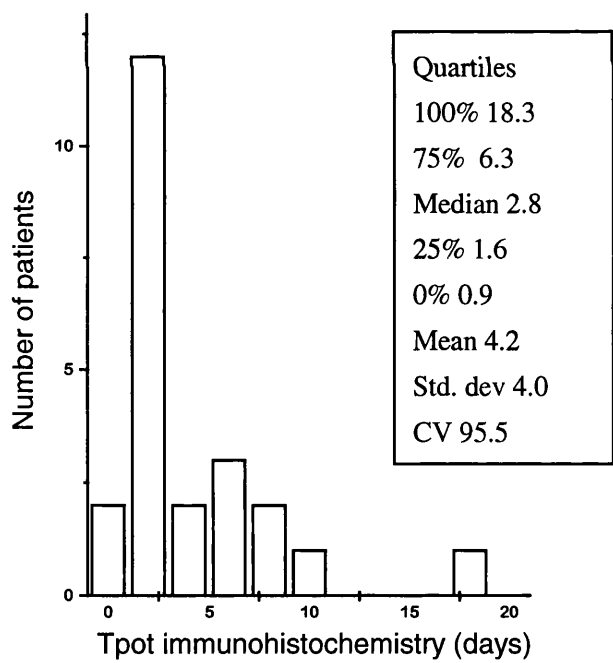


Figure 3.9. The distribution of Tpot(im).

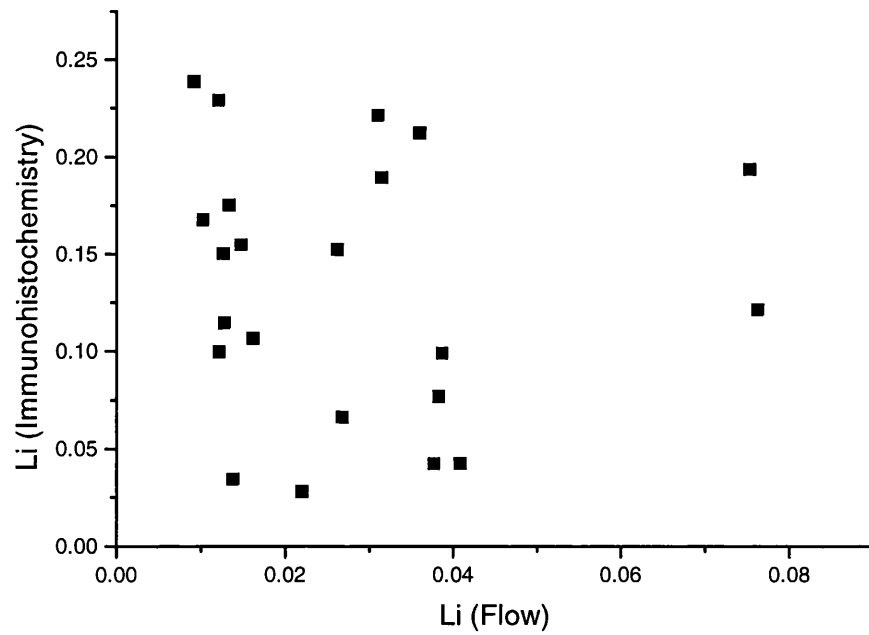


Figure 3.10a No relationship is seen between the Tpot (flow cytometry) and Tpot (im).

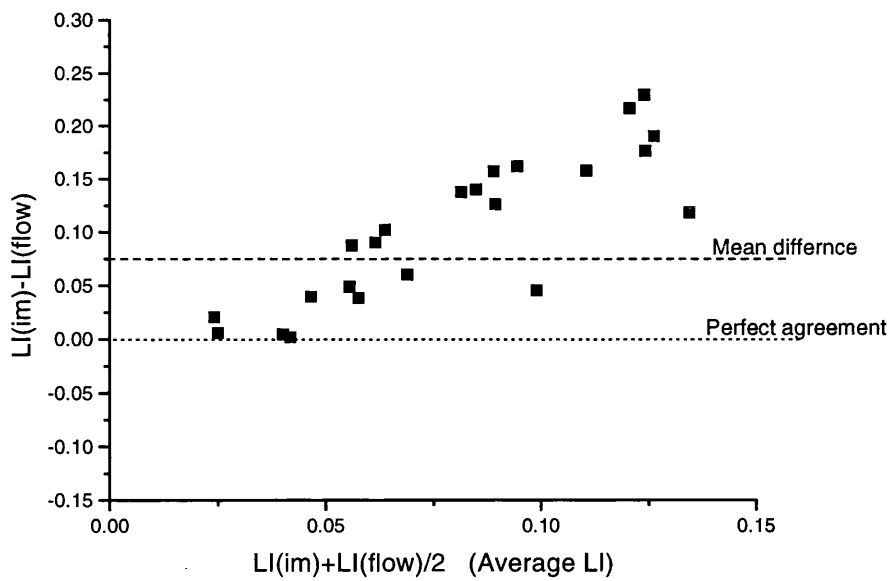


Figure 3.10b. A Bland Altman analysis of the difference between the LI(im) and LI (flow cytometry)

In the absence of a relationship between the LI (im) and LI (flow cytometry) there was no relationship between the LI (im) and Tpot (flow cytometry). (Spearman rank order correlation $\rho=0.9$) However according to the calculation for Tpot, the LI (im) was inversely related to the Tpot (im). This relationship is described by an exponential decay curve (Figure 3.11). This curve was however is influenced by 1 data point. If this is ignored (data point circled) then a linear relationship is seen.

There appeared to be a relationship between Ts and Tpot (im) (Figure 3.12) This may have been due to the reduced variation in LI(im) compared to LI (flow cytometry).

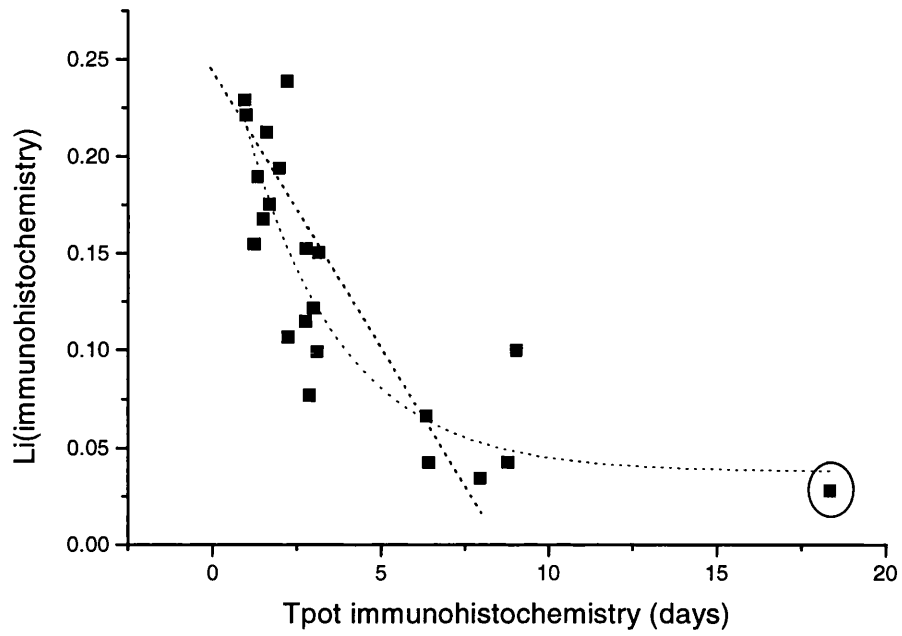


Figure 3.11. The inverse relationship between Tpot (im) and Li (im)

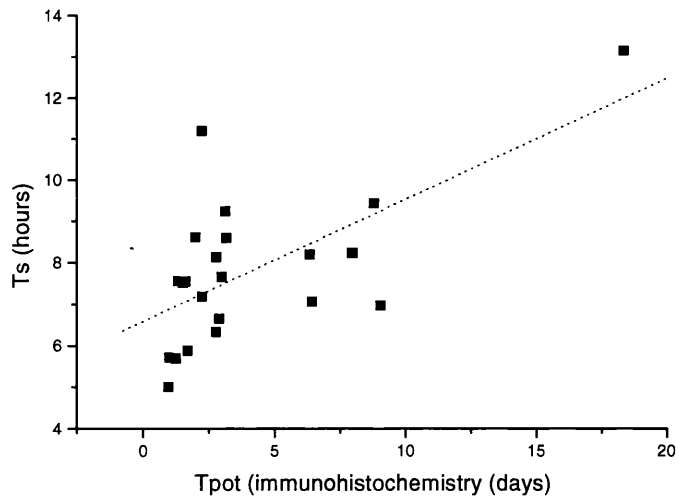


Figure 3.12. The relationship of the Ts with the Tpot (im)

3.3.3.2 *The growth fraction*

The GF was calculated by Ki-67 immunohistochemistry and in parallel with the LI there was marked variation between tumours. The values ranged from 0.07 to 0.49. (Figure 3.13). The GF correlated with the LI measured by immunohistochemistry and is seen as a linear relationship (Figure 3.14), but not with the LI (flow cytometry). (Spearman RHO $p=0.001$ and $p=0.3$ respectively.)

The GF also correlated with the Tpot (im) (Spearman RHO $p=0.0006$). An exponential decay curve describes this relationship. This relationship is influenced by one particular data point. If this data point is ignored (circled in Figure 3.15) then a linear relationship can describe the relationship.

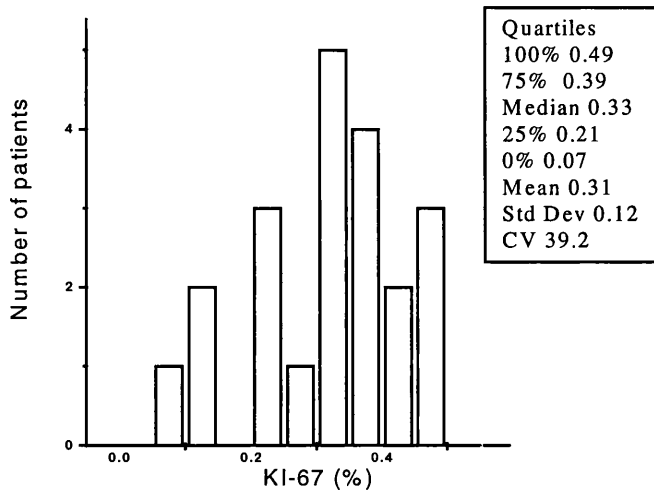


Figure 3.13. The distribution of GF.

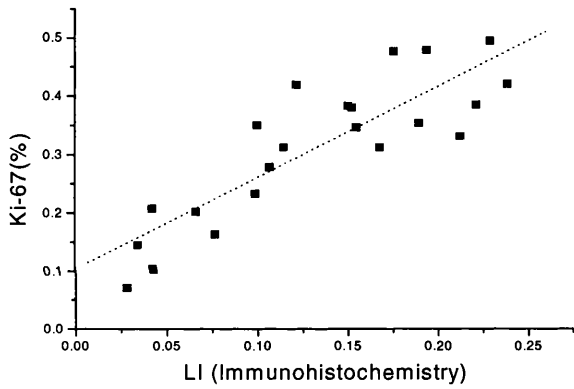


Figure 3.14. The linear relationship of LI(im) and GF measured by Ki67 immunohistochemistry. The fit line is described by $Ki-67 = 0.1 + 1.6 LI$

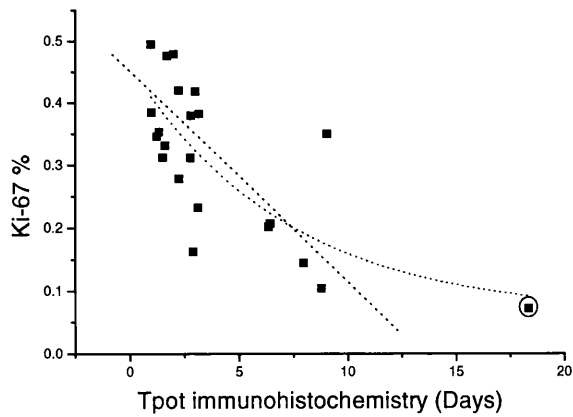


Figure 3.15. The relationship of the Tpot (im) with the GF.

3.3.3.3 The cell cycle time

The cell cycle duration was derived from the GF and LI by the formula:

$$T_c = \frac{\log_e(GF+1)}{\log_e 2}$$

Two cell cycle durations could therefore be calculated. Firstly from the LI (im) and secondly from the LI(flow cytometry). The results are illustrated in Table 3.2.

	TC (FLOW DATA) DAYS.	TC (IMMUNO DATA) DAYS.
Range (days)	1.1-20.7	0.5-3.9
Mean (days)	5.2	1.2
Standard deviation	4.4	0.8
Median (days)	3.9	1.1
CV	84.0	63.1

Table 3.2. The T_c derived from the immunohistochemical and flow cytometry data.

The range and variation is less for the immunohistochemistry data compared to the flow cytometry data. This is because the Figures are derived and the LI measured by immunohistochemistry has less variation than the LI measured by flow cytometry. Although the Figures are mathematically related it there was no direct relationship of the T_c with the T_{pot}, GF or LI . Although it appeared that there was a relationship between the T_s and T_c. (Figure 3.16).This relationship was described by the formula:

$$T_s = 5.8 + 2.3T_c(im)$$

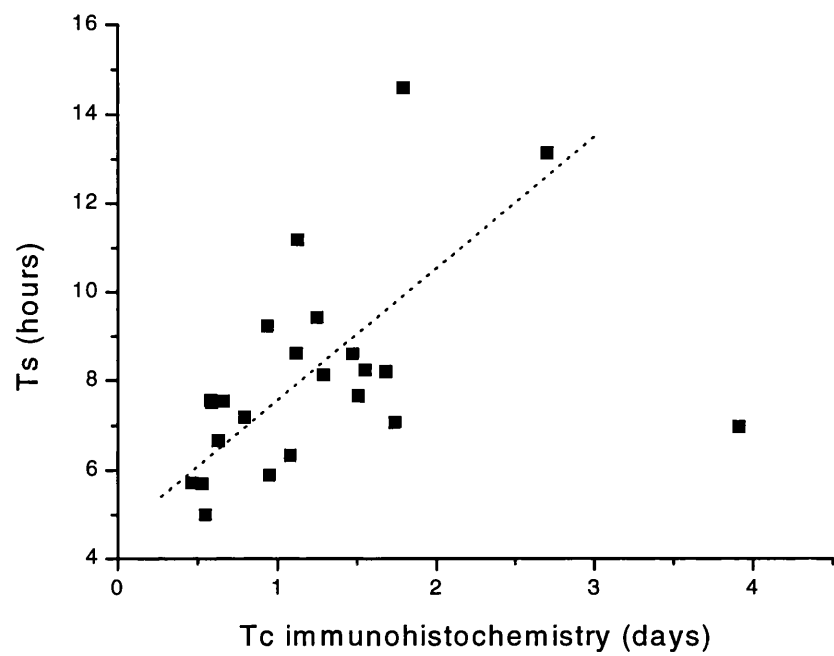


Figure 3.16. The relationship of Ts with Tc(im)

3.3.3.4 Cell production Spatial arrangement of proliferating cells

The spatial arrangement of the proliferating compartment of cells was documented following immunohistochemical analysis of the GF and the LI. 4 distinctive proliferation patterns were described:

- 1) **Marginal** represented specimens in which proliferating cells were restricted to the peripheral cell layers of the tumour islands. (Figure 3.17).
- 2) **Marginal diffuse** pattern where a tendency for peripheral staining was combined with a diffuse pattern throughout the remainder of the tumour area which diminishes centrally. (Figure 3.18).
- 2) **Mixed**, in which combinations of two or more of the distinct patterns co-existed within the tumour specimen. (Figure 3.19).
- 4) **Random** distribution was found in many tumours which consisted of diffuse staining throughout the tumour cells without any obvious concentration of proliferating cells in the palisading or other cell layers. (Figure 3.20).

BRDURD PROLIFERATION PATTERN	KI-67 PROLIFERATION PATTERN				Total
	MARGINAL	MARGINAL DIFFUSE	RANDOM	MIX	
Marginal	0	1	1	0	2
Marginal diffuse	0	7	6	1	14
Random	0	3	3	0	6
Mix	0	0	0	1	1
Total	0	11	10	2	23

Table 3.3. The proliferation patterns seen in BCC.

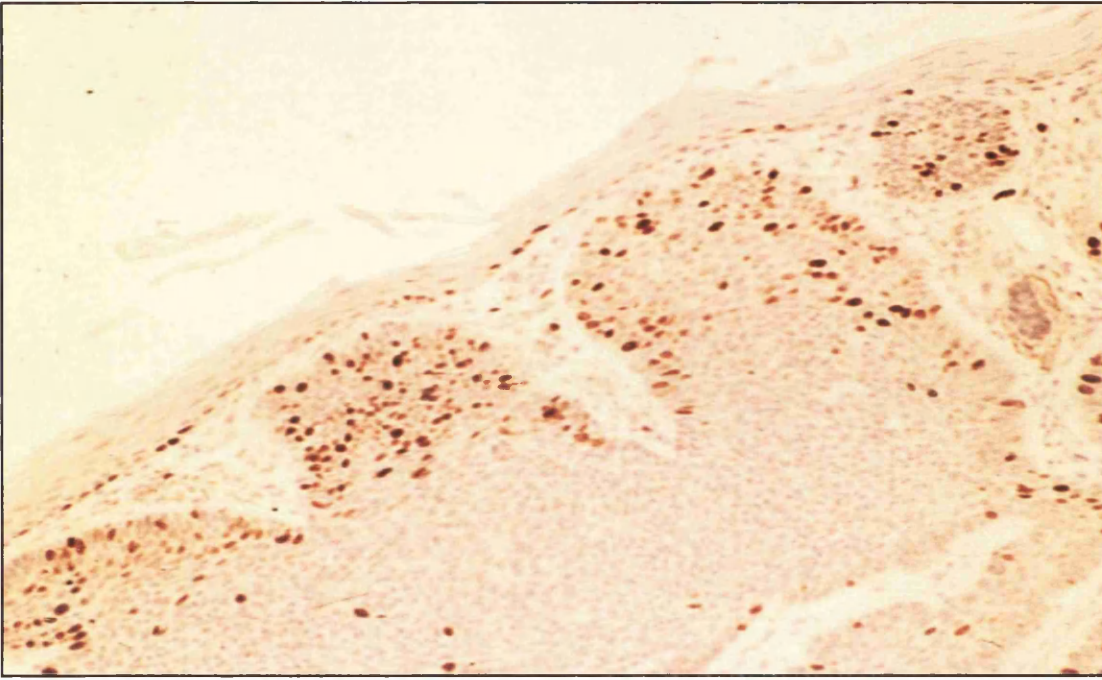


Figure 3.19 Mixed proliferation pattern.

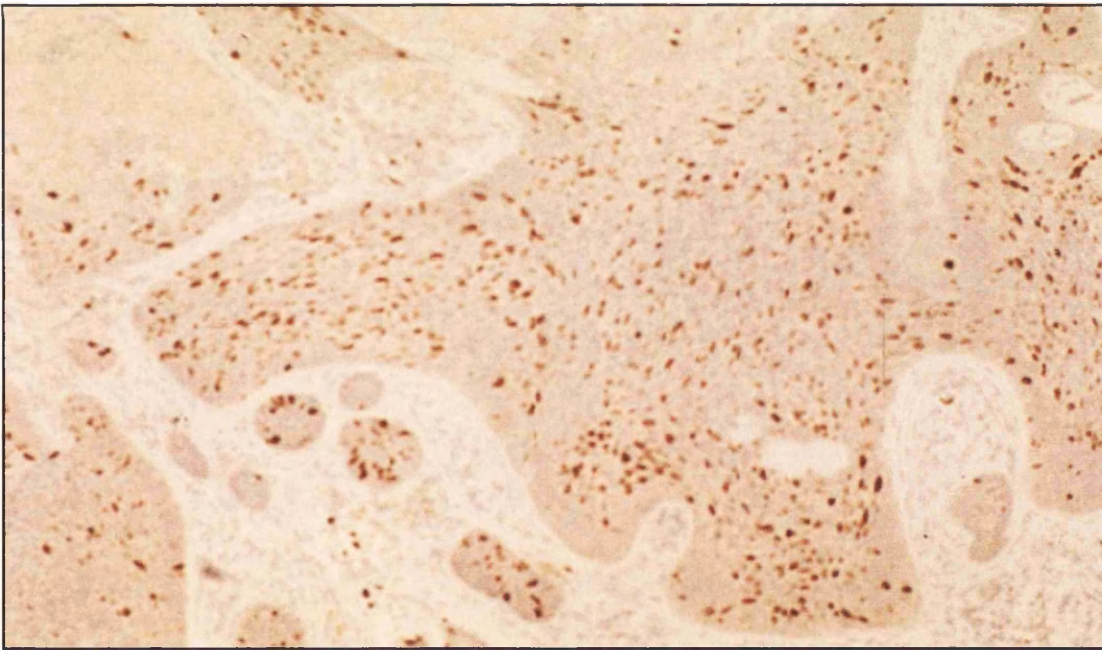


Figure 3.20 Random proliferation pattern

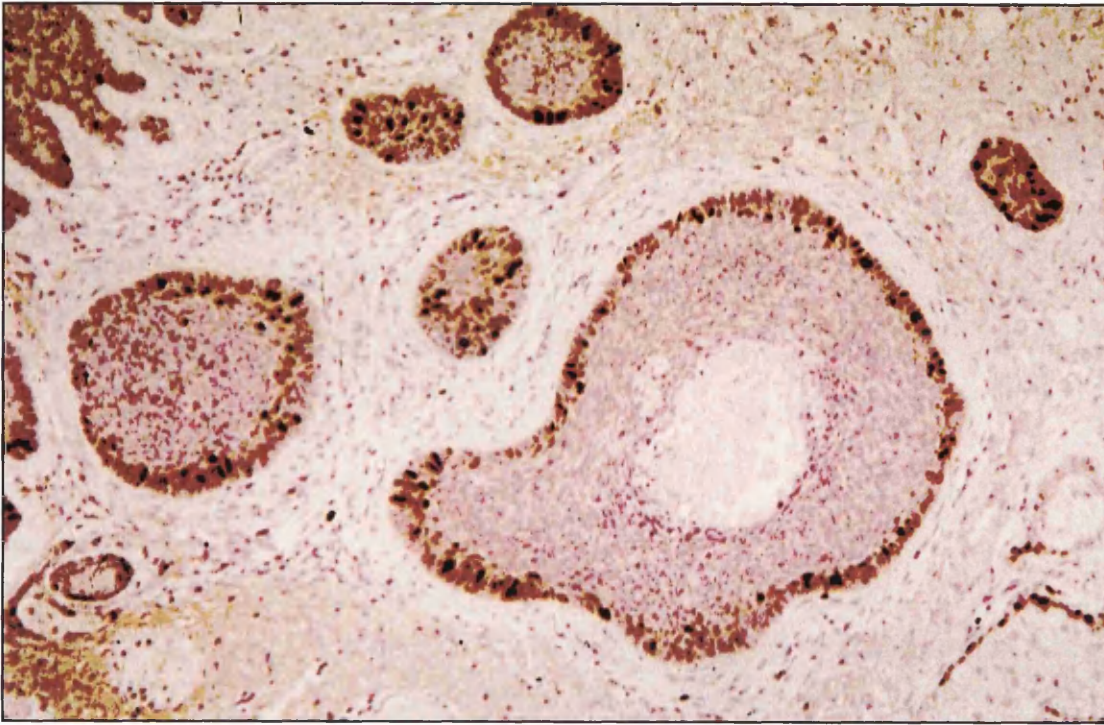


Figure 3.17 Marginal proliferation pattern

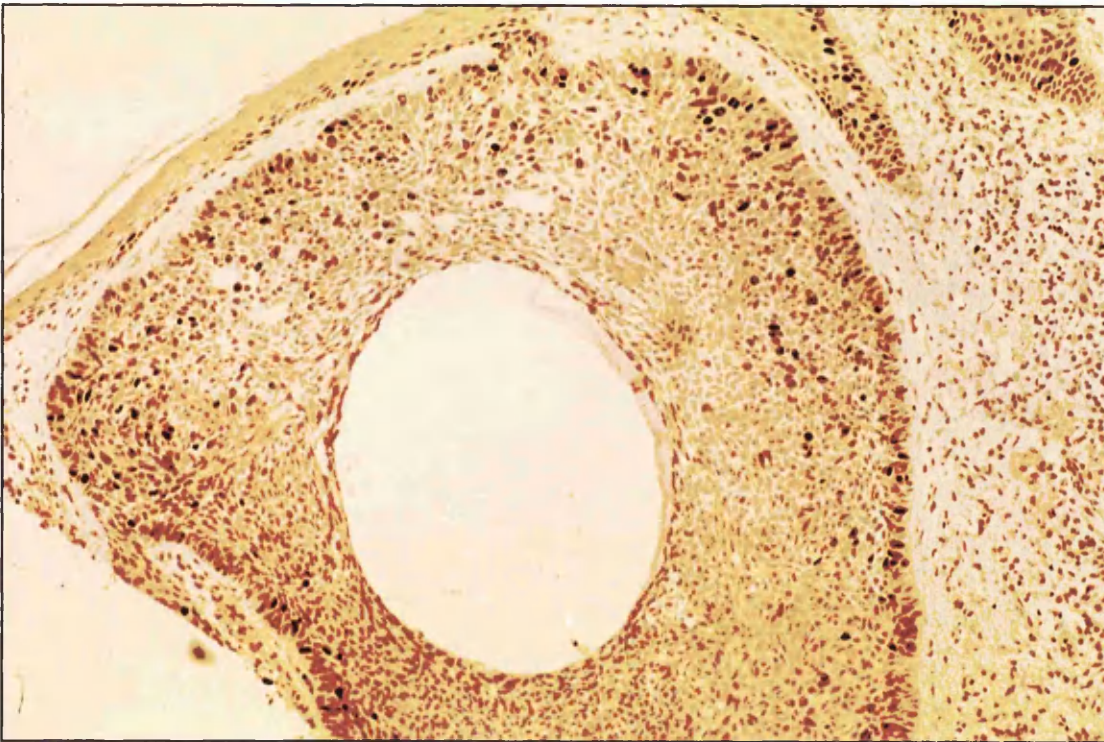


Figure 3.18 Marginal diffuse proliferation pattern

The commonest pattern for both Ki67 and BrdUrd was the marginal diffuse pattern followed by a random pattern. The marginal and mixed patterns were much less common. (Table 3.3). In general there was agreement between the proliferation of Ki-67 and of BrdUrd labelling. There was more Ki-67 labelling than that of BrdUrd and therefore one tumour that appeared to have a marginal diffuse pattern with BrdUrd showed a random pattern by ki-67 immunohistochemistry.

There was a trend that the ki-67 proliferation pattern of the random type had a longer Tpot(im) than the mixed and marginal diffuse types (Wilcoxon $p=0.06$.) The reason for this is not straightforward since there was no relationship between the Ki-67 proliferation pattern and the LI, Ts or GF. (Figure 3.21) There was no relationship between the BrdUrd proliferation pattern and the cell production parameters.

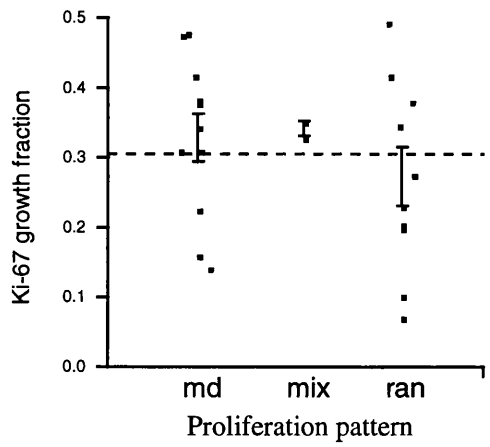


Figure 3.21 a) The relationship of the proliferation pattern with the growth fraction

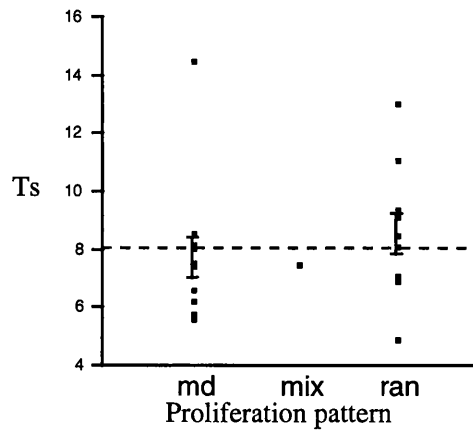


Figure 3.21 b) The relationship of the proliferation pattern with the Ts

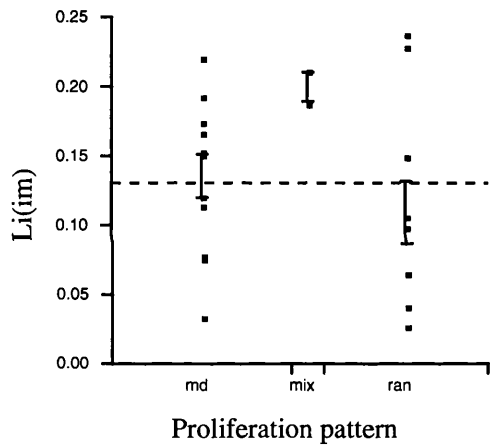


Figure 3.21 c) The relationship of the proliferation pattern with the Li (im)

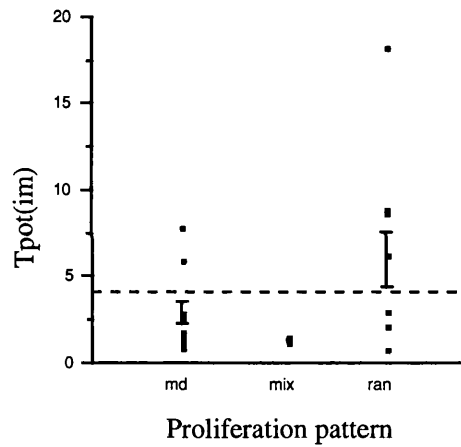


Figure 3.21 d) The relationship of the proliferation pattern with the Tpot(im)

3.3.4 Cell loss

3.3.4.1 Morphology

3.3.4.1.1 Apoptosis

Apoptosis was counted according to the morphological characteristics described in Chapter 2 (Figure 2.2). The distribution of the apoptotic indices is shown in Figure 3.22. The mean apoptotic index was 2% with a range of 1%-5%. There was no apparent relationship between the apoptotic index and the cell production parameters. (Figures 3.23-3.25)

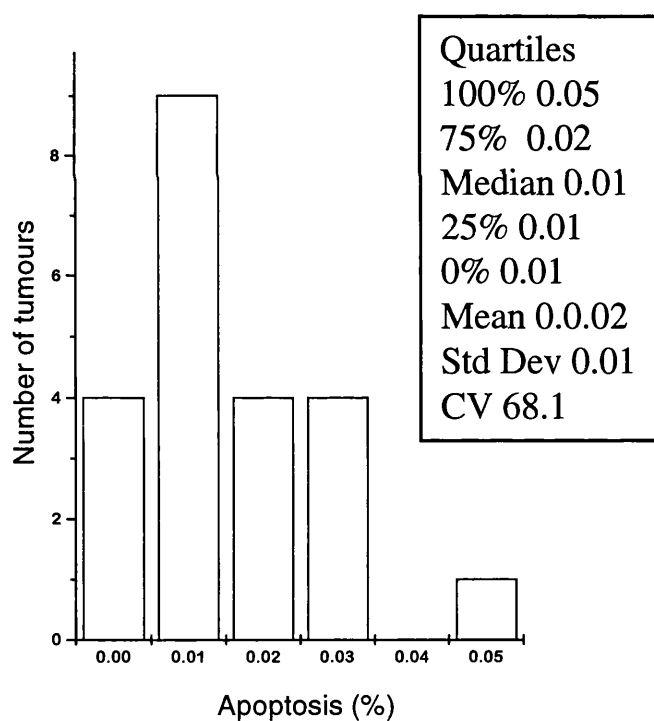


Figure 3.22. The distribution of apoptotic index.

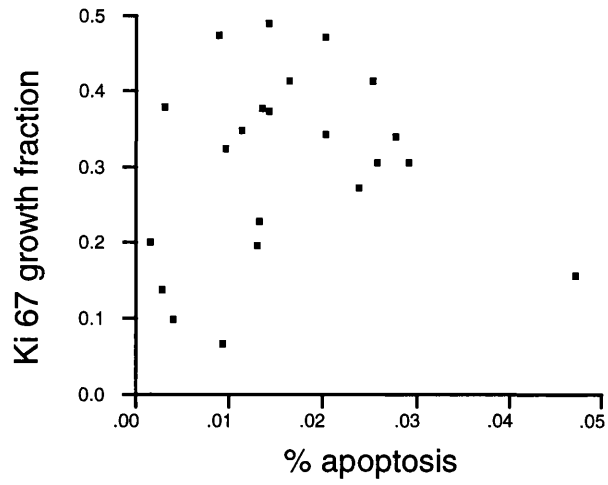


Figure 3.23 The relationship of apoptosis with the growth fraction.

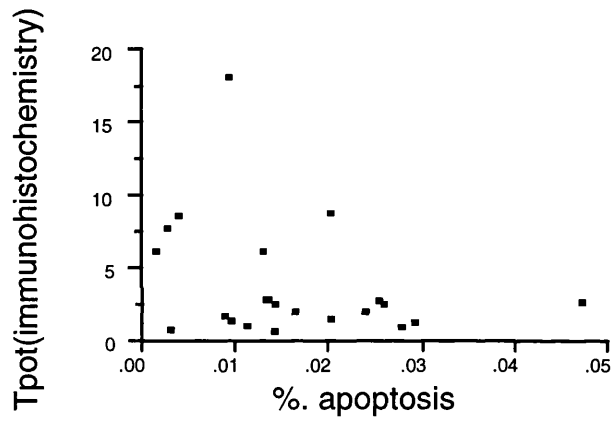


Figure 3.24 The relationship of apoptosis with the Tpot(immunohistochemistry)

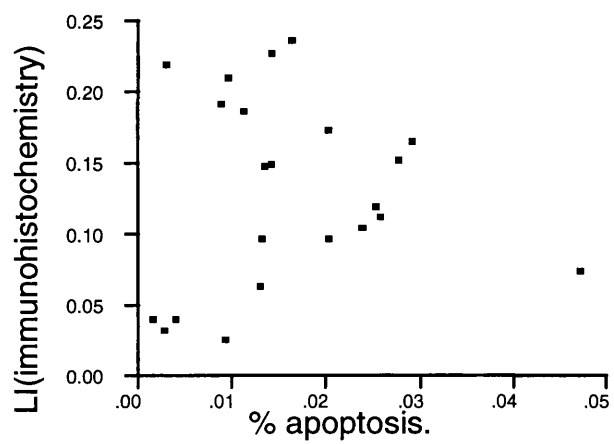


Figure 3.25 The relationship of apoptosis with the LI(immunohistochemistry)

3.3.4.1.2 Necrosis

Necrosis was assessed semi-quantitatively. In general necrosis was not a prominent feature seen in the BCCs with 60% showing no visible necrosis. 35% of the patients had (+) of necrosis and in half of these this was associated with cyst formation. Only 1 patient had (++) of necrosis. This was a primary tumour of the ear. The necrosis appeared to be associated with ulceration. There was no relationship of necrosis to tumour position, type or size. No relationship existed between the cell production parameters or apoptosis with necrosis.

3.3.4.1.3 Ulceration

62% of the tumours had discontinuity of the epithelium over the surface (ulceration). Ulceration was always associated with necrosis (9 of 14 patients with ulceration had visible necrosis) although there were 5 patients with ulceration but no necrosis. There was no relationship between tumour size, position or type. No relationship existed with the cell production or cell loss parameters.

3.3.4.2 Immunohistochemistry

3.3.4.2.1 *bcl-2* immunohistochemistry

bcl-2 oncoprotein expression was scored according to the criteria in chapter 2. The data is summarised in Table 3.4. (Figure 3.26a-c)

82 % of tumours stained positively for *bcl-2* oncoprotein with 64% of tumours having 50% or greater area with positive staining. 46% of the tumours stained with moderate intensity or greater. There was no relationship between the apoptotic index and the *bcl-2* score. A relationship was found between *bcl-2* staining and the GF and LI. Tumours with mild or absent immunostaining had a higher GF and LI than those with

moderate or strong bcl-2 immunostaining. (Wilcoxon/Kruskal-Wallis $p=0.03$ and $p=0.07$ respectively.) (Figure 3.27)

% Positivity	Score	Number	Probability	Intensity	Score	Number	probability
None	0	4	0.18	None	0	4	0.18
0-25	1	4	0.18	Weak	1	8	0.36
26-50	2	2	0.09	Moderate	2	8	0.36
51-75	3	4	0.18	Strong	3	2	0.09
76-100	4	8	0.36				

Table 3.4. bcl-2 immunohistochemistry in BCC.

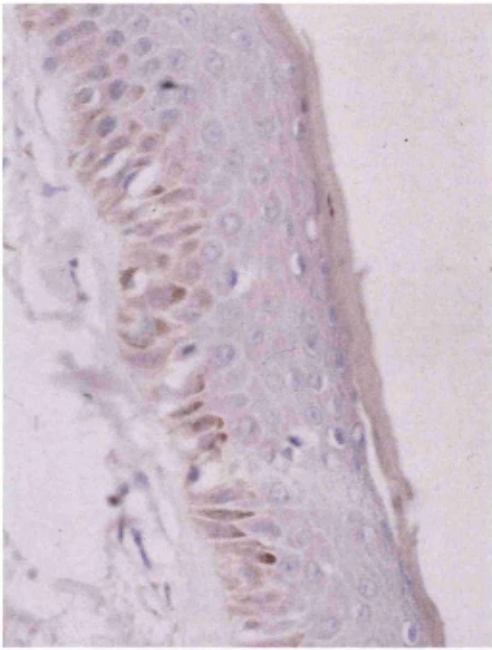


Figure 3.26a. Positive bcl-2 staining in the epidermal basal layer.

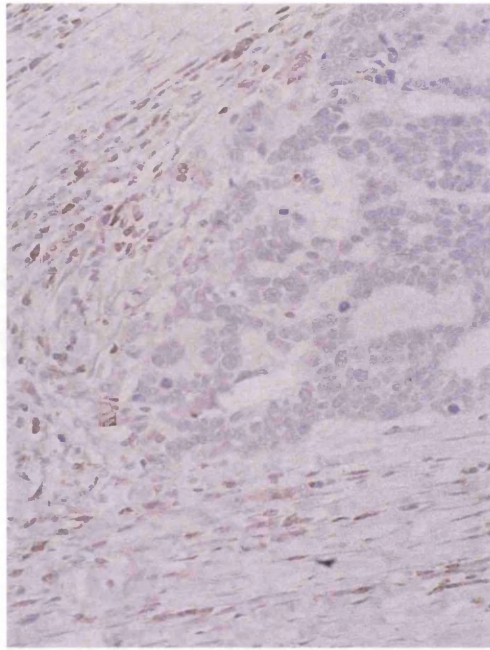


Figure 3.26b. Negative bcl-2 staining with the lymphocytes staining psitively.

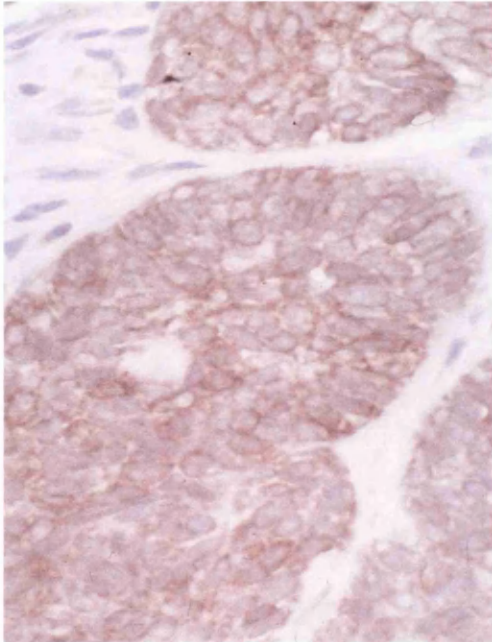


Figure 3.26c Positive bcl-2 staining

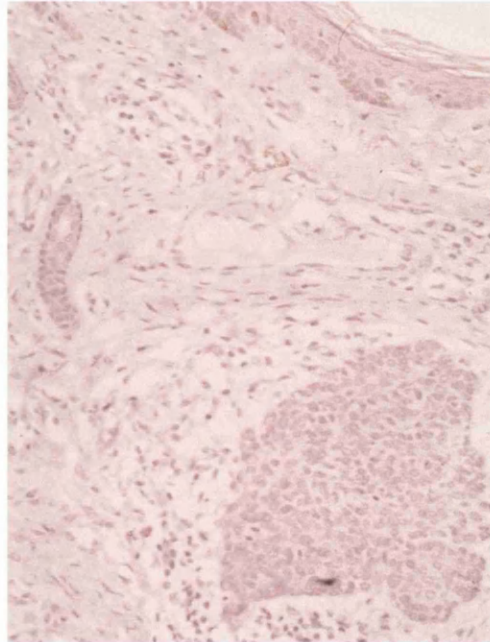


Figure 3.27d Negative bax staining.

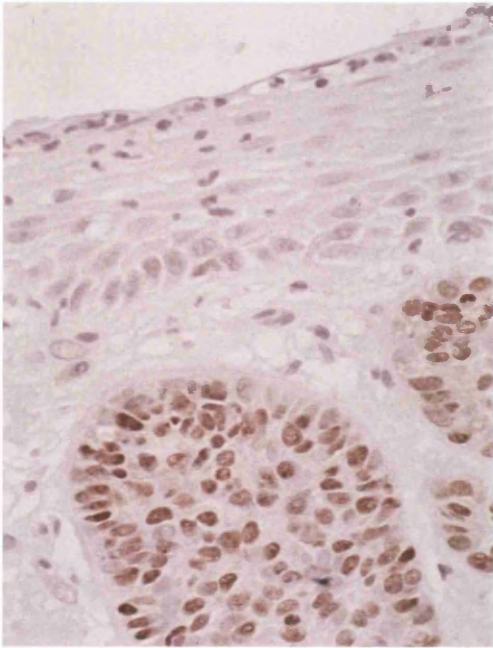


Figure 3.26e Positive p53 staining

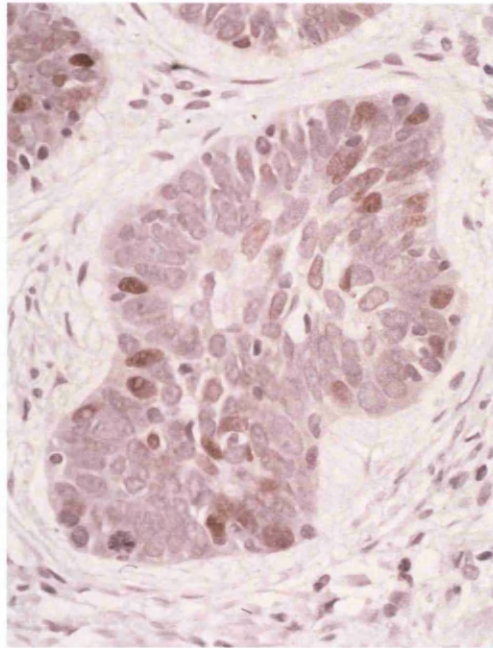


Figure 3.26f Negative p53 staining

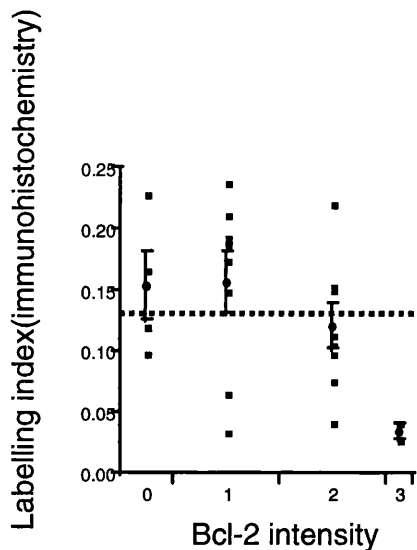


Figure 3.27a. The relationship of bcl-2 to the LI

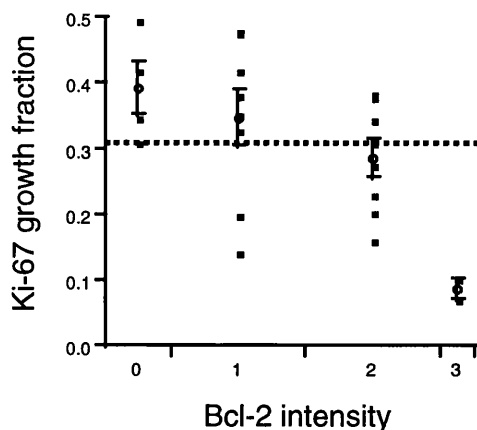


Figure 3.27b. The relationship of bcl-2 to the GF.

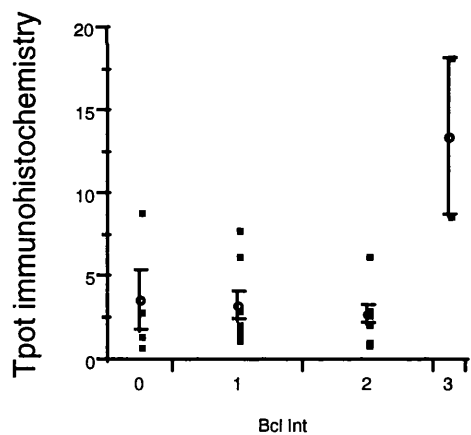


Figure 3.27c The relationship of bcl-2 to the Tpot(im).

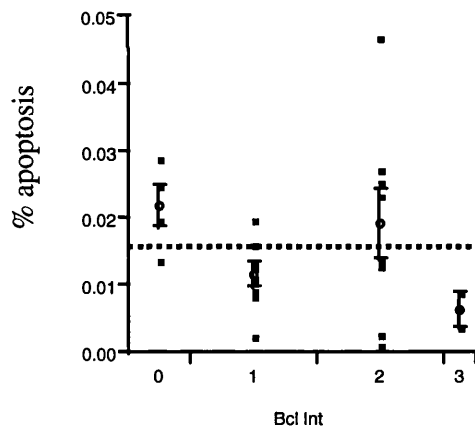


Figure 3.27d The relationship of bcl-2 to the apoptotic index.

3.3.4.2.2 *bax immunohistochemistry*

bax oncoprotein expression was quantified according to similar criteria. The results are in Table 3.5. (Figure 3.26d)

% positivity	Score	Number	Probability	Intensity	Score	Number	probability
None	0	17	0.77	None	0	17	0.77
0-29	1	3	0.13	Weak	1	0	0
30-49	2	1	0.05	Moderate	2	5	0.23
50-79	3	1	0.05	Strong	3	0	0
80-100	4	0	0				

Table 3.5. *bax* oncoprotein immunohistochemistry.

77% of tumours were immunonegative for *bax* oncoprotein. There was therefore much less variation than the *bcl-2* oncoprotein staining. No relationship was found between the *bcl-2* and *bax* oncoprotein staining. On contrary to expectations, high levels of *bax* oncoprotein staining was not related to higher apoptotic indices or other cell proliferation parameters. .

3.3.4.2.3 *p53 Immunohistochemistry*

There was a range of percentage positivity for *p53* immunostaining although the majority of tumours stained with less than 29% (62%) or with 80-100% (25%) (Figure 3.26e-f). Few tumours exhibited staining characteristics between these values (12%). The distribution is illustrated in Figure 3.28.

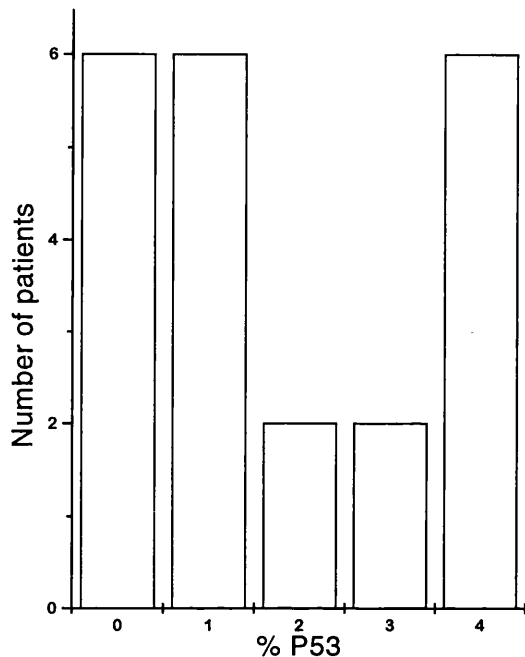


Figure 3.28. The distribution of p53 immunohistochemistry in BCC.

p53 expression was not related to the cell loss parameters. i.e the apoptotic index, bcl-2 or bax immunohistochemistry. A trend of a higher growth fraction in those tumours with greater than 50% p53 expression ($p=0.07$) was found although this did not reach statistical significance. This trend was not supported by the LI data. There was no correlation between p53 and Tc, Tpot or Ts.

3.3.4.3 Relationship of cell proliferation and cell loss with clinical parameters

Although the main aim of this chapter was to study the interrelationships between cell production, cell loss and the regulating oncoproteins, these factors were also compared with the clinical parameters such as patient age, sex, tumour type, position size and recurrence status. These results are a preliminary study that involves small patient numbers and the findings must therefore be interpreted accordingly. The relationship of some of the proliferative parameters are investigated more closely in the following chapters. Patient age and sex were not related to the kinetic parameters.

The growth pattern of the tumours appeared related to the cell production and cell loss parameters although due to the small cohort of patients the values did not reach statistical significance. The infiltrative tumours were more proliferative than the nodular tumours with a higher LI and GF and shorter Ts and Tpot. (Table 3.6). The infiltrative tumours also tended to express less bcl-2 and have a higher apoptotic index and express more p53 than the nodular type.

Growth pattern	Mean LI % (SE)	Mean GF % (SE)	Mean Tpot days (SE)	Mean Ts hours (SE)	Mean apoptotic index % (SE)	% moderate / strong bcl-2	% moderate / strong p53
Infiltrative	16.9 (0.03)	39.7 (0.05)	2.9 (2.9)	7.2 (0.8)	0.02 (0.004)	20	60
Micronod	8.9 (0.03)	27.5 (0.05)	3.7 (2.0)	6.9 (1.0)	0.02 (0.005)	75	50
Nodular	11.7 (0.02)	26.0 (0.26)	5.3 (1.2)	9.4 (0.6)	0.01 (0.003)	45	27
Superficia 1	22.1 (0.06)	33.5 (0.1)	1.7 (4.1)	5.9 (2.0)	0.01 (0.007)	50	0

Table 3.6. The relationship of cell production and cell loss with histological subtypes of BCC.

There was no relationship between the tumour position, size or recurrence status with the proliferative or cell loss parameters.

3.4 Discussion

Considerable efforts are being made to investigate the processes central to the control of cellular proliferation of human tumours as it has become increasingly clear that abnormalities in these pathways are central to tumourigenesis. Furthermore, the

information provided from these studies may have both a therapeutic and prognostic relevance (Tubiana and Courdi 1989; Wilson, Dische et al. 1995). Therefore, cell proliferation was used as a starting point from which to assess the cell biology of BCC. Tumour growth is determined by 3 main variables (1) The Tc (2)The GF and (3) The cell loss factor. Historically the growth fraction has been difficult to measure directly and could only be derived, therefore the LI was traditionally measured by assessing the uptake of DNA precursors. Subsequently, the concept of the Tpot was developed as a dynamic measurement of cell proliferation rate rather than the static measurements such as the LI(Steel 1977). This is a theoretical measurement of cell proliferation in the absence of cell loss. Prior to the application of flow cytometry, and the introduction of BrdUrd to the study of cell kinetics, measurement of these parameters was both difficult and tedious, requiring the use of radioactive precursors and multiple biopsies to construct P.L.M curves (Quastler and Sherman 1959). Additionally, it took weeks or months for the results to be available.

Our understanding of tumour cell kinetics has been substantially increased by the use of flow cytometry and BrdUrd incorporation. (Begg, McNally et al. 1985; Wilson 1991; Wilson 1994). The Tpot can be calculated from the LI and Ts from a single observation. This is more acceptable to the patient and allows for rapid analysis without the requirement of a radioactive precursor. The Tpot is considered to provide a more superior and more complete index of proliferation than LI or Ts in isolation. (Wilson 1993).

The aim of this study was to develop a complete picture of the cell proliferation in BCC and to understand the interactions of cell production and cell loss. This was to provide a platform for other studies which would investigate more closely the relationship of some cell loss and cell production parameters with clinical and histological data. The cohort of patients in this study was relatively small due to the withdrawal of the licence of BrdUrd. The patients in this cohort tended to be old with a mean age of 81 and included a greater number of aggressive (infiltrative) tumours that would normally be expected in a consecutive series. This was a result of the selection procedure in which in-patients were favoured rather than day cases due to the requirement of a time interval between injection and biopsy.

None of the tumours studied in this relatively small series were aneuploid. Other studies have also revealed low incidences of aneuploid BCCs. (Buchner, Hiddemann et al. 1985; Fortier Beaulieu, Laquerriere et al. 1994; Robinson, A.W. et al. 1996). In one study examining 509 BCCs, aneuploidy was noted in 19% of tumours. This is in contrast to SCC in which approximately 80% are aneuploid (Barlogie and Raber 1983). DNA aneuploidy has been shown to be related to histological subtype with increased aneuploidy in infiltrative, morpheic or multifocal histological patterns or with keratinisation or metatypical differentiations. (Herzberg, Garcia et al. 1993; Fortier Beaulieu, Laquerriere et al. 1994) Chromosomal abnormalities in BCCs may therefore relate to local invasiveness. There has been very few reports in the literature regarding translocations and other DNA derangements. DNA aneuploidy and tetraploidy has been associated with metastasis. (Tubiana and Courdi 1989) Although the mechanisms for the absence of metastatic dissemination in BCC are not understood, this finding would support a concept that there is less gross abnormality in DNA in BCC than in SCC. As a result some elements of the normal constraints of tumour growth are retained in BCC which prevents the proliferation of the malignant cells in metastatic sites.

It appears from these results that BCC is a highly proliferative tumour with a short T_s and high LI. Consequently the T_{pot} was short. There is also a large variation of LI and T_s and consequently T_{pot} in BCC. The T_s was calculated by the relative movement method from flow cytometry. The median T_s value was 7.2 hours (range 5.0-14.6 hours). This compares well to the tissue of origin of BCCs. T_s values of keratinocytes have been documented at 5.9-16 hours (Leigh, Lane et al. 1993). These results are in contrast to the value of T_s of BCC of 20 hours calculated by Weinstein and Frost and the consideration that the T_s of BCC is double that of normal skin by Heenen and Galand. This value, however was derived from multiple tumours and range of values for individual tumours could not be calculated (Weinstein and Frost 1970). It is possible that the patient selection bias of the 2 studies may account for these differences. In this study more aggressive tumours in older patients were selected while Weinstein and Frost selected patients with multiple nodular or superficial tumours.

The LI was calculated by flow cytometry and immunohistochemistry and varied according to the technique of measurement. The measurement by flow cytometry resulted in lower values than by immunohistochemistry (median 0.03 vs 0.14) with less variance (CV 50.7 vs 64.7). However there was no relationship between individual results measured by flow cytometry or immunohistochemistry. The most probable explanation for this finding is that tumour and non tumour cells cannot be differentiated by flow cytometry because of the diploid DNA content of both populations. This results in a dilution of the labelled cells since there was relatively proliferation seen in the peritumoural stroma. In contrast, the labelled and unlabeled cells in tumour islands can be counted by immunohistochemistry and the dilution factor of non tumour populations is eliminated. We would therefore consider that the immunohistochemistry data is a more valid measurement of the LI rather than the flow cytometry data. This is supported by the strong linear relationship of the immunohistochemical LI compared to the GF and a poor correlation of the LI (flow cytometry) compared to the GF. The Linear relationship between the LI and GF is described by $LI = -0.015 + 0.047GF$. This suggests that about half of the cells in the cell cycle are in the S phase. Assuming that the cells pass around the cell cycle at a constant rate it would also suggest that the T_s is approximately half of the T_c .

There was a range of potential doubling time derived from the LI and T_s . These ranged from 0.9 days to 18.3 days for the data derived from immunohistochemistry and 3.4-40.8 days with flow cytometric derived data. For the reasons discussed above we would consider the immunohistochemistry derived data to best represent the true T_{pot} . This would result in a median T_{ot} of 2.8 days and a mean T_{pot} of 4.2 days. Clearly this represents a very fast proliferating tumour type when compared to other tumours. (Table 3.7) The T_s value for BCC was noted to be shorter than all of the other tumours measured by an identical technique in the same laboratory. It would appear that that T_s increases throughout the upper aerodigestive with tumours of the oral mucosa having shorter median T_s than those derived from the oesophageal or bronchial mucosa. Tumours of the cervix tended to have a longer T_s whilst melanomas and BCCs had shorter T_s possibly reflecting the proliferative status of the

skin. The relatively short Ts for BCC is in keeping with the finding that diploid tumours have a shorter Ts than aneuploid tumours (Wilson 1991).

Table 3.7 also reveals the marked differences the estimation of LI by flow cytometry and immunohistochemistry. The underestimation of the LI by flow cytometry especially in diploid tumours has been previously documented.(Wilson, Dische et al. 1995). Consequently the Tpot (Flow cytometry) for BCC is relatively long compared to other tumours. This is a disproportionately low value due to all of the BCCs being diploid. However the Tpot(im) for BCC is relatively fast for BCC and on a par with other tumours.

Tumour type	Number of patients	% aneuploid	Li(flow) %	Ts Hhs	Tpot (flow) Days	Li(im) %	Tpot (im) Days
BCC	24	0	2.6	7.6	11.6	14.2	2.8
Head / Neck	132	48.2	6.8	10	5.2	14.2	2.4
Lung	28	81.6	8.6	15.3	6.5	18.5	2.2
Oesophagus	48	63.8	8.3	13.1	5.3	22.4	1.9
Cervix	25	68	10.2	15.8	4.5	19.8	2.0
Melanoma	38	50	6.7	11.4	6.9	12.5	3.0
Colorectal	100	65	9.0	13.1	3.9	N/K	N/K

Table 3.7. A comparison of the proliferative parameters of BCC compared to other tumours measured by flow cytometry and immunohistochemistry. (Personal communication G. D. Wilson).

Although the Tpot is derived mathematically from the LI and Ts, it is the LI which has the dominant effect. This is because the Ts has less variation than the LI by flow cytometry or immunohistochemistry. (CV 27.8 vs 64.7 and 50.7 respectively) A strong inverse relationship is therefore seen between the Tpot and the LI. The effect of the Ts measurement on the Tpot (flow cytometry) was only seen in tumours with a low LI. At a LI of less than 0.02 there was a linear relationship between the variables. A stronger linear relationship was found between Ts and Tpot (im) because there was less variation in the LI (im) compared to the LI (flow cytometry). The GF was also inversely proportional to the Tpot and the relationship could be described by an exponential decay curve (similar to the relationship of the LI with the Tpot).

The cell cycle time was derived and not measured directly and is therefore subject to the variations in the LI, Ts and GF. The mean and median cell cycle time was 1.2 and 1.1 days respectively with a range of 0.5-3.9 days. The variation of Tc between different tumours may represent true variation in Tc or the variation within the values from which the Tc is derived. (Tpot and GF) The Tc derived in this study was shorter than the value measured by Weinstein and Frost who calculated as 217 hours (9.4 days) but are in agreement with other solid tumours in which the cell cycle time is documented between 1 and 4 days. (Tubiana and Courdi 1989) In support of the linear relationship between the LI and GF there was also a correlation between the Tc and Ts (Spearman RHO $p=ns$) ie the duration of the S phase is directly proportional to the cell cycle duration. This relationship in the Ts and Tc has also been previously reported (Mendelsohn 1975; Tubiana, Pejovic et al. 1981).

The spatial arrangement of proliferating cells has been previously explored in BCC. Weinstein and Frost considered that the proliferating cells were a relatively homogeneous population while Grimwood reported that the proliferating cells were situated at the periphery of transplanted tumour nodules (Weinstein and Frost 1970; Grimwood, Ferris et al. 1986). This study suggests that there is a range of proliferations patterns from a purely marginal pattern ranging to a random pattern with an intermediate marginal diffuse pattern. It is possible that these patterns reflect the range of behaviour seen in BCC and this relationship is explored further in Chapter 5.

We are unable to satisfactorily explain the correlation between the Tpot and the proliferation patterns in the absence of a relationship between the Ts and LI.

The paradoxically high rates of cell production in BCC have been previously documented. This study has confirmed this finding by using alternative methods. It has been suggested that apoptosis may explain this dichotomy (Kerr and Searle 1972). The apoptotic index in this study ranged from 0.01- 0.05 with a median and mean value of 0.01 and 0.02 respectively. In keeping with the slow clinical growth characteristics of this tumour the cell loss rate must therefore be assumed to be high. For the tumour to remain static, cell production must equal cell loss. The cell production rate is the inverse of the Tpot and can be calculated from the LI divided by the Ts. The median cell production rate is therefore 1.8% per hour (Mean 1.8%, CV 62.5). The speed of the apoptotic process will therefore determine the relative contribution of this apoptotic rate to the kinetics of BCC. It has been postulated that the whole apoptotic process is rapid. Depending on the circumstances the core of the process may take a few minutes or up to many hours (Wyllie 1985), following this the apoptotic debris are phagocytosed which may take a few hours. In this study the morphologically recognisable apoptotic bodies have been quantified which represents the end stage of chromatin condensation and characterises the core process. Therefore the rates of cell production of 1.8% per hour would match the apoptotic index of 1% assuming this process takes minutes to a few hours. It is conceivable that apoptosis alone may explain the slow clinical growth of BCC. There was no direct relationship demonstrable between apoptotic index and the cell production rate.

Other mechanisms of cell loss may include necrosis or desquamation. Necrosis was analysed semi quantitatively in this series and it was apparent that it is not a prominent feature in the majority of BCC as only 40 % showed signs of necrosis. Necrosis was always associated with ulceration and this might suggest that it is the mechanism for cyst formation in some tumours. There was no relationship with tumour size and cell production parameters. This suggests that necrosis in the BCCs studied in this group is not due to rapid growth exceeding the blood supply of the tumour population of cells although due to the small sample size the data has to be interpreted with caution. Necrosis was not associated with apoptosis. This reflects the study design since only

spontaneous necrosis was counted and not cells around necrotic foci that may represent apoptosis triggered by necrosis. Ulceration is a common feature in BCC and was found in 62% of BCCs. Ulceration was always associated with necrosis. Whether the underlying necrosis causes loss of the epidermis or visa versa is unknown. It is clear, however that both ulceration and necrosis to a lesser extent, may both contribute to the cell loss factor in BCC. The extent of their contribution is unclear since it is apparent that apoptosis alone may account for the discrepancy between the measure cell kinetics and the slow observed clinical growth rate.

The control of apoptosis is regulated by the bcl-2 family of oncoproteins and by other tumour suppressor genes such as p53. In this study, the relationship between these oncoproteins was investigated and also the correlation of these cell death proteins with cell production. The cell survival/cell death switch is determined by the ratio of bcl-2 to bax. These ratios are in turn controlled by p53. The group of tumours in this study tended to be immunopositive for bcl-2 with 82 % positive staining. This finding is in agreement with other studies (Cerroni and Kerl 1994; Morales Ducret, van de Rijn et al. 1995; Rodriguez Villanueva, Colome et al. 1995; Verhaegh, Sanders et al. 1995; Wikonkal, Berg et al. 1997). This study also found that the majority of BCCs do not express bax oncoprotein (77%) In isolation this would suggest that gene expression in BCC favours an anti apoptotic phenotype which is conflicting to the high proliferation/apoptosis hypothesis previously postulated. However, there are also many other oncoproteins that determine the survival/apoptosis balance such as Bak, Bcl-xs, Bad, Bcl-w and Bcl-xl. The contributions of these proteins may explain these conflicting findings. Additionally the relative concentration of these factors may not be the only determinant of function as they can be functionally modified by post translational mechanisms and furthermore the dimerization status of these proteins was unknown. Consequently the individual bcl-2 or bax intensities or percentage positivities were not related to the apoptotic index nor was there any correlation between the ratio of bcl-2 to bax with the apoptotic index. Although bcl-2 does not exert control over cell production rates there was a significant relationship between the bcl-2 and the GF and a strong trend with the LI. Tumours with little or absent bcl-2 staining had a higher GF and LI than those with moderate or strong bcl-2 staining. (Wilcoxon / Kruskal-Wallis $p=0.03$ and 0.07 respectively.) It is possible that this

may reflect the stem cell of origin of some BCCs. These cells are slow cycling cells that tend to express bcl-2.

The p53 tumour suppressor protein exerts control over cell production and cell loss. DNA damage results in the activation of p53 that causes cell cycle arrest and also induces apoptosis by its actions on bcl-2 and bax. P53 staining in BCC was found to be either less than 20% (54.5% of the patients) or greater than 80% (27% of the patients) with only 18% of the patients with intermediate values. It is uncertain whether the accumulation of p53 represents a mutation of the gene or stabilisation by other mechanisms. There may be accumulation of wild type p53 in response to DNA damage, due to increased stability of mutated p53 or conversely there may be mutations with negative staining (Campbell, Quinn et al. 1993). It is of interest however that about half of the tumours did stain positively for p53 and that it is reported that approximately 40-56% of BCCs harbour p53 mutations (Gailani, Leffell et al. 1996). The data supports the concept that the accumulation of p53 may be due to a mutated or dysfunctional protein since there was a trend that these tumours had a higher GF reflecting some loss of cell cycle control. This relationship, however, was not supported by the other cell production parameters measured. The relationship between p53 accumulation and cell loss was unclear in this study because no relationship was found between bcl-2, bax or apoptotic index.

Although the main aim of this chapter was to study the cell biology of BCC by exploring the interactions of cell production and cell loss, it was also possible to correlate this data with some of the clinical and histological information. The numbers were small and therefore should be interpreted as a preliminary study for the more detailed analysis in the following chapters. Patient factors had no relationship with the proliferative parameters suggesting that these are determined by inherent tumour biology. Although the values did not reach statistical significance it appeared that the growth pattern was related to cell production and cell loss rates with the infiltrative tumours tending to be more proliferative with a higher LI, GF and shorter T_{pot} than the nodular type of tumours. The infiltrative tumours also had more apoptosis and expressed less bcl-2 than the nodular tumours. These findings are explored further in

later chapters since these differences may in part be responsible for the differing behaviours of the various BCC subtypes.

Chapter 4

Clinicopathological Characteristics of Primary, Recurrent and Horrifying Basal Cell Carcinoma.

4.1 Introduction

Approximately 90% of BCCs are cured by simple procedures at first attempt. However, in a significant number of patients, clinical problems are posed by recurrent lesions. Rarely, some tumours do not adhere to these general characteristics and become increasingly destructive and have been termed aggressive, (Vico, Fourez et al. 1995); (Jacobs, Rippey et al. 1982) mutilating (McGurk and Edwards 1984), (Dvoretzky, Fisher et al. 1978; Schwartz, Vickerman et al. 1979), giant (Bianchini and Wolter 1987) (Schwartz, De Jager et al. 1986) or horrifying tumours (Jackson and Adams 1973). Of these terms, horrifying is defined the most clearly. The ability to predict potentially problem tumours is of fundamental importance in the management of BCC. The clinical behaviour of BCC may be determined by a variety of factors. Firstly, patient factors such as their age, and time of presentation may effect the outcome of a tumour. Secondly, treatment factors such as modality and adequacy of treatment may influence recurrence. Thirdly, tumour factors such as tumour type and position may be important. It would appear that histological subtype may be related to tumour behaviour (Thackary 1951; Sloane 1977), however confusion has arisen in BCC due to the existence of a variety of different subtypes and non uniformity of classifications. Furthermore, it is uncertain whether certain subtypes are intrinsically more aggressive than others or that different subtypes are more commonly incompletely excised.

The aim of this chapter was to evaluate some of the patient, tumour and treatment factors that may determine the outcome of BCC. In particular, the role of the tumour

subtype was studied by dividing the series according the growth pattern and differentiation. These patients groups have subsequently been analysed in the following chapters in which the tumour biology is examined more closely by evaluating the proliferative characteristics of the tumours.

4.2 Materials and methods

Primary, recurrent and horrifying tumours were selected according to the criteria in Chapter 2. Clinicopathological information as described in Chapter 2 was extracted from the patients notes. An attempt was made to acquire all of the original and subsequent specimens of recurrent and horrifying tumours from other hospitals. The tumours were all re-examined by haemotoxylin and eosin staining in order to attain the growth pattern and differentiation status as described in Chapter 2, since this information was rarely recorded by the histologist. Most tumours displayed a mixture of differentiation subtypes and therefore only the predominant characteristic was recorded. The completeness of excision was obtained from the histology reports.

4.3 Results

4.3.1 Patients

4.3.1.1 Presentation

170 tumours were analysed. There were 88 primary, 46 recurrent and 36 horrifying tumours.

The criteria for the classification of horrifying tumours has been described in Chapter 2. The horrifying tumours could either present as horrifying due to patient neglect or present at a normal time (described as early) and develop into a horrifying tumour by way of multiple recurrences. In this series 21 presented late and 15 presented early.

The complete history of the tumour was recorded in 15 of the late presenting group. The patients admitted to having the tumour from 1-23 years (Mean 9.1 years.)

4.3.1.2 Age and sex

The age at first presentation was recorded for the primary tumours. The age of presentation of the original tumour was recorded for the recurrent group. Some of the horrifying tumours presented very late and admitted that they had tumour from between 1 and 20 years (21 patients). The age of onset of the original tumour was estimated from the information given by the patients.

Table 4.1 shows the horrifying tumours occurred at a significantly younger age than presentation in the primary group. (Kruskal-Wallis $p=0.01$) There was no difference between the recurrent and primary group. The sex distribution was equal in the primary tumours but there was a greater proportion of males in the recurrent and horrifying groups.

	Primary	Recurrent	Horrifying
Number of patients	88	46	36
% Male	48.9	65.2	66.7
% Female	51.1	34.8	33.3
Median age	71.5	71	61.0*
Mean age	69.7	66.9	61.0
Range age	38.0-94.0	32.0-86.0	29.0-84.0
75% quartile age	79.0	75.75	76.0
25% quartile age	63.0	60.0	47.0
Standard deviation age	12.4	13.1	15.6
C.V. age	17.7	19.6	25.5
Normal Distribution age	no	no	no

Table 4.1. The age distribution of BCC in the clinical groups.

4.3.2 Tumours

4.3.2.1 Tumour size

Tumour diameter estimations were obtained from the patient notes and from the histology report. The size of the tumours for each group are presented in Table 4.2. The recurrent tumours, at first presentation, and early presenting horrifying tumours were larger than the primary tumours. (Kruskal-Wallis $p < 0.01$ and $p = 0.01$ respectively). The late presenting horrifying tumours by definition were all very large. (median size 70mm)

	Primary	Recurrent	Horrifying early presenting	Horrifying Late presenting
Number of patients	88	46	(10)/16	(19)/20
Median (mm)	8.0	10.0*	13.5*	70
Mean (mm)	9.7	12.6	17.0	82.1
Range (mm)	2.0-42	4.0-30	7.0-60	15.0-200
75% quartile (mm)	10.2	15.0	16.2	80.0
25% quartile (mm)	5.0	8.0	8.0	50.0
Standard deviation	7.4	6.5	15.7	51.6
C.V.	76.6	51.2	92.1	62.8
Normal Distribution	no	no	no	no

Table 4.2. The tumour size for each clinical group. The numbers in brackets represent the number of patients in which the tumour size was recorded.

4.3.2.2 Anatomical tumour position

As can be seen from Table 4.3, the primary and recurrent tumours occurred predominantly on the upper and middle face while the horrifying tumours were most commonly found on the middle face. A disproportionately high percentage of

horrifying were situated on the scalp (14%). Recurrent or horrifying tumours were rarely found on the trunk or limbs.

	PRIMARY	RECURRENT	HORRIFYING
Scalp	1.2	2.3	13.9
Face-upper	28.9	38.6	16.7
Face-middle	25.3	38.6	63.9
Face-lower	10.8	4.6	2.8
Ear	4.8	4.6	0
Neck	2.4	4.6	0
Trunk	15.7	4.6	2.8
Upper limb	1.2	0	0
Lower limb	10.8	2.3	0

Table 4.3. The anatomical position of tumours expressed as the percentage of tumours in each clinical group.

4.3.2.3 Histological growth pattern

The histological growth patterns of each clinical group is shown in 4. 1. The primary tumours were most commonly of the nodular subtype (50%) with less infiltrative or morpheic tumours (18.2% and 4.6% respectively). The horrifying group tended to be of infiltrative subtype most commonly (47.1% for both early and late presenting groups), although, nodular tumours were also included irrespective of time of presentation. The recurrent group contained an intermediate mixture of histological growth patterns.

There was no age or sex difference between the histological types of BCC (Kruskal-Wallis $p=0.8$ and Chi square $p=0.4$ respectively).

From Figure 4.2. it can be seen that there were positional differences between the histological variants of BCC. The superficial type were found most commonly on the

trunk and account for 43.8% of tumours in this position. The nodular and infiltrative tumours were distributed evenly throughout all sites. The morpheic tumours were found on the scalp, upper, middle and lower face. No morpheic tumours were found on the limbs or trunk.

Overall, there were no differences found between the median sizes for each histological growth pattern at first presentation, excluding the tumours in the late presenting, horrifying group. The median sizes were as follows infiltrative 10.0mm, micronodular 10.0mm, superficial 10.0 mm, morpheic 10.0mm, nodular 8.0mm. However, on examination of the primary tumours only, there were significant differences between the groups. The median size of the infiltrative (12mm) and morpheic (10mm) and superficial tumours (10mm) were larger than the micronodular (8mm) or nodular tumours (6mm) (Kruskal-Wallis $p=0.001$).

There was also little difference in the size of each growth pattern between the clinical groups. The nodular tumours were the only group in which the tumour size was related to the outcome. Non-recurrent tumours were significantly smaller than recurrent tumours. (Table 4.4.)

Growth pattern	Prim mm	Rec. mm	Hor. Early mm	p value	Hor. Late mm
Nodular	6	10	15	>0.001	60
Micronodular	8	10	20	0.2	50
Infiltrative	12	10	11	0.7	70
Mor.pheic	10	25	7	0.2	75
Superficial	10	15	-	0.1	-

Table 4.4 showing the differences in median size of each growth pattern with respect to the clinical outcome. The p value for the size difference (Kruskal-Wallis) does not include the late presenting horrifying group.

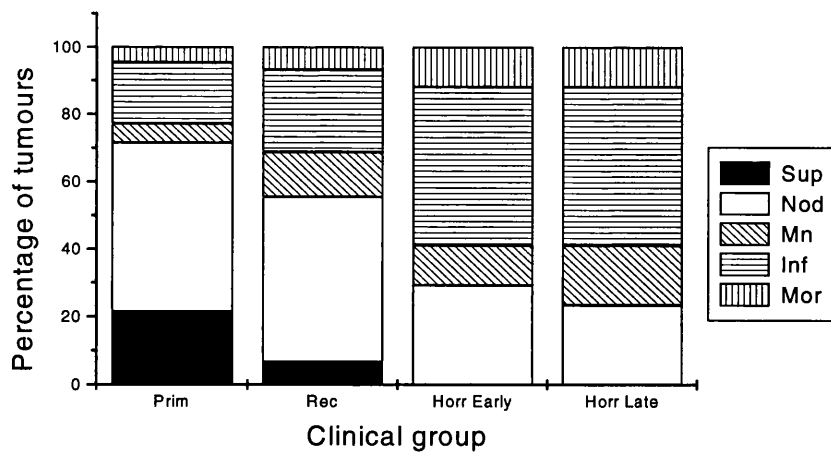


Figure 4.1. The histological growth patterns within each clinical group.

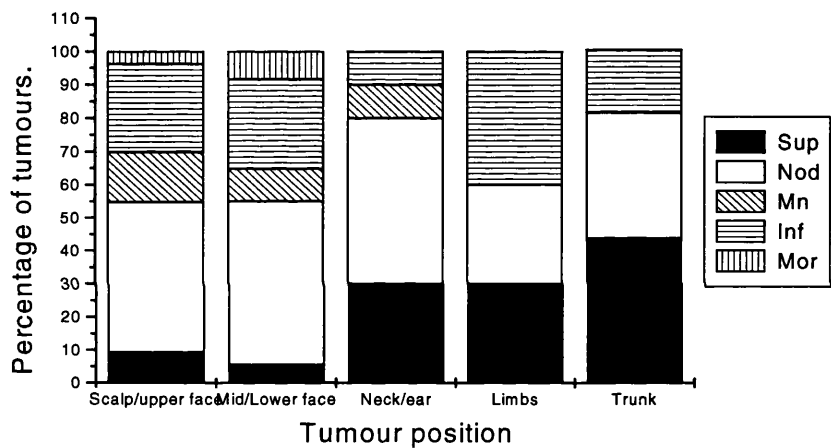


Figure 4.2. The variation in histological growth pattern with tumour position. (Includes all groups of patients).

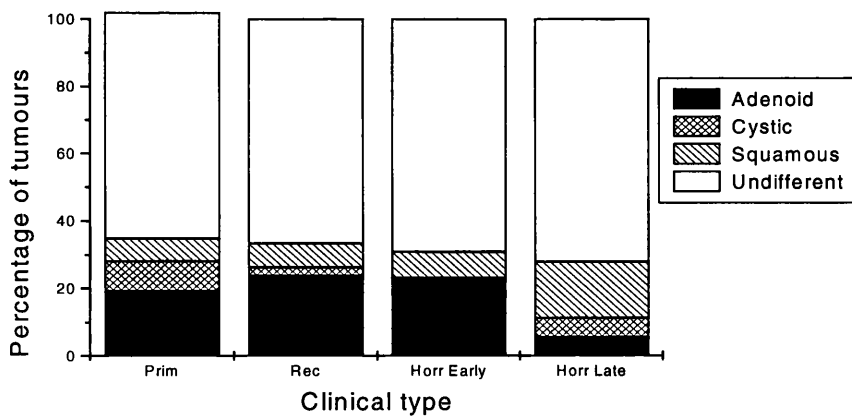


Figure 4.3. The differentiation status of the tumours in each clinical subtype.

4.3.2.4 Histological differentiation

An assessment of the differentiation status of each tumour was made as described in Chapter 2. Most tumours were heterogeneous and exhibited more than type of differentiation subtype. Therefore the predominant differentiation subtype was recorded in each tumour.(Table 4.5). The majority of tumours were classified as undifferentiated (67.5%) and 19.4% displayed adenoid features. Although cystic change and squamous metaplasia is considered a common feature of BCC, these were scarcely the most predominant feature in this series. Infiltrative, morpheic, micronodular and superficial tumours were mostly undifferentiated (73%, 100%, 80%, 91% respectively). Nodular tumours displayed the greatest diversity of differentiation types with only 49% being predominantly undifferentiated. There was very little difference between the differentiation status and the clinical subtype. (Figure 4.3).

	Superficial	Nodular	Micronod.	Infiltrative	Morpheic	Total
Adenoid	9.1	29.6	6.7	17.1	0.0	19.4
Cystic	0.0	11.3	0.0	0.0	0.0	5.0
Squamous metaplasia	0.0	9.9	13.3	9.8	0.0	8.1
Undifferentiated.	90.9	49.3	80.0	73.1	100.0	67.5

Table 4.5. The predominant differentiation in the histological types of BCC. The figures are percentages of tumours in each histological subgroup.

4.3.2.5 *Change of histological growth pattern with tumour progression*

37 successive specimens from recurrent tumours and 18 from horrifying tumours were available for analysis of the change in histological growth pattern with tumour progression. The growth patterns of the first and last biopsies are summarised in Table 4.6. Nodular tumours, in both recurrent and horrifying groups tended to remain nodular (60% of cases) but some progressed to other tumour subtypes, the most common being infiltrative. Infiltrative tumours often remained infiltrative but approximately half appeared nodular in subsequent recurrence. It is uncertain whether the infiltrative - nodular changes or visa versa represents progression to a different phenotype or that a mixed nodulo infiltrative subtype exists such that the observed changes represent sampling error. The finding that there is less change in the horrifying tumours, which are larger and therefore are examined by more sections, suggest the latter might be a plausible explanation. Sampling error may also be a mechanism by which a completely excised tumour may recur, when in reality inadequate sampling of the tumour margin resulted in a false complete excision histological report. A common feature in the horrifying subgroup was the progression of the micronodular tumours to become infiltrative tumours. Superficial tumours could also progress to a more aggressive infiltrative or micronodular phenotype.

RECURRENT TUMOURS					
	Final histological growth pattern				
Initial growth pattern	Nodular	Micronodular	Infiltrative	Superficial	Morpheic
Nodular (18)	11	2	3	2	-
Micronod. (6)	-	5	1	-	-
Infiltrative (9)	4	-	5	-	-
Morpheic (2)	-	-	-	-	2
Superficial (2)	-	1	1	-	-
HORRIFYING TUMOURS					
Nodular (6)	4	1	1	-	-
Micronod. (4)	-	1	3	-	-
Infiltrative (7)	-	1	5		1
Morpheic (1)	-	-	1	-	-

Table 4.6. The progression of the histological subtypes from the first available biopsy to the last biopsy. The numbers in parentheses represents the total numbers of tumours in each subtype.

4.4 Outcome

4.4.1 Tumour size and tumour control

As previously discussed tumour size at presentation was significantly related to the development of a recurrent or early presenting horrifying tumour. However size was not related to incomplete excision. Excluding the horrifying late presenting tumours, the median size in completely excised tumours was 9mm compared to 10mm in incompletely excised tumours (Kruskal-Wallis $p=0.1$). On further analysis, isolation of histological subtypes demonstrated that the size was related to incomplete excision

in the nodular subgroup of tumours. (Table 4.7). However the numbers are small in all groups. Larger tumours more commonly recurred following complete excision compared to smaller tumours, (median 8.0mm vs 12.5mm Kruskal-Wallis $p=0.05$.) Recurrence or the development of a horrifying tumour after radiotherapy was also related to size. The median size of non recurrent tumours was 4.5mm compared to 7.5 mm for the recurrent group and 9mm for the early presenting horrifying group. These were statistically different to the non recurrent group (Kruskal-Wallis $p=0.03$ and $p=0.02$ respectively).

	COMPLETE	INCOMPLETE	P
Nod (38:12)	6	10	0.03
Mn (4:3)	10	10	1
Inf (18:4)	10	11	0.9
Mor (5)	12	-	-
Sup (16:2))	10	13	0.2

Table 4.7. Tumour size and subtype in relation to the completeness of excision. The figures in parentheses represents the numbers in each group respectively.

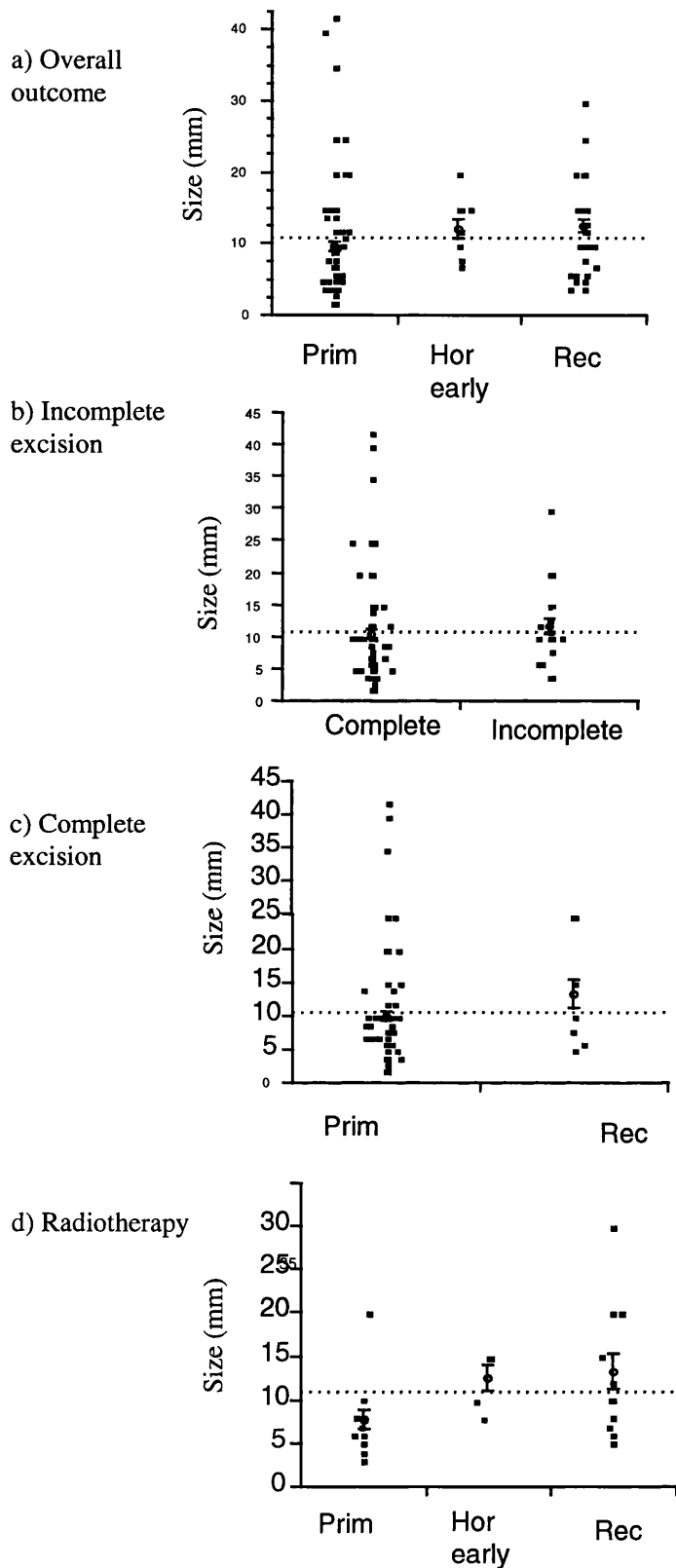


Figure 4.4. The relationship of initial tumour size to outcome (a overall, b following incomplete excision, c following complete excision, d following radiotherapy). Error bars represent SEM and the dotted lines represents the median value.

4.4.2 Treatment and tumour control

The initial treatment for each clinical group is shown in Table 4.8. Primary tumours in this series were predominantly surgically treated (79.5%) although a significant proportion received a course of radiotherapy (14.8%). There was one tumour that was reported to be incompletely excised, that had no further treatment, and did not recur. All of these patients had local tumour control for 5 years.

	Primary (88)	Recurrent (46)	Horrifying early (16)	Horrifying late (20)
Radiotherapy	14.8 (13)	32.6 (15)	62.5 (10)	20.0 (4)
Cryotherapy	0	4.4 (2)	0	0
Curettage	0	6.5 (3)	12.5 (2)	0
Surgery-Complete	79.5 (70)	17.4 (8)	0	50 (10)
Surgery-Close	2.3 (2)	6.5 (3)	0	5 (1)
Surgery Incomplete	1.1 (1)	30.4 (14)	12.5 (2)	0
Surgery Incomplete and re excision	2.3 (2)	2.2 (1)	0	10 (2)
Surgery incomplete and radiotherapy			6.3 (1)	15 (3)
Unknown			6.3 (1)	

Table 4.8 The initial treatment for each tumour group. Numbers in bold represent percentages of each patient group while the numbers in parentheses represent actual patient numbers.

Recurrent tumours referred to Mount Vernon were more commonly characterised by incomplete excision (30.4%) or treatment by radiotherapy (32.6%), as the initial treatment, most commonly. One patient had incomplete excision with immediate re excision but still developed a recurrence and 8 patients developed a recurrence despite a report of complete excision. 5 patients had either curettage or cryotherapy. 35 of the

recurrent tumours had 1 recurrence event, 10 had 2 and 1 patient had 4 subsequent relapses.

The early presenting horrifying tumours most commonly received primary radiotherapy as first treatment (10 patients). The patients initially treated with either curettage or incomplete excision were then administered radiotherapy for the treatment of the first recurrence. Subsequent recurrences were usually managed by repeated surgical excision most commonly although, in some instances, this was combined with radiotherapy. Of the 15 early presenting horrifying tumours, 4 had 3 recurrences, 4 had 4 recurrences, 4 between 5 and 10 recurrences and 3 patients had greater than 10 recurrences. Ultimately 5 patients achieved tumour control (follow up 5-16 years, mean 10 years), 3 patients died of local recurrence and 6 patients are alive with macroscopic or microscopic disease. 1 patient died with metastatic disease.

From the preceding paragraphs it can be seen that the treatment modality and adequacy were very important determinants of outcome in BCC. Examining the surgically treated tumours, it can be seen that complete excision was achieved in the treatment in 79.5% of primary tumours and only 17.5% of recurrent tumours. Importantly, no early presenting horrifying tumours initially had complete excision. The high proportion of complete excisions in non recurrent compared to recurrent tumours is highly significant (Chi square $p < 0.001$). Only 2.3 % of the primary tumours had incomplete excision compared to 31.1% of the recurrent tumours and 25% of the early presenting horrifying tumours. These differences were highly significant (Chi square $p > 0.0001$ and $p = 0.0003$ respectively).

Interestingly less of the primary tumours received radiotherapy compared to the recurrent tumours (14.8% versus 32.6%); this difference reached statistical significance (Chi square $p = 0.05$). 62.5% of the early presenting horrifying tumours had radiotherapy. This was significantly higher than both the primary or recurrent tumours (Chi square $p > 0.0001$ and $p = 0.02$).

The late presenting horrifying patients had primary surgery most commonly (16 patients) except for 2 patients who refused surgery and underwent radiotherapy. 2

patients had elective radiotherapy. Of the late presenting, horrifying tumours, 2 had 1 recurrence, 1 had 2 recurrences and 1 had 3 recurrences. 3 patients developed metastatic disease and died. 1 patient died of local recurrence and there was one intra operative death. 6 patients are alive with microscopic or macroscopic disease and 11 patients have local control of their tumour (follow up 1-16 years, mean 8 years).

4.4.3 Treatment failure and histological growth pattern

4.4.3.1 Histology and incomplete excision

Table 4.9 summarises the incomplete excision rates for each histological subtype. The incomplete excision rate for the infiltrative and morpheic and micronodular tumours is higher than that reported by Sexton (Sexton, Jones et al. 1990) who reported incomplete excision rates for these tumours at 26.5%, 33.3% and 18.6%. This is because the series is biased by recurrent and horrifying tumours in which incomplete excision was more common. Irrespective of this, there was also a high proportion of incompletely excised nodular tumours. This finding reflects poor initially surgical management resulting in a high proportion of recurrent nodular tumours. It is an important finding that the incompletely excised nodular tumours were larger than the completely excised groups.

Growth pattern.	Complete exn		Incomplete exn		Incomplete exn rate
	%	median size mm	%	median size mm	
Mor	54.5% (6)	12	18.2% (2)	12	33.3%
Inf	51.2% (21)	10	17% (7)	11	33.3%
Mn	40.0% (6)	12	20.0% (3)	10	50%
Nod	54.1% (40)	6	16.2% (12)	10	33.3%
Sup	68.2% (15)	10	9.1% (2)	13	13%

Table 4.9. Incomplete excision rates for the histological subgroups of BCC. The numbers represent the percentages of each growth pattern treated by a particular treatment modality treatment group. The percentages do not add up to 100% because some treatment modalities are excluded.

4.4.3.2 Recurrence following complete excision

The likely hood of recurrence following complete excision is demonstrated in Table 4.10. This shows that the morpheic and infiltrative subtype recur more commonly following complete excision than the nodular variety. This was statistically significant for the morpheic tumours. Due to the relatively small numbers this did not reach significance for the infiltrative tumours.

Growth pattern.	Primary	Recurrent	P value (Fisher's exact) compared to nodular
Mor (5)	40%	60%	0.01
Inf (19)	79%	21%	0.1
Mn (4)	100%	0%	1
Nod (39)	92%	8%	-
Sup (16)	94%	6%	1

Table 4.10 Recurrence following complete excision. The Percentage of each growth pattern that recur following complete excision is shown. The actual number of tumours is shown in the left hand column.

4.4.3.3 Recurrence following radiotherapy

Table 4.11 shows the proportion of each growth pattern that became recurrent or horrifying following radiotherapy. The numbers were small and therefore statistical comparisons were not appropriate. However, the data demonstrates that a large proportion of the infiltrative tumours become recurrent or horrifying following radiotherapy. The data also demonstrates that a proportion of nodular tumours recurred or became horrifying following radiotherapy. The differences are not due to size differences in the subtypes treated by the different treatment modalities. (Table 4.12).

Growth pattern.	Primary	Recurrent	Horrific early presenting
Mor (2)	100%	0%	0%
Inf (10)	10%	50%	40%
Mn (5)	20%	20%	60%
Nod (16)	38%	25%	37%
Sup (3)	100%	0%	0%

Table 4.11 The recurrence of histological subtypes following radiotherapy.

Growth pattern.	Complete exn	Incomplete exn	Dxt
Mor	12	12	10
Inf	10	11	10
Mn	12	10	15
Nod	6	10	8
Sup	10	13	6

Table 4.12. The median size (mm) of tumours treated by a particular treatment modality.

4.4.3.4 Outcome in relation to histological subtype and treatment modality

Figure 4.5 it can be seen that the percentages of each histological subtype were similar for each treatment group and of similar size (see Table 4.11) suggesting that the differences in outcome seen between treatment modalities does not represent tumour selection. Figure 4.6 shows that recurrent tumours tended to arise because of incompletely excised nodular tumours (9 patients), although 3 patients did develop a

recurrence despite complete excision. There were 7 infiltrative or morpheic recurrences despite complete excision. This represents sampling error due to the difficulty in sampling all of the edge of tumours with conventional histological methods. 13 of the recurrent tumours arose following radiotherapy. These recurrences were of infiltrative, nodular or micronodular tumours. The horrifying tumours developed following primary radiotherapy of nodular or infiltrative tumours or following incomplete excision of infiltrative or morpheic tumours. The tumours with incomplete excision were treated subsequently with radiotherapy. Why some patients who developed a recurrence following radiotherapy are cured whilst others then progress to develop horrifying tumours is unknown. It is conceivable that this is due individual biological differences between tumours and to alterations in local defences allowing deeper infiltration. In an attempt to identify tumours more prone to incomplete excision or to recurrence following complete excision or radiotherapy some of the biological characteristics of the tumours were examined in the subsequent Chapters.

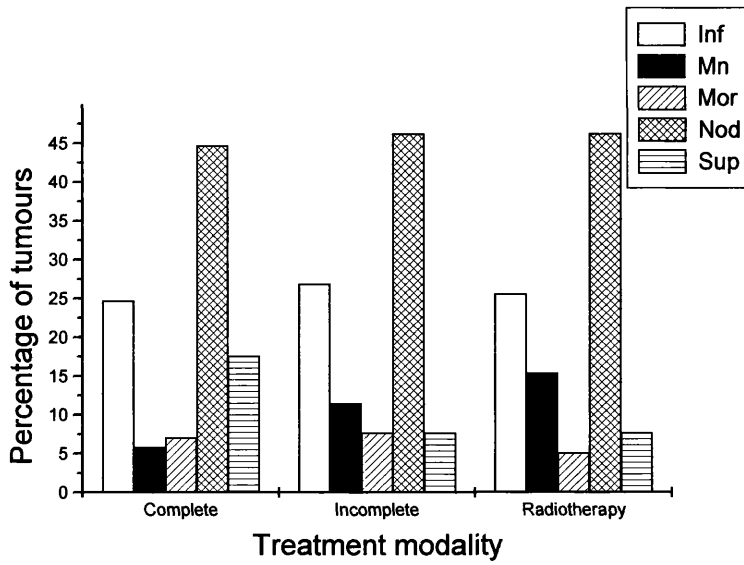


Figure 4.5. The proportion of each growth pattern treated by each treatment modality.

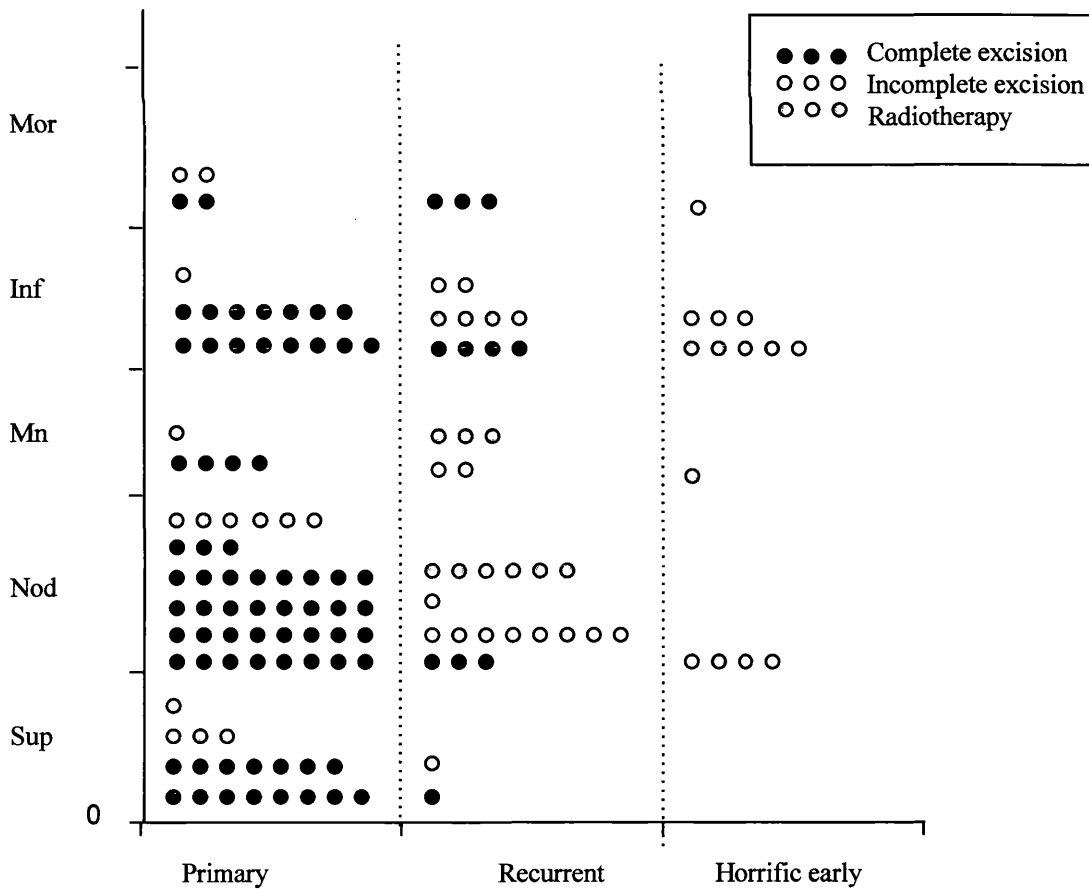


Figure 4.6. The outcome of each tumour subtype treated by surgery or radiotherapy.

4.5 Discussion

This chapter has analysed some of the patient, tumour and treatment factors that may determine the development of a recurrent or horrifying tumour phenotype. These factors have been compared with nonrecurrent tumours. In this study, the primary tumours were selected from a consecutive series of tumours at Mount Vernon Hospital. These tumours may therefore be a different population to those usually referred to a dermatologist for treatment and those reported in other series. The median age of 70 and equal sex distribution matched other series. (Kopf 1979) The tumour size at presentation and anatomical position also reflected other published series. 75% of the primary tumours were 10.2 mm or less and the majority were on the middle or upper face with the lower face being the least common facial position. 27 % were not on the face which is slightly higher than other series. 10% of the BCCs were on the lower limb. This may reflect referral patterns since these patients may require a skin graft since the lower limb lacks excess skin, thus hindering direct closure of wounds. Alternatively this may reflect increasing numbers of non facial BCC due to habitual sun exposure changes. Histologically, the primary tumours in this group contained more infiltrative tumours than other series with 18% being infiltrative and 5% being morpheic. Other series report 7%-8% infiltrative and 2%-9% morpheic tumours (Sexton, Jones et al. 1990). This may be due, in part, to the greater number of lower limb tumours of which 40% were infiltrative. In agreement with other studies, approximately 20% of lesions were superficial which is the commonest tumour of the trunk. As these tumours were referred to Mount Vernon Hospital for primary treatment. They were managed by surgical excision or radiotherapy rather than other modalities favoured by dermatologists.

The recurrent tumour phenotype has been inconsistently related to patient age and sex. Increasing age was associated with recurrence in patients treated with curettage and cautery but not radiotherapy or surgery in one study. (Kopf 1979) Another study found recurrence higher in young females while other studies have found a greater incidence in young men (Rigel, Robins et al. 1981). In this study there were slightly more

recurrences in male patients compared to the sex distribution in the series of primary tumours. No age differences were established between non recurrent or recurrent tumours.

In this study, the original tumour of the recurrent group were recorded as being significantly larger than non recurrent tumours (median 10mm vs. 8 mm $p=0.01$). It was conceivable that this may be due to the larger tumours being inadequately excised because their subclinical extension was greater than smaller tumours and therefore resulting in a greater recurrence rate (Burg, Hirsch et al. 1975; Salasche and Amonette 1981; Breuninger, Rassner et al. 1984; Wolf and Zitelli 1987). The conventional 2 mm excision may not have been large enough for these tumours or these tumours may have had the excision margin compromised for cosmetic reasons. This concept was supported by the finding that size was related to incomplete excision in the nodular tumours, although this was not the case in all of the other tumour subtypes. Size was also related to recurrence following radiotherapy. Larger tumours became recurrent or horrifying following radiotherapy compared to the non recurrent group. The size difference demonstrated between different clinical groups may have also resulted from larger tumours recurring post-complete excision. This recurrence is most probably due to the inadequate sampling of BCCs by the histological methods used. The majority of BCCs grow asymmetrically and therefore midline sections do not accurately determine the true excision margin (Breuninger and Dietz 1991). This was also supported by the finding that the infiltrative and morpheic tumour subtype recurred more commonly following complete excision. The consequence of this is that a protrusion of tumour at the edge of the specimen being missed than in the nodular subtype. The possibility that the larger tumours may be those of a more aggressive phenotype (infiltrative or morpheic) was not supported by this data since the median size of all of the histological subtypes was the same. However in the primary group the infiltrative tumours tended to be slightly larger than the nodular tumours.

As a caveat, it cannot be ruled out that the significant size difference may be spurious due the inaccuracies of the data source. It is not common for the tumour size to be measured accurately, an estimation is usually recorded. The histology report provided an alternative source of tumour size estimation although again the tumour margins

pose a difficulties for the histologist on a post excisional specimen due to avasularity and colour loss. This questions the accuracy of this data source. However these sources of error would be present in both the primary and recurrent group.

The finding that the greatest proportion of recurrent tumours were in the mid and upper facial region (38.6% and 38.6%) is in agreement with other studies (Lang and Maize 1986; Silverman, Kopf et al. 1992; Dixon, Lee et al. 1993) and suggests that either the tumours in this position are more resistant to treatment or that these tend to be inadequately treated in favour of cosmesis. 57% of tumours in the mid or lower face are of nodular subtype with 41% being infiltrative or morpheic. This suggests that a higher proportion of aggressive tumour subtypes may be found in this position. It is probable that these subtypes are commonly incompletely treated since they have a larger subclinical extension than the nodular type. However higher proportions of infiltrative subtypes were found in other parts of the body where recurrence rates were low. It is most likely that conservative excision in favour of cosmesis is a more prominent feature for facial rather than other BCCs. This is difficult to substantiate in the absence of the information regarding the exact excision margin. This may explain why there are relatively high numbers of incompletely excised nodular tumours in the recurrent group. It is a conservative excision rather than an aggressive tumour that results in the recurrence of the nodular tumours. However, overall, it is most likely that the cause of the higher incidence of recurrence or of horrifying tumours in this region is the combination of a higher number of infiltrative subtypes combined with conservative excision.

This study has demonstrated the importance of medical factors in determining the outcome of BCC, although the data has to be interpreted with care due to the bias from the selection methods. Incomplete excision was significantly related to recurrence or the development of a horrifying tumour. Approximately one third of the recurrent tumours and two thirds of the horrifying tumours had initial incomplete excision. This study demonstrated that there were high rates of incomplete excision of all types of BCC, unlike other published series in which the infiltrative and morpheic subtypes have the highest incomplete excision rates (Sexton, Jones et al. 1990). This reflects the referral patterns of recurrent BCCs to Mount Vernon hospital. Many of

these tumours were initially treated by non surgical specialities. Since a high proportion of the BCCs were on the face, excision margins were most likely compromised in favour of cosmesis. Hence, there were high incomplete excision rates, even of the nodular tumours. Also a high proportion of the recurrent and horrifying tumours had primary radiotherapy (32.6% and 62.5%). However, it is difficult to argue that complete excision is the sole treatment of choice because a relatively high proportion of completely excised BCCs recurred (23.9%). Although none of these became horrifying.

Incomplete excision cannot be attributed as the sole cause of all of the recurrences since 8 of these had complete excision. This may represent the development of new primary tumours, although it is unlikely that these would appear in exactly the same position. It would be most likely that this represents a false complete excision report. The failure of present methods of histological assessment has been reported previously since analysis of vertical sections rather than horizontal sections (as in Mohs technique) samples only a small proportion of the tumour margins. Furthermore, it has been demonstrated that 70 % of tumours grow asymmetrically and therefore midline or cruciate sampling of the tumour during histological assessment may miss a incompletely excised tumour margin (Breuninger, Schippert et al. 1989). The suggestion that these tumours were in fact incompletely excised is supported by the finding that 6 of the 8 recurrent tumours that recurred, despite complete excision, were of infiltrative or morpheic subtype. A small spicule of tumour extension can easily be missed due to inadequate sampling (Lang and Maize 1986). One recurrent lesion had a close surgical margin recorded. In reality, this infiltrative tumour was most probably incompletely excised and the close margin represented sampling error. This is seen in table 4.8 that analyses the initial treatment modality for each tumour subtype. A high proportion of infiltrative and morpheic tumours that recurred had previously been reported to have complete excision.

A significant finding was the high proportion of the tumours that recurred following primary radiotherapy (15/46). This may reflect referral patterns at Mount Vernon since the radiotherapists may favour referral of the recurrent tumours to the Plastic Surgeons. Recurrence rates for radiotherapy have been recorded between 4.1% (Knox,

Freeman et al. 1967) to 31% (Nordman and Nordman 1978) Size but not age, sex, site, duration or time span treated have been found to be independent prognostic makers for recurrence with 1 author reporting a 4.4% recurrence for tumours less than 1cm rising to 9.5% for larger tumours (Silverman, Kopf et al. 1992). The mean size for the radiotherapy recurrences was 13.3 mm (range 5-30mm) This may be, in part responsible for the treatment failures. There is little literature relating the tumour subtype to radiotherapy curability. In this series 4 of the tumours were infiltrative, 3 micronodular and 6 were nodular. The relatively high proportion of infiltrative tumours may suggest that these are less clinically responsive to radiotherapy than other subtypes. This is confirmed in Table 4.10. Of the 10 infiltrative tumours treated by radiotherapy, only 1 was cured, 5 recurred and 4 became horrifying. It is also possible that the nodular tumours may have also had a infiltrative component since the pre radiotherapy biopsy is often very small and therefore the whole tumour is not sampled. A distinct nodulo- infiltrative subtype of BCC has been proposed by (Sloane 1977). It is probable that BCCs with this intermediate subgroup exist since on examining successive recurrence biopsies some tumours changed from a nodular subtype to an infiltrative subtype or visa-versa with progression. This probably represents sampling error since this was a less common event in the horrifying tumours in which multiple sections were often available for examination.

The development of a horrifying or aggressive phenotype is an unusual event for BCC. Many terms have been developed such as aggressive, ,(Vico, Fourez et al. 1995); (Jacobs, Rippey et al. 1982) mutilating (McGurk and Edwards 1984); (Dvoretzky, Fisher et al. 1978; Schwartz, Vickerman et al. 1979), giant(Bianchini and Wolter 1987) (Schwartz, De Jager et al. 1986) or horrifying tumours (Jackson and Adams 1973). At present it is unknown why some tumours behave in this fashion while others follow an apparently benign course. Many factors have been considered important in the evolution of a horrifying tumour: Firstly, patient factors such as age (Leffell, Headington et al. 1991), race (Itayemi, Abioye et al. 1979) time of presentation (Randle, Roenigk et al. 1993), host immunity (Weimar, Ceilley et al. 1980); (Oram, Orengo et al. 1995) and the degree of exposure to environmental or other carcinogens (Spoor, Lindo et al. 1977). Secondly, tumour factors such as the intrinsic tumour aggressiveness determined by the biology of each individual. Thirdly

treatment factors such as modality(Randle, Roenigk et al. 1993) (radiotherapy or surgery), adequacy of treatment or the accuracy of the histological report in terms of the margins of tumour excision. No single or universal factor or combination of factors have been causally related to horrifying lesions. Some papers suggest they are histologically indistinguishable(Jackson and Adams 1973), others implicate reduced host immunity (Jackson and Adams 1973); (Weimar, Ceilley et al. 1980) or exposure to x-irradiation as important aetiological factors (Spoor, Lindo et al. 1977). The predominant histological difference associated with aggressive tumours has been the prevalence of an infiltrative growth pattern (Jacobs, Rippey et al. 1982) (Sloane 1977). Recent studies have demonstrated altered p53 and bcl-2 oncoprotein expression, stromal and nuclear differences and altered neovascularization of infiltrative or histologically aggressive tumours compared to non aggressive tumours (De Rosa, Staibano et al. 1992; De Rosa, Staibano et al. 1993; De Rosa, Barra et al. 1994). These studies, however, relate to morphologically aggressive tumours rather than to a clinical group of patients with horrifying or aggressive BCC. It remains equivocal whether these differences imply that horrifying tumours result from a biologically more aggressive subtype or the fact that certain types, especially infiltrative tumours, are more commonly incompletely excised and the changes result from tumour progression. The question remains as to whether all BCCs could potentially become horrifying if left unchecked or whether some tumours possess certain biological features that determine the development of this particularly aggressive tumour phenotype

In this study, in common with Jackson's findings (Jackson and Adams 1973), the age of onset of the tumours was younger for the horrifying subtype compared to both the horrifying or recurrent subgroup. This finding may reflect that these patients may be less resistant to the development and progression of BCCs. It has been demonstrated that young patients who develop BCCs develop aggressive tumours(Leffell, Headington et al. 1991) and that they have reduced DNA repair capacity than normal patients (Grossman and Wei 1995). However the mean age of onset was comparably old (61 years) when compared to these series where young included patients under 35 years of age. This finding may also not reflect a true difference since the age of presentation of the recurrent and primary tumours was recorded and the duration was

unknown. Some of the patients may have had the BCCs for several years which may diminish the age disparity between the groups.

The finding that the horrifying tumours were more common in men than women has also been established by other authors. (Jacobs, Rippey et al. 1982; Randle, Roenigk et al. 1993, Jackson, 1973 #16447). It is possible that this is related to the exposure of environmental carcinogens that may result in an aggressive tumour phenotype which is more common in males. It has been reported, however, that there is a preponderance of large BCCs of the scalp in women. (Binstock, Stegman et al. 1981).

Patient neglect was also a common feature in this series of patients (20/36) with a mean time of 9.1 years to seek medical help. In Randle's series of giant tumours approximately 40% had been present for greater than 5 years. 11 of these neglected tumours were infiltrative or morpheic histological growth pattern, but also 6 were of the nodular subtype. It is well recognised that infiltrative BCCs can invade deeply and cause tissue destruction but the nodular subtype are considered to be a less destructive type of BCC. It is unknown whether all nodular BCCs could become horrific if neglected or whether these represent a histologically identical subset of the nodular type that are intrinsically more aggressive. This may represent the nodulo-infiltrative subtype previously described that is difficult to recognise on a pre radiotherapy biopsy. This question of morphologically similar BCCs with differing biologies is studied in the following Chapters by the application of biological markers to the tumour subtypes. It is important, however that 16 of the 36 patients developed horrifying tumours despite early medical intervention. 68% of a series of giant BCCs had received prior treatment but there was no information regarding the size or type of the original tumours (Randle, Roenigk et al. 1993). The median size of the original tumours in this study was 13.5mm which was larger than the primary or recurrent group. This might have contributed to the failure of initial treatment. 3 patients had incomplete excision and 2 failed curettage. It is probable that the excision margins were compromised for cosmetic purposes since all of the early presenting group were on the face. 8 of the early presenting, horrifying tumours had radiotherapy as primary treatment; 1 had incomplete excision followed by radiotherapy. The role of radiotherapy in the development of horrifying BCC is uncertain. 50% of Jackson's

series and 16% of Randle's series had primary radiotherapy as initial treatment. It is probable that tumours which are predominantly infiltrative are less clinically responsive to radiotherapy than smaller, more superficial tumours. The combined data of the recurrent and early presenting horrifying tumours (Figure 4.6.) supports this proposal. 5 of 10 of the early presenting horrifying tumours that underwent primary radiotherapy were of an infiltrative growth pattern, 4 were nodular and 1 was micronodular. It is also possible that residual tumour may also be able to infiltrate more extensively due to the loss of normal local barriers such as impaired local immune systems. This makes further treatment by surgery more challenging since the tumours may infiltrate through periosteum or perichondrium thus resulting in multiple recurrences. This may account for the progression of the nodular subtype to become horrifying.

In conclusion, this Chapter has analysed the clinical and histological characteristics of the patient population investigated in this thesis. Primary, recurrent and horrifying tumours have been evaluated. These clinical types seem to be determined by firstly some patient factors such as age and sex. Secondly by some tumour factors such as type and size and thirdly, by some treatment factors such as modality and adequacy of treatment. The following chapters evaluate tumour biology more closely by studying a variety of tumour markers such that potentially problem tumours may then be identifiable at an early stage allowing for improved treatment and follow up planning.

Chapter 5

The clinical and histological significance of the growth fraction in basal cell carcinoma.

5.1 Introduction

From the descriptions in Chapter 1 it is clear that BCC is an extremely diverse tumour. Histologically, there is much variation with some tumours tending to rounded islands of growth (nodular type) or to lateral spread (superficial type) or for deep invasion (infiltrative subtype). Some tumours, such as the morpheic type, cause a dense fibrotic reaction while the stroma in the nodular type is less fibrotic. Some tumours tend to remain in large nodules while others break up into small clusters of cells (the micronodular type) or into cords of cells seen in the morpheic variety. In tandem with the variety of histological subtypes, a wide range of variation of proliferative measurements were found in Chapter 1. The first aim of this chapter is to establish whether cell proliferation may be partly responsible for the histological variations seen in BCC.

Clinically, there is also a variety of tumour behaviours. Some tumours tend to grow slowly and are relatively non-destructive and cured by simple procedures. Other tumours tend to recur, some of these may infiltrate deeply or widely requiring more complex treatments. Some tumours are particularly destructive and may be especially resistant to treatment and require elaborate surgery and reconstruction. As discussed in Chapter 1, there are many factors that determine the clinical outcome of BCC, such as patient age, tumour type and position and the treatment modality. However the relationship between these factors is not clear cut. It is also evident that traditional histological classifications do not provide adequate prognostic criteria to identify the potentially recurrent or aggressive tumour since some nodular tumours that are usually considered the least invasive form of BCC can progress to become horrific. Cellular

proliferation is a fundamental determinant of tumour growth behaviour and unchecked cell division facilitates the acquisition of further genetic modification which may include the propensity to infiltrate and metastasise (Tubiana and Courdi). It is apparent that the assessment of the proliferative status of tumours is an important prognostic indicator in patients with breast, ovarian, bladder, lung cancers and with lymphoproliferative disorders (Tubiana and Courdi 1989). The aim of this chapter was to correlate the clinical and histological characteristics of this tumour with an index of cell proliferation. Proliferation measurements may then help to predict potentially problem tumours at an early stage.

As discussed in Chapter 3, there are many parameters that can be evaluated as a measure of cell proliferation. In this chapter, we have used immunohistochemical detection of the Ki-67 antigen, using the MIB-1 monoclonal antibody, to assess the growth fraction in BCCs. This is simple to perform and can be used on archival paraffin sections without the requirement of the administration of a radioactive precursor or thymidine analogue. Furthermore, the growth fraction measurements had a linear relationship with the LI and also correlated with the Tpo t(immunohistochemistry) (Figures 3.14 and 3.15). Information of clinical relevance of ki-67 has been proposed in a number of situations particularly breast cancer (Veronese, Gambacorta et al. 1993), head and neck cancer, (Sahin, Ro et al. 1991) non Hodgkin's lymphoma (Miller, Grogan et al. 1994) and oesophageal carcinoma (Youssef, Matsuda et al. 1995). It is for these reasons that Ki-67 was chosen for the study of this patient group.

5.2 Materials and Methods

Primary, recurrent and horrifying BCCs defined according to the criteria in Chapter 2 were identified and the all of the available paraffin blocks were retrieved. The clinical data was collected from the patients notes. An attempt was made to collect all successive blocks from patients with recurrent tumours, although this was not always possible. The tumours were classified according to the histological growth pattern and

differentiation previously described. Immunohistochemical staining was performed on 5µm sections with the MIB-1 antibody following antigen retrieval (Section 2.7.1). The number of positive cells were counted in relation to the negative cells in the tumour islands. 10 high power field were counted per tumour on a grid on a T.V monitor attached to the microscope (x100) .

5.3 Results

5.3.1. Distribution of growth fraction in BCC

Figure 5.1 shows the distribution of ki-67 growth fractions in the first available specimen of each of the BCCs included in this study. The values ranged from 0.05-0.77 with a mean and median value of 0.32 and 0.31 respectively. The distribution of these values corresponded to a normal distribution. (Shapiro-Wilk $p>0.05$)

5.3.2. Relationship of the growth fraction with tumour size and position

Analysis all of the clinical groups revealed that there was no relationship between the tumour size and the growth fraction (Figure 5.2). However, when analysing the primary tumours only (Figure 5.3), there was a significant increase in the GF with increasing tumour size (anova $p=0.004$). The mean GF for the tumours sized 0-4mm was 0.20, while the mean growth fraction of tumours greater than 20mm was 0.39. These differences arise because the infiltrative and morpheic tumours were larger than the nodular tumours in the series of primary tumours (Chapter4). There were no differences between the growth fraction and the patient age (anova $p=0.7$), sex (t-test $p=0.4$), or tumour position (anova $p=0.2$).

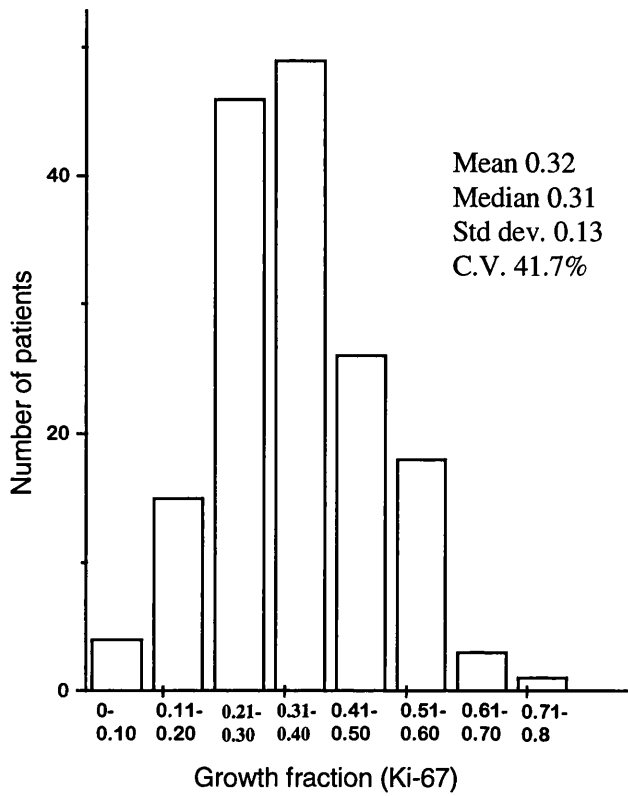


Figure 5.1. The distribution of growth fraction in the original biopsy of all of the BCCs.

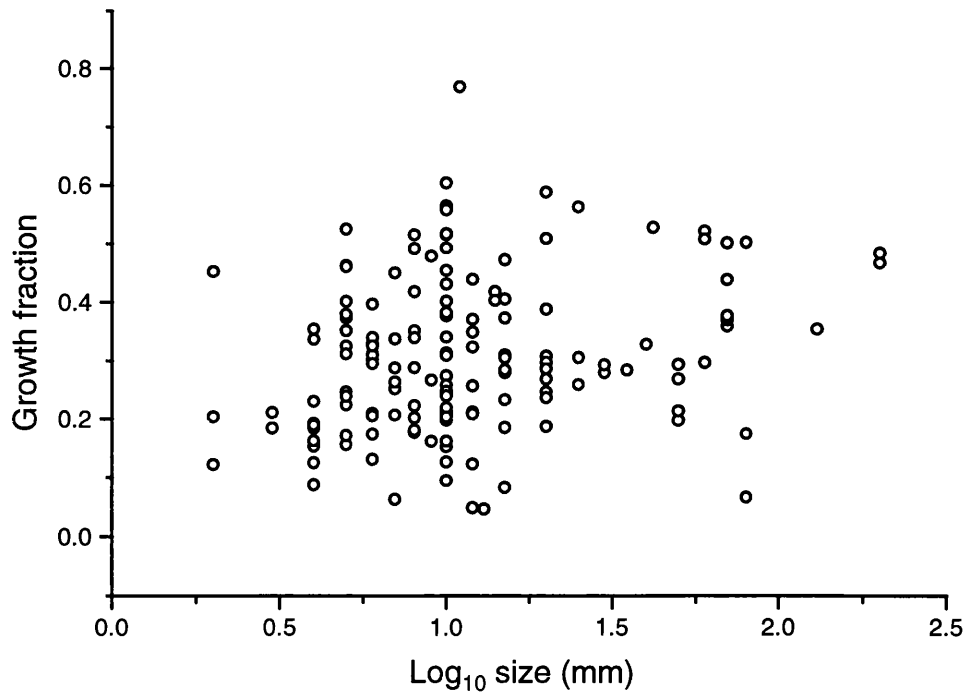


Figure 5.2. The relationship between tumour size and the growth fraction for all clinical tumour types is plotted on a log scale for clarity.

5.3.3. Relationship of the growth fraction with histological growth pattern

The level of proliferation found within each tumour growth pattern shown in Figure 5.4. This demonstrated significant differences between the groups ($p < 0.0001$). The overall ranking from low through to the highest growth fractions was nodular, micronodular, superficial, infiltrating and morpheic. The mean growth fractions for each group were 0.26, 0.29, 0.34, 0.39 and 0.40 respectively. The infiltrating and morpheic tumours had a significantly higher growth fraction than the micronodular ($p = 0.007$, $p = 0.04$) and nodular tumour groups ($p > 0.0001$, $p = 0.0002$) (Figure 5.5a-b). Superficial tumours were only significantly different from nodular tumours ($p = 0.005$).

5.3.4. Relationship of the growth fraction to differentiation

There was no difference in the proliferative characteristics of the tumours as a function of the type of differentiation present in the specimen. (Figure 5.6). The mean growth fraction for each differentiation subtype was: adenoid 0.27, cystic, 0.29, squamous metaplasia 0.33, undifferentiated 0.33.

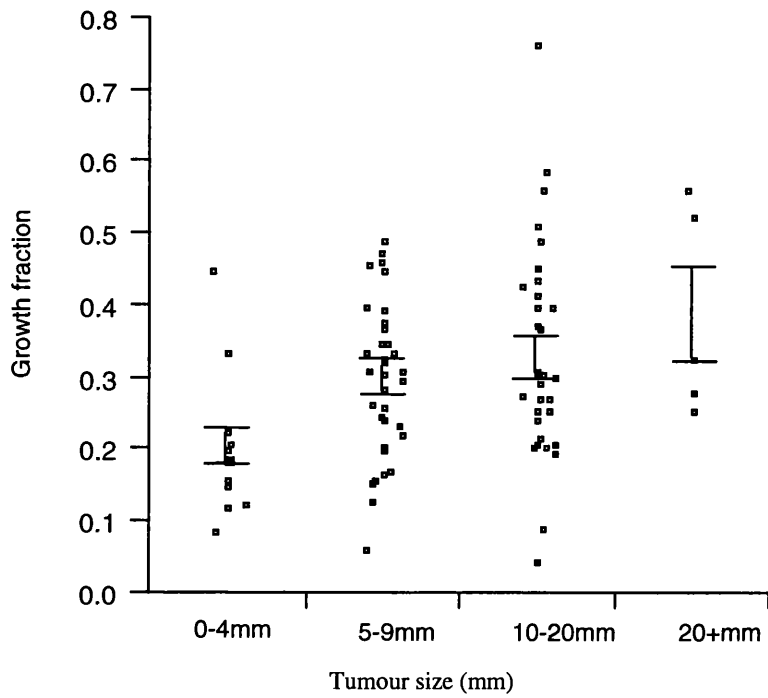


Figure 5.3. The relationship of tumour size with the growth fraction in primary tumours. Error bars represent SEMs

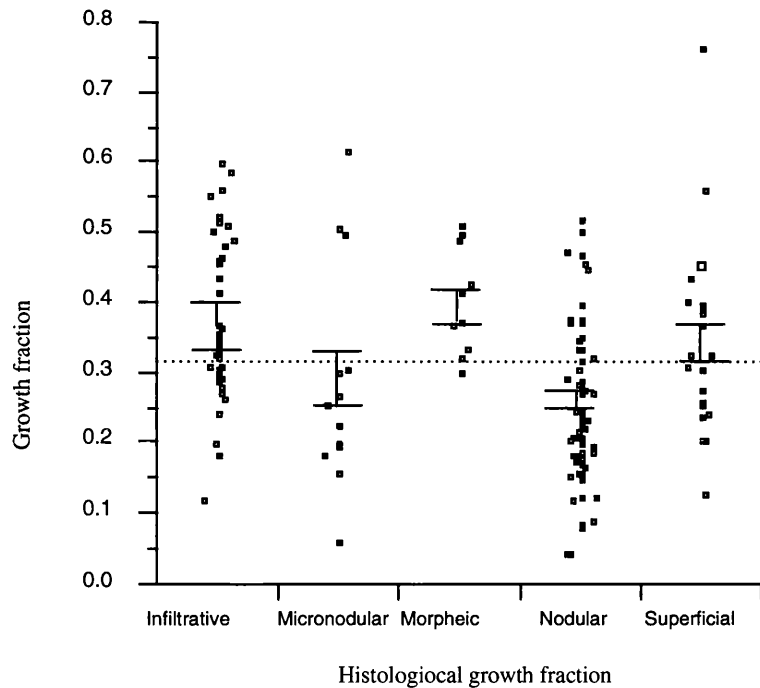


Figure 5.4. The growth fraction within each histological growth pattern. Error bars represent SEMs. The median value represented with a dotted line.

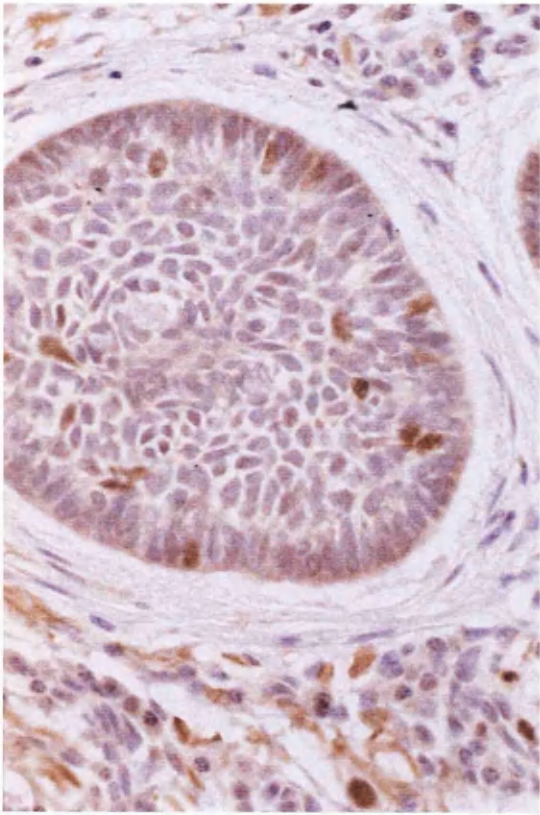


Figure 5.5a A nodular BCC with a low GF.

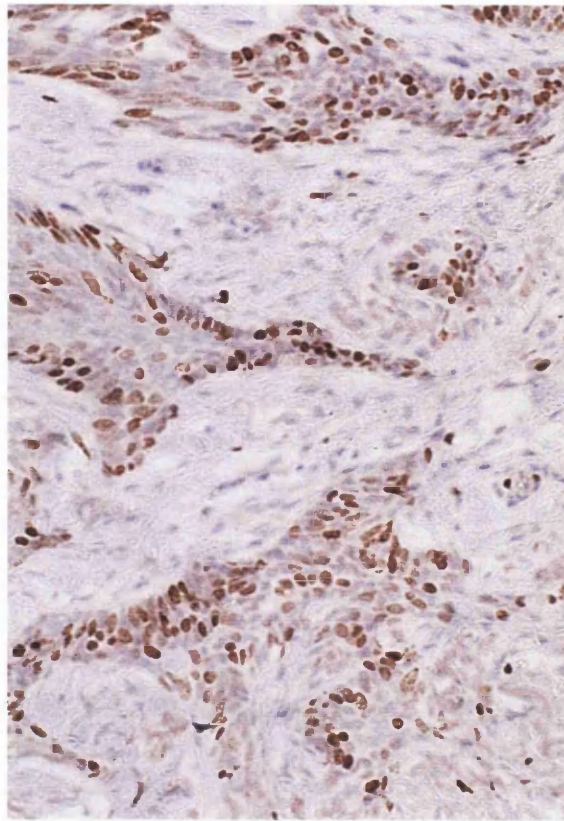


Figure 5.5b An infiltrative BCC with a high GF

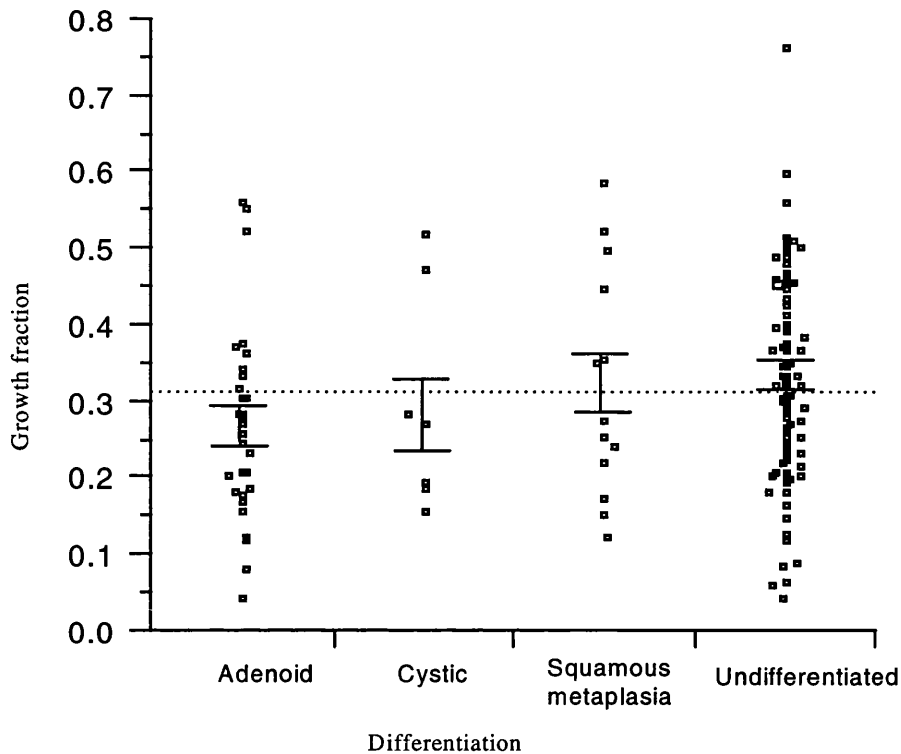


Figure 5.6. The growth fraction as a function of each differentiation subtype of BCC. Error bars represent standard error of the means. The dotted line represents the median value.

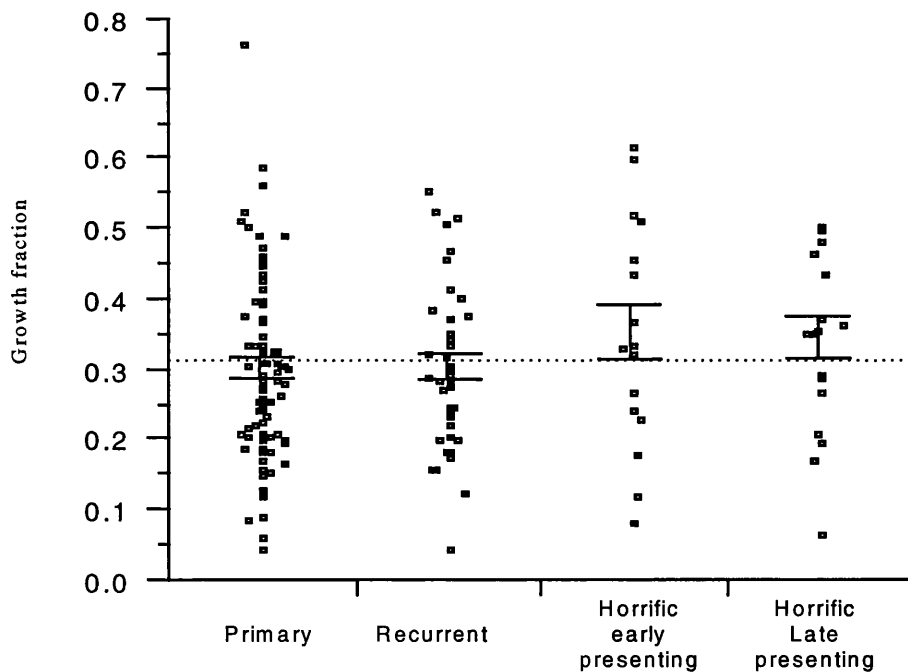


Figure 5.7. The growth fraction for each clinical subtype of BCC. Error bars represent standard error of the means. The dotted line represents the median value.

5.3.5. Relationship of the growth fraction to clinical behaviour

Figure 5.7 shows the relationship of the growth fraction with the first available biopsy of each clinical group of patients. Although the means for the primary and recurrent group (0.31 and 0.31) were lower than for the horrifying group (early presenting mean 0.36, late presenting 0.35) this did not reach statistical significance (anova $p=0.4$).

In order to determine whether the growth fractions of tumours of similar subtype were different for the recurrent and horrifying groups, these were compared in table 5.1. A nodular or infiltrative tumour that becomes recurrent or horrifying is not different in terms of the growth fraction to a non-recurrent tumour.

	PRIMARY	RECURRENT	HORRIFYING	ANOVA P VALUE
Nodular	0.26	0.27	0.27	0.8
Micronodular	0.21	0.29	0.37	0.3
Infiltrative	0.31	0.31	0.35	0.2
Morpheic	0.45	0.34	0.39	0.2
Superficial	0.34	0.35	-	0.9

Table 5.1. Comparison of the growth fraction of histologically similar groups for primary, recurrent and horrifying tumours.

5.3.6. The relationship of treatment failure with the growth fraction

In order to establish whether the growth fraction could predict recurrence, the growth fraction for each clinical group treated with a particular therapeutic modality was compared. The large number of treatment modalities resulted in a small number of observations in each group. 33 patients had radiotherapy and from Figure 5.8 it can be seen that the outcome following radiotherapy could not be predicted from the growth

fraction. The growth fraction was not different for the non recurrent or recurrent BCCs (including the early presenting horrifying group). (Anova $p=0.5$)

The growth fraction was also compared for all patients treated with excisional surgery. Firstly irrespective of completeness of excision and secondly for complete surgical excision only. For all surgical margins (Figure 5.9) there was no difference in the growth fraction of non recurrent or recurrent tumours (Anova $p=1$). For those tumours that were completely excised (Figure 5.10) the growth fraction was higher for the recurrent tumours (0.30 and 0.34 respectively but this did not reach statistical significance (Anova $p=0.4$).

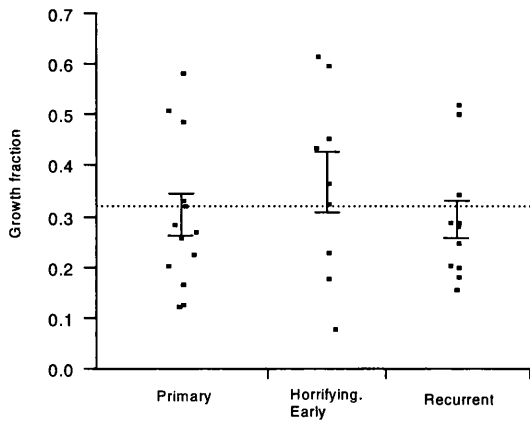


Figure 5.8. The growth fraction of patients treated with primary radiotherapy. The dotted line is the median and error bars the SEM.

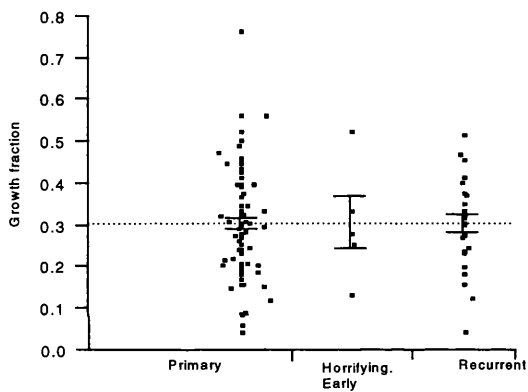


Figure 5.9. The growth fraction of tumours treated by surgery. (Both complete and incomplete excision). The dotted line is the median and error bars the SEM.

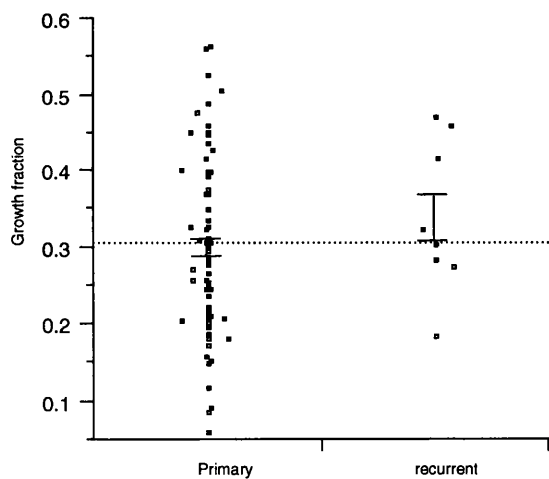


Figure 5.10. The relation ship of the growth fraction to the outcome of completely excised BCCs. The dotted line is the median and error bars the SEM.

5.3.7. The relationship of the growth fraction with tumour progression

The growth fractions of sequential specimens was measured for the recurrent and horrifying tumours. Sequential specimens were available for 36 recurrent tumours. The progression of the growth fraction of the recurrent tumours is illustrated in Figure 5.11 and 5.11. It can be seen that the initial growth fraction may increase or decrease irrespective of the initial value. Figure 5.11 shows the change in the growth fraction with recurrence expressed as a proportion of the original growth fraction for ease of comparison. Overall 21 tumours had increased growth fraction on recurrence and 15 had a reduced growth fraction. However, statistically there was no difference in the growth fraction between the first and second biopsies. The mean of biopsy 1 was 0.31 and for biopsy 2 was 0.32 (paired t test $p=0.5$). There was also no difference between the first and last available biopsies (mean growth fraction of the last biopsy 0.33, $p=0.3$).

The change in growth fraction in the horrifying group is shown in Figure 5.13 and 5.14. In 13 patients the growth fraction increased with tumour progression and in 5 patients the growth fraction fell. Overall there was no difference between the growth fraction of the first and last available biopsies (mean 0.31 and 0.34 respectively. Paired t test $p=0.5$).

An attempt was made to evaluate the change of growth fraction in relationship to the treatment type. This was only possible in the recurrent tumours. The horrifying tumours tended to have multiple treatments with surgery and radiotherapy combined. In the recurrent group 10 patients had radiotherapy and 26 had surgery as primary treatment. The fractional change of the growth fraction in comparison to the first available biopsy were no different for radiotherapy or for surgery (mean 0.28 and 0.34 respectively, $p=0.4$).

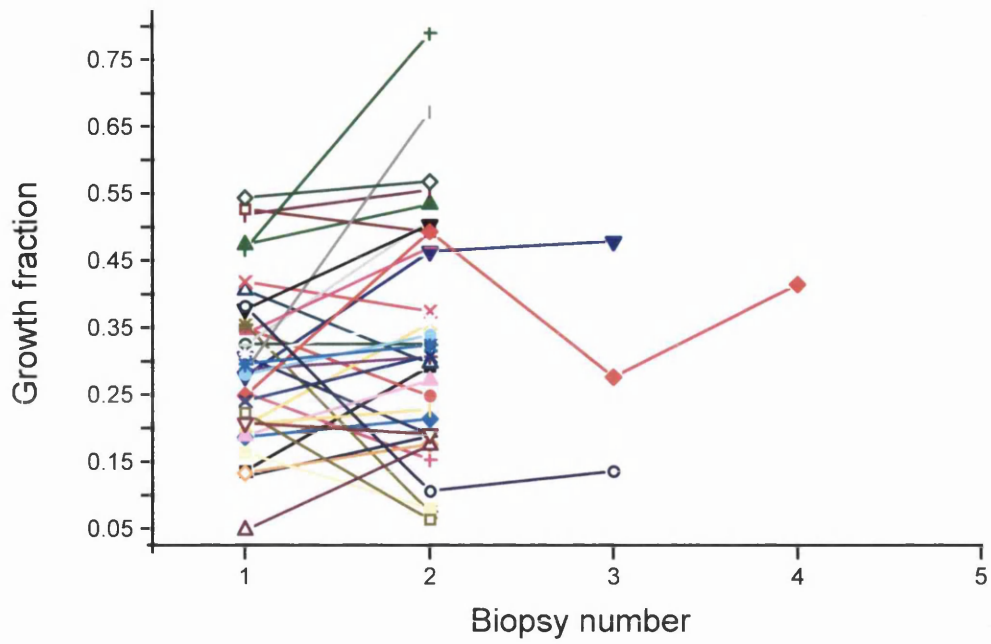


Figure 5.11. The growth fraction of successive biopsies of recurrent BCC.

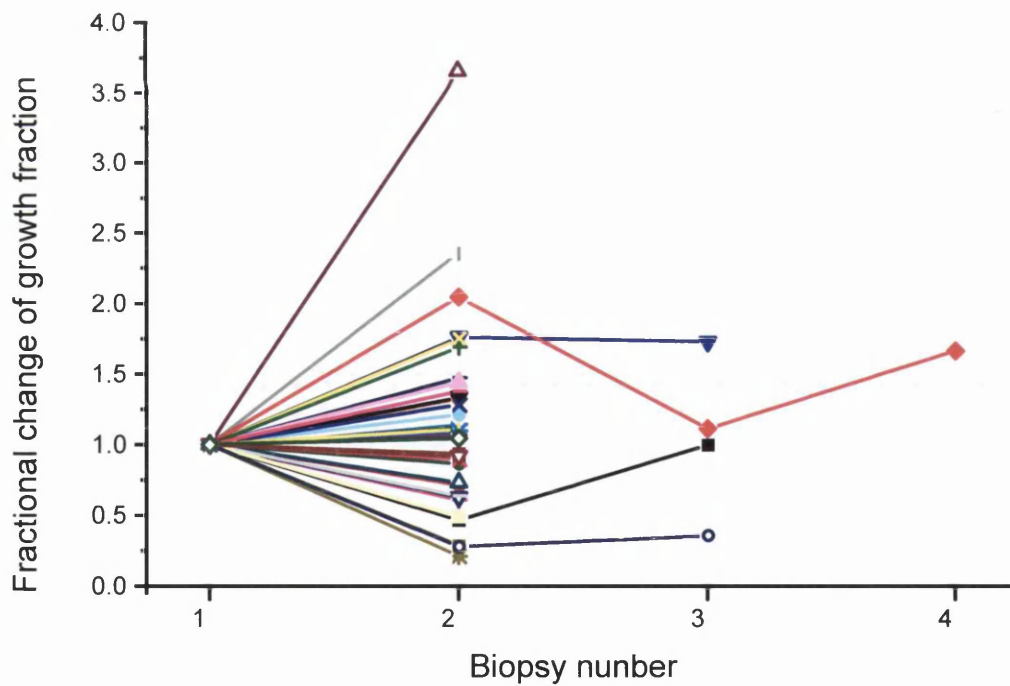


Figure 5.12. The growth fraction of successive biopsies of recurrent tumours expressed as a proportion of the original growth fraction.

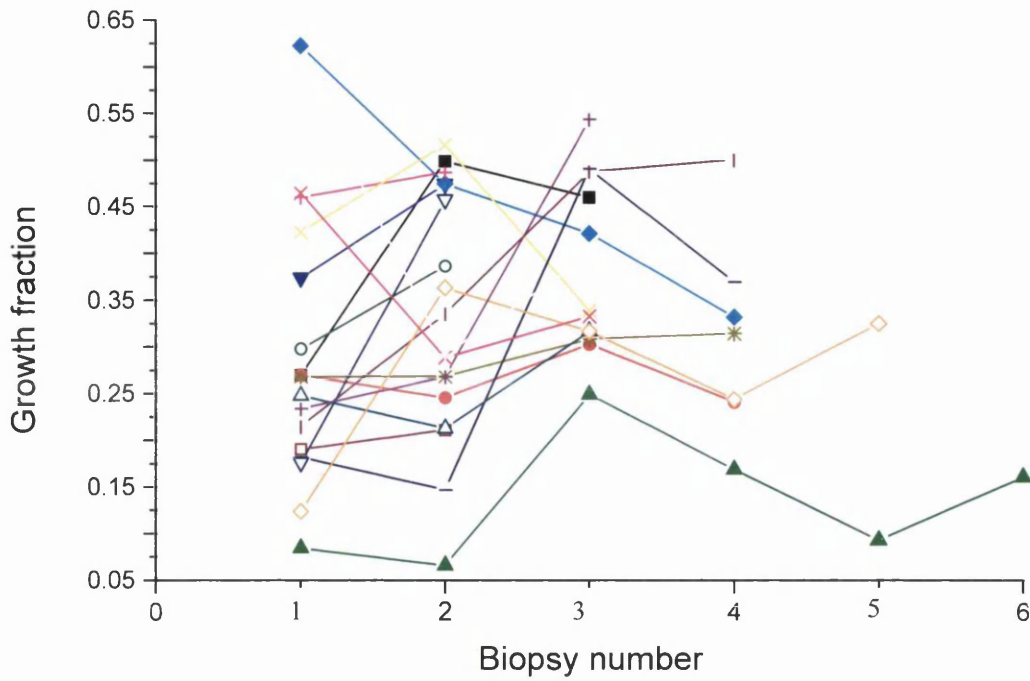


Figure 5.13. The change in growth fraction of horrifying BCC with successive biopsies.

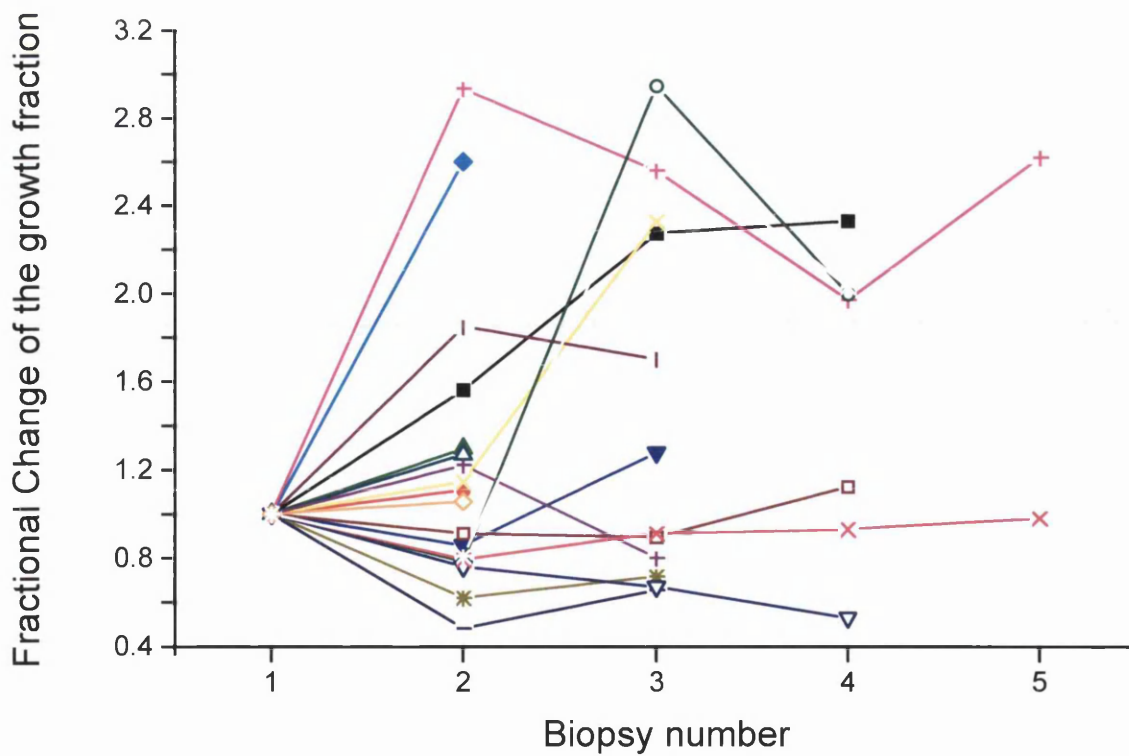


Figure 5.15. The growth fraction of successive biopsies of recurrent tumours expressed as a proportion of the original growth fraction.

5.3.8. Ki-67 proliferation patterns.

The spatial arrangement of proliferating cells was recorded according to the proliferation patterns described in Chapter 3. The frequency and size of each proliferation pattern is illustrated in Table 5.2. The random pattern was the commonest and accounted for 51.9% of tumours, while the marginal pattern was the least common. The mean size increased from the marginal tumours up to the random pattern; this was not statistically significant because of some very large random pattern tumours elevating the mean size. Therefore there was no significant difference in the median sizes. The number of tumours with a marginal pattern was small (4 tumours) and therefore the median of 5.5mm was not significantly different to the other patterns.

PROLIFERATION PATTERN	PERCENTAGE	MEAN SIZE (MM)	MEDIAN SIZE (MM)
Marginal	2.5%	7.0	5.5
Marginal diffuse	31.0%	15.7	10
Mix	14.5%	18.0	10
Random	51.9%	22.6	10

Table 5.2. The frequency of the proliferation patterns and their relationship with tumour size.

The distribution of the proliferation patterns for each histological subtype is illustrated in Figure 5.15. Nodular tumours were of marginal diffuse or random pattern most commonly. Infiltrative and morpheic tumours were predominantly of random pattern. The marginal diffuse pattern was the commonest proliferation pattern in the micronodular tumours. Superficial tumours had equal distributions of mixed, random or marginal diffuse patterns.

The distribution of the proliferation patterns for each type of differentiation is illustrated in Figure 5.16. Adenoid tumours were most commonly of a random pattern.

Cystic tumours contained an equal admixture of proliferation patterns except the rarer marginal pattern. It appeared that the marginal pattern occurred in small nodules of undifferentiated tumour or those with cystic areas. However, this is not apparent in the results because only the predominant differentiation status is recorded. Most tumours with cystic areas consisted predominantly of other differentiation subtype.

There were differences between the proliferation patterns in different clinical groups but this could be attributed to the high proportion of infiltrative tumours seen in the horrific group. This resulted in a higher percentage of random pattern tumours seen in the horrifying group than either the primary or recurrent tumours (Figure 5.18).

The growth fraction varied with the proliferation pattern. (Figure 5.16) In ascending order the marginal (11.6%), marginal diffuse (29.5%), the random (32.5%) and the mixed (34.0%). The only group that reach statistical significance from the other patterns was the marginal group ($p=0.008$).

5.3.9. Proliferation pattern and treatment failure

The relationship of the proliferation pattern to the outcome after radiotherapy and surgery is illustrated in Figure 5.19. and Figure 5.20. The proliferation pattern was not a predictor of outcome for either treatment modality. (Radiotherapy chi square $p=0.4$, complete excision chi square $p=0.3$).

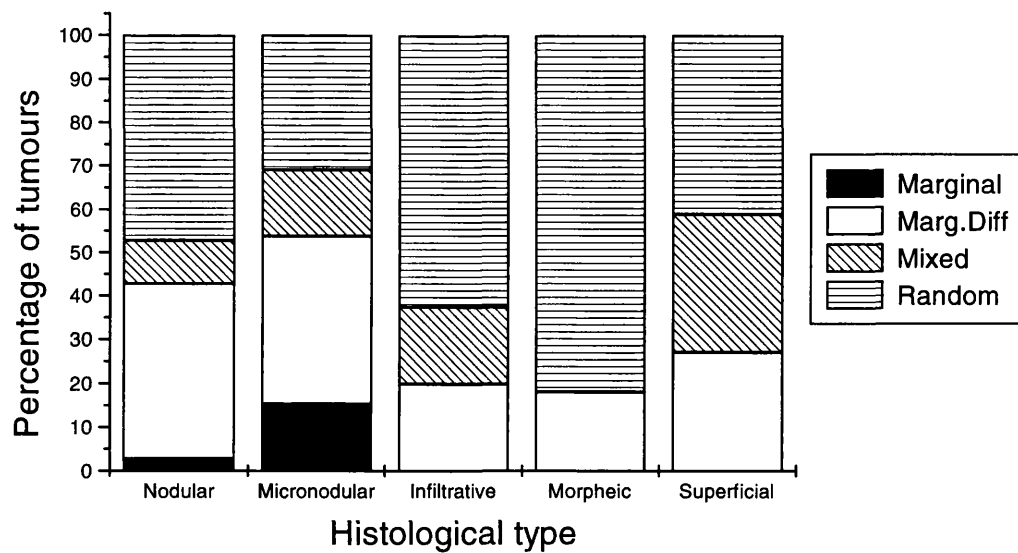


Figure 5.15. The proliferation patterns of the histological subtypes of BCC.

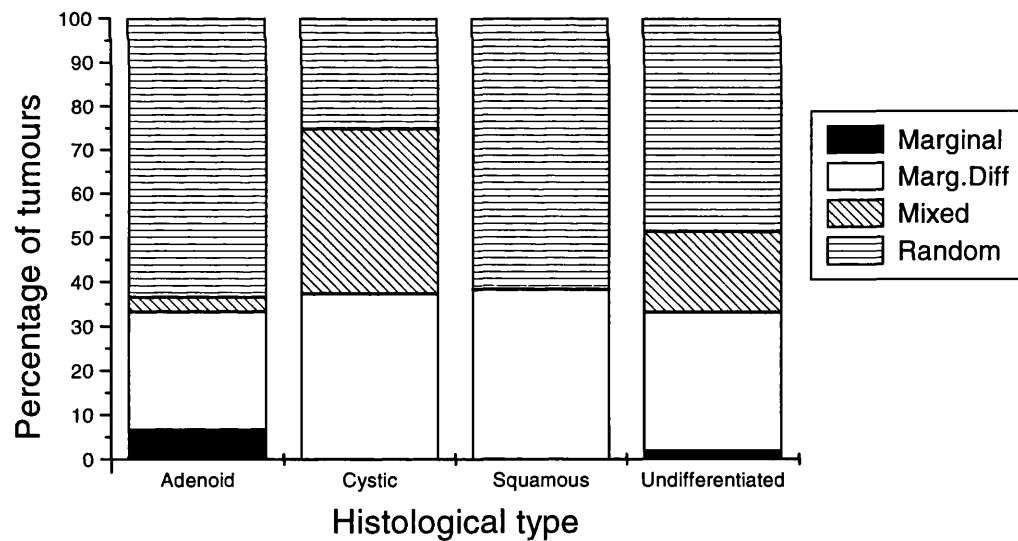


Figure 5.16. The proliferation patterns for the differentiation subtypes of BCC.

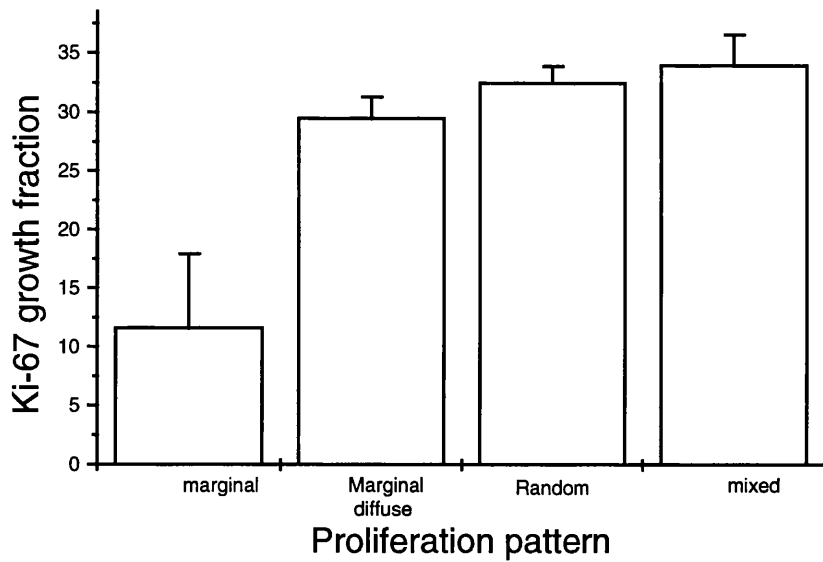


Figure 5.17. The mean growth fraction for each proliferation pattern.

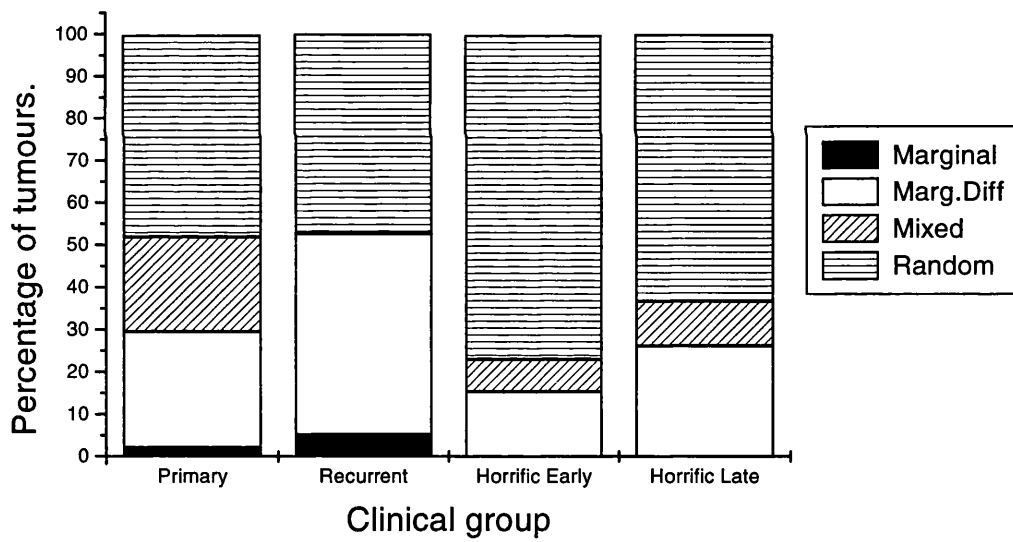


Figure 5.18. The proliferation patterns for each clinical subtype of BCC.

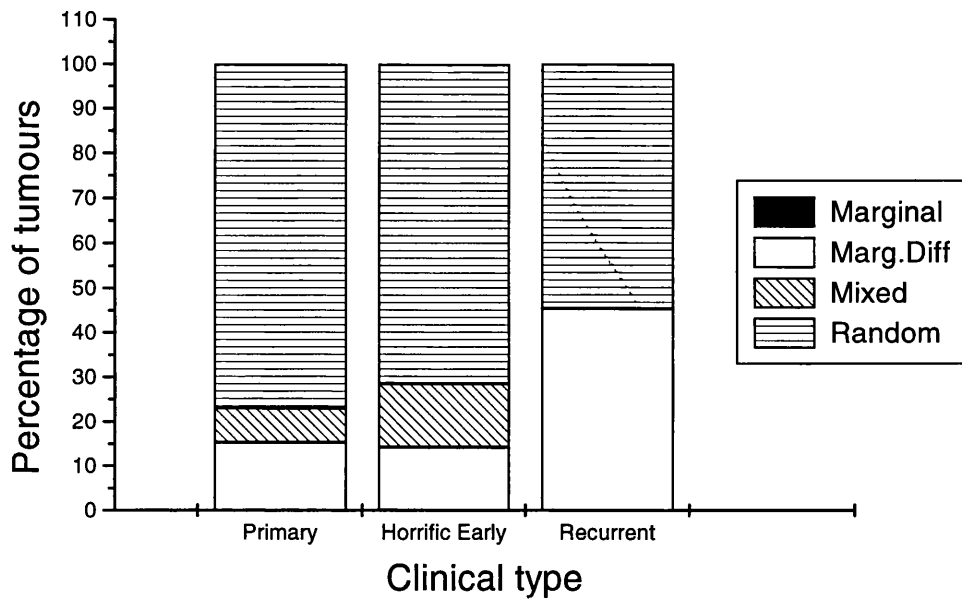


Figure 5.19. The effect of the proliferation pattern on the outcome for radiotherapy.

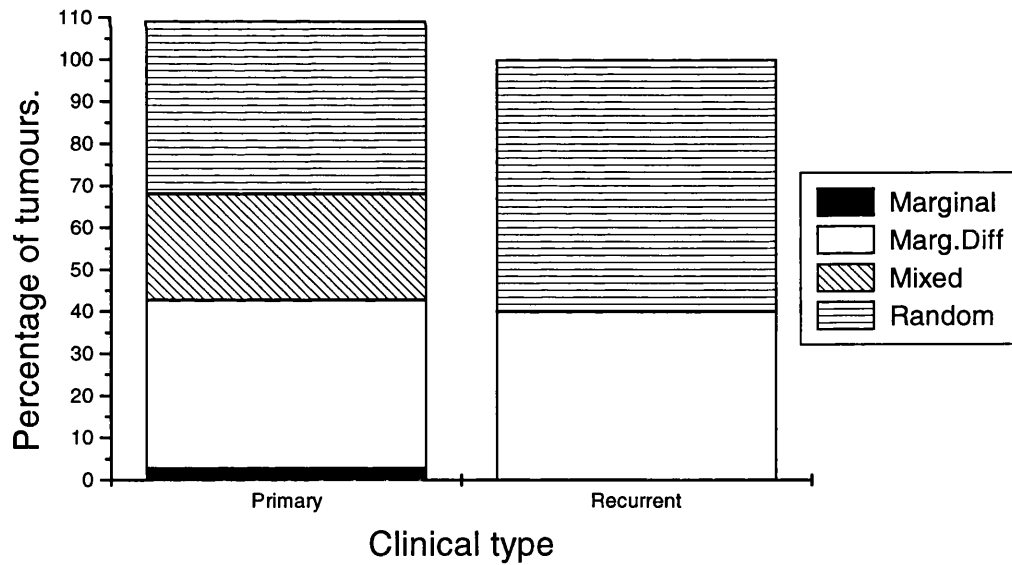


Figure 5.20. The Influence of the proliferation pattern with outcome for complete excision.

5.3.10. Heterogeneity of proliferation

Ten high power fields were counted at random in each tissue section and the average number of cells assessed per specimen was 1801. Within each specimen there was considerable variability in Ki-67 positivity, the overall coefficient of variation (C.V.) was 36.4% (range 5.4%- 95.8%). However, there were no significant differences in heterogeneity of proliferation amongst the different growth patterns ..(Anova $p=0.3$). The C.Vs, in ascending order, were 32.6% (morpheic), 34.1% (infiltrating), 36.3% (superficial), 39.8% (nodular) and 42.9% (micronodular). The C.V did not vary with the predominant differentiation status either (anova $p=0.7$) The C.V for each differentiation subtype was adenoid 36.7%, cystic 37.5%, squamous metaplasia 42.4%, and undifferentiated 37.3%. There was also no difference in heterogeneity between each clinical group (See Figure 5.21.).

The interesting aspect of heterogeneity was the inverse relationship with Ki-67 growth fraction, the more proliferative tumours exhibited less variation. (See Figure 5.22.). Tumours with a low growth fraction had the greatest C.V, while tumours with a high growth fraction had a lower C.V. This is in part explained by the pattern of proliferation. The tumours with a marginal proliferation pattern (also with the lowest growth fraction) had the greatest C.V 64.0% when compared to the other patterns. ($p=0.004$) This was because these tumours exhibited marginal staining only with no central proliferation. However there were only a small number of tumours displaying this proliferation pattern. The C.V in the marginal diffuse pattern (38.2%) was higher than the mixed pattern (33.9%) and random pattern (37.4%) but this did not reach statistical significance. There was no difference in the C.V as a function of tumour size or position.

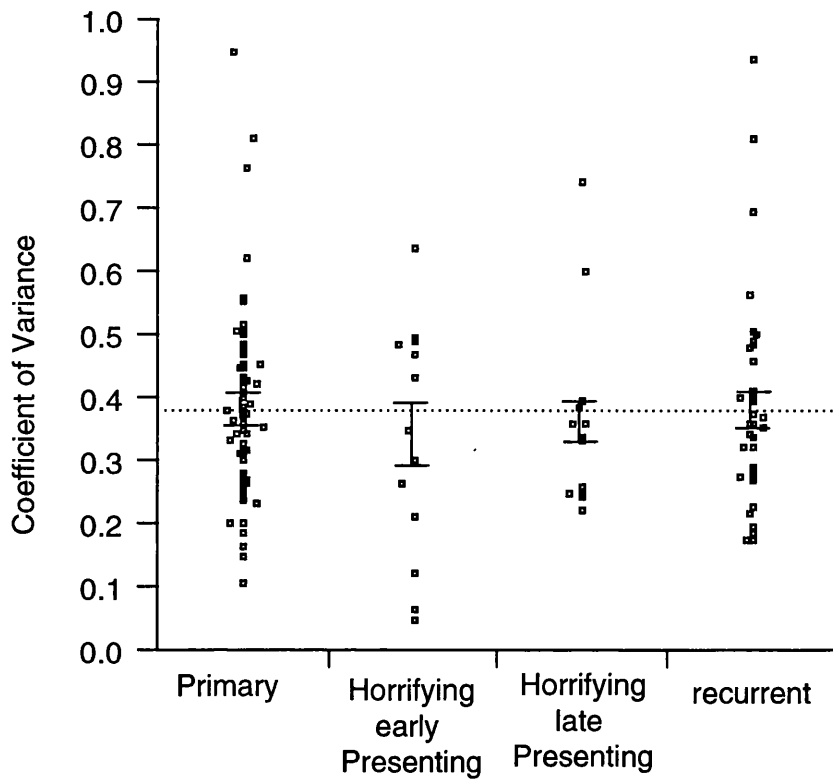


Figure 5.21. The coefficient of variance for each clinical subtype of BCC.

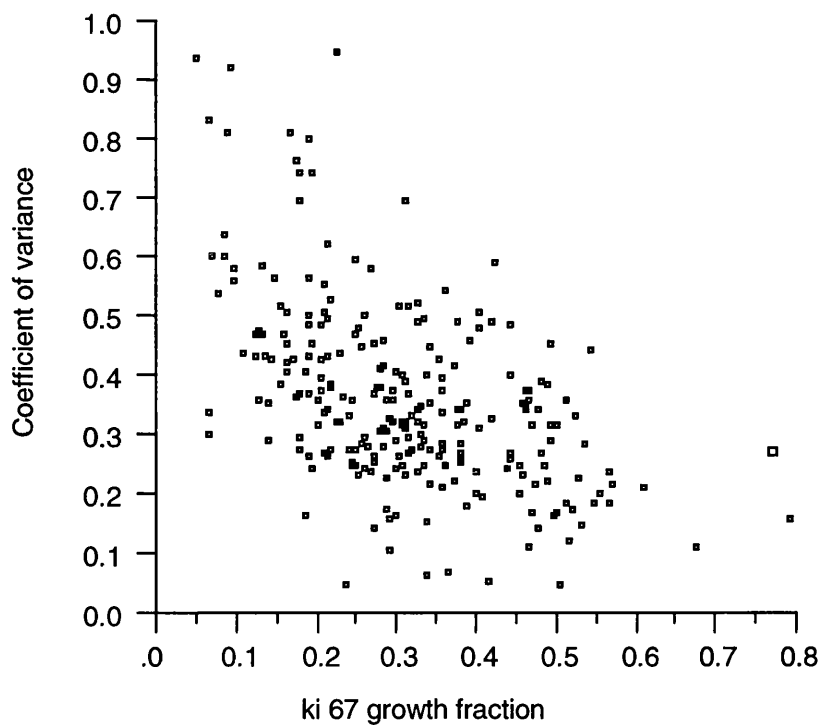


Figure 5.22. The inverse relationship of the coefficient of Variance with the Ki-67 growth fraction

5.4 Discussion

This study confirms the findings of Chapter 3 that BCC is a highly proliferative tumour. It demonstrates that the Ki-67 growth fraction displays a wide variation (range 0.05-0.77, C.V 0.42) in different types of BCC and that, in general, the overall value could be considered high for a slowly growing tumour (Mean 0.32). Only one other study has formerly investigated Ki-67 expression in a variety of primary BCC's and their findings are in agreement with the observations made in this study. (Baum, Meurer et al. 1993) Indeed, in 62 BCC, the mean value of Ki-67 growth fraction was slightly higher than our own findings (0.33 versus 0.32). Consideration of both present and past evidence clearly demonstrate that BCC proliferates as rapidly as many other solid tumours. The Ki-67 growth fraction of 0.32 is greater than breast (0.201) (Barnard, Hall et al. 1987) melanoma (0.20), (Fogt, Vortmeyer et al. 1995) and gastric cancer (0.248), (Porschen, Kriegel et al. 1991) and of a similar magnitude to cervix (0.30), (Wong 1994) and head and neck (0.35), (Ogawa 1992) and oesophagus, colon and rectum (0.357, 0.376, 0.343) (Porschen, Kriegel et al. 1991).

In this study we found a significant difference in growth fraction between the nodular histological growth patterns and the infiltrating, morpheic and, to a lesser extent, superficial lesions. The morpheic and infiltrative subtypes having the highest growth fractions. The infiltrating and morpheic growth patterns are more prone to recurrence and these results may explain the findings of Healy et al (Healy, Angus et al. 1995), who found a higher growth fraction in BCC which recurred after complete excision, although the authors state that their tumour groups were matched for histological subtype. However, micronodular tumours, which may also be prone to incomplete excision and subsequent recurrence were characterised by a relatively low growth fraction suggesting that proliferation alone cannot account for aggressive behaviour. The finding of a significant difference between histological growth patterns in BCC is contrary to the findings of Baum and Healy (Baum, Meurer et al. 1993; Healy, Angus et al. 1995). This is partly due to the small numbers (Healy's series had only 1 morpheic tumour) and also partly due to the ambiguous classification systems that were utilised. Baum's series were divided into nodular, fibrosing and superficial while

Healy's group used the terms nodular, adenoid and morpheic. In this study we also classified the tumours according to the most predominant differentiation subtype using a Lever type classification. If this system was used, there was no difference in the growth fraction between each type. Not only is this classification difficult to use because most tumours display more than one differentiation subtype but it has little bearing on behaviour in that it does not have prognostic information and does not differentiate between biological differences such as cellular proliferation characteristics. A classification according to the growth pattern described is useable and carries prognostic and biological information.

This study also investigated whether the growth fraction may be a marker of recurrence for BCC. Healy et al found that the growth fraction of the original tumours of recurrent BCCs was higher than non recurrent tumours. In Healy's study only tumours that were reportedly completely excised were included. This present study included all recurrent tumours irrespective of the initial treatment modality since it has been reported that not all incompletely excised lesions recur. In this study there was no difference between the growth fraction of recurrent and non recurrent tumours which is in conflict with Healy's results. It is most probable that this is because the tumour population in this study represents a more heterogeneous group. Healy's study may have indirectly selected the infiltrative subtypes since they may appear completely excised on vertical section when, in fact, they may be incompletely excised. The growth fraction was therefore higher in the recurrent group compared to a non recurrent group. Consideration of the data in Chapter 4 would suggest that even though the infiltrative and morpheic subtypes represented a greater proportion of the recurrent than the primary group (31.1% vs 22.8%) this was not enough to result in significant differences in the growth fraction. The inclusion of inadequately treated nodular tumours (with a lower growth fraction) resulted in there being no difference between the primary and recurrent group.

The development of a horrifying BCC is a relatively rare event and many factors have been postulated to be associated with this development. These have been discussed in Chapter 4. There is no study at present investigating the cellular biology of these tumours. It was conceivable that the growth fraction may determine the behaviour of

these tumours indicating the existence of a particularly aggressive subset of BCC. However, the growth fraction, measured by Ki 67 immunohistochemistry, did not support this hypothesis. There was no significant difference between the growth fraction of the primary, recurrent or horrifying subgroups. There was however, a small increase in the overall growth fraction of the horrifying group because of the preponderance of infiltrative tumours in this subgroup but this did not reach statistical significance. Furthermore, when the growth fraction of each histological subgroup is compared, there is no evidence to suggest that infiltrative tumours which become horrific are biologically different to an primary or recurrent e tumours of this type. The same is true for all of the other subtypes. Late presentation and tumour neglect has been a common feature of horrifying lesions. In this series only half of the patients presented late. The other half presented early with non horrific tumours which recurred and became horrific as the tumour progressed. It would not be unreasonable to expect that the biology of the tumours of these 2 groups may be different. The early presenting group consisting of inherently aggressive tumours which were resistant to treatment, and the late presenting group consisting of less aggressive tumours. Histologically, there was a similar mix of histological growth patterns in each group with the infiltrating type equally divided and there was no difference in the growth fraction between the early and late presenting groups. Clearly this does not support these expectations of differing biologies.

The finding that infiltrative, and morpheic, in either horrifying or non horrifying groups have a higher growth fraction is highly significant and supports the view that these tumours are biologically different and may contribute to their more invasive behaviour which results in a higher incomplete excision rate and recurrence rate. Their volume doubling time may be faster and therefore they may be able to penetrate into deeper structures in a shorter time than other tumour subtypes. Their subclinical extension may therefore be greater resulting in a higher incomplete excision rate. This may result in greater tissue destruction. Hence a larger proportion of the horrifying tumour type were of an infiltrative growth pattern. An alternative explanation for the pathogenesis of horrifying tumours would be that gradual tumour progression, with successive recurrence or with time, may result in a more aggressive phenotype. However analysis of sequential specimens from patients with recurring tumours and

multiply recurrent horrifying tumours failed to demonstrate a change in growth fraction with tumour progression. This does not support a concept that recurrence and tumour progression results in a intrinsically more aggressive tumour. It is most probable that multiple recurrences results in progressive tumour growth into deeper planes such as bone or cartilage. As a consequence, these tumours are technically more challenging to completely eradicate. This is compounded by the majority of tumours being on the face which further limits the ease of complete excision.

In Chapter 4, it was also suggested that the infiltrative growth pattern may be relatively less clinically responsive to radiotherapy compared to other subtypes. It is possible that this may, in part, be mediated by the proliferative status of these subtypes possessing the ability to repopulate more readily after radiotherapy than the less proliferative tumours subtypes. This may account for their recurrence following radiotherapy and the difficulty in maintaining tumour control after radiotherapy recurrence.

The spatial arrangement of the proliferating compartment of cells was studied for all of the tumour types. The proliferation patterns have been described in Chapter 3. The random pattern of proliferation was the commonest pattern and found most commonly in the morpheic (81%) and infiltrative (62%) histological growth patterns. This is in contrast to radioactive thymidine labelling studies (Grimwood, Ferris et al. 1986) or by PCNA immunohistochemistry (Stamp, Nasim et al. 1993) whereby the proliferating cells were found predominantly at the periphery of the tumour nodules. The higher labelling index of Ki-67 in comparison to radioactive thymidine or PCNA may account for these differences since there is increased central staining in comparison to peripheral staining. Additionally the high proportion of infiltrative tumours with random pattern staining in this series may also contribute. In this study 31% of tumours did have a marginal diffuse pattern and 2.5% a marginal pattern. These proliferation patterns were found most commonly in the nodular or micronodular histological types. It would seem apparent therefore that the location of proliferation may contribute to the histological growth pattern. This relationship was responsible for the high proportion of random pattern tumours being found in the horrifying group compared to primary or recurrent tumours.

There was less of a relationship between the differentiation status and proliferation pattern, although the tumours with cystic differentiation contained the highest proportion of the mixed pattern and the least tumours with a random pattern of proliferation. This was because proliferation around cysts tended to be of a marginal or marginal diffuse pattern while elsewhere in the specimen there were random patterns of differentiation. Overall, there was no clear relationship between the differentiation status and the proliferation pattern. This latter observation calls into question whether BCC exhibit true differentiation. In head and neck squamous cell cancer, a tumour with well defined differentiation criteria, a relationship between proliferation pattern and differentiation status has been identified with marginal and marginal diffuse being most prevalent in well/moderately differentiated tumours and associated with less rapid proliferation (Wilson, Dische et al. 1995). This relationship does not exist in BCC because the differentiation criteria are less well defined. It is debatable whether BCCs show a high level of differentiation towards adnexal primordia or are inherently undifferentiated (Stamp, Nasim et al. 1993). In BCC, there is no doubt that certain subtypes, particularly nodular and, to a lesser extent, infiltrating and superficial, display a variety of differentiated features such as cyst formation and adenoid and squamous characteristics, yet we found no correlation with either the number or location of proliferating cells. This observation, in conjunction with the both the heterogeneity of proliferation and diversity of growth and proliferation patterns found within individual BCC support the concept of a mutable tumour. Episodic progression and regression of BCC (Franchimont, Pierard et al. 1982), as evidenced by areas of necrosis and confluent apoptosis, cellular attempts to differentiate and variable proliferation, may be the result of a changing balance between tumour and stroma/dermal-derived cellular signals.

This study also aimed to establish whether the growth fraction or proliferation pattern could predict recurrence following a particular treatment modality. Only surgery and radiotherapy could be evaluated because the numbers of patients undergoing other therapeutic modalities such a curettage or cryotherapy were too small. The growth fraction or proliferation patterns alone could not predict recurrence following radiotherapy. This finding is surprising when the results of Chapter 4 are taken into

consideration. i.e. the observation that the commonest cause of treatment failure for the infiltrative tumours was radiotherapy (47%) would suggest a high growth fraction may be associated with post radiotherapy recurrence. However, the commonest cause of treatment failure of the nodular tumours was also radiotherapy (38.5%) and therefore there was no relationship between the proliferative indices and outcome. For tumours that were completely excised the growth fraction was higher for the recurrent group although this was not statistically significant and the patient numbers were relatively small. This finding was due to there being a greater proportion of infiltrative tumours in the recurrent group compared to the primary group. (33.3% vs 21.1%) but there was also a proportion of nodular tumours in the recurrent group which may have accounted for the statistically insignificant result.

In conclusion we have combined both a classification of tumour histological growth pattern with differentiation and augmented this clinico-histological description with information on the magnitude and organisation of the proliferative compartment of BCC. Many different classifications for BCC have been suggested based on differentiation (Lever 1971), clinico-pathological criteria (Emmett 1990) or descriptive histology (Wade and Ackerman 1978). The most useful classification, for clinical purposes, must include information on the histological growth pattern first emphasised by Thackray, (Thackary 1951) Sloane (Sloane 1977) and Sexton (Sexton, Jones et al. 1990). From the previous chapter we found that this classification system carries some prognostic significance with the infiltrative and morpheic subtype tending to inadequate treatment, recurrence or the development of a horrifying lesion. Measurement of the growth fraction has suggested that there are definite biological differences between tumour subtypes with the infiltrative and morpheic variety of tumour having the highest growth fraction. This may be a mechanism by which these subtypes infiltrate more deeply and therefore tend to be clinically more aggressive. The growth fraction however was not prognostically significant because there are many other factors that contribute to the outcome such as time of presentation, treatment type and the adequacy of a particular treatment determined by tumour selection and the technical ability of the treatment operator.

Chapter 6

p53 and bcl-2 protein expression in basal cell carcinoma. The relationship to histology and clinical outcome.

6.1 Introduction

p53 and the Bcl-2 family are intimately related as pivotal participants in the regulatory mechanisms of bax mediated apoptosis following DNA damage. The balance of cell proliferation in relationship to cell loss determines the growth characteristics of a tumour. Overexpression of p53 oncoprotein has been identified in 0-92% of BCCs (Wikonkal, Berg et al. 1997) and about 50% of p53 mutations have the UV induced signature. It is therefore possible that the relationship of p53 and bcl-2 expression may determine the histological and clinical subtype of BCC. In fact, overexpression of p53 has been associated with histologically aggressive variants of BCC (De Rosa, Staibano et al. 1993). The inter-relationships of these characteristics may also provide prognostic information regarding the outcome following treatment.

The aim of this part of the study was therefore to evaluate p53 and bcl-2 expression in the cohort of primary, recurrent and horrifying BCC and to establish whether these measurements may provide insight into the histological and clinical variety of BCC and also may supply prognostic information.

6.2 Methods

An immunohistochemical analysis of p53 and bcl-2 oncoprotein analysis was performed on the cohort of patients previously described in Chapter 3. Mutant and

wild type p53 was detected with the mouse monoclonal antibody (Dako p53, clone DO-7). A mouse monoclonal antibody for human bcl-2 (Dako) was used. 5 µm sections were stained by the methods described in Chapter 2. The results were scored semi quantitatively by the scoring system described in Chapter 2.

6.3 Results

6.3.1 Bcl-2 oncoprotein expression

bcl-2 results were available for 153 specimens. There were 82 primary, 40 recurrent and 31 horrifying tumours.

bcl-2 expression is illustrated in Figure 6.1. The intensity scoring resulted in a 4 point system (0-3) while the percentage scoring resulted in a 5 point system (0-4). In many tumours there was 100% of the tumour area stained although this was not always a strong in intensity. This resulted in a normal distribution for the intensity score but a non parametric distribution for the percentage scoring.

6.3.1.1 Relation ship to patient sex, age and tumour position and size.

There was no relationship of the bcl-2 intensity or percentage score with the patient age or sex or tumour position or size.

6.3.1.2 Relationship to histopathological growth pattern and differentiation.

There were differences in the intensity and percentage of bcl-2 staining between the different growth patterns of BCC (Figures 6.2 and 6.3). In general, the intensity and percentage of staining fell from the most intensely stained to the least intensely stained in the following order; superficial, micronodular, nodular, infiltrative and

morpheic (Chi square, intensity $p=0.02$, percentage $p=0.09$). A mean score for intensity and percentage staining can be calculated for each growth pattern of tumour. These are illustrated in Table 6.1.

The bcl-2 scores were also evaluated in relation to the differentiation status of the tumours (See Figure 6.4 and 6.5.). There was little variation or differences between the differentiation subtypes (Chi square: Intensity $p=0.6$, percentage $p=0.3$) although it was of interest that the tumours with squamous metaplasia had the smallest mean score for bcl-2 staining. The mean scores are illustrated in Table 6.2.

GROWTH PATTERN	MEAN PERCENTAGE BCL-2 SCORE	MEAN INTENSITY BCL-2 SCORE
Superficial	2.9	2.0
Micronodular	2.7	1.7
Nodular	2.6	1.5
Infiltrative	2.1	1.3
Morpheic	1.9	1.3

Table 6.1 Intensity and percentage staining of bcl-2 as a function of the growth pattern.

DIFFERENTIATION STATUS	MEAN PERCENTAGE BCL-2 SCORE	MEAN INTENSITY BCL-2 SCORE
Adenoid	2.35	1.59
Cystic	2.81	1.81
Squamous Metaplasia	2.31	1.26
Undifferentiated	2.46	1.50

Table 6.2. Intensity and percentage staining of bcl-2 as a function of the differentiation status.

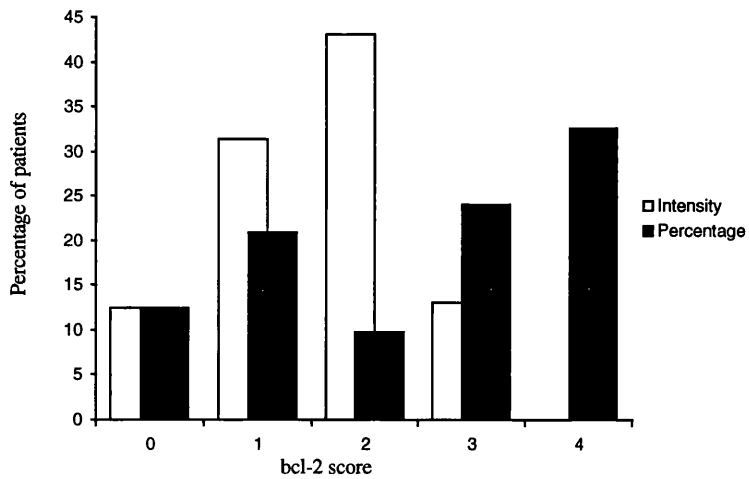


Figure 6.1. The overall distribution of bcl-2 staining in 153 BCCs of mixed clinical and histopathological type.

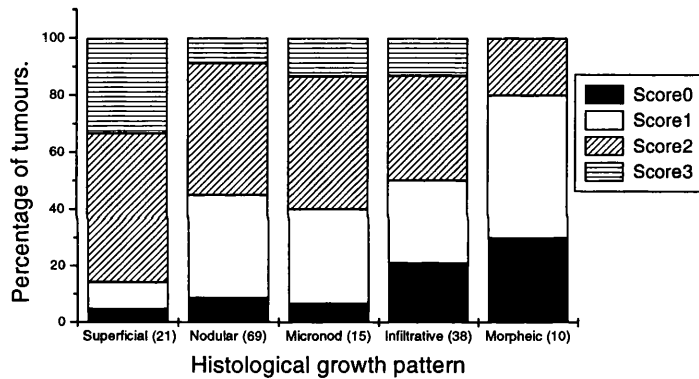


Figure 6.2. The intensity of bcl-2 immunostaining in each histological growth pattern of BCC. (Figure in parenthesis represent the total number of tumours)

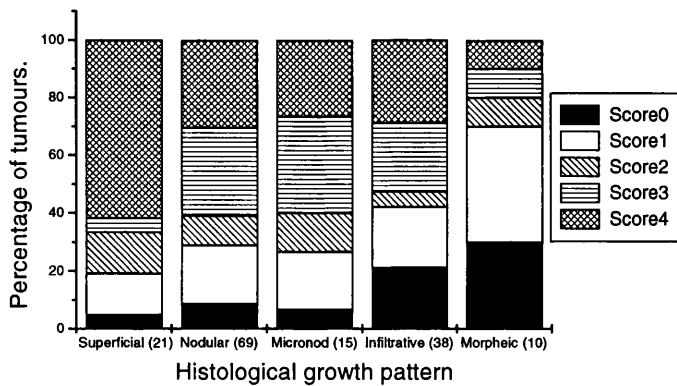


Figure 6.3. The percentage of bcl-2 immunostaining in each histological growth pattern of BCC. (Figure in parenthesis represent the total number of tumours)

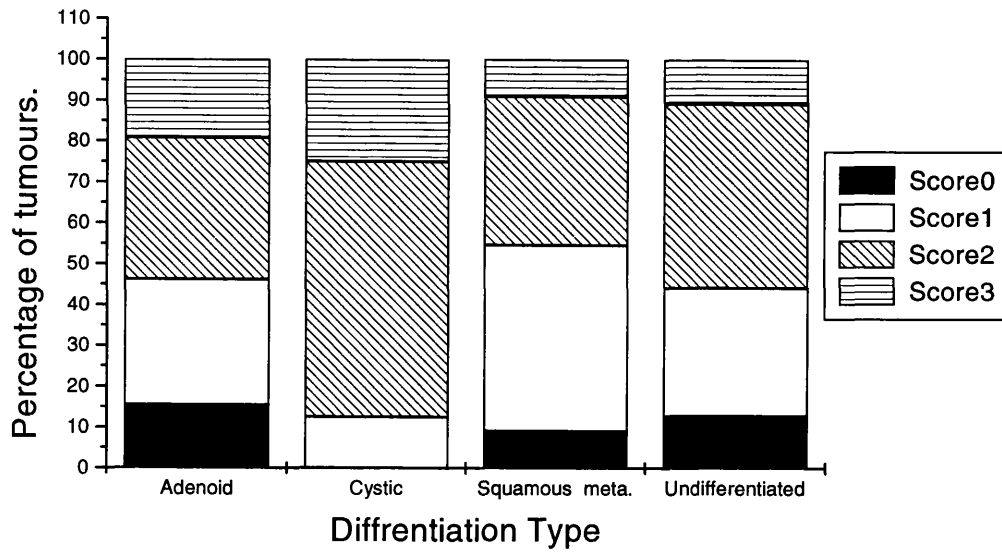


Figure 6.4. The intensity score of bcl-2 immunostaining in each differentiation subgroup of BCC.

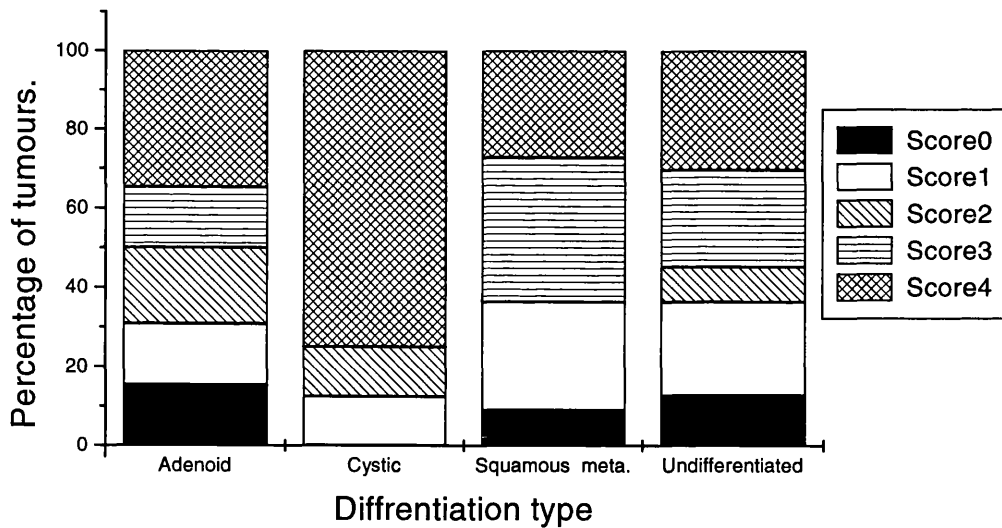


Figure 6.5. The percentage score of bcl-2 immunostaining in each differentiation subgroup of BCC.

6.3.1.3 Relationship to clinical outcome

There was a trend that the bcl-2 expression was related to the clinical type (Chi square Intensity $p=0.08$, percentage $p=0.09$) (Figures 6.6 and 6.7). It was apparent that the recurrent tumours expressed less bcl-2 oncoprotein than the primary tumours. 58% of the recurrent tumours had low or no bcl-2 expression (intensity score 0 or 1) compared to 40% of the primary tumours. This relationship was less pronounced when examining the percentage staining (43% scoring 2 or less versus 50% in the recurrent group).

Conversely there was no difference between the early presenting horrifying tumours compared to the primary tumours.

The late presenting horrifying tumours tended to express more bcl-2 oncoprotein than all of the groups with only 25% with none or weak bl-2 expression. This relationship was also exhibited with the bcl-2 percentage scores.

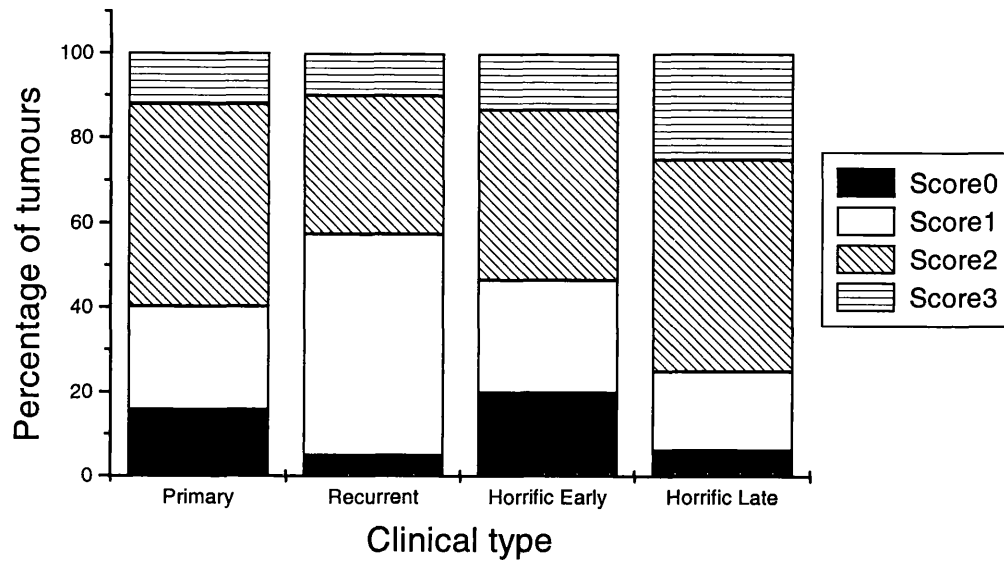


Figure 6.6. bcl-2 immunostaining intensity in relation to the clinical subtype.

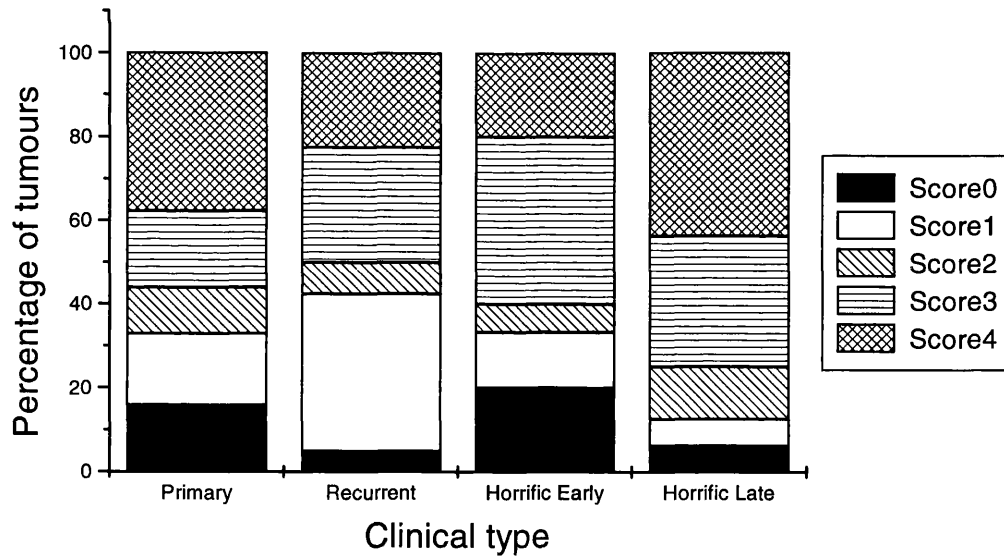


Figure 6.7. bcl-2 immunostaining intensity in relation to the clinical subtype.

6.3.1.4 Relationship to outcome following treatment modality.

The effect of bcl-2 status on the outcome following radiotherapy and complete excision could be assessed although in some groups there were small numbers which severely limits the significance of this relationship. Table 6.3 shows the relationship with primary radiotherapy. In this small cohort of patients neither bcl-2 intensity or percentage area staining was statistically related to the outcome (Chi square $p=0.7$ and $p=0.3$ respectively).

The relationship between bcl-2 expression and outcome following complete excision is shown in Table 6.4. There was a significant association between bcl-2 intensity and recurrence following complete excision. There was less bcl-2 expression in the recurrent group compared to the non recurrent group (80% with low or no staining compared to 40%) (Chi square $p=0.009$). This relationship was also present, but of less significance, with bcl-2 percentage area (Chi square $p=0.1$). There were no horrifying tumours developing after early complete excision.

	PRIMARY (8)	RECURRENT (11)	HORRIFIC EARLY (9)
Intensity 0 and 1	37.5%	36.3%	55.6%
Intensity 2 and 3	63.5%	63.7%	44.4%
Percentage 0 and 1	37.5%	27.3%	22.2%
Percentage 2, 3, 4	62.5%	72.7%	77.8%

Table 6.3. The relationship between bcl-2 expression and outcome following radiotherapy. The actual numbers are in parentheses.

	PRIMARY (70)	RECURRENT (10)
Intensity 0 and 1	40%	80% *
Intensity 2 and 3	60%	20% *
Percentage 0 and 1	32.9%	60%
Percentage 2, 3, 4	67.1%	40%

Table 6.4. The relationship between bcl-2 expression and outcome following complete excision. The actual numbers are in parentheses.

6.3.1.5 Tumour progression and bcl-2.

There were sequential specimens available in only 26 recurrent BCCs and 7 horrifying BCCs. The change in bcl-2 in sequential specimens of the recurrent and horrifying BCCs is illustrated in Figures 6.8 and 6.9. In the recurrent group, 13 scores remained the same, 8 increased and 5 decreased. Statistically, there was no significant difference between the first and last available biopsy (Chi square $p=0.2$). In the horrifying group, only 1 bcl-2 score stayed the same, 4 increased and 2 decreased. Again, there was no significant difference between the first and last biopsy of the horrifying tumours. (Chi square $p=0.1$). Therefore there were individual differences in bcl-2 expression with tumour progression but overall there was no significant change.

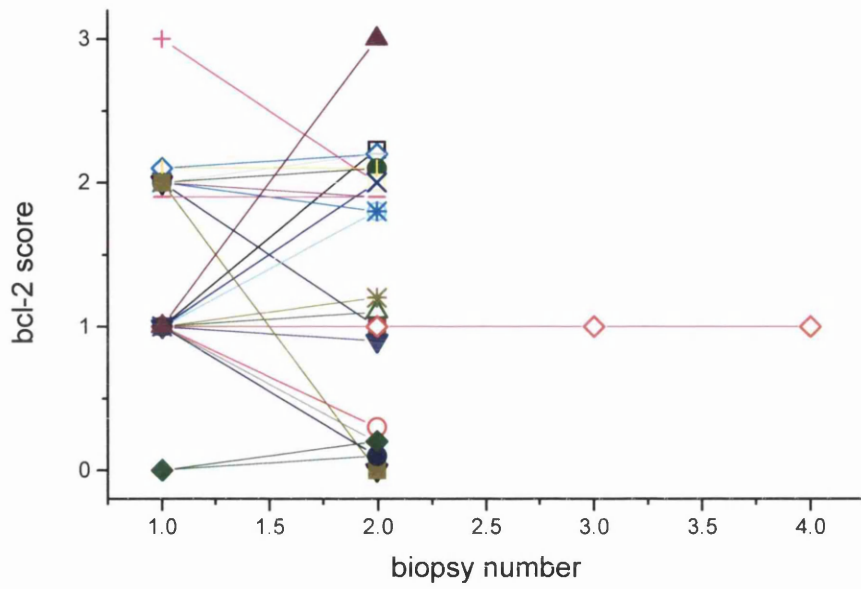


Figure 6.8. The change in bcl-2 expression with tumour progression in recurrent tumours.

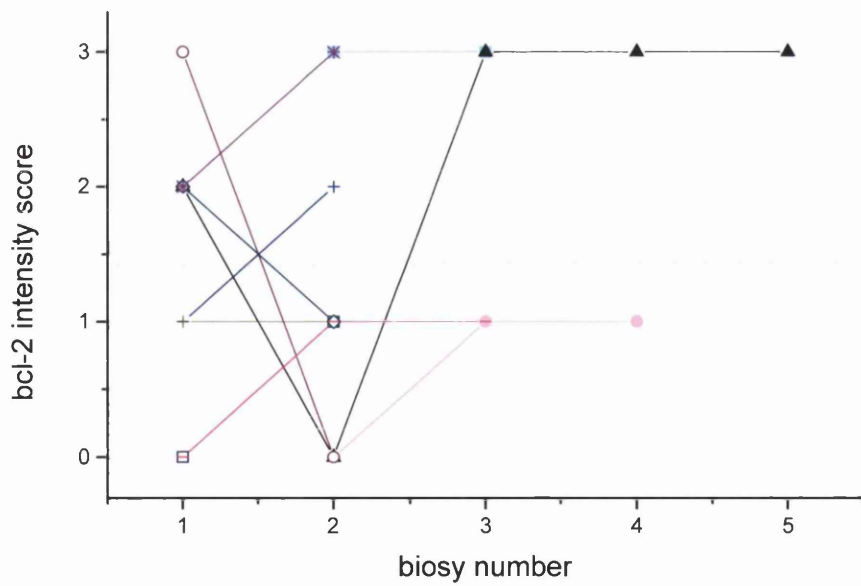


Figure 6.9. The change in bcl-2 expression with tumour progression in horrifying tumours.

6.3.2. p53 oncoprotein expression

The percentage area of p53 oncoprotein expression was scored rather than the intensity. This was because p53 stained the nuclei rather than the cytoplasm and that although there were some differences in the intensity between tumours this was not as obvious as found with the cytoplasmic staining of bcl-2. Results were available for 154 tumours. There were 84 primary, 39 recurrent, 14 early presenting horrifying tumours and 17 late presenting horrifying tumours. The distribution of p53 staining is shown in Figure 6.10. This represents a binomial distribution with 50% of the tumours with none or less than 30% area of p53 staining and 39% of tumours with 50% or greater of their area p53 positive. Only 11 % had intermediate staining.

6.3.2.1 Relation ship to patient sex, age and tumour position and size.

Division of p53 expression score into 2 categories of either low (less than 30% area staining) or high (greater than 30% staining) revealed an inverse relationship between patient age and p53 immunostaining. The older the patient, the less the p53 expression in the tumours (Spearman Rho -0.16, p=0.05) This relationship was also seen if the patients were divided into age categories in Table 6.5. The tumours of patients of less than 50 years old express less p53 than the older groups (Chi square p=0.01). The proportion of patients with greater than 30% positivity increase with increasing age.

	<50 YRS. (18)	50-70YRS (48)	70-80YRS (49)	80+YRS (30)
Low p53 (< 30%)	16.7%	47.9%	53.1%	63.3%
High p53 (>30%)	83.3%	52.1%	46.9%	36.7%

Table 6.5. The inverse relationship of patient age with p53 expression.

There was no relationship between position of the tumour with respect to sun exposure or with tumour size or patient sex.

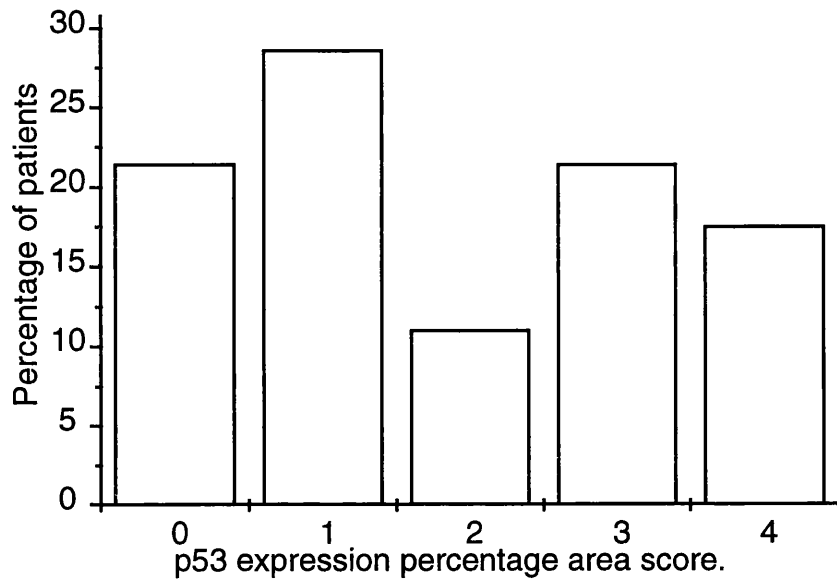


Figure 6.10. The distribution of p53 oncoprotein expression in 154 BCCs.

6.3.2.2 The relationship with tumour histology

From Figure 6.11 it can be seen that the level of p53 expression was not related to the histological growth pattern (Chi square $p=0.4$). Furthermore if the score was graded as high or low there remains no difference between the growth patterns. Similarly there was no statistical difference in the p53 immunostaining and the differentiation status of the tumours (Chi square $p=0.9$) (Figure 6.12.).

6.3.2.3 The relationship with clinical outcome

There were no differences of the p53 expression between the clinical subtypes of BCC. P53 expression was therefore not related to the overall outcome (Figure 6.13). In order to assess the effect of the p53 expression on outcome following surgery or radiotherapy the outcome for each treatment was analysed (Figures 6.14 and 6.15). Recurrence following radiotherapy was associated with a lower p53 expression than non recurrent tumours. 60% of primary tumours expressed greater than 30% area positive to p53 compared to 38% of recurrent tumours and 50% of early presenting horrifying tumours. The numbers were small and were therefore not statistically significant (Chi square $p=0.7$). Recurrence following complete excision surgery appeared to be associated with increased p53 expression. 75% of the recurrent tumours expressing greater than 30% of their are positive for p53 versus 53% of the non recurrent tumours. Again this did not achieve statistical significance (Chi square $p=0.4$).

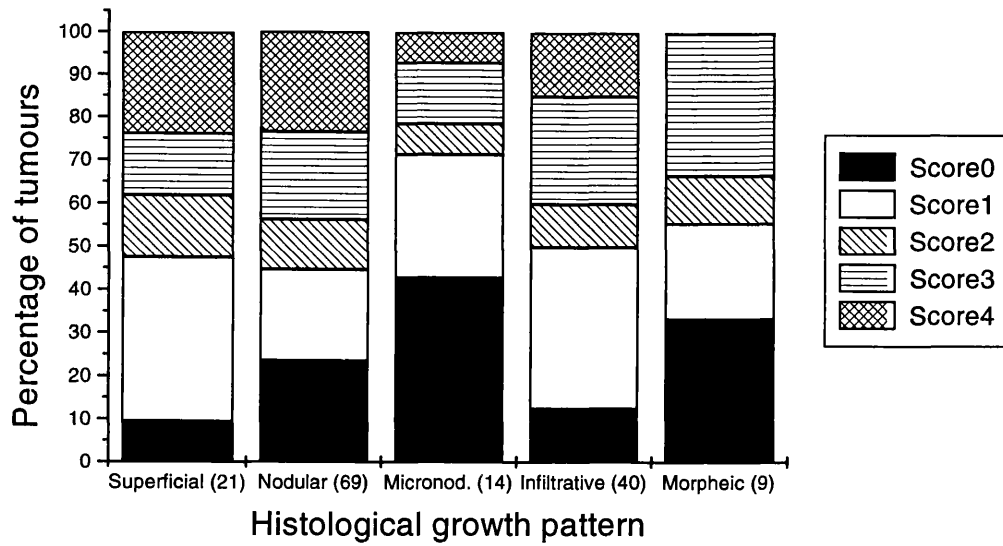


Figure 6.11. The relationship between the histological growth pattern and p53 expression. Individual score are shown in the legend. Total numbers of patients in parentheses.

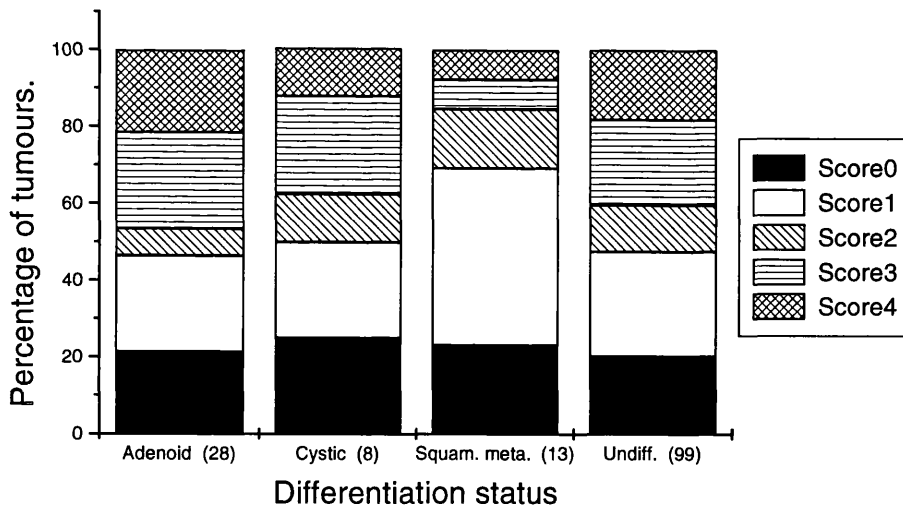


Figure 6.12. The relationship between the differentiation status and p53 expression. Individual score are shown in the legend. Total numbers of patients in parentheses.

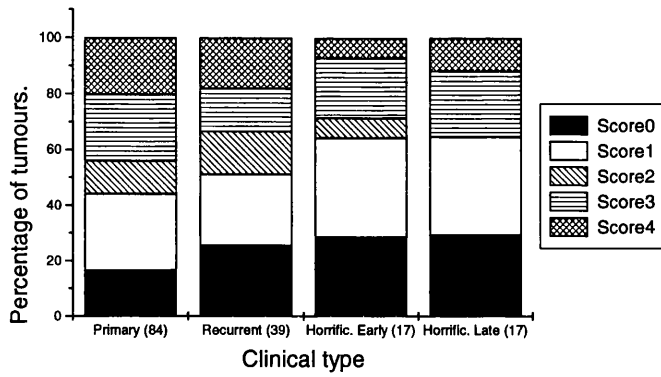


Figure 6.13. The relationship of p53 score to overall outcome for all treatment modalities. Total numbers of patients in parentheses.

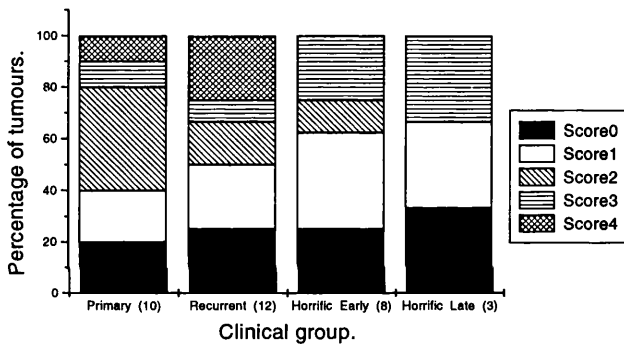


Figure 6.14. The relationship of p53 score to overall outcome for patients treated with primary radiotherapy. Total numbers of patients in parentheses.

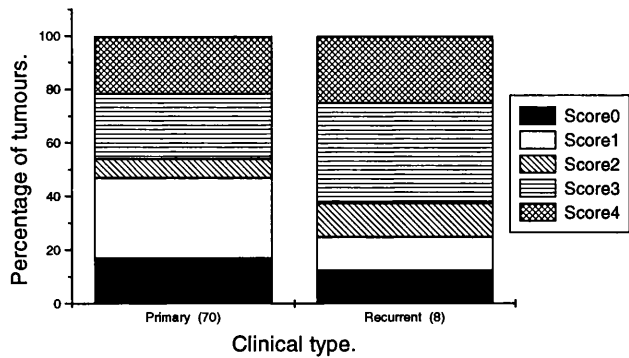


Figure 6.15. The relationship of p53 score to overall outcome for patients treated with complete excision. Total numbers of patients in parentheses.

6.3.2.4 P53 expression and tumour progression

There were few sequential specimens available with p53 immunohistochemistry. (See Figures 6.16 and 6.17) Of these there were 11 recurrent tumours and 12 horrifying tumours. p53 tended to increase in the recurrent group of tumours. In 8 tumours, p53 expression increased, it decreased in one specimen and remained the same in 2 specimens. Similarly, in the horrifying group, p53 expression increased in 7 specimens, remained the same in 3 specimens and decreased in 2 specimens. Due to the small numbers there was no overall statistical significance. (Recurrent: Chi square $p=0.2$. Horrific: Chi square $p=0.4$)

6.3.2.5 The relationship of bcl-2 and p53

There was no demonstrable correlation between p53 and percentage or bcl2 intensity are score. (See Figure 6.18 and 6.19).

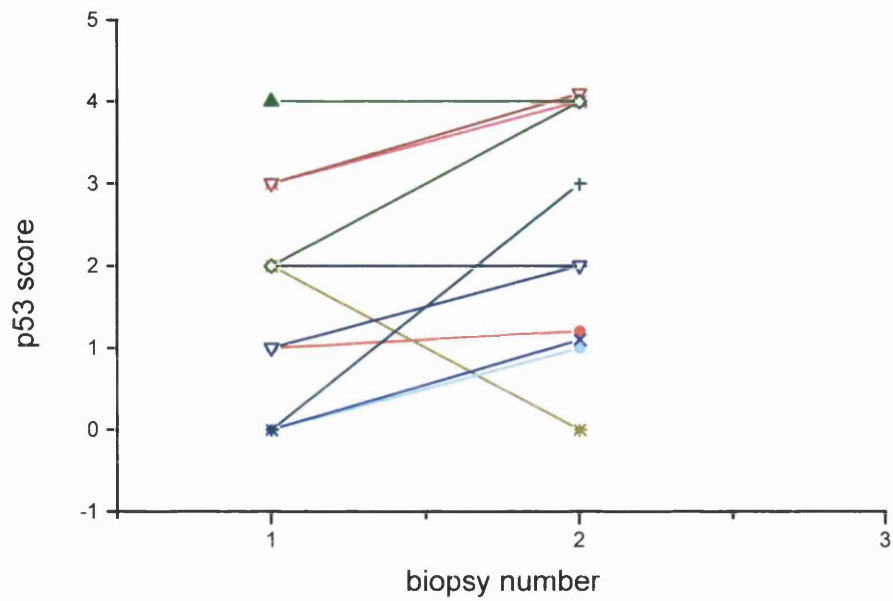


Figure 6.16. The progression of p53 expression in recurrent BCCs.

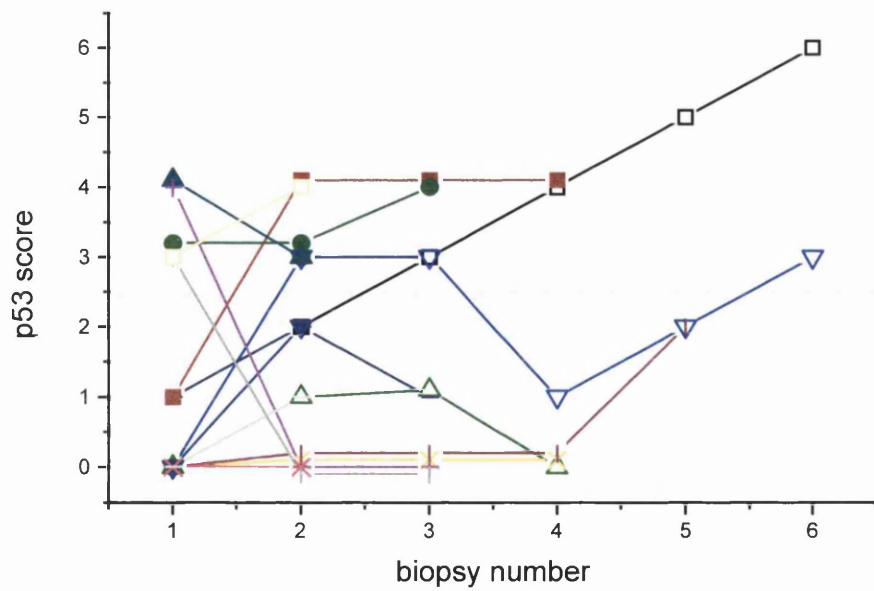


Figure 6.17. The progression of p53 expression in Horrifying BCCs.

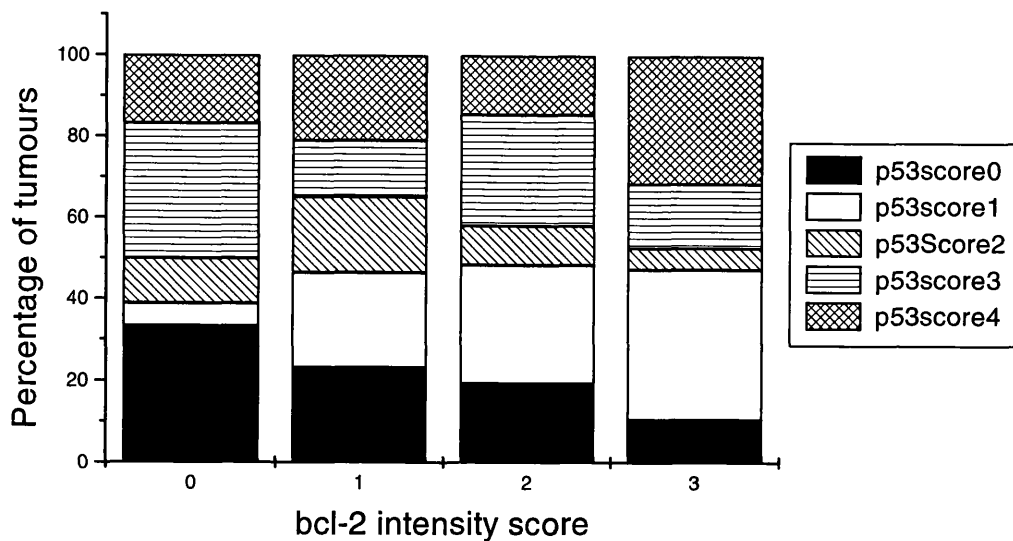


Figure 6.18. The relationship of bcl-2 intensity to the percentage immunostaining of p53 in 142 BCCs.

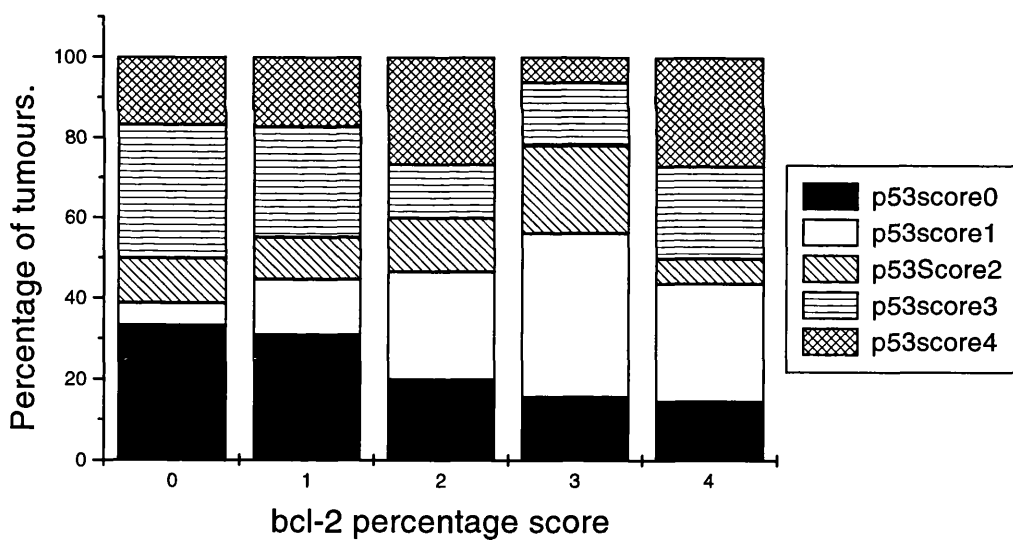


Figure 6.19. The relationship of percentage bcl-2 immunostaining to the percentage immunostaining of p53 in 142 BCCs.

6.4 Discussion

This Chapter investigated the roles of p53 and bcl-2 expression in BCC. These genes are responsible for cell cycle and apoptotic control which are central processes governing tumour growth. They therefore might behave as important determinants of clinical and histological behaviour. The p53 protein is a transcription factor that contributes to maintaining the integrity of the genome by initiating G1 arrest after genotoxic damage allowing time for DNA repair to be effected. Also as a transcription factor for bax, (Miyashita and Reed 1995) p53 can drive cells with an overtly damaged genome into apoptosis thereby eliminating dysfunctional and potentially transformed cells. This defence mechanism however can be overcome by mutagenic agents, especially UV radiation, which is postulated as being an important aetiological agent for BCC. Mutant p53 is no longer able to fulfil its physiological role and therefore becomes an accessory to malignant transformation by preventing apoptosis of potentially malignant cells.

Mutation hotspots occur in the DNA binding domain of the p53 gene. This results in a protein that is more stable than the wild type protein resulting in p53 accumulation. Antibodies such as the D-07 clone bind to an epitope in a conserved region near the N-terminus. These antibodies will therefore detect accumulation of wild type and mutant p53 protein. Because the mutant is more stable than wild type p53, increased immunohistochemical staining usually is found with mutant p53. False negative results may occur if i) both p53 alleles have been deleted resulting in no protein production. ii) The protein is stabilised but its concentration fails to reach the level of detectability. iii) The configuration of the mutated p53 protein is such that it is no longer recognisable by the antibody. False positive results can also occur if normal p53 accumulates in cells to a higher than normal concentration. This relationship has been investigated further. In one study, only 4 of 8 BCC with a mutation were immuno-positive and of 19 immuno-positive samples, only 4 had a detectable mutation by PCR sequencing of exons 5-8 of the p53 protein (Campbell, Quinn et al. 1993). It should be noted that that false negative reactions may not in reality be false if incomplete stabilisation correlates with incomplete loss of function. The same may be

true for false positives, if build up of normal p53 protein can occur with loss of function for example due to abnormalities of p53 ligands.

Although the true relationship between immunohistochemistry and p53 dysfunction is uncertain, overexpression of p53 has been shown to be associated with histologically aggressive histological subtypes in small published series of BCC (De Rosa, Staibano et al. 1993; Barrett, Smith et al. 1997). The relationship with clinical outcome has not been investigated. p53 and bcl-2 are intimately related since bcl-2 suppresses apoptosis by binding to the bax protein. Bax homodimers promote apoptosis while bax/bcl-2 heterodimers inhibits apoptosis. The balance of bcl-2 and bax regulates apoptosis. The phenomenon of high bcl-2 expression has been shown in B cell chronic lymphocytic leukaemia, follicular centre cell lymphomas, in high grade B cell neoplasms, in Hodgkin's lymphoma and other cancers. Normal tissues also express bcl-2 such as the embryonal hair germ which has morphological similarities to BCC (Crowson, Magro et al. 1996). In view of the certain biological analogies between low grade lymphoproliferative disease and the indolent growth of BCC, and the morphological similarities between the latter and the embryonal hair germ and the follicular bulge from which BCC is meant to arise (Ponten, Ren et al. 1994), it has been postulated that bcl-2 may be preferentially expressed in indolent growth patterns of BCC (Crowson, Magro et al. 1996). Small studies have revealed a negative correlation between p53 and bcl-2 and also have suggested that weak bcl-2 expression is associated with histologically aggressive tumours (Crowson, Magro et al. 1996; Wikonkal, Berg et al. 1997).

In this immunohistochemical study, a semi quantitative scoring system was utilised for both p53 and bcl-2 staining. There was differential expression of both bcl-2 and p53 oncoproteins among the variety of clinical and histological variants. In this study 88 % of the BCCs stained positively for bcl-2 oncoprotein which is similar to the 93% previously reported. (Crowson, Magro et al. 1996). Some studies have reported 100% of BCCs staining positively for bcl-2 [Verhaegh, 1995 #10793; Cerroni, 1994 #10898(Morales Ducret, van de Rijn et al. 1995)] This may be because the numbers were relatively small (15 and 20 respectively in these series) and there may have been less histological variation than present in this study. One other study reported 76% of

BCCs expressing Bcl-2 (Wikonkal, Berg et al. 1997). The significance of the high levels of bcl-2 expression has, in part, been discussed in Chapter 3. It may simply reflect the basal cell tissue of origin of BCC, since the basal layer of the epidermis stains positively for bcl-2. Alternatively it may represent a mechanism for the loss of growth control in BCC. From Chapter 3 it can be seen that the cell kinetic parameters of BCC are similar to normal epidermis and high bcl-2 expression may suggest that there is tumour expansion due to reduced cell loss compared to non neoplastic tissue. However, we have found relatively high levels of spontaneous apoptosis that may account for the high proliferative rate seen in BCC. Another suggested mechanism for the high bcl-2 expression is that it may represent a homeostatic response to high apoptotic rates (Morales Ducret, van de Rijn et al. 1995). No chromosomal translocation that may result in upregulation of bcl-2, as seen in B-CLL, has been demonstrated although there are only a limited number of studies investigating this further possible mechanism (Kawasaki, Caldeira et al. 1991; Morales Ducret, van de Rijn et al. 1995). The comparison of low bcl-2 expression found in SCC is of great interest. This may reflect a different cell of origin but also may also account for an alternative pathway of neoplastic transformation. This may determine the differing cell biology and behaviour of SCC compared to BCC.

There has been a wide range of p53 expression reported for BCC. One study reported no p53 expression in BCCs (Gusterson, Anbazhagan et al. 1991), although the polyclonal antibodies, CM1 and JG8, and the monoclonal antibody, 1081, were utilised which appear to be less sensitive than the D-07 monoclonal antibody. One large series reported 42% of BCCs being positive for p53 when stained with the D07 clone.(Ro, Cooper et al. 1993). In this series, we graded the scoring system. 21% were completely negative, however 28% had a scant amount of staining (less than 30% positive cells). It is therefore difficult to draw a direct comparison between the results, since it is uncertain whether the low scoring tumours would have been documented as positive or negative by other authors. In this series, we found a binomial distribution with the majority of tumours with either low or high staining with relatively few tumours of intermediate staining. It would seem sensible that the low staining group could be considered negative and the high staining group considered positive for a

comparison to be drawn. If this is the case, then the results compare well with the study mentioned above since 50% of the tumours would be considered positive.

It is uncertain whether 50% of the tumours in this study harbour a p53 mutation or have accumulated p53 due to other mechanisms. Detectable p53 mutations have been found in approximately 50% of BCCs using DNA sequencing (Rady, Scinicariello et al. 1992; Ziegler, Leffell et al. 1993; van der Riet, Karp et al. 1994). Another study found only 15% of tumours with p53 mutations (Campbell, Quinn et al. 1993) on sequencing. It is considered that these mutations are UV-induced since they are of the characteristic UVR induced signature (G:C or A:T transition opposite a dipyridamine site, or single strand breaks).

The role of UV induced p53 mutations in BCC is not as clear in BCC as in cutaneous SCC. In SCC, there is strong epidemiological evidence that cumulative exposure of sunlight is correlated with increasing risk. There is also a progression of the disease from premalignant conditions such as actinic keratosis and Bowen's disease leading to overt SCC. Furthermore, it has been demonstrated that malignant conversion of these lesions is associated with mutations in p53 (Ro, Cooper et al. 1993). This suggests that sun induced p53 mutations are an important early step in the development of SCC.

In BCC, the epidemiological evidence of sun exposure is less strong and less tumours harbour p53 mutations. Furthermore, most BCCs irrespective of type and size show allelic loss of at the site of the patched gene on chromosome 9q suggesting that this might be a more important tumour suppressor gene in the pathogenesis of BCC. In addition it has been shown that the 9q allele loss is not related to the sun induced p53 mutations (Gailani, Leffell et al. 1996). It is therefore possible that p53 mutation may be either an alternative pathway for evolution of BCC or that p53 is a later epigenetic mutation as a result of tumour progression. (van der Riet, Karp et al. 1994). In either case, the p53 mutation may behave as an important prognostic marker. The possibility of p53 dependant and p53 independent pathways have been previously postulated. D'Errico (1997) found that a high proportion of BCCs in old patients had p53 mutations and suggested that this was due to reduced DNA repair capacity with age combined with chronic sun exposure results in p53 mutations. Low numbers of p53

mutations were found in young patients with BCC suggesting that genetically susceptible individuals developed BCCs through a p53-independent pathway (D. Errico, Calcagnile et al. 1997). The data from this present study is at variance with D'Errico's results. An inverse relationship was found with patient age and p53 immunohistochemistry. Young patients tended to express more p53 mutations than the tumours of older patients. It is uncertain whether this reflects differences due to the English versus Italian populations, or is due to variations in tumour subtype or it may reflect the experimental variation due to immunohistochemical analysis versus DNA sequencing. Furthermore, in this study, there was no relationship between tumour position and p53 expression. Sun exposed sites were not more likely to have tumours expressing p53 than non sun exposed sites.

In this study there were differences in bcl-2 expression between the different histological growth patterns. Superficial tumours expressed the most intense bcl-2 staining and infiltrative and morpheic tumours expressed the least intense bcl-2 staining. Nodular and micronodular had an intermediate bcl-2 expression. These differences were significant (Chi square $p=0.02$). This relationship of bcl-2 expression was also found with the percentage area scoring system but was less significant and showed a trend only (Chi square $p=0.09$) This differential expression of bcl-2 with histological growth pattern is in agreement with one other study (Crowson, Magro et al. 1996), although the numbers were larger in this present study and the classification of the tumour types was also slightly different. The nodular and superficial type are generally considered the least aggressive form of BCC and their bcl-2 staining resembles normal skin structures with which they bear structural and histological similarities. The more aggressive tumour subtype such as the infiltrative and morpheic variety express the least bcl-2. Lack of bcl-2 expression is more akin to the staining seen in SCCs and therefore loss of bcl-2 in BCC may therefore be associated with a more aggressive biological behaviour which is found more commonly with SCC. bcl-2 may therefore be involved in the genesis and progression of BCC. bcl-2 expression predisposes to the accumulation of excessive numbers of neoplastic epithelial cells derived from the stem cell compartment and this may result in the development of the more indolent nodular or superficial BCC. The protection from apoptosis by bcl-2 may therefore increase the risk of mutation from UV exposure. A second hit may

occur possibly involving p53 or other genes that results with progression into a more aggressive histological variant of BCC. Whether bcl-2 expression is lost in this process, due to mutation, or by cytokine induced down regulation is unknown. This proposed mechanism is similar to a process seen in bcl-2 expressing transgenic mice. Resting B lymphocytes progress into diffuse malignant large cell lymphomata after a second hit involving c-myc. The data from this study, however, would not support a concept that the p53 mutation is the second hit that confers tumour progression from a non aggressive histological type to an aggressive subtype since there was no relationship between p53 expression and histological growth pattern. Furthermore, there was no inverse relationship between p53 and bcl-2 expression that would be expected if this theory was the case. Other studies have found a relationship between p53 and histological growth pattern and have demonstrated increased p53 immunohistochemical staining in aggressive histological variants (De Rosa, Staibano et al. 1993) and also an inverse relationship between bcl-2 (Wikonkal, Berg et al. 1997) and p53 expression.

The question remains as to whether the aggressive histological subtypes are a result of tumour progression of more indolent BCCs or whether their histological growth pattern is predetermined from tumourigenesis. Histological data (from Chapter 4) and the observation that the p53 and bcl-2 immunohistochemical patterns did not change with each recurrence, would support the latter option. However the tumours with multiple subsequent specimens due to recurrence could, by definition, be described as already having the aggressive phenotype; Further tumour progression having occurred prior to the first biopsy.

There was no statistical relationship between the differentiation status of the tumours and either bcl-2 or p53 expression. Any real difference may be partly obscured by only the predominant differentiation type being documented and not oncoprotein expression for each differentiation subtype of each part of a tumour. It was of interest, that the tumours displaying squamous metaplasia displayed the least bcl-2 expression than the other subtypes. A staining pattern more akin to SCCs. It would therefore be expected that this subtype should express more p53 than the other differentiation

subtypes. This relationship was not maintained. In fact the tumours exhibiting predominantly squamous metaplasia expressed less p53 than the other subtypes.

The p53 and bcl-2 expression was examined according to the clinical outcome irrespective of the histological subtype. There was a trend that recurrent tumours expressed less bcl-2 than non-recurrent tumours. However, this relationship did not hold for the early presenting horrifying tumours. This discrepancy may be explained when bcl-2 is analysed according to the outcome for each treatment modality. Recurrence following complete excision, was significantly related to low bcl-2 expression ($p=0.009$). However, bcl-2 expression failed to predict outcome following radiotherapy. Since the majority of primary tumours were treated by complete excision and did not recur this would select tumours with a higher bcl-2 expression. The recurrent tumours were initially treated by a variety of therapeutic modalities, including complete excision. This would reduce the overall bcl-2 expression in the recurrent group although this effect was diluted by post-radiotherapy recurrences with a variety of bcl-2 scores. The early presenting, horrifying tumours were most commonly treated by primary radiotherapy. This group had a bcl-2 score that was not different to the primary tumours since bcl-2 was no prognostic for post radiotherapy recurrence.

The question of why bcl-2 is prognostic in surgery but not radiotherapy needs to be addressed. This might be explained by the finding that the infiltrative and morpheic subtype recur most commonly following complete excision and it is these tumours that express the least bcl-2. Of the tumours that recurred following radiotherapy, there were only 31% of infiltrative or morpheic types and therefore the bcl-2 score was not as low enough to produce a significant result.

p53 failed to significantly differentiate between the clinical outcome of individual or all treatment modalities. This was, in part, due to small numbers of patients but the most likely reason was that there was no relationship of p53 expression to histological subtype and as a result there was little relationship to clinical outcome.

In conclusion this study has examined the relationship of p53 and bcl-2 protein expression with the clinical and histological variation that is seen in BCC. In general, BCC expresses bcl-2 protein which reflects the stem cell of origin of the tumour and may also contribute to the growth kinetics. Differential bcl-2 protein expression was related to the histological subtype of BCC and therefore was also related to clinical outcome especially following complete excision. Approximately half of the tumours expressed p53 protein by immunohistochemistry which possibly reflects the degree of UV involvement in the aetiology of individual tumours but may also be a result of tumour progression. The importance of p53 protein expression to the biology of BCC is uncertain since there was no relationship to histological subtype outcome or to overall outcome.

Chapter 7

Evaluation of a novel treatment modality for basal cell carcinoma. The optomechanically flash scanned carbon dioxide laser.

7.1 Introduction

A large number of treatment modalities have been described for BCC such as excision, (Bart, Schrage et al. 1978; Blomqvist, Eriksson et al. 1982; Silverman, Kopf et al. 1992) curettage and electrodesiccation (Knox, Freeman et al. 1967; Crissey 1971; Kopf, Bart et al. 1977; Edens, Bartlow et al. 1983; Salasche 1983; Salasche 1984; Spiller and Spiller 1984), curettage (McDaniel 1978; McDaniel 1983; Salasche 1983; Salasche 1984; Spiller and Spiller 1984), Mohs surgery (Mohs 1970; Mohs 1971; Robins and Amonette 1972; Burg, Hirsch et al. 1975; Hien, Prawer et al. 1981; d'Aubermont and Bennett 1985; Callahan, Monheit et al. 1989; Frankel 1990; Sahl, Yessenow et al. 1994), cryosurgery, (Bullock, Beard et al. 1976; McLean, Haynes et al. 1978; Allen, McGill et al. 1979; Rosen and Vered 1979; Hall, Leppard et al. 1986; Biro and Price 1990; Dachow Siwiec 1990) radiation, (Mustarde 1970; Murphy 1971; Goldschmidt 1976; Harwood 1986; Mazon, Ghalie et al. 1986; Goldberg and Rubin 1989) topical 5 fluouracil, (Achten, Van Oost et al. 1970; Reymann 1970; Litwin, Ryan et al. 1972) retinoids (Peck, Gross et al. 1982; Lippman, Shimm et al. 1988) and photodynamic therapy (Wilson, Mang et al. 1989; Hintschich, Feyh et al. 1993; Cairnduff, Stringer et al. 1994; Svanberg, Andersson et al. 1994; Lui, Salasche et al. 1995). However, tumour recurrence remains a significant clinical problem and ranges from 0.7% - 30% depending on treatment modality, length of follow up, patient and tumour selection (Rowe, Carroll et al. 1989). Failure to recognise lateral subclinical growth and deep extension of the tumour is a common cause of treatment failure and may, in a small number of cases, result in tumour invasion into deeper tissue planes or vital structures such as the eye or into bone. (Jackson and Adams 1973; Jacobs,

Rippey et al. 1982). Lateral subclinical extension and depth of growth is determined by the type of the BCC, primary or recurrent growth and size of the lesion and it is important that these factors are taken into consideration when deciding treatment modality and excision margin (Burg, Hirsch et al. 1975; Salasche and Amonette 1981; Breuninger and Dietz 1991).

Carbon dioxide laser ablation has more recently been advocated for the treatment of BCCs especially those of superficial clinical type (Adams and Price 1979; Wheeland, Bailin et al. 1987; Fitzpatrick and Goldman 1995; Gloster and Roenigk 1995). The efficacy of carbon dioxide ablation is reported in terms of low short term recurrence rates (Adams and Price 1979; Wheeland, Bailin et al. 1987). This would seem inadequate to assess the efficacy of a new treatment since the history of recurrence of BCC is long with approximately one third recurring from five to ten years after primary treatment (Rowe, Carroll et al. 1989). Additionally, incomplete ablation has not been sufficiently assessed. Despite the uncertainty of the relationship of incomplete excision and tumour recurrence, complete ablation of this potentially locally invasive tumour should remain the treatment goal (Pascal, Hobby et al. 1968; Koplin and Zarem 1980; Hauben, Zirkin et al. 1982; De Silva and Dellon 1985; Dellon, DeSilva et al. 1985; Richmond and Davie 1987). The aim of part of this thesis this study was to assess more thoroughly the use of the carbon dioxide laser as an additional tool to manage BCC by examining the completeness of tumour ablation. This was achieved by formal excision of the tumour bed immediately after ablation and histological examination of the specimen. Three important issues arise. Firstly; can the carbon dioxide laser completely ablate BCCs? Secondly, which types of BCC are suitable for this procedure? Thirdly what extent of ablation is required? Prognostic markers were not evaluated in this patient group because recurrence was not being evaluated. Furthermore, the previous Chapters have demonstrated that the completeness of excision is the most important determinant of outcome and that prognostic markers have limited value in assessing outcome for surgical treatment modalities.

7.2 Method

7.2.1 Laser

The Sharplan 1020 carbon dioxide laser in combination with the Swiftlase scanner was used in all cases at a power of 10 W (Figure 7.1). The carbon dioxide laser operates in the far infrared spectrum at a wavelength of 10,600 nm. Selective photothermolysis utilizing water as the chromophore causes rapid heating and vaporization of intra and extracellular water resulting in tissue destruction. Thermal diffusion into the surrounding tissue causes unwanted thermal necrosis and char resulting in unacceptable scarring. This is kept to a minimum by the Swiftlase scanner. Thermal diffusion is time dependent and can be reduced if the laser energy is delivered to any one spot for less than the thermal relaxation time of less than one msec. (Hobbs, Bailin et al. 1987; Fitzpatrick and Goldman 1995). The Swiftlase operates by scanning the laser beam rapidly over the tissue area in a spiral type pattern that maintains laser exposure, of any spot, to less than a msec, thus, reducing thermal diffusion (Figure 7.1).

7.2.2 Patients

The study was a prospective trial. Patients were selected from the waiting list for excision biopsy of BCCs under local anesthetic at the Mount Vernon Hospital Regional Plastic Surgery Unit. We arranged 5 sessions of 8 - 13 patients and analyzed the results between each session. Initially, patient selection was kept to a minimum to optimize our ablation technique and to establish a feel for the capabilities of this method. We excluded patients with lesions close to the eye, large infiltrative or morpheic tumours or problem, recurrent tumours. As the study progressed we attempted to select patients with more suitable lesions and later we relaxed our selection criteria to define the limits of this treatment.

7.2.3 Classification of tumours

The tumours were divided into the following recognised clinical groups (Goldberg 1996). Superficial which appear as finely scaled and wrinkled superficial ulcerations that may resemble subacute or chronic dermatitis. Nodular resemble a pearly translucent papule or nodule that may be ulcerated or pigmented. The infiltrative tumours are flatter tend to invade deeply. They may have a morpheic or fibrotic component associated.

Following biopsy the tumours were classified according to the histological growth pattern described in Chapter 2.

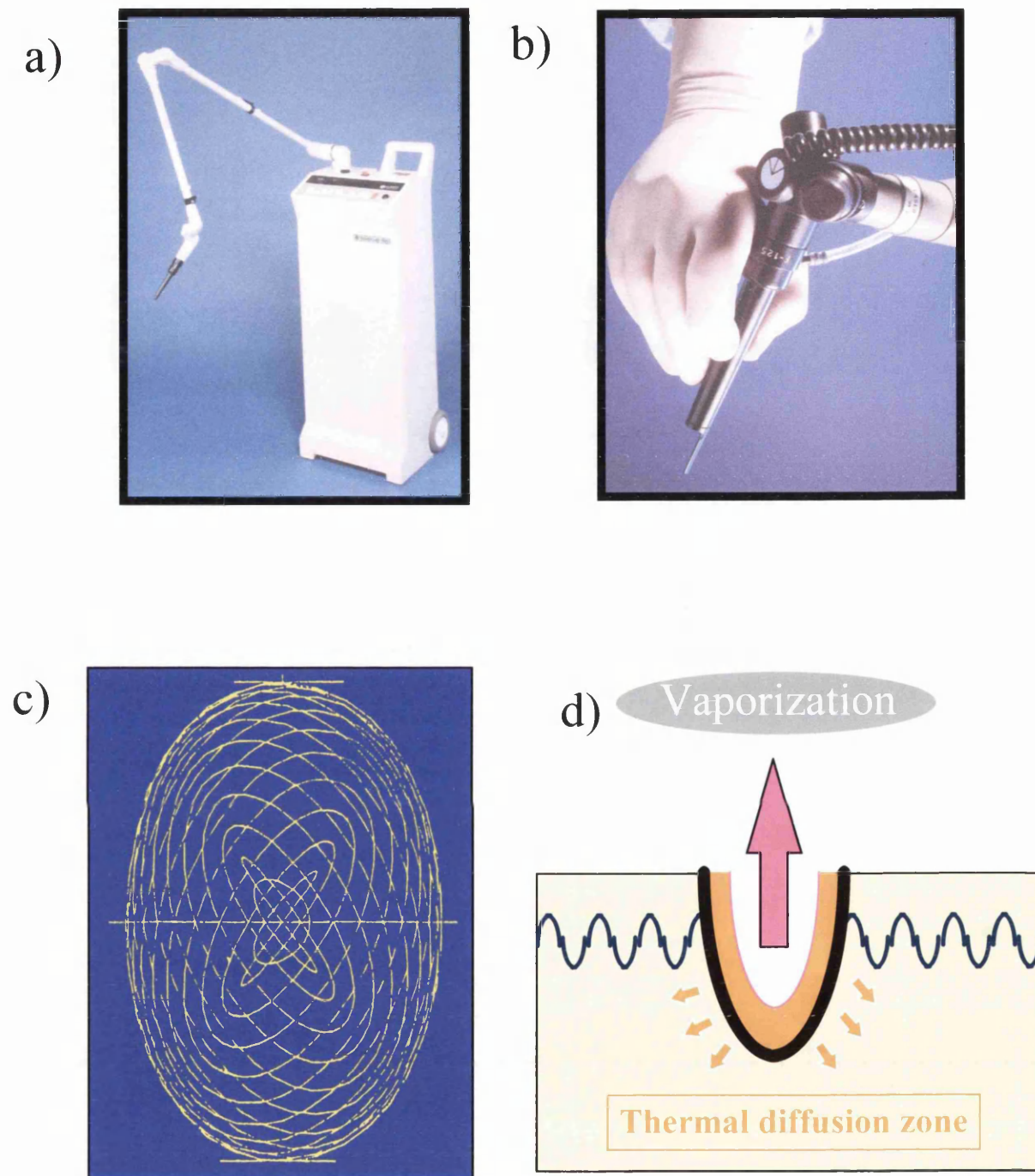


Figure 7.1 a) The sharplan 1020 carbon dioxide laser.

b) The swift scanner.

c) The spiral scanning pattern.

d) Illustrating tissue vaporisation.

7.2.4 Procedure

Patients attended theatre as for a local anesthetic excision biopsy. Tumour clinical type, size and position were recorded. The tumour edge, excision margin and reconstruction design (if required) were marked under theatre lighting. The area was then infiltrated with 1% lignocaine. A 2 mm punch biopsy, to confirm the diagnosis and histological growth pattern was taken. The tumour was then lasered and the time taken was recorded. Initially the depth was determined according to a modification of Wheeland's technique described to ablate BCCs with curettage in combination with the carbon dioxide laser. (Wheeland, Bailin et al. 1987). This technique was designed to control the depth of ablation by visualizing the residual tumour. After the first pass of the laser the char is wiped away with a damp gauze. The residual tumour can be identified as a slightly pinkish residue within the confines of the whiter dermis. A second pass with the laser is performed. The char is the wiped away revealing a smaller area of pinkish residual tumour. This process is repeated. Each time the residual tumour diminishes in size, until no remaining tumour is seen (Figure 7.2). Initially there was a high failure rate which questions the reliability of this method to determine ablation depth. This was, in part, due to the blood from the punch biopsy hindering clear visualization of the tumour bed. Additionally if ablation continues through the dermis into the subcutaneous tissue, the distinction of the pinkish tumour against the whitish dermis disappears and the sign becomes less dependable. Following our early failures we therefore modified the technique and ablated at least 2 further passes after the disappearance of the tumour. This resulted in ablation to the deep dermis or subcutaneous tissue in the majority of the later cases.

Following tumour ablation, the tumour bed was excised with a cuff of surrounding tissue and the defect reconstructed as for a normal excision biopsy. The surgery time was recorded so that the laser and surgery times could be compared. The excision specimen was then histologically examined for residual tumour in the vicinity of the crater. Outcome was measured as the completeness of tumour ablation and the presence of residual tumour. (Table 1 and Figure 3). Ablation depth was recorded with

reference to the level of the deepest part of the crater in the dermis, subcutaneous tissue or deeper structure such as muscle.

Outcome	
Complete ablation	No residual tumour
Incomplete ablation -deep margin	Tumour residue at base of ablation crater
Incomplete ablation -lateral margin	Tumour residue at edge of crater
Smudge	Small area of poorly demarcated dark staining cells within the heat artifact which may represent residual tumour of questionable viability

Table 7.1. The possible outcomes following laser ablation and histological examination of the excision specimen.

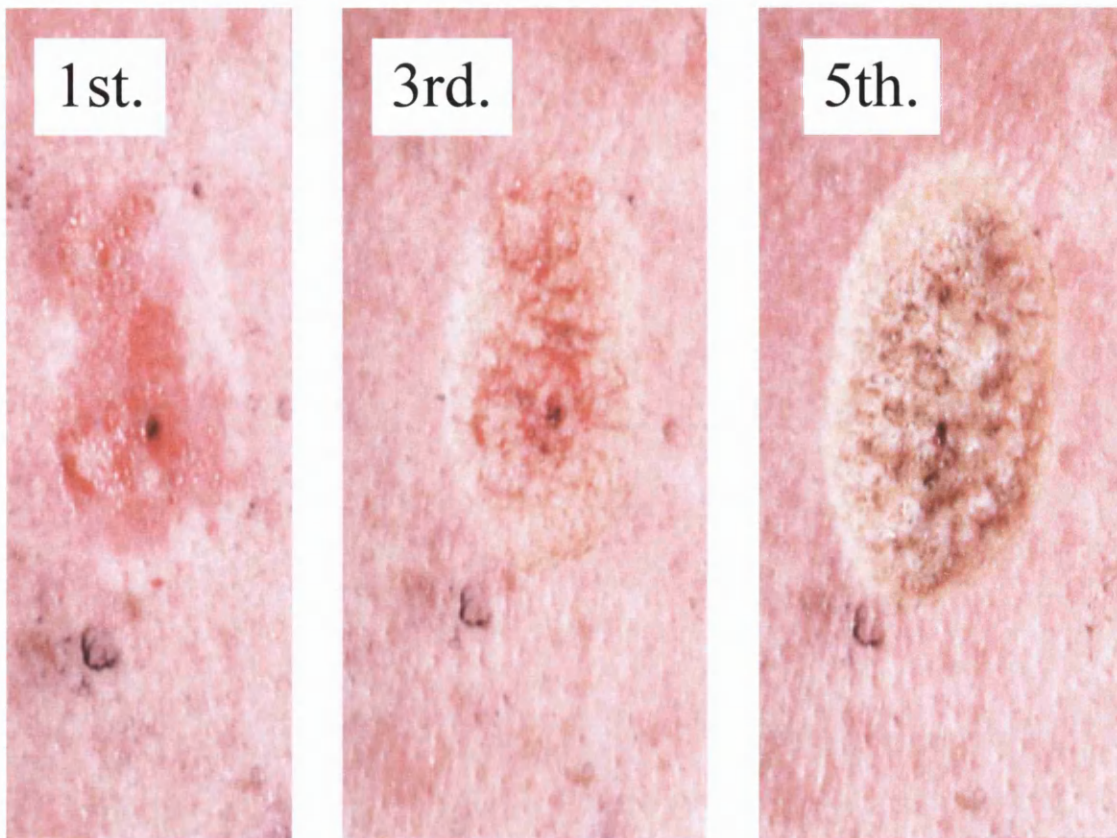


Figure 7.2. Laser ablation of a BCC demonstrating Wheeland's sign. With successive passes of the laser the residual tumour, seen a pinkish colour within the white dermis, diminishes in size until it disappears.

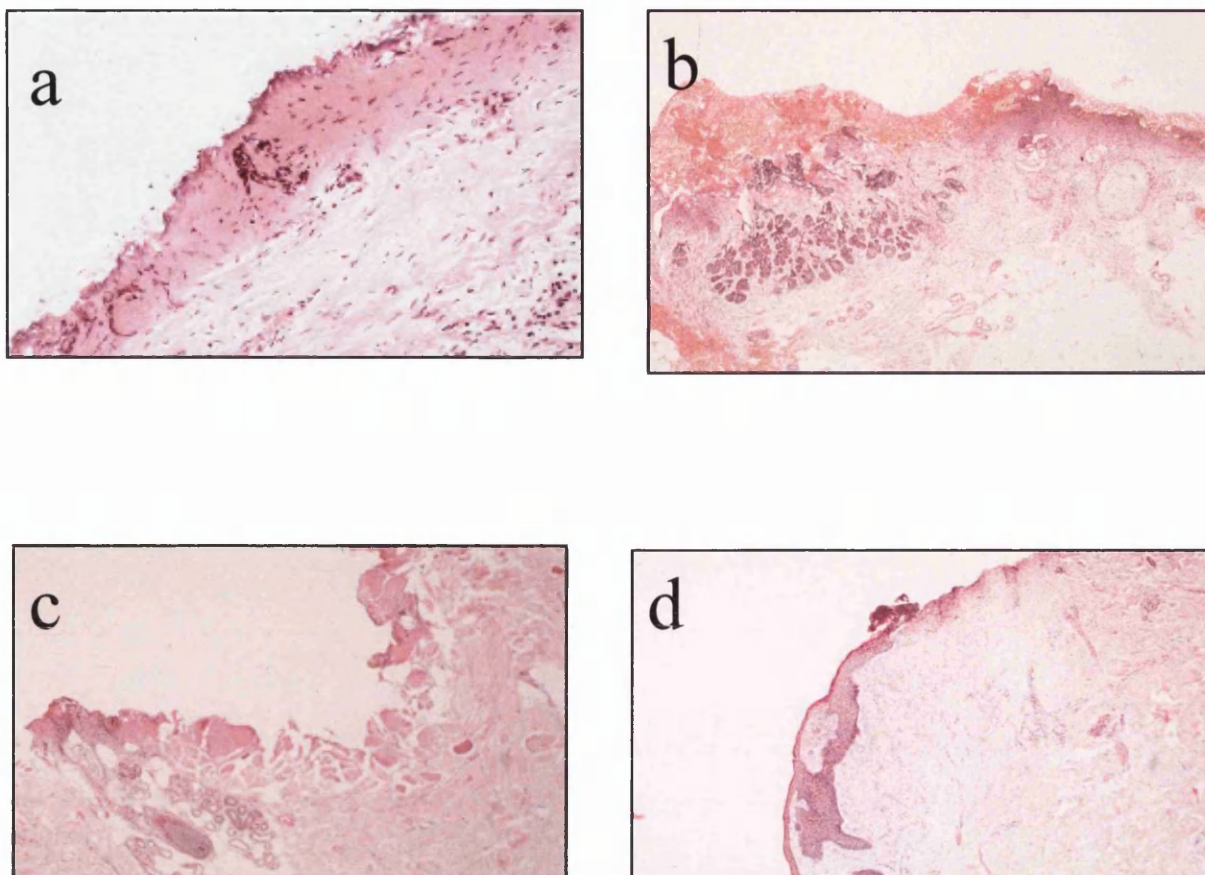


Figure 7.3. Photomicrographs of the excised crater following laser ablation of basal cell carcinoma.

- a). Complete ablation of the tumour with heat artefact on the crater base (x100).
- b). Incompletely ablated basal cell carcinoma with residual tumour in the base of the crater. The blood from the biopsy is seen obscuring the residual tumour (x75).
- c). A “smudge” of dark staining tissue that may represent viable or non viable residual tumour (X400).
- d). Residual tumour at the edge of a crater in a patient with Gorlin’s syndrome (x45).

7.3 Results

7.3.1 Patients and tumours

35 patients with 51 BCCs were involved in the study. There were 12 females and 23 males. 3 patients with 8 tumours had Gorlin's syndrome. The age range was 69 years (Range from 43 to 100 years). 50 of the tumours were primary and 1 was recurrent. The size ranged from 4 - 35 mm in diameter (mean 13.6, median 10mm). The clinical classification of the tumours was as follows: 28 were nodular, 21 superficial and 2 were infiltrative. The superficial tumours tended to be situated on the trunk and limbs while the nodular tumours presented in all regions but were situated more commonly on the face. (Figure 4)

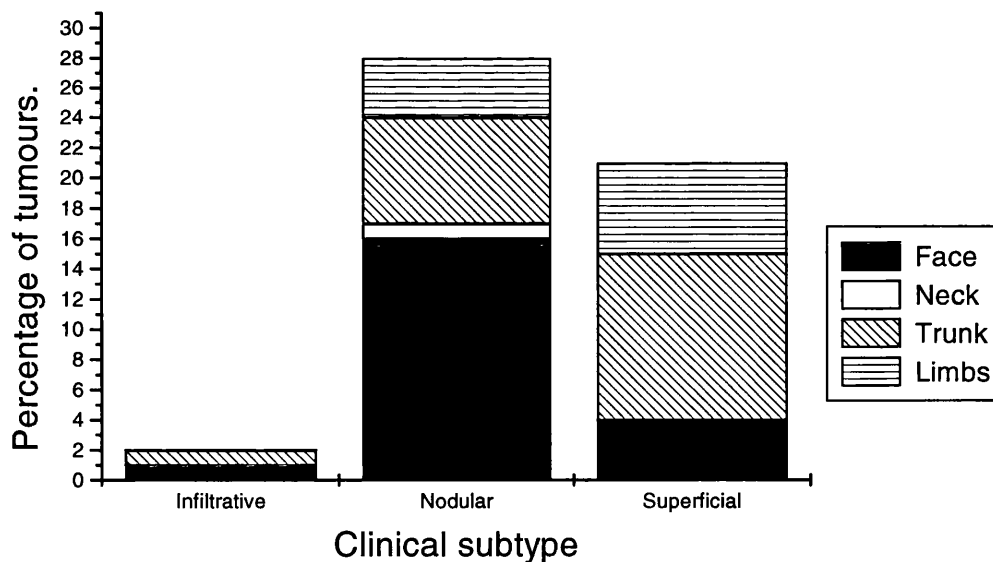


Figure 7.4. The relationship of tumour clinical type to position.

7.3.2 Outcome

7.3.2.1 The learning curve

Overall there was a 67% complete ablation rate (34/51). There were 14 incomplete ablations. 10 with tumour residue at the deep margin of the crater and 4 with residue laterally. Additionally 3 patients had smudges of material that may represent viable or non viable tumour. For analysis this was considered as incomplete ablation at the deep margin. There was a very distinct learning curve. Initially there was a 20 % complete ablation rate which rose to 80% or over in the latter sessions (See table 2).

7.3.2.2 Ablation width and incomplete lateral margins.

The ablation margin was calculated from the difference between the tumour size and crater size. The mean margin was 3.4mm (range 1-10, median 3 mm) The ablation margin did not significantly alter between sessions (Anova for trend $p=0.20$, DF (1,36) $F=1.37$). 4 tumours were incompletely ablated at the lateral margin. There was no statistical difference between the margins of tumours with complete or incomplete lateral margins. (Complete lateral margin mean 3.7mm, incomplete lateral margin mean 2.0mm. Student t test $p=0.28$), although there was no tumour with a complete lateral margin if the laser ablation margin was greater than the mean of 3.4mm. 3 of 4 patients with tumour residue laterally, occurred in patients with Gorlins' syndrome. The other patient had a clinically nodular tumour of 12mm which was ablated with a 2 mm margin.

7.3.2.3 Ablation depth and incomplete deep margins

Ablation depth progressively increased and contributed mostly to the learning curve. In the first session 86 % of tumours ablated to the upper or middle dermis and only 14 % to the lower dermis or subcutaneous tissue.. (Table 2). In the last session 10 % were ablated to the upper or middle dermis and 90% to the lower dermis or subcutaneous tissue.. (Chi square for trend $p= 0.0005$, Chi square=12.08, DF=1). Overall 13 tumours had residual tumour or smudge remaining at the deep margin. Ablation depth

was significantly related to tumour clearance at the deep margin including smudges. (Chi square $p=0.006$, Chi square =16.17, DF=5) Increasing ablation depth from the upper, middle, lower dermis to subcutaneous tissue resulted in increasing clear deep margins (including smudge) from 40% to 66% to 93% to 92% respectively. (Chi square for trend $p=0.017$, chi square =5.71, DF=1). One large tumour ablated to muscle was incomplete on the deep margin. All of the 4 tumours with lateral residue were lasered to the lower dermis (3 patients) or subcutaneous tissue (1 patient.)

Session	Ablation depth							Ablation margin				Clinical type		
	Upper dermis	Middle dermis	Lower dermis	Subcutis	Muscle	Unknown	Clear	Deep residue	Lateral residue	Smudge	Sup	Nod	Inf	
1 (8)	43%	43%	0%	14%	0%	0%	25%	75%	0%	0%	13%	87%	0%	
2 (13)	23%	46%	23%	7%	0%	0%	53%	15%	23%	9%	46%	54%	0%	
3 (11)	0%	45%	36%	9%	9%	9%	91%	9%	0%	0%	36%	64%	0%	
4 (9)	11%	0%	33%	56%	0%	0%	78%	0%	11%	11%	56%	22%	22%	
5 (10)	0%	10%	40%	50%	0%	0%	80%	20%	0%	0%	50%	50%	0%	

Table 7.2. Data showing the percentage tumour clearance for each ablation session, ablation depth, and tumour type. The numbers in parenthesis represents the tumours lasered in each session.

7.3.2.4 Tumour clinical type

Tumour type altered as the study progressed (See Table 7.2). In the first session 13% of the tumours were superficial and 87% were nodular, while in the last session 50% were superficial and 50% were nodular. Tumour type also contributed to the learning curve, as our selection criteria were not particularly strict since we were attempting to find the limitations of this technique. Tumour clearance for all margins (deep, lateral and smudge) was significantly related to tumour type. (Chi square $p=0.01$, chi square =8.89, DF=2). Only 3 of 21 superficial tumours were incompletely ablated (1 smudge, 1 deep and 1 lateral margin) In comparison 14 of 28 nodular tumours were incompletely ablated (10 deep margin, 3 lateral margin and 2 smudges.) Both of the clinically infiltrative tumours were ablated to the subcutis and had clear margins.

7.3.2.5 The relationship between clinical type, depth of ablation and tumour size

2 of 21 superficial tumours were incompletely ablated at the deep margin (including 1 smudge). The ablation depth in both tumours was to the upper dermis. There were no incomplete deep margins or smudges in any of the 17 superficial tumours that were ablated to the middle dermis or deeper. (See Figure 7.5) There was 1 lateral incomplete margin of a tumour in a Gorlin's patient which was ablated to the lower dermis. This 8mm tumour was ablated with a 2mm margin.

In the nodular tumours deep residue or smudges occurred at all ablation levels although this diminished from 100% failure in the upper dermis to 13% in the subcutis. (Chi square for trends $p=0.016$, Chi square =5.76, DF=1) In order to investigate whether there was a subgroup of nodular tumours suitable for laser ablation, the tumours were divided according to the size (above or below the median of 10mm) and into those ablated into the upper or middle dermis compared to those ablated to the lower dermis, subcutis and muscle. For nodular tumours, ablated to upper or middle dermis, there was a high incomplete ablation rate for both large (>10mm) or small (<10mm) tumours (80% and 67 % respectively). When large

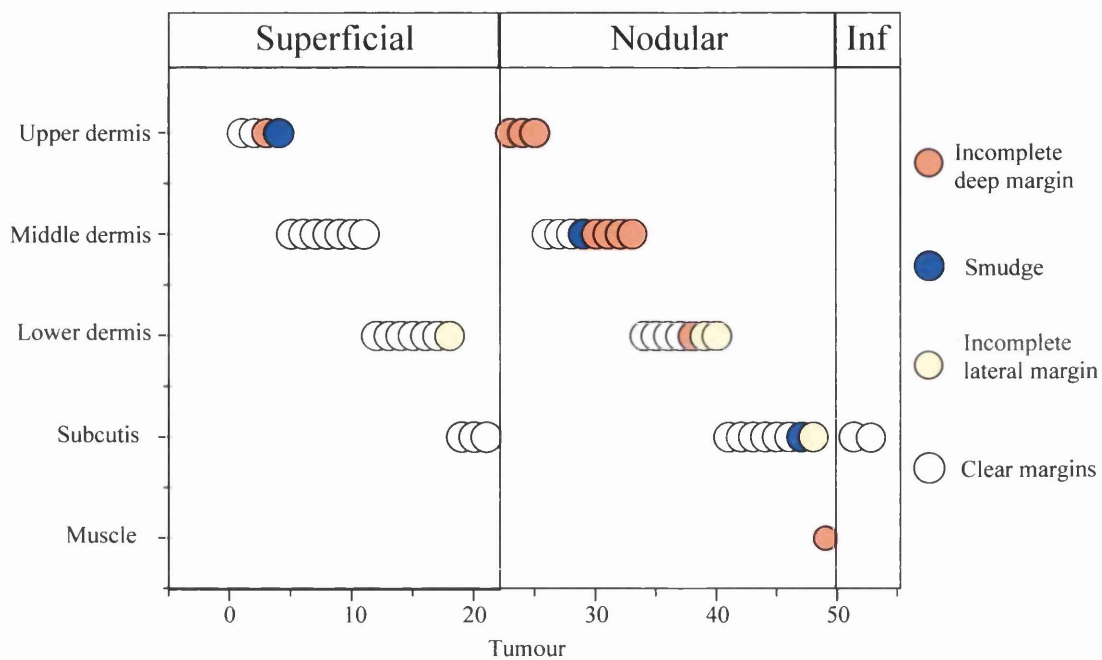


Figure 7.5. The relationship of tumour clinical type to clearance at variable ablation depths.

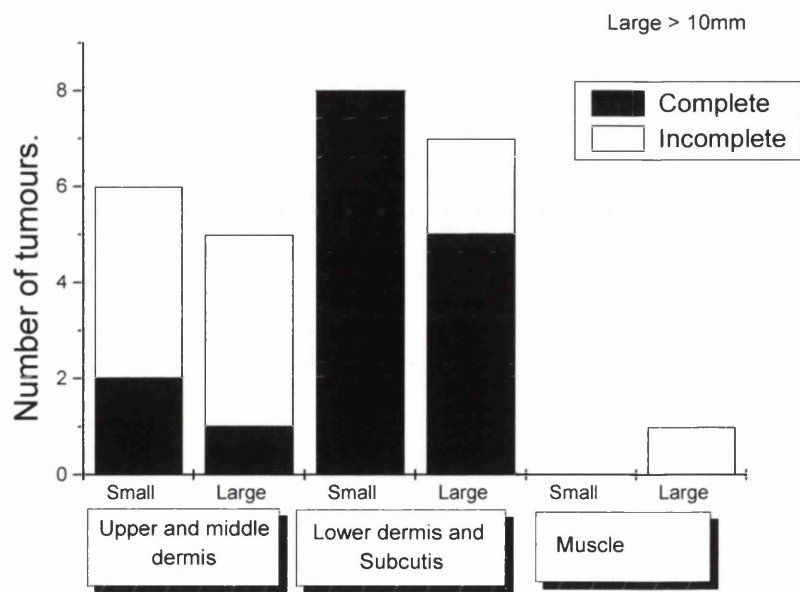


Figure 7.6. The relationship between the size of nodular tumours, ablation depth and clearance. This demonstrates that all of the small nodular tumours were ablated at the level of the lower dermis or deeper.

7.3.2.6 The relationship between clinical type and histological growth pattern.

The clinical and histological classifications could be compared in order to determine whether our ability to assess the tumours clinically may hinder patient selection for laser ablation. (See Table 7.3) It can be seen that there is not always agreement between the clinical and histological classification. Superficial tumours may histologically also include nodular or micronodular tumours. Macroscopically nodular tumours may microscopically include all types but importantly they may have an infiltrative component. (2 tumours). 2 of 3 histologically infiltrative tumours were misinterpreted as being clinically of nodular type and were both incompletely ablated. (Both were larger than 10mm).

Table 7.4 demonstrates the incomplete ablation rate (for the deep margin including smudge) as a determinant of clinical classification. This table also the incomplete ablation rates depending whether the clinical classification is in agreement or disagreement with the histological classification. This investigates whether the patients can be selected on clinical grounds for laser ablation. The data demonstrates that clinically superficial tumours, irrespective of their histological subtype can reliably be ablated provided they are lasered to a depth of the middle dermis or deeper. It appears that clinically superficial tumours are thin (irrespective of whether they are of superficial, micronodular or nodular histological growth pattern). On the other hand it can be seen that macroscopically nodular tumours cannot reliably be ablated irrespective of their microscopic subtype.

Histological type	Clinical type		
	Infiltrative	Nodular	Superficial
Infiltrative	1	2	0
Micronodular	0	9	3
Nodular	1	12	4
Superficial	0	4	14

Table 7.3. A comparison of clinical and histological types of basal cell carcinoma

		% OF TUMOURS WITH INCOMPLETE DEEP MARGIN OR SMUDGE .	
	Ablation depth	Superficial	Nodular
Clinical type	upper	50%	100%
	middle	0%	62%
	Lower	0%	20%
	subcutis	0%	85%
	muscle	-	100%
Histology in agreement with clinical type	upper	33%	100%
	middle	0%	75%
	Lower	0%	75%
	subcutis	0%	0%
	muscle	-	100%
Histology disagreement with clinical type	upper	100%	100%
	middle	0%	100%
	Lower	0%	60%
	subcutis	0%	16%
	muscle	-	-

Table 7.4. The incomplete ablation rate as a determinant of whether the clinical or histological classification are in agreement. (Lateral incomplete margins have been excluded).

7.3.2.7 Laser verses excision times.

Surgery and laser time (excluding local anesthetic time) were compared for each tumour. The laser time ranged from 1.5 -6 minutes (mean 3.7, median 4 minutes). The operation time ranged from 7-50 minutes (mean 17.1 median 15 minutes). The difference was highly significant (Paired t test $p > 0.0001$)

7.4 Discussion

Recent improvements in laser technology have resulted in small, user friendly and portable lasers suitable for the ablation of cutaneous lesions either by excision or vaporization. Scanning and ultrapulse techniques have reduced thermal diffusion and unwanted thermal necrosis extending outward from the ablation zone thereby minimizing local tissue damage, facilitating healing and reducing scarring. Although the ease and speed of this treatment modality seem very attractive, it is important that the efficacy, in terms of cancer clearance, is fully assessed before laser ablation of certain skin malignancies becomes commonplace.

Publications concerning the effectiveness of the carbon dioxide laser to treat basal cell carcinoma reveal diverse results. In one study 50% local recurrence at 1 year was attributed to cautious use of the laser, whilst another study demonstrated no recurrence in 370 superficial BCC with a 20 month follow-up (Wheeland, Bailin et al. 1987) There is no study critically examining the effectiveness of carbon dioxide laser ablation on a variety of histological subtypes by formally excising the tumour bed after laser ablation.

In this study, 51 BCCs of mixed subtype were ablated to a variety of different depths, then the tumour bed excised and histologically examined. Initially control of the depth of ablation was aided by a modification of the Wheeland sign.(Wheeland, Bailin et al. 1987) Unfortunately by utilising this method, we tended to ablate too superficially which resulted in incomplete ablation at the deep margin of the tumours. This was in

part due to the biopsy causing bleeding which obscured the subtle but visible tumour - dermal interface. As the study progressed we tended to ablate more deeply by ablating at least 2 passes beyond the disappearance of the tumour-dermal interface. This resulted in ablation to the lower dermis or subcutaneous tissue in the majority of cases. The increased ablation depth substantially contributed to the learning curve and improved complete ablation rates from 40% to 92%. Some tumours, that would not normally be suitable for laser ablation, such as the larger nodular tumours or those with an infiltrative component, were also selected to attempt to define the limits of this technique. We found that once ablation reaches the subcutaneous tissue, control of the depth of ablation becomes difficult because the contrast of white dermis and pink tumour was lost.

Tumour selection is of paramount importance if laser ablation is to be an effective treatment modality for BCC. In this study tumours which clinically appeared to be of superficial type could be effectively treated if they were ablated to a depth of the middle dermis or deeper. The 2 of 21 superficial tumours with incomplete deep margins were ablated to the upper third of the dermis only. The remaining 19 that had been ablated to the middle dermis or deeper and had clear margins. However, nodular tumours, are not all suitable for carbon dioxide laser ablation. Overall 39% (11/28) were incompletely ablated on the deep margin and 10% incomplete laterally. However if the tumours were selected for size it was found that small nodular tumours of less than 10mm could be ablated completely if ablated to the lower dermis or subcutaneous tissue (8 of 8) tumours. With ablation to this level, the advantage of laser treatment is diminished since re epitheliasation from adnexal structures will be substantially reduced resulting in delayed wound healing. Ablation to the lower dermis or subcutaneous tissue or deeper (to muscle) for larger tumours (>10mm) is not reliable There was a 37% incomplete ablation rate. We ablated 2 infiltrative tumours completely while attempting to stretch this technique and define the boundaries. We do not recommend this as good practice since this type of tumour, if inadequately treated, tends towards deep infiltration. Control of the margins by excision and formal histological assessment is recommended. With laser therapy, histological evidence of the margins is not available.

By comparing the clinical and histological classification we found that 2 clinically nodular tumours were of infiltrative histological growth pattern. Both were incompletely ablated despite ablation to the middle dermis or subcutaneous tissue. Both were larger than 10mm and would therefore be outside our recommendation for ablation of nodular tumours, however this questions whether nodular tumours should be ablated by this technique if it is possible for histologically infiltrative tumours to be unintentionally included. The group of clinically superficial tumours included 3 micronodular and 4 nodular histological growth patterns. Despite this difference all of these tumours were completely ablated. This suggests that the histologically micronodular or nodular tumours that appear to be macroscopically of superficial clinical type are thin tumours making them suitable for carbon dioxide ablation.

Lateral incomplete ablation occurred in 4 tumours, despite ablation margins ranging from 2 to 3 mm; 3 of these patients had Gorlins syndrome. Wider ablation may not always be possible or beneficial in this group of patients since the tumours are often so numerous that they encroach on each other. The differentiation clinically between normal skin and tumour may prove difficult in these patients due to multiple previous surgical episodes, scarring and ever-growing crops of new tumours. Our experience suggests that this group benefit from the ease and speed of laser ablation and attend regularly treating 8-110 BCCs in each session (Grobbelaar, Horlock et al. (In Press)). A combined approach utilising the laser and retinoids, to reduce tumour size and prevent new tumour formation, (Hodak, Ginzburg et al. 1987) may prove beneficial in the future. The other tumour with a incomplete lateral margin was a 12mm nodular tumour. As with deep incomplete ablation we would recommend that only small BCC of less than 10mm should be ablated which may avoid this problem.

A comparison of laser and excision time unequivocally demonstrated the rapidity of laser ablation compared to conventional surgery. Laser therapy was at least 3 times faster than surgery and would appear to be time and cost efficient. This is especially advantageous and in the groups of patients with multiple BCCs.

This study was not able to examine scarring following carbon dioxide laser ablation of BCC since all of the tumour beds were formally excised and reconstructed. We would expect that ablation depths to the middle dermis would result in complete re-epithialisation within 2 weeks. For lesions on the trunk and back the scars would compare well or be better than sutured closures in these areas that are known for scar stretching. It is for this reason that superficial BCCs, which occur most commonly on the trunk and limbs, would be most suitable. On the other hand small nodular BCC, which require deeper ablation, and occur more commonly on the head and neck, an area which heals well with surgical closure, would be less suitable due to delayed healing and inferior scarring than conventional excision.

In conclusion we have critically examined the role of the carbon dioxide laser to completely ablate BCCs by excising the tumour bed immediately after laser treatment. Complete ablation was significantly related to tumour subtype and to ablation depth ($p=0.01$ and 0.006). This study suggests that the clinically superficial tumours, irrespective of histological growth pattern are the most suitable tumours for this treatment. If they are ablated to the middle dermis or deeper, 100% had clear deep margins. We would expect rapid healing and favourable cosmetic results since the majority of this tumour type occur on the trunk and limbs. In contrast, nodular tumours cannot all be reliably ablated with the carbon dioxide laser. A small subset of nodular tumours, which are less than 10mm, had clear margins if they are ablated to the lower dermis or deeper. We would expect delayed healing at this ablation depth and poorer cosmesis in these tumours which occur more commonly on the head and neck. Furthermore histologically infiltrative tumours, which may macroscopically appear to be of nodular clinical type, could result in unrecognised incomplete ablation allowing deep infiltration. Incomplete ablation at the lateral margin was associated most commonly in patients with Gorlin's and was not significantly related to ablation margin. This fast treatment modality would be most beneficial to patients with multiple superficial BCCs.

Chapter 8

General discussion.

8.1 Introduction

It is apparent that BCC is an extremely heterogeneous tumour with huge variation in clinical and histological appearance. This tumour is treated by a wide variety of medical specialities including General practitioners, Dermatologists, Radiotherapists, Plastic Surgeons and ENT Surgeons. As a result a range of therapeutic modalities have been described to treat BCC. The majority of tumours are cured at first attempt but approximately 10% recur and a small proportion of these may progress to become multiply recurrent or may require disfiguring surgery for the chance of cure. A small proportion of tumours may behave extremely aggressively and become horrifying. The determination of the most suitable treatment modality and the analysis of causes for treatment failure is complicated by the lack of a simple uniform histological classification and reliable prognostic markers for BCC.

The behaviour pattern of BCC is determined by a many variables. Firstly, patient factors such as susceptibility to the development of a BCC influenced by skin type, immunity and susceptibility genes (DNA repair mechanisms). Also the behaviour of the patient such as the exposure to carcinogens e.g. UV light. The patient may also present late to the doctor with advanced disease which will also affect outcome in BCC. Secondly, medical factors will also influence the outcome in patients. The choice of therapeutic modality and the skill of the operator may alter the course of a tumour. Outcome is also determined by the accuracy of the histological report. Thirdly, tumour behaviour is controlled by the intrinsic invasive properties of each individual tumour. This is determined by the underlying cell biological processes that determines the clinical and histological variation seen in BCCs.

Despite BCC being the commonest cancer world wide, there is little understanding of the underlying genetic processes that result in the deregulation in cellular proliferation resulting in malignant transformation. From the preceding paragraph it can be seen that these factors are of fundamental importance in the clinical, histological and behavioural variation in BCC. Therefore the main aim of this thesis was to examine cell proliferation and key regulatory pathways and to correlate these with histology and clinical outcome. Furthermore, the data was available to assess the implication of some of the medical factors, such as treatment type and adequacy in relation to histological and biological variables.

8.2 Cell production in BCC

8.2.1 Rate measurements

Disrupted regulation of cell proliferation and cell loss is central to tumour development and growth and is the end result of genetic modification by the deregulation of oncogenes and tumour suppressor genes. This was therefore the starting point from which to investigate the cell biology of BCC. The study of proliferation may prove of considerable importance and value in three main areas. Firstly, it is an important biological variable that may allow further insight into the significance of cell proliferation to tumour growth, and this may be of particular value when studied in conjunction with other putative genetic or biological regulatory factors of cell turnover. A further, equally valuable, benefit is the prospect that it may yield information of prognostic significance in addition to that already available from histological criteria.

A variety of state or rate proliferative measurements were evaluated. There are few rate type measurements of cell proliferation in BCCs published. (Weinstein and Frost 1970; Heenen, Lambert et al. 1975; Galand and Heenan 1988). These authors have used tritiated thymidine and studied only small patient numbers (Heenen, Lambert et al. 1975) or have pooled data from multiple tumours (Weinstein and Frost 1970;

Galand and Heenen 1988) in order to calculate an S-phase duration. These studies have suggested that that T_s in BCC is approximately double that of normal skin at about 20 hours. This present study used modern, more powerful methods to calculate the cell proliferation rates in individual tumours by utilising *in vivo* bromodeoxyuridine labelling analysed by flow cytometry. A T_s duration was calculated by the relative movement method (Begg AC., McNally N.J. et al. 1985) ranging from 5.0-14.6 hours (median 7.6). This is faster than the findings of previous authors but is equal to the S-phase duration of normal skin which ranges from 5.9-16.0 hours.(Leigh, Lane et al. 1993)This is in keeping with the concept that the T_s duration of a tumour is similar to the tissue from which it is derived.

The concept of the T_{pot} has subsequently been developed as an additional proliferative rate measurement that also accounts for the number of cells in cycle in conjunction with the cell cycle time and is the theoretical measurement of cell proliferation in the absence of the cell loss. In this study the labelling index was measured by 2 methods. Firstly, by flow cytometry and secondly, by immunohistochemistry. These methods revealed very different results. This was because flow cytometry could not distinguish tumour and non tumour (i.e. stromal) cells in the absence of an aneuploid tumour population. Therefore the number of labelled cells was diluted by non proliferating stromal cells which reduced the LI by a mean value of 10%. However, as the different tumours had varying degrees of stromal cells within them this value was not a constant and therefore the LI calculated by immunohistochemistry was not related to the LI calculated by flow cytometry. We therefore considered the LI(im) a more reliable method than the flow cytometric technique. This was supported by a highly significant linear relationship of the LI(im) with the GF (from Ki-67 immunohistochemistry) which was absent using the LI (flow cytometry). The T_{pot} values ranged from 4 days to 18.3 days with a median value of 2.8 days. Therefore BCC is a highly proliferative tumour with a T_{pot} in the same range as other more aggressive epithelial cancers (Table 3.7).

8.2.2 State measurements

Static measurements of cell proliferation were also evaluated in BCCs by measuring both the BrdUrd LI and the Ki-67 growth fraction of the patients studied prospectively and also a large cohort of archival specimens (Ki-67 immunohistochemistry only). These results also confirmed that BCC is a highly proliferative tumour with a labelling index ranging from 0.07- 0.23 and a median of 0.14. The labelling index was directly related to the growth fraction by a linear relationship. The growth fraction was in agreement with other studies (Baum, Meurer et al. 1993; Healy, Angus et al. 1995) was high, ranging from 0.05 - 0.77 with a mean of 0.32. Indeed This value was greater than reported in many other solid tumours such as breast, gastric cancer or melanoma (Barnard, Hall et al. 1987) (Porschen R, Kreigel et al. 1991; Fogt F, A.O. et al. 1995.) Within tumours there was considerable variation of Ki-67 positivity with a mean C.V of 36.4%. The variation in positivity was described by 4 different proliferation patterns namely marginal (2.5%) , marginal diffuse (31%), random pattern (51%) or mixed (14.5%). The mean C.V. for the BrdUrd LI was 50.7% and in general there was agreement between the BrdUrd and Ki-67 proliferation patterns. It was difficult to determine the factors that controlled the proliferation patterns. There was some relationship with tumours size since the marginal tumours were smaller. Proliferation pattern was not related to p53 or bcl-2 expression. It is possible that the proliferation patterns are related to the stromal-tumour interactions by cytokines that either inhibit or promote apoptosis since it has been demonstrated that TGF- β is associated with increased proliferation at the periphery of some BCCs (Stamp, Nasim et al. 1993).

8.3 Cell loss in BCC

The proliferation data highlights the paradox of slow clinical growth in BCC whilst cell production rates are high. This in part may be due to the fact that BCC is a very heterogeneous population of tumours. While some do grow slowly others may develop more rapidly. However, this is not the entire answer since the aggressive

phenotype is less common and would not account for a median Tpot value of 2.8 days even taking into account the patient population selected in this study population. (In patients rather than day cases.) We therefore examined the issue of cell loss by assessing apoptosis, necrosis and desquamation from ulceration.

It has been suggested that high rates of apoptosis are found in BCCs and that this accounts for the balance of high cell production and slow tumour growth (Kerr and Searle 1972). However few studies have quantified these measurements. In this study apoptosis was quantified by counting apoptotic cells defined by morphological criteria. The proportion of apoptotic cells ranged from 0.01- 0.05 with a median value of 0.01. This value is of similar magnitude to other epithelial or skin cancers such as bladder transitional cell carcinoma and melanoma (Mooney, Ruis Peris et al. 1995; King, Matteson et al. 1996). It was difficult to equate the contribution of this level of cell loss with the degree of cell production since, at present, there is no rate measurement of apoptosis. It is suggested that the process, as seen morphologically based on nuclear and chromatin condensation, lasts minutes to hours (Wyllie A.H. 1993). Therefore, the cell loss from spontaneous apoptosis would approximate a rate of 1%-5% per hour. For the tumour to remain static this would have to equal the cell production rate. A median cell production rate of 1.8% per hour was calculated from the LI divided by the Ts. These calculations suggest that the spontaneous apoptosis alone could account for the slow clinical growth of BCC.

Necrosis, as a mechanism for cell loss, was present in only 40% of BCCs. In approximately half of the patients this was associated with cyst formation. The mechanism of cyst formation has been previously described as a result of degeneration of stroma or due to sebaceous differentiation (Lever 1971). All patients with necrosis had some degree of ulceration. In all, 62 % of the tumours had ulceration at the surface which represents an addition route for cell loss. Ulceration could occur without necrosis. It is possible that the ulceration in these tumours may be due to other cause such as trauma or scratching.

The growth kinetics of BCC can therefore be explained by the relative high rates of cell production being balanced by cell loss due to spontaneous apoptosis, necrosis and

desquamation from ulceration. In order to understand these processes further the genetic control of cell production and cell loss was evaluated by studying three important genes, namely bcl-2, bax and p53.

8.4 Regulation of cell proliferation and cell loss in BCC

The cell survival/apoptosis switch is determined by the relative quantities of bcl-2 and bax and associated proteins. bax homodimers promote apoptosis while bax/bcl-2 heterodimers suppress apoptosis. The p53 tumour suppressor gene is one of many pathways that can activate apoptosis. In response to DNA damage, p53 can either cause G1 cell cycle arrest or, by up regulating the transcription of bax, can initiate apoptosis. In this study bcl-2 and p53 were measured in relation to the cell production in Chapter 3 and relation to histology and outcome in Chapter 6. Bax protein was evaluated in a smaller number of tumours in relation to cell proliferation in Chapter 3.

8.4.1 Bcl-2 family

Bcl-2 protein was expressed in 88% of the 153 tumours analysed in Chapter 6 and in 82% of the 22 tumours analysed in Chapter 3. Differential expression was noted between tumours ranging from mild to strong staining. High bcl-2 expression might reflect the stem cell origin of BCC since bcl-2 is detectable in basal layer of normal epidermis but not in the more superficial layers. (Raskin 1997). Bcl-2 expression is switched off in terminally differentiated cells. (Raskin 1997). The results concerning apoptosis and bcl-2 expression in this thesis represents a dogma. Why should there be overexpression of the anti apoptotic oncogene bcl-2 yet there is a high rate of cell loss by apoptosis. This was addressed by measuring bax protein expression since high level of bax would compensate for overexpression of bcl-2 and tip the balance in favour of apoptosis. Bax may be the key apoptotic regulator in BCC rather than bcl-2. Contrary to expectations, the majority of BCCs (77%) did not express bax protein and the bax and bcl-2 protein expression were not related. Additionally, there was no relationship between bax expression and the apoptotic index suggesting that increased

bax expression was not the basis for spontaneous apoptosis in BCC. The driving force behind the apoptosis in BCC therefore remains unknown. It is possible that the relative concentrations of bax and bcl-2 is not the only determinant of the process as they can be functionally modified by post translational modification. However, it is most likely that the survival/apoptosis balance is determined by other oncoproteins. These may either be member of the bcl-2 family such as bak, bcl-xs, bad, bcl-w and bcl-xl. Other possibilities such as the induction of apoptosis by cytotoxic lymphocytes by mechanisms that bypass the action of bcl-2 or the action of IL-2 or interferon gamma up regulating the fas ligand that may overwhelm bcl-2 (Raskin 1997).

An interesting relationship was found between bcl-2 expression and cellular proliferation. In Chapter 2, tumours with little or absent bcl-2 staining had a significantly higher GF or LI than those with moderate or strong staining although the numbers were small. This relationship was maintained if the bcl-2 expression of the larger cohort of patients (total 142) was compared with the GF (Chapters 5 and 6 respectively). Tumours with low (mild or no intensity) bcl-2 staining had a significantly higher GF than those with a high (moderate or strong) bcl-2 intensity. (t test: $p=0.005$) This is illustrated in Figure 8.1. It is uncertain why this relationship should exist. It is possible that the tumours expressing high bcl-2 expression may mirror the behaviour of the slow cycling stem cells which are postulated as the cell of origin of BCC.

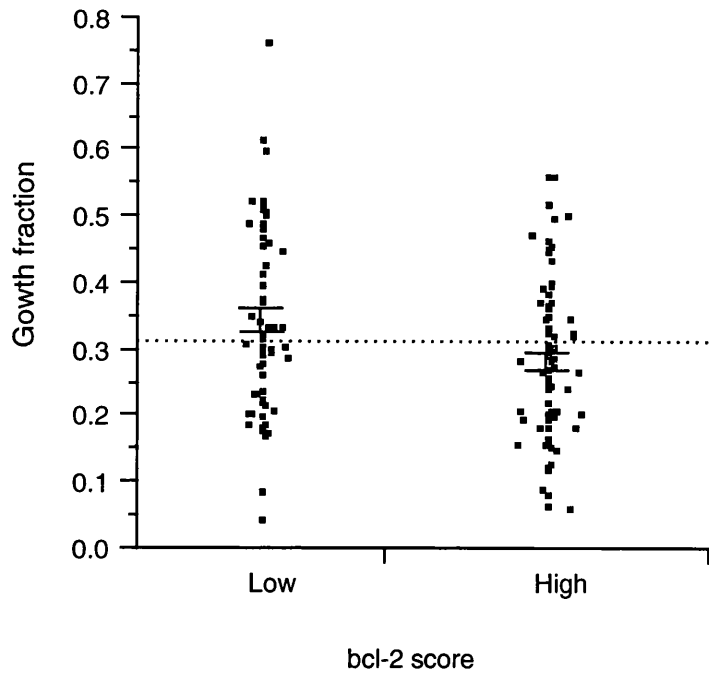


Figure 8.1. The relationship between bcl-2 expression and the growth fraction in 142 BCCs. (Error bars represent standard error of the means.)

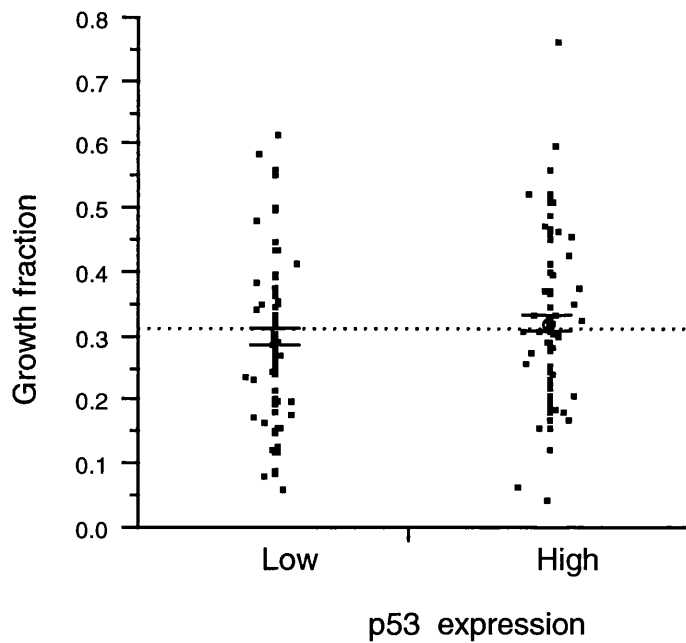


Figure 8.2. The relationship between p53 expression and the growth fraction in 153 BCCs. (Error bars represent standard error of the means.)

8.4. 2 p53

Examination of p53 expression was further evidence to support the concept that the bcl-2/bax pathway is not the main determinant of apoptosis and cell proliferation in BCC. A clear relationship would be expected between p53 and bcl-2 and bax expression if this pathway was important in the control of apoptosis in BCC.

p53 expression tended to show a binomial distribution with either high (>50%) or low (<25%) staining with relatively few tumours with intermediate staining. This may reflect the clonal population of the tumour populations accumulating 53 protein or harbouring a p53 mutation. Tumours with intermediate staining may represent either tumours with more than one clonal population or the accumulation of p53 mutations in some cells due to tumour progression or UV damage induced expression of wt p53.

Half of the 154 tumours studied in Chapter 6 stained positively (>25%area) for p53. The positive staining represents the accumulation of mutant or wild type p53 since the antibody D-07 was used. From this study we are unable to be certain that those tumours which stained positively contained mutant p53 or had accumulated normal p53. It has been shown that p53 immunopositivity is not always associated with a mutation and immunonegative tumours may contain a p53 mutation (Campbell, Quinn et al. 1993). The finding that 50% of the tumours accumulated p53 is in agreement with other authors who have demonstrated 48-50% of BCCs having p53 mutations (Rady, Scinicariello et al. 1992; Moles, Moyret et al. 1993; van der Riet, Karp et al. 1994). It is considered that the p53 mutation is caused by UV radiation since they are of the characteristic mutation signature (Ziegler, Leffell et al. 1993; Gailani, Leffell et al. 1996) and usually only one p53 allele, rather than both, is mutated. It is therefore possible that a sunlight induced mutation may mutate one p53 allele. This sequesters the other normal allele by a dominant negative mechanism and therefore prevents p53 from causing G1 arrest in response to DNA damage. This would release the cell from the normal regulatory growth restraints and allow increased cellular proliferation and reduced apoptosis. This would also facilitate the accumulation of further genetic

damage which would not be repaired or eliminated by the apoptotic process thereby promoting tumour progression.

In keeping with this concept, there was a trend that the growth fraction was slightly higher in tumours with a high p53 expression in the small cohort of patients in Chapter 2 (0.28 for the low p53 expression versus 0.37 for high p53 expression. t test $p=0.07$). This relationship can be examined further by combining the results from Chapters 5 and 6. A small difference was seen in the growth fractions (0.30 versus 0.32) but this was not statistically significant. (t test $p=0.2$) (See Figure 8.2). It is possible that the absence of a clear relationship between p53 expression and cell proliferation is because not all of the p53 positive tumours had mutated or dysfunctional p53 and there was therefore some degree of growth control in p53 positive tumours. For example some cells may have accumulated wild type p53 as a response to DNA damage or due to stabilisation by other mechanisms. It should also be noted that there are many other cell cycle control mechanisms exist in addition to p53, and therefore it is unlikely that a clear relationship will be seen with cell proliferation and p53 expression.

There was no relationship found between the p53 expression and the apoptotic index suggesting that p53 is not the main determinant of apoptosis in BCC although the numbers studied were small. Furthermore a low apoptotic index would be expected if there were a high proportion of tumours harbouring dysfunctional p53. Although the determination of the relative quantity of apoptosis was simple in this study, it would seem that the level of apoptosis was not low. It is uncertain whether the apoptosis is being determined by a p53 independent pathway or that the p53 in BCCs is functional and therefore relatively high rates of apoptosis are found.

If p53 was an important determinant of the apoptotic rate in BCC, then a clear relationship between p53, bax and bcl-2 would be expected. In Chapter 2 this was not the case, although the numbers in the study were small. Bax and bcl-2 expression were not related to p53 expression. If p53 were functional then DNA damage would result in the increased transcription of bax relative to bcl-2 such that apoptosis would be switched on.

This, therefore, questions the role of p53 mutations as a key genetic abnormality in BCC. If a p53 mutation was a prerequisite for the development of BCC then the majority of BCCs would harbour p53 mutations. Evidence from this study and from other authors does not support this concept since, only about 50% of BCCs harbour mutations in mutation hotspots (exons 5-8) (van der Riet, Karp et al. 1994). This does not exclude the possibility that there are mutations in other regions of the gene. However these would be unlikely to account for the other 50% of tumours. It is conceivable that there may be more than one genetic pathway that results in the development of a BCC and that some are caused by p53 dependant pathways whilst others are p53 independent. This concept has been postulated by other authors who considered that the UV induced tumours in sun exposed areas on older patients are a result of p53 mutations while BCCs in younger patients in less sun-exposed sites may be due to 9q allele loss (D. Errico, Calcagnile et al. 1997).. The data from this study did not support this concept since younger patients had significantly greater p53 staining than older patients (Chi square $p=0.01$).

More recently there is evidence to suggest that aberrations in the *patched* gene is the main genetic route to BCC development. The *patched* gene is a putative tumour suppressor gene that is responsible for Gorlin's syndrome and has recently been proposed as the gatekeeper gene for BCC since it is mutated in the majority of BCCs irrespective of type and size. (Bale ; Farndon, Morris et al. ; Gailani, Leffell et al. 1996; Gailani and Bale 1997). This results in loss of the negative feedback that controls patched and results in overexpression of patched mRNA in all BCCs o far studied (Unden, Zaphiropoulos et al. 1997). The exact mechanism of the function of *patched* on the control of cell proliferation and cell loss is unknown although this group of genes can alter cell proliferation via effectors such as the Wnt and TGF β proteins. The downstream result of the *patched* gene mutation may therefore be an important determinant of cell proliferation and apoptotic rate that may therefore explain the absence of a clear relationship between these factors and p53, bax and bcl-2. p53 mutations may be involved with progression in some tumours and may therefore influence histology and the behaviour of BCCs. The influence of p53, and

bcl-2 and cellular proliferation on histology and behaviour was examined in the Chapters 4,5,and 6 of this thesis.

8.5 Histology of BCC

BCC exhibits a huge variation in clinical and histological appearance. From the comparison of clinical and histological subtype in Chapter 7 it can be seen that the histological type cannot always be predicted from the clinical appearance (Table 7.3). This clinical and histological variation has hindered the development of a uniform histological classification system that can easily describe individual tumours and therefore a multitude of methods have been utilised. As a result, confusion can arise in the literature since like cannot be compared with like if different classification are used. One of the most common classifications used is that described by Lever (Lever 1971). While this system is fully comprehensive, it is difficult to isolate any tumour in a single subgroup. This is because it is based on the differentiation subtype of each tumour and most tumours are heterogeneous and display a variety of differentiation states within them. Consequently some studies found the histological appearance of BCC of little prognostic value (Jackson and Adams 1973) (Pascal, Hobby et al. 1968) (Shanoff, Spira et al. 1967).

The importance of the growth pattern as a determinant of the behaviour of BCC followed the work by Thackary and Sloane (Thackary 1951; Sloane 1977) and this has provided the basis for many applicable and clinically useful classification systems (Jacobs, Rippey et al. 1982; Emmett 1990; Sexton, Jones et al. 1990). These authors emphasised the differences between the well circumscribed nodular variety from the infiltrative variety which have less well developed peripheral palisading often combined with a sclerotic and fibrotic stroma. In this present study, we have developed a novel classification system based on the principles of the growth pattern combined with a further description of the commonest differentiation subtype. This system best describes the spectrum of BCCs and we have related this to histology and outcome. Because there is a spectrum, there may be some degree of overlap between

categories, however, there proved to be a minimal number of tumours in which this was a problem.

8.5.1 The relationship of patient age, sex and tumour positions to histology

At present there is incomplete understanding of the factors that determine the histological diversity of BCC. In this study, there were no age or sex differences between the histological types. This is at variance with one study that found a greater number of infiltrative tumours in young patients (Leffell, Headington et al. 1991). It has been demonstrated that young patients who develop BCCs have impaired DNA repair (Wei, Matanoski et al. 1993). It is conceivable that these patients develop specific mutations that determine the development of aggressive type histological patterns since these types of tumours have been associated with different oncogene expression in comparison to less aggressive variants. This will be examined in more detail later.

In this study, there were marked differences in histological type with tumour position. In both Chapters 4 and 7, the superficial type was found most commonly on the trunk, although the other varieties were evenly distributed. This may represent differences in the causative aetioloical agent of BCCs in various positions. For example, the trunk has been considered a less sun exposed site. The mutation spectrum of the aetiological agents may vary and result in different mutations of different oncogenes resulting in variations in the growth pattern. However the habit and degree of sun exposure of each patient was not recorded and therefore it is uncertain whether the aetiological agent for the BCCs in different positions altered. Alternatively, the histological variation may be a result of local epigenetic influences due to dermal thickness and the number and type of adnexal structures altering the cytokine milieu. A further alternative explanation is that the histological growth pattern may represent alteration in the stem cell of origin. As an example, tumours on the scalp may be more likely to result from a stem cell of a hair follicle rather than an interfollicular stem cell from a

less hairy region. Finally the variation in histology may represent changes due to tumour progression. i.e. all tumours were initially identical but have changed due to tumour progression from accumulation of further mutations. However, this is not supported by the fact that there was relatively little change in the growth patterns in those tumours with multiple successive specimens.

8.5.2 The tumour biology of the histological subtypes of BCC

In this study there was some biological variation demonstrable between the different growth patterns. This would be expected since, as discussed above, the growth pattern is probably a result of the interplay between differing mutations of several oncogenes and additional influence of cytokines from the peritumoural tissue.

An important finding was that the infiltrative and morpheic tumours were significantly more proliferative than the nodular tumours. A small preliminary study of this relationship was performed in Chapter 2. The infiltrative tumours were found to have a greater LI, GF and shorter Ts and Tpot than the nodular tumours. Additionally, there was a higher apoptotic index in the infiltrative tumours. (Table 3.6, chapter 3). This relationship was confirmed in the larger patient series in Chapter 5. The infiltrative and morpheic tumours had a significantly higher growth fraction than the nodular or micronodular tumours. Superficial tumours were of intermediate growth fraction. This finding is at variance with other authors (Baum, Meurer et al. 1993; Healy, Angus et al. 1995). However this reflects the inadequacies of categorisation by other classification systems, since, if the tumours in this study were classified according to differentiation, then no difference was seen. Other authors have demonstrated proliferative differences between histological variants of BCC (Toth, Guenther et al. 1996; Barrett, Smith et al. 1997). One reason why the infiltrative and morpheic tumours should be more proliferative is that they were the least differentiated of all tumour types (See Chapter 4) and terminal differentiation will result in a cell exiting of the cell cycle.

The growth pattern was not only determined by the relative numbers of proliferating cells within them but also by the spatial arrangement of the proliferating compartment. The random proliferation pattern was found most commonly in the infiltrative (70% of tumours) or morpheic tumours (80% of tumours) while the marginal diffuse pattern was found most commonly in the nodular (40%) or micronodular tumours (40%). The results do not demonstrate that particular proliferation patterns are associated with specific differentiation states. This is due to the finding that most tumours contain more than one differentiation subtype. It was apparent, however, that marginal or marginal diffuse patterns were associated with attempts at cystic type differentiation. Therefore the nodular tumours, that exhibited the greatest amount of differentiation, tended to have more marginal diffuse proliferation than the less well differentiated infiltrative or morpheic types.

The preliminary finding in Chapter 3 which suggested that the infiltrative tumours exhibited more apoptosis and less bcl-2 protein expression than the nodular tumours was also further substantiated in Chapter 6. There was a progressive loss of bcl-2 from the superficial or nodular subtypes to the morpheic and infiltrative subtype, although the greatest difference was seen with the morpheic tumours. Increased apoptosis in these groups is in keeping with loss of bcl-2. The underlying cause of the loss of bcl-2 expression is unknown. It is conceivable that this may be due to the interaction and down regulation by other genes such as p53, since some authors have suggested increased p53 expression in histologically aggressive BCCs (De Rosa, Staibano et al. 1993). However this study does not support this hypothesis for two reasons. Firstly, there was no relationship between p53 expression with histological growth pattern. Secondly there was no relationship between p53 and bcl-2 expression in Chapter 6, although, as a caveat, in smaller patient numbers in Chapter 2 it appeared that the infiltrative tumours were more commonly p53 positive.

The consequence of loss of bcl-2 expression is postulated to produce an increasingly invasive tumour with local infiltration more akin to squamous cell carcinomas. It is of interest that SCCs do not express bcl-2. (Verhaegh, Sanders et al. 1995) and lack of bcl-2 was associated with squamous metaplasia in this study. The association of loss

of bcl-2 and invasion is probably a result of a combination of genetic and epigenetic interactions that represents the further loss of growth control.

8.6 Clinical outcome in BCC

One of the aims of this thesis was to assess histological and biological parameters in BCC such that they could be correlated with outcome and evaluated as possible prognostic markers. Traditionally, this would be evaluated by Kaplan Meyer analysis of survival and disease free intervals. In basal cell carcinomas these values are less well defined since most patients survive and are cured, or others continue to survive with a slowly progressing tumour. In recurrent tumours, the patients are often rereviewed at regular intervals with the possibility of a recurrence which makes the determination of disease free interval difficult to assess. The data in this study was, therefore, analysed according to three endpoints; no recurrence, recurrence or the development of a horrifying tumour. As previously mentioned, the outcome is dependant on patient factors, treatment factors and on tumour biology. It was evident that the horrifying groups could result from multiple treatment failures (early presenting) or from patient neglect (late presenting) and therefore this group was subdivided. The horrifying group are unusual and pose a formidable management problem and have therefore been of great interest.

8.6.1 Relationship of patient factors to outcome

Late presentation was associated with 20 of 36 horrifying tumours. It could also be argued that the recurrent tumours presented later than the primary tumours since they were 2mm larger at initial presentation (10 versus 8mm). The finding that horrifying tumours, at first appearance, was more prevalent in younger patients is also a significant finding. Although there was no overall relationship of histological growth pattern with age, these tumours represent the infiltrative tumours presenting in younger people. This fact, combined with some treatment factors (discussed later),

was probably important in contributing to the outcome in this patient group. Recurrent and horrifying tumours were more common in males than females. The reason for this finding is difficult to elucidate in the absence of any biological differences being found between tumours of males and females. It is conceivable that this may be due to a combination of later presentation in males combined with some treatment factors. eg the males were treated with radiotherapy more commonly than female (28% versus 17%) and the females had a greater proportion of complete excision (61% versus 49%) although incomplete excision were of similar magnitude in each group (12 versus 13% in each group).

8.6.2 Medical factors and outcome

Treatment modality and adequacy were very important determinants of outcome in BCC. Complete excision was reported in 79.5% of primary tumours and only 17.5% of recurrent tumours. None early presenting horrifying tumours initially had complete excision. The high proportion of complete excisions in non-recurrent compared to recurrent tumours is highly significant (Chi square $p < 0.001$). It was an important finding that 23.9% of recurrences were associated with complete excision initially. It is unlikely that these were new primary tumours occurring because they were found in an identical position to the primary and successive biopsies of recurrent tumours were usually of a similar histological subtype. It is most likely that this finding represents inadequate sampling of the tumours by conventional histology, although this study does not prove this issue. Tongues of tumour cells that protrude asymmetrically from the tumour will not be sampled by midline sections and therefore result in a false complete excision. This is supported by the finding that larger tumours and infiltrative or morpheic subtypes tended to recur more commonly after a report of complete excision.

Only 2.3 % of the primary tumours had incomplete excision compared to 31.1% of the recurrent tumours and 25% of the early presenting horrifying tumours. These differences were highly significant (Chi square $p > 0.0001$ and $p = 0.0003$ respectively). This is at variance to other studies that suggest that approximately 30-40% of

incompletely excised tumours recur (Hauben, Zirkin et al. 1982; De Silva and Dellon 1985; Dellon, DeSilva et al. 1985; Richmond and Davie 1987) This is probably partly due to the selection of patients in this study. If the population size of non recurrent tumours had been larger then there may have been a greater proportion of non recurrent incompletely excised tumours. However, bearing in mind the finding that incomplete excision is an important determinant of recurrence, it would appear unlikely residual tumour would not result in recurrence. It has not been demonstrated that there was residual tumour in situ with a with the studies showing low rates of recurrence following incomplete excision (Hauben, Zirkin et al. 1982; De Silva and Dellon 1985; Dellon, DeSilva et al. 1985; Richmond and Davie 1987) This group probably represents sampling error resulting in a false incomplete excision margin in some patients with nodular type tumours that shell out of their stroma and appear incomplete. This would explain the findings of Dellon (Dellon, DeSilva et al. 1985) who demonstrated low recurrence rates for tumours with well defined peripheral pallasading.

Significantly fewer of the primary tumours had radiotherapy compared to the recurrent tumours (14.8% versus 32.6%). This difference reached statistical significance (Chi square $p=0.05$). This may reflect referral patterns since relatively few of this series of primary tumours had radiotherapy and the post radiotherapy recurrences tended to be referred to plastic surgery for further treatment. 62.5% of the early presenting, horrifying tumours received radiotherapy. This was significantly higher than both the primary or recurrent tumours (Chi square $p>0,0001$ and $p=0.02$). This finding is not because the radiotherapy treated tumours were more aggressive initially as they were of a similar size and histological subtypes to the other treatment groups.

These results demonstrate the importance of the treatment modality on the outcome. We therefore examined the outcome for each histological type of tumour and also correlated outcome with some of the biological variables. This would evaluate the differences in the tumours treated with each treatment modality.

8.6.3 Histology and outcome

There are conflicting reports relating histology to outcome in BCC. Some studies have associated recurrence with particular histological variants (Thackary 1951; Sloane 1977; Hauben, Zirkin et al. 1982; Dellon 1985; Dixon, Lee et al. 1989; Emmett 1990; Dixon, Lee et al. 1993) while others find no association. (Pascal, Hobby et al. 1968) (Shanoff, Spira et al. 1967) This is in part due to the inadequacies in histological classification but also due to the influence of other variables such as adequacy of treatment which has been demonstrated as a major determinant of outcome. In this study the non recurrent group were biased towards an aggressive histological subtype due to their referral to plastic surgeons. This was apparent when comparing the range of histological subtypes in this series with other reported studies. Anatomical variation was also an important factor since tumours on the lower limb, which may require skin grafting due to the paucity of excess skin, were commonly of infiltrative subtype. Irrespective of this bias, there was a small excess of morpheic and infiltrative tumours in the recurrent group and a significantly larger proportion of infiltrative tumours in the both of the horrifying subtypes suggesting that histological growth pattern is important. In comparison, the finding that there was not as many infiltrative and morpheic tumours in the recurrent group as expected reflects the influence of treatment factors. This is demonstrated by the presence of a high proportion of incompletely excised nodular tumours found in the recurrent and horrifying groups. The higher proportion of nodular tumours represents differences in the skill of the surgeon and also the finding that these are common on the face which may result in a conservative excision in favour of cosmesis.

8.6.4 Medical factors and histology

The incomplete excision rate for the infiltrative and morpheic and micronodular tumours is higher than that reported by Sexton (Sexton, Jones et al. 1990) who reported incomplete excision rates for these tumours at 26.5%, 33.3% and 18.6%. This had arisen because the present series is biased by recurrent and horrifying tumours in which incomplete excision is more common. Irrespective of this, there is a high proportion of incompletely excised nodular tumours. This finding reflects poor

initial surgical management resulting in a high proportion of recurrent nodular tumours. It is an important finding that the incompletely excised nodular tumours were larger than those in the completely excised group. This might be due to a more extensive subclinical extension of the larger nodular tumours. This was demonstrated by Breuninger who showed a 30% incomplete excision rate in tumours greater than 10mm when excised with a 2mm margin. (Breuninger and Dietz 1991) This issue is also compounded by the nodular tumours being found most commonly on the face.

8.6.5 Outcome in relation to histology and treatment modality

It is apparent that the recurrence was determined by tumour and surgical factors. These are summarised in Figure 4.6. Surgical factors determined the recurrence of nodular tumours since Incomplete excision of nodular tumours resulted in recurrence. Tumour factors may have played a more dominant role in the recurrence of those that recurred following complete excision. This was probably due to sampling error as a result of the irregular microarchitecture of particular tumours especially the large and infiltrative tumours. Larger tumours also tended to recur more commonly. Firstly because the larger nodular tumours tended to be incompletely excised more commonly but also because larger tumours tended to recur following complete excision or radiotherapy. Radiotherapy also seemed to be associated more commonly with recurrence, however this may represent referral patterns. However radiotherapy was most commonly associated with the development of a horrifying tumour. Whether this was due to unknown patient factors or the possibility that radiotherapy may alter the normal local defences allowing deep infiltration is unknown.

8.7 Biological markers of outcome in BCC

The three biological markers, namely Ki-67, bcl-2 and p53, were evaluated with the clinical parameters such as complete excision or recurrence. This was to assess their value as potential prognostic markers.. A summary of the relationships of these parameters shown in Table 8.1.

	Ki-67	Prol. Pattern	bcl-2	P53	Size
Complete / incomp exn	Anova p=ns	chi square p=ns	chi square p=ns	chi square p=ns	anova p=ns nod. p=0.03
Overall outcome	anova p=0.4	chi square p=ns	chi square p=0.04 rec. p=ns hor	chi square p=ns	anova p=0.02 rec p=0.3 hor
Rec. post comp. exn	anova p=ns	chi square p=ns	chi square p=0.009	chi square p=ns	anova p=ns
Rec. post Dxt	anova p=ns	chi square p=ns	p=ns	chi square p=ns	anova p=0.02 rec p=0.02 hor

Table 8.1. The statistical relationship of Ki-67, p53 and bcl-2 to clinical outcome.

Ki-67 was not able to predict overall outcome either in all tumours or in any of the individual histological subtypes. When examining the outcome for each treatment modality, the growth fraction was higher for the recurrent completely excised tumours than the non recurrent tumours. This was because more of these tumours were infiltrative. However, because larger nodular tumours also recurred following complete excision there was no overall statistical difference. The growth fraction was not able to predict completeness of excision since there was no difference in the growth fraction of the completely or incompletely excised tumours (mean = 0.310 and 0.314.) The growth fraction was not different for outcome following radiotherapy. This was slightly surprising, since in Chapter 4, there was a suggestion that the infiltrative tumours appeared to be more common in the recurrent and horrifying groups. When examining the patterns of proliferation, similar results were found. The patterns of proliferation could not predict overall outcome or the outcome following a particular treatment modality.

Bcl-2 expression revealed variable results in predicting the outcome in BCC. It was apparent that recurrent BCCs expressed less bcl-2 than primary tumours. This may be explained by the finding that infiltrative or morpheic tumours expressed less bcl-2 and these types are more prone to recurrence. However, there was no difference in the early presenting horrifying tumours and non recurrent tumours. This discrepancy is explained by the finding that bcl-2 was a predictor for outcome following complete excision ($p=0.009$) but not for radiotherapy. A large proportion of the early presenting horrifying tumours were initially treated with radiotherapy and therefore bcl-2 did not predict the outcome. Bcl-2 would therefore not be a clinically useful investigation since these patients are the group that would be the most useful to identify at an early stage.

p53 failed to predict outcome in any of the patient groups. This was because there was no apparent relationship between p53 and histology. It is most likely that there should be an underlying relationship between p53 mutation and histological subtype or invasive capability but this is not demonstrated by using the D-07 antibody. antibody identifies accumulation of both wild type and mutated p53 and positive staining is therefore the result of a variety of genetic or epigenetic influences some of which may be correlated with favourable outcome and others with a poor outcome.

These data indicate that the outcome in BCC is not related solely to the tumour biology. Outcome is related to a combination of patient, treatment and tumour factors. The relative importance of each of these factors being different in each circumstance. For example a small nodular tumour may recur if treated inadequately or a larger infiltrative tumour may not recur if treated well. The variety and adequacy of treatment regimens found in BCC is heavily influenced by the face being the most common anatomical site. Therefore, tumour size becomes a prognostic feature. It is difficult, for cosmetic considerations, not to influence the treatment adequacy. Incomplete excision is therefore commonplace. This is unlike melanoma in which there are standardised treatment regimens and incomplete excision is uncommon and there biological considerations become an important determinant of outcome. Furthermore, local control is most influenced by treatment adequacy while metastatic

dissemination is determined by biological factors. Biological measurements in BCC are therefore less important predictors of outcome.

The role of prognostic markers in BCC are therefore limited. Adequate excision alone will provide a cure. This is determined by surgical skill and reconstructive capability combined with adequate histological sampling of the margins of the tumour. Complete excision, macroscopically and microscopically, should therefore be assured. The role of radiotherapy in the treatment of BCC is uncertain. Cure rates are reported to equal those of surgery. However, a high proportion of the early presenting horrifying tumours underwent primary radiotherapy in this study. There was no evidence that these tumours were biologically more aggressive than non horrifying tumours. Whether this is due to post treatment biological changes in the tumour or that the local immunological control of the tumour by the body alters is unknown. Another complication of radiotherapy might be that recognition of tumour margins both macroscopically and microscopically is compromised thereby making complete excision harder to assure. This may result in further recurrence. Further investigation is required if radiotherapy regimens are to become standardised for BCC and prognostic markers may play a more important role in determining which tumours may or may not respond to this modality.

8.8 Evaluation of the optomechanically flash scanned carbon dioxide laser

In the absence of biochemical markers of outcome of recurrence for BCC, the results of this treatment were evaluated by the completeness of excision. This clinical study closely examined the clinical and histological subtypes treated by the laser. The clinically superficial tumours, which may include a subset of microscopically nodular or micronodular tumours, could reliably be treated by the carbon dioxide laser as they are confined to the upper parts of the dermis. Residual tumour could reliably be seen within the confines of the dermis by the Wheeland method and therefore ablation depth could be carefully controlled. Some nodular tumours could also be

reliably treated. In agreement with Chapter 4 Size was an important factor in this subtype. Small tumours (less than 1cm) did not penetrate through the dermis and could, therefore, be ablated provided that they were lasered to a depth of the deep dermis or deeper. Larger tumour extended more deeply and therefore could not reliably be ablated. In one tumour there was still residual tumour even at ablation depths to the muscles. It is possible that this may be a mechanism by which tumour size was related to failure of radiotherapy. The larger nodular tumours may penetrate more deeply than expected to a level in excess of the depth that superficial x irradiation can penetrate. However this is speculation since the type of X irradiation was not always recorded and therefore not documented in this study. It is also possible that some tumours which appeared nodular were of infiltrative histological subtype and penetrated more deeply than was apparent. The finding that some tumours which appear nodular may be of variable histological subtype has not previously been reported and could be an important determinant of failure in patients treated with curettage. While nodular tumours may shell out easily some tumours that appear nodular and are treated by this modality do not shell out and thus infiltrative tumour may remain in situ.

The speed of the carbon dioxide laser makes this a very attractive modality to treat patients with multiple BCCs especially those with Gorlin's syndrome. However due to the multitude of tumours in these patients it is difficult to ablate these lesions with clear lateral margins since the majority of the patients skin may contain small foci of BCC. However this is common to all methods of treatment in these patients and we have found that the laser is superior since a large number of tumours can be treated in one sitting which is beneficial for overall tumour control(Grobbelaar, Horlock et al. (In Press).

In conclusion, this study has evaluated some of the biological processes that determine the growth characteristics of BCC. Biologically, histologically and clinically BCC is an extremely heterogeneous group of tumours characterised by rapid cell proliferation balanced with equally high rates of cell loss We have related these factors to the outcome in this tumour. We have found that the tumour biology is not a major

determinant of recurrence. Medical factors such as the type and adequacy of treatment are also important. It is most likely that, due to the occurrence of a large proportion of these tumours on the face such that cosmetic considerations may override oncological principles. This has resulted in the development of a large number of ablative, excisional or other treatment modalities. Analysis of the efficacy of each of these has been hindered by the lack of a classification system and the long natural history of the development of recurrence. This study has demonstrated the importance of the completeness of excision in the prevention of recurrence, however, certain tumours appear to recur despite complete excision. This is probably due to the difficulty of sampling the tumour margins of poorly circumscribed nodular or infiltrative tumours by non Mohs histological methods. Late presentation of all tumour subtypes may also determine the development of a horrifying phenotype. As a consequence of the involvement of a multitude of factors in determining outcome, biological markers were not prognostic. In BCC local tumour control is determined by factors determining complete ablation or removal of the tumour. Tumour size, position and excisional margin are therefore important. Provided reconstructive uncertainty can be overcome by the skills of the surgeon, then basic oncological principles can be followed, even on cosmetically sensitive areas such as the face. Incomplete excision rates should therefore be reduced and the development of a horrifying tumour in patients that present early should be eliminated. The treatment of BCC requires standardisation to reduce inadequate treatment as a common cause of treatment failure.

8.9 Future studies

The underlying genetic abnormalities that determine the development of BCC have not yet been fully elucidated. Recent evidence suggests that the patched gene is the key mutation that determines the development of BCC (Stone, Hynes et al. 1996; Dean 1997; Gailani and Bale 1997; Uden, Zaphiropoulos et al. 1997). Further studies evaluating whether all tumours have mutations in this gene or whether there are other pathways of tumour development would be of considerable benefit in understanding the growth control of BCC. Analysis of the mutation spectrum in this gene may also

shed light on the aetiology of BCC. Identification and characterisation of these genetic aberrations may then provide a potential target for a novel gene therapy approach for therapy. Re-introduction of the wild type *patched* gene into tumours may then restore proliferative control into these tumours. This studies would require the development of satisfactory cell culture techniques and animal models for BCC. At present research into the cell biology of BCC has been hampered by the lack of these techniques.

A subgroup of patients who develop multiple BCCs may have an identifiable susceptibility gene, like the Gorlin's patients, they may harbour a mutation in one patched gene that may reduce the tumour suppressor function. These patients may be identified at a young age and prevention of excessive sun exposure implemented.

At present there is little understanding of the mechanism of growth control in the range of histological variants of BCC. It is conceivable that this is determined by the tumour/stromal interactions. Investigation of the expression of cytokines such as TGF and EGF in the stroma may provide insight into these events.

At present, prognostic markers for BCC are not indicated because treatment factors determine the outcome to a greater extent than tumour biology. Advances in management initially requires the standardisation and improvement of local treatment to prevent incomplete excision and recurrence. Adequate documentation of tumour subtype, size and of the exact treatment regimen will provide data for analysis of each treatment modality. Improvement in standard histological sampling of BCCs may also reduce the number of local recurrences following a report of complete excision. The adoption of clinically useful histological classification systems will also provide additional data to assess the adequacy of treatment type or excision margin. Having standardised and optimised the therapy of BCC, then the biology of each tumour will become of greater importance in determining outcome and prognostic markers will become more relevant.

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