THE GENETICS OF BASAL CELL CARCINOMA OF THE SKIN

Thesis

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Dr Sally de Zwaan MBBS (Hons), BSc

Westmead Institute for Cancer Research at the
Westmead Millennium Institute, University of Sydney
Westmead NSW 2145 Australia

Statement of Originality

I hereby declare that this submission is my own work. To the best of my knowledge and belief, no data presented in this dissertation has been previously published or reported, other than the oral presentations that carry my name as co-author; experiments and statistical analyses presented in this thesis were performed by myself, except in the instances where due acknowledgement has been made in the text.

Sally de Zwaan
August 2007

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List of Abbreviations

A	Adenine
a	red-green reflectance
ACTH	Corticotropin
b	yellow-blue reflectance
BCC	Basal Cell Carcinoma
BMPs	Bone Morphogenic Proteins
bp	Base pair
C	Cytosine
CCR	Coriell Cell Repository
CDKN2A	Cyclin Dependent Kinase N2A
CHIP	Children's Hospital Informatics Program
ci	Cubitus Interruptus
CI	Confidence Interval
CIE	Commission International de l'Eclairage 1976
CMV	Cytomegalovirus
CYP	Cytochrome P450
dHPLC	Denaturing High Performance Liquid Chromatography
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EV	Epidermodysplasia Verruciformis
G	Guanine
Gli	Glioma Associated Oncogene
GST	Glutathione-S-Transferase
H20	Water
HapMap	International HapMap Project
hh	Hedgehog

HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
IVS	Intronic variance sequence
KCl	Potassium Chloride
L	luminance
Leu	Leucine
MAPK	MAP Kinase gene
MC1R	Melanocortin 1 Receptor gene
$MgCl_2$	Magnesium Chloride
MM	Malignant Melanoma
mM	millimoles
μL	Microlitres
MPP	Multiple Presentation Phenotype
NBCCS	Nevoid Basal Cell Carcinoma Syndrome
NIHPDR	National Institute of Health Polymorphism Discovery Resource
NMSC	Nonmelanoma Skin Cancer
NSW	New South Wales
OR	Odds Ratio
p14ARF	p14 alternate reading frame
PCR	Polymerase Chain Reaction
POMC	Proopiomelanocortin
Pro	Proline
PTCH	Patched 1 gene
QLD	Queensland, Australia
RCT	Randomised Controlled Trial
RHC	Red Hair Colour
RR	Relative Risk
RTRs	Renal Transplant Recipients
SCC	Squamous Cell Carcinoma
SCFA	Skin and Cancer Foundation Australia
SD	Standard Deviation

SE	Standard Error
smo	smoothened
SNP	Single Nucleotide Polymorphism
T	Thymine
TGFβ	Tumour Growth Factor β
Tris-HCL	Tris (hydroxymethyl) aminomethane Hydrochloride
UK	United Kingdom
USA	United States of America
UVA	Ultraviolet A radiation
UVB	Ultraviolet B radiation
UVR	Ultraviolet Radiation
VIC	Victoria, Australia
WA	Western Australia, Australia
WICVAR	Centre for Genome Resarch at the Whitehead Institute, Cambridge MA
XP	Xeroderma Pigmentosum
α	Alpha
β	Beta
γ	Gamma

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Publications arising from this thesis

de Zwaan, SE, Mann, GJ "Familial Clustering of Early-Onset Basal Cell Carcinoma of the Skin", *Journal of Investigative Dermatology* (In press)
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Abstract

BCC is the commonest cancer in European-derived populations and Australia has the highest recorded incidence in the world, creating enormous individual and societal cost in management of this disease. The incidence of this cancer has been increasing internationally, with evidence of a 1 to 2% rise in incidence in Australia per year over the last two decades.

The main four epidemiological risk factors for the development of BCC are ultraviolet radiation (UVR) exposure, increasing age, male sex, and inability to tan. The pattern and timing of UVR exposure is important to BCC risk, with childhood and intermittent UVR exposure both associated with an increased risk. The complex of inherited characteristics making up an individual's 'sun sensitivity' is also important in determining BCC risk.

Very little is known about population genetic susceptibility to BCC outside of the rare genodermatosis Gorlin syndrome. Mutations in the tumour suppressor gene *patched* (*PTCH*) are responsible for this BCC predisposition syndrome and the molecular pathway and target genes of this highly conserved pathway are well described. Derangments in this pathway occur in sporadic BCC development, and the *PTCH* gene is an obvious candidate to contribute to non-syndromic susceptibility to BCC.

The melanocortin 1 receptor (MC1R) locus is known to be involved in pigmentary traits and the cutaneous response to UVR, and variants have been associated with skin cancer risk. Many other genes have been considered with respect to population BCC risk and include p53, HPV, GSTs, and HLAs. There is preliminary evidence for specific familial aggregation of BCC, but very little known about the causes.

56 individuals who developed BCC under the age of 40 in the year 2000 were recruited from the Skin and Cancer Foundation of Australia's database. This represents the youngest 7 – 8% of Australians with BCC from a database that captures approximately 10% of Sydney's BCCs. 212 of their first degree relatives were also recruited, including 89 parents and 123 siblings of these 56 probands.

All subjects were interviewed with respect to their cancer history and all reports of cancer verified with histopathological reports where possible. The oldest unaffected sibling for each proband (where available) was designated as an intra-family control. All cases and control siblings filled out a questionnaire regarding their pigmentary and sun sensitivity factors and underwent a skin examination by a trained examiner. Peripheral blood was collected from these cases and controls for genotyping of *PTCH*. All the exons of *PTCH* for which mutations have been documented in Gorlin patients were amplified using PCR. PCR products were screened for mutations using dHPLC, and all detectable variants sequenced.

Prevalence of BCC and SCC for the Australian population was estimated from incidence data using a novel statistical approach. Familial aggregation of BCC, SCC and MM occurred within the 56 families studied here. The majority of families with aggregation of skin cancer had a combination of SCC and BCC, however nearly one fifth of families in this study had aggregation of BCC to the exclusion of SCC or MM, suggesting that BCC-specific risk factors are also likely to be at work. Skin cancer risks for first-degree relatives of people with early onset BCC were calculated: sisters and mothers of people with early-onset BCC had a 2-fold increased risk of BCC; brothers had a 5-fold increased risk of BCC; and sisters and fathers of people with early-onset BCC had over four times the prevalence of SCC than that expected. For melanoma, the increased risk was significant for male relatives only, with a 10-fold increased risk for brothers of people with early-onset BCC and 3-fold for fathers.

On skin examination of cases and controls, several phenotypic factors were significantly associated with BCC risk. These included increasing risk of BCC with having fair, easy-burning skin (ie decreasing skin phototype), and with having signs of cumulative sun damage to the skin in the form of actinic keratoses. Signs reflecting the combination of pigmentary characteristics and sun exposure - in the form of arm freckling and solar lentigines - also gave subjects a significantly increased risk BCC. Constitutive red-green reflectance of the skin was associated with decreased risk of BCC, as measured by spectrophotometery. Other non-significant trends were seen that may become significant in larger studies including associations of BCC with propensity to burn, moderate tanning

ability and an inability to tan. No convincing trend for risk of BCC was seen with the pigmentary variables of hair or eye colour, and a non-significant reduced risk of BCC was associated with increasing numbers of seborrhoeic keratoses.

Twenty *PTCH* exons (exons 2, 3, 5 to 18, and 20 to 23) were screened, accounting for 97% of the coding regions with published mutations in *PTCH*. Nine of these 20 exons were found to harbour single nucleotide polymorphisms (SNPs), seen on dHPLC as variant melting curves and confirmed on direct sequencing. SNPs frequencies were not significantly different to published population frequencies, or to Australian general population frequencies where SNP database population data was unavailable. Assuming a Poisson distribution, and having observed no mutations in a sample of 56, we can be 97.5% confident that if there are any *PTCH* mutations contributing to early-onset BCC in the Australian population, then their prevalence is less than 5.1%.

Overall, this study provides evidence that familial aggregation of BCC is occurring, that first-degree relatives are at increased risk of all three types of skin cancer, and that a combination of environmental and genetic risk factors are likely to be responsible. The *PTCH* gene is excluded as a major cause of this increased susceptibility to BCC in particular and skin cancer in general. The weaknesses of the study design are explored, the possible clinical relevance of the data is examined, and future directions for research into the genetics of basal cell carcinoma are discussed.

Chapter 1: Introduction and Background

1.1. Introduction

Basal cell carcinoma (BCC) is the commonest cancer in fair-skinned populations. Non-melanoma skin cancers (NMSC) make up 80% of all new cancers in Australia, presenting an enormous individual and financial cost to Australians: treating these cancers accounted for 42% of cancer related medical costs in 1993 and cost the Australian community $280 million that year. Great gains have been made over recent decades regarding the understanding of epidemiological associations and risks for BCC, with much of this research based in Australia where the incidence is the highest in the world. Yet, unlike other common cancers such as breast and colon cancer, very little is known about population genetic susceptibility to BCC outside one rare familial syndrome. This project studies early-onset cases and their relatives in an attempt to further elucidate the causes of BCC, the commonest of cancers.

The term NMSC encompasses both BCC and squamous cell carcinoma (SCC): both are common tumours derived from the same keratinocyte cell population in the skin, although they have distinct biological and pathological features. There are other NMSCs apart from these including Merkel's cell carcinoma and atypical fibroxanthoma, however as these are so rare, the term NMSC is usually used to refer to BCC and SCC only.

BCC is more than twice as common as SCC in Australia [1], and although very locally destructive, rarely metastasizes. SCC does metastasize, usually after a long latency, and is responsible for nearly all the 400 deaths per year attributable to NMSC. Neither cancer has any direct biological relationship with malignant melanoma (MM), which arises instead from neural crest-derived pigment producing cells that migrate into the skin during early development.

Sunlight is known to be one of the major causes of all three types of skin cancer, through the mutagenic effects of ultraviolet radiation (UVR). Other major epidemiological associations include advancing age, male sex (especially in older groups), and a

decreased ability to tan. Pigmentary characteristics such as skin, hair, and eye colour also contribute to risk. The timing and pattern of sun exposure are known to be important for BCC risk: exposure in childhood and teenage years increase an individual's later risk, and intermittent exposure such as that received on summer holidays increases risk more than continuous exposure such as that encountered in an outdoor occupation. Knowledge of these important epidemiological associations allowed the Australian government to mount an important public health preventative campaign in the early 1980's called 'slip, slop, slap' that encouraged awareness of the dangers of sun exposure, and the importance of preventative measures ('slip on a t-shirt, slop on some sunscreen and slap on a hat'). This message appears to have permeated the community's consciousness over the last few decades: some attribute the decline of skin cancer in younger age groups to the success of such campaigns [1].

Most chronic diseases are found to have both genetic and environmental aetiological components, with multiple factors interacting in complex ways and often expressed as risk phenotypes. There is no reason to believe why BCC would not be subject to the same aetiological forces. Familial aggregation studies have been important bases to explore the combination of inherited and exogenous risk factors in cancer [2]. Historically, there seems to have been a reluctance to approach family studies of NMSC. Perhaps the obvious importance of sun exposure in the aetiology of BCC has discouraged interest in additional genetic influences, or it has been assumed that any familial aggregation of BCC would merely reflect sharing of high-risk skin types. There are also methodological difficulties in such studies. First, the high burden of disease makes mandatory reporting of NMSC to cancer registries cumbersome, and makes it harder to demonstrate increased incidence in relatives. Second, the fact that NMSC are often destructively treated reduces the available data from histological confirmation, which is important in verifying cancer reports in probands and relatives.

Although much work has been done on rare genodermatoses involving BCC, very little is known about genetic susceptibility to BCC outside these settings. A small Australian study looking at familial clustering of NMSC that noted apparent differences in susceptibility to BCC versus SCC between NMSC pedigrees, suggesting differing genetic

influences [3]. However, there have been no larger-scale studies of this phenomenon. Gorlin syndrome is one of the rare familial syndromes that has received research attention, and involves autosomal dominant inheritance of marked susceptibility to BCC and a range of developmental defects. Mutations in the cell growth regulatory gene *patched* (*PTCH*), when present in the germline are responsible for this disorder. They have also been found in a high percentage of sporadic tumours, suggesting a critical involvement of this pathway in BCC tumour development generally. Furthermore, mice with Gorlin syndrome-like knockouts of one copy of *PTCH* develop BCCs if exposed to UVR, mimicking the human situation [4]. No studies have yet investigated whether germline mutations or less highly penetrant variants of this gene might account for part of non-syndromic BCC susceptibility in the general population.

1.2. BCC and its management

1.2.1. Cell of origin

BCCs are composed of lobules of basaloid cells with hyperchromatic nuclei and scant cytoplasm that are pluripotential cells derived, or closely related to, basal keratinocytes and follicular or epidermal stem cells [5]. The cell of origin of the basal cell carcinoma has been contentious however typing of cytokeratins within tumour cells suggests a follicular origin [6, 7]. Metastasis occurs rarely, at a rate of 1 in 1000 to 35000 and this infrequency is probably due in part to the dependency of the tumour for a specific dermal connective tissue stroma for its growth [8, 9].

1.2.2. Body sites affected

BCC virtually never arises in stratified squamous epithelium other than the skin and only extremely rarely affects the mucosal surfaces of the body [10, 11]. All over the world, BCC is most commonly seen on the skin of the head and neck [12, 13] and is almost always more common in men than in women [14]. In fact, in light-skinned European-derived populations the rate is between 18% and 40% higher in men than women [15, 16]. Recent studies in Australia have shown that rates of BCC may be higher in women of younger age groups and in men of older age groups [1]. Detailed body-site specific

rates of BCC incidences are usually adjusted for the actual surface proportion covered by the site to allow comparison and are thus also called ‘relative body densities’ [14]. The relative body densities of BCC incidences in Australia can be seen in a large series from a population based study in Townsville in Northern Queensland [17]. Highest densities of BCC for both genders in this series were on the lip, orbit, and nasolabial fold. The next highest were on the ear, nose, and cheek, then forehead, eyebrow, chin, jaw and pre-auricular region. Following these regions, the neck and shoulders showed high densities as did the posterior trunk in men and the sun-exposed surface of the arms in women. For both genders the less sun-exposed body sites such as heels and thighs showed the lowest incidence rates. For all body sites except buttocks and genital region, incidence rates for women were lower than for men. Male preponderance was especially noticeable for the scalp which may be reasonably attributed to hair loss in men giving rise to increased exposure of the scalp to solar radiation. The near absence of BCC from the backs of the hands has generally assumed to be due to the nature of the skin rather than to complex effects of sun exposure.

1.2.3. BCC classification

There is no generally agreed classification of BCC in Australia however the commonest subtypes reported are grouped by histological growth pattern [18]; nodular, superficial, and morpheaform/sclerosing. BCCs are more likely to be nodular on the head and neck and superficial spreading on the trunk and upper limbs [19, 20]. The most common is the nodular type which appears as a dome-shaped shiny transluscent (pearly) papule with a telangiectatic surface, often with surrounding actinic damage. It may become crusted, ulcerated or pigmented, and usually has a firm consistency. Superficial BCC presents as a bright pink, shiny, usually well defined erythematous scaly plaque, with a rolled edge that shows slow centrifugal growth; areas of erosion or crusting may also arise in these lesions. Morpheaform or sclerosing BCC tends to be more aggressive with a whitish colour, poorly defined margins and an indurated consistency. It is often more difficult to detect clinically and therefore more extensive upon diagnosis, often with wide subclinical extension [21].

1.2.4. BCC treatment

1.2.4.1. Treatment options

BCCs often become more aggressive with recurrence and therefore the first priority in the treatment of BCC is complete eradication of the tumour. This is done with the additional goals of restoration of function and optimal cosmetic result. Shave or punch biopsies may be obtained for a tissue diagnosis prior to destructive or excisional therapy, however in practice, a prior tissue diagnosis is often not obtained. Surgical excision remains the gold standard treatment against which other treatments are judged, and may be done with or without Moh's micrographic surgery. Other surgical treatments include curettage and diathermy, and cryotherapy. Non-surgical treatment options include radiotherapy, topical treatments such as 5-fluorouracil, photodynamic therapy, laser therapy, imiquimod 5% cream, and intralesional/perilesional cytokines. The choice of therapy depends on the characteristics of the individual tumour, patient preference, and the resources available [19, 21].

1.2.4.2. Surgical treatments

Surgical excision can often be done by excising a simple ellipse around the tumour under local anaesthetic with a margin of 3-4mm. Moh's microscopically controlled excision is considered the treatment of choice if the tumour is very large, recurrent, poorly defined, or displays an aggressive histological pattern. It is also indicated for tumours in locations associated with a high risk of recurrence such as the periorbital and facial areas, and for those in areas of cosmetic importance. In this technique, frozen sections of the lesion undergo histopathological examination during surgery allowing the entire excision margin to be visualised. These are interpreted by the operator to better allow for complete excision and to minimise removal of normal surrounding tissue [21]. Curettage and diathermy give good results when reserved for small, well-defined, relatively superficial tumours and its success is highly operator-dependent. Cryotherapy also tends to be used on small superficial tumours. These destructive treatments are simpler than excision however they prevent the histopathological verification of these cancers and also verification of the adequacy of excision by visualization of tumour margins. Furthermore,

destructive treatments and the concomitant uncertainty of diagnosis make the estimation of true incidence of this type of cancer very difficult. The resulting wound can take longer to heal from curettage or cryotherapy than wounds resulting from excision. Five year cure rates for primary BCC with surgical techniques are in the order of 95% [22, 23], and closer to 99% for Moh's micrographic surgery on a primary tumour [24].

1.2.4.3. Non-surgical treatments

Non-surgical techniques are increasingly employed for the treatment of BCC. Radiotherapy has been used on all stages of BCC with results comparable to surgery [25-27]. It requires a number of weeks for receiving then healing, and is associated with some long term sequelae including scarring and increased neoplasia risk. It is therefore usually reserved for either: (a) extensive lesions where major surgery is not appropriate, or (b) elderly patients in sites that are difficult to reconstruct and would be associated with considerable morbidity, as the concerns regarding long term sequelae of radiotherapy are small [28]. Radiotherapy can be also be used to complement surgery for cases of persistent, recurrent or advanced tumours [5]. Topical treatments for BCC can give good cosmetic results and can be effective treatment in superficial BCCs. 5-fluorouracil cream is useful in the management of multiple superficial BCCs on the trunk and limbs [29]. Imiquimod functions as an immune system modulator and has recently shown to be efficacious in the treatment of superficial BCC [27, 30, 31]. Its antitumour properties are related to its stimulation of interferons and other cytokines, and of antigen presenting cell function. Intralesional injection of recombinant interferon-α has been only partially successful with reported cure rates varying between greatly [5, 31, 32]. Topical photodynamic treatment may be useful for the treatment of superficial BCC where standard surgical intervention poses difficulties, for example in patients who have bleeding disorders or pacemakers. It involves the application of a systemic or topical photsensitising chemical which leads to photodestruction when the skin is subsequently exposed to light [33]. Laser therapy may offer some advantages in the precision of tissue removal; however the majority of tumours are better managed by the less expensive commonly available techniques. Intralesional interferon, photodynamic therapy, and laser

therapy should be considered investigational until confirmed and reproducible outcomes are established in prospective trials [5].

1.3. Incidence

1.3.1. World burden of NMSC

Basal cell carcinoma of the skin is the most common malignancy in populations of European origin including North America and Australia. The world burden of nonmelanoma skin cancer (NMSC; BCC and squamous cell carcinoma or SCC) is enormous: worldwide, NMSC is three times more common than lung cancer, the next most common cancer [34]; the incidence of NMSC alone in the United States is approximately equal to that of *all* non-cutaneous cancers combined [35]. Nonmelanoma skin cancer is by far the most common cancer in Australia where it outnumbers all other forms of cancer by 3 to 1. We spend more on treating NMSC than any other cancer, costing the Australian health sector $280 million in 1993/1994 [36]. Australia has the highest incidence of NMSC in the world with Western Australia having the highest ever recorded incidence [16].

1.3.2. Australian incidence and prevalence data

1.3.2.1. Methods of data collection

Data on the prevalence and incidence of NMSC in Australia has been collected using a variety of methodologies and with different subsections of the Australian population, making the results very difficult to compare directly. These include national surveys, state-based surveys, analysis of state-based cancer registry data, and smaller regional community studies. One of the most important differences in methodology between studies is the decision to include lesions treated destructively prior to histopathological confirmation. The inclusion of lesions treated destructively reduces the risk of underestimation of incidence figures, but it does so at an increased risk of inclusion of misdiagnosed lesions; the misdiagnosis rate by experienced Australian dermatologists has been estimated at as high as 41% for BCC and even higher for SCC [37, 38].

Periodic surveys such as the Australia-wide survey of NMSC by Staples [1] can provide good estimates of incidence and prevalence in broad groupings of histological type and population characteristics. The series by Staples et al [1] is the most recent and extensive national survey of NMSC incidence in Australia giving figures for the incidence of BCC and SCC age-adjusted to the World standard population. This series also allows data analysis by individual States and by three zones of latitude. It looked at all cancers reported over one year including those with a clinical diagnosis only. National telephone surveys of households randomly selected by the electoral roll were performed in 1985, 1990, and 1995, and reports of treated skin cancer in the previous 12 months were verified clinically and histopathologically if possible.

Cancer registries provide a sufficient sample size over extended time periods to permit a detailed description of skin cancer patterns and time trends at specific body sub sites, within narrow demographic categories and at different latitudes. Unfortunately, NMSC is unsuitable for routine cancer registration in Australia due to the sheer numbers of them within the Australian community, the way that many lesions are treated destructively without histopathological confirmation, and the fact that this often occurs outside a hospital setting. The only cancer registries to have recorded NMSC in Australia were State based and have since ceased recording these cancers [39-41]. The Tasmanian Cancer Registry collected data between 1978 and 1987 [39]. It only included histopathologically confirmed lesions or those referred for radiotherapy, and only the first BCC treated per year. The Queensland state-based study involved reporting all treated NMSCs during a six month period in 1984 by general practitioners and hospital outpatient departments in four representative areas of the State [40]. Cancers that were confirmed histopathologically as well as those treated destructively were included.

Studies of small communities such as those performed in Townsville in Queensland [17], Maryborough in Victoria [42], and Geraldton in Western Australia [16] have the advantage of allowing longitudinal assessment including detailed skin examination and risk factor assessments. Individuals can be screened and then followed up over time to detect any incident malignant lesions. This method may give a more accurate assessment of the incidence within that population than relying on subjects' recall of treated lesions

because it less likely to miss unreported or undiagnosed tumours. It is an expensive and time consuming method of data collection and therefore impractical for larger studies.

1.3.2.2. Comparison of Australian incidence data

Important information about the incidence, epidemiology and risk factors for BCC have been obtained by combining information from all of these studies. A comparison of the figures can be seen in table 1.1, with a summary of the national survey data in figure 1.1. These incidence figures are alarmingly high and differ quite dramatically according to location, sex, and methodology of the study. The figures from the carefully-screened community studies include the largest reported incidence for BCC in the world to date from Geraldton in Western Australia. Where incidence was measured over time it is clear that incidence figures of both BCC and SCC in Australia have been increasing, with a rise of 1 to 2% per year over the periods studies. Interestingly, the national survey showed a slight decrease in incidence of BCC in people under 40 years of age which may reflect changes in attitude and behaviour towards sun exposure, perhaps due in part to extensive public health campaigns operating in Australia for the last 20 years [43].

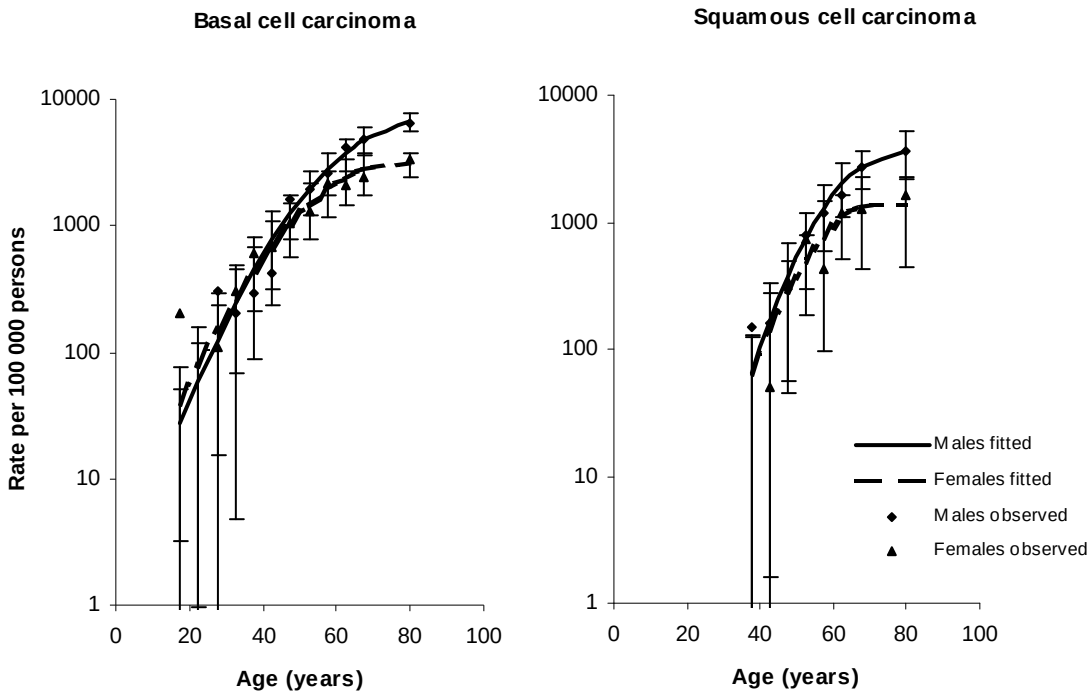
All of the Australian studies show increasing incidence with age and higher incidences for men than for women with the exception of a slightly larger incidence in women than men in the under-40s. This sex difference may be influenced by differences in ultraviolet radiation (UVR) exposure between younger men and women and/or by differences in rate of presentation for medical treatment. All studies also showed higher incidence of BCC than SCC, with incidence of BCC ranging from between 2.5 and 4 times the incidence of SCC. A latitudinal gradient emerged within the national survey and between the State-based surveys where incidence is clearly greater for populations closer to the equator.

Table 1.1 Comparison of measured incidences of BCC found in Australian studies

Population studied	**Type of study**	**Time period**	**Males per 100,000***	**Females per 100,00***
Australia [1]	National survey	1995	955	629
Tasmania [39]	State cancer registry	1978-1987	219	110
Queensland [40]	State cancer registry	1984	1474	713
Nambour, QLD [44]	Community survey	1986	2074	1579
Townsville, North QLD [17]	Community survey	1996-1997	2058	1195
Maryborough, VIC [42]	Community survey	1982-1986	2244	1069
Geraldton, WA [16]	Community survey	1987-1992	7067	3379

*age-standardised rate

Figure 1.1 Observed rates and age-specific incidence curves with 95% confidence intervals for BCC and SCC for males and females from 1995 Australia.
(Created with permission from corrected raw data by M Staples from data reported in ***[1]****)*



1.3.3. International incidence and prevalence data

1.3.3.1. International cancer registry data

There are more cancer registries that include NMSC in countries other than Australia which is likely to be due to the lower incidence and therefore reduced burden of diagnosis and reporting. Despite this reduced burden, these registries sometimes still have difficulty obtaining accurate records for NMSC [45]. The incidence figures for NMSC determined internationally are universally lower than those seen in Australian populations although a trend towards increasing incidence is also seen in these disparate populations. Cancer registries that include data on NMSC have operated in Nordic countries[46], Southern European regions [47], Slovakia [13], New Hampshire [48] and Southeastern Arizona [49]in the United States, the Swiss canton of Vaud [50], Singapore[51], and the Netherlands [52]. Smaller population based incidence studies have also been performed on populations in every continent.

Table 1.2 Comparison of incidence BCC worldwide per 100,000 *

Location	**BCC Men**	**BCC Women**
Australia [1]	955	629
United States [53]	247	150
United Kingdom [54]	128	105
Finland [55]	49	45
Switzerland [50]	52	38
Netherlands [52]	53	38
Slovakia [13]	38	29
Hawaii [56]	576	298

*World age-standardised rate

1.3.3.2. Differences between Australian and International incidence data

The most dramatic difference between the data collected in these countries compared with Australian data is the 5-100 times lower rate of BCC incidence seen in European-derived populations outside Australia (see table 1.2). For instance the incidence of BCC in Slovakia in the period 1993 to 1995 was estimated at 38 per 100,000 for men and 29

for women [13]. In Finland from 1991 to 1995 it was 49 per 100,000 for men and 45 for women [55]. We can compare this to the national Australian survey which reported a combined (men and women) figure of 788 for the same time period. The Australian national survey figures are likely to be larger than Australian cancer registry figures by a factor of three. For BCC in 1985 Staples et al [1] report an overall age-standardised incidence of 333 per 100,000 for greater than 37°S latitude. Tasmania lies in a region entirely greater than 40°S latitude and the registry's comparable total incidence figure from a similar time in history was 111, exactly one third the nationally estimated figure. We should therefore reduce the survey figure from 788 to 263 to make it comparable to any cancer registry estimate. This estimate still remains at least five-fold larger than those seen in Northern Europe.

Another difference between the Australian and international data on incidence is the variation in male to female ratios. Most studies conducted on Australian populations show greater male than female incidence, although there is evidence that this ratio is reversing in younger cohorts [1, 57]. The only populations to record incidences greater in women than in men are in Asia, and include studies on Korean [58] and Japanese populations [12]. This may be due to differences in genetic or environmental risk factors between the different populations, or more likely, a combination of both.

1.3.3.3. Similarities between Australian and international incidence data

Similarities also exist between Australian and international studies, the most striking of which is a trend towards increasing incidence of BCC. Annual percentage increases of three to 6% have been reliably recorded across a number of studies of European-derived populations, with similar rates of increase for BCC and SCC [59]. The New Hampshire Skin Cancer Study Group from the United States of America report an increase of more than 80% in incidence from 1979 to 1994 in both men and women [60]. Incidence data from the Eindhoven Cancer Registry in the Netherlands collected between 1973 and 2000 also show a rapid increase in incidence for both sexes with figures doubling over this period [61]. Over these last few decades, figures have doubled in Sweden [62], increased by 70% in men and 65% in women in Slovakia [13], and increased steadily in Singapore at a rate of 3% per year for nearly 30 years [63].

It is clear that people with darker pigmentation have a lower incidence of BCC than lighter-skinned people living in the same environment. For example, in Kenya the incidence of BCC is 0.65 per 100,000 for residents with darkly pigmented skin and 585 per 100,000 for the European-derived Africans living in the same community, indicating a substantially increased risk in the fairer-skinned residents [64]. In Qatar in the Middle East, the incidence of BCC is also higher in Europeans living in there compared with locally born residents [65]. Non-Hispanic whites in Southeastern Arizona have 11 times the incidence of BCC compared with Hispanic whites [49].

An obvious trend that emerges from incidence studies is that the risk of BCC increases as one gets closer to the equator. Within Australia, the nationwide survey by Staples et al [1] gives the most conclusive evidence of this as it samples population from the entire continent of Australia and looks specifically at this question. Over the decade studied, a latitudinal gradient in incidence of BCC remains evident with a trend to significantly higher BCC incidence as latitude decreases. Incidence in latitudes less than 29°S remained approximately three times higher than those greater than 37°S, and in 1995 this difference was 3.1:1 for men and 4.2:1 for women. Other within-study latitudinal trends are evident in the Queensland state-based study [40] which shows a significantly increased incidence of BCC in the Northern as opposed to the Southern parts of the State. The only exception to this is the Gold Coast which has been estimated to have an incidence similar to that of the Northern part of the State. This is likely to be due to the complex nature of the Gold Coast population which is affected by the selective migration of sun-lovers and by year-round tourism with the sun and beach the main attractions. The differences in incidences quoted in the separate community studies also display a latitudinal gradient although these studies are not ideally suited to looking at such relationships due to the vast differences in collection and measurement between them.

This inverse relationship between latitude and BCC incidence is also evident overseas, with greater incidences reported with lower latitudes in Sweden, Norway and Finland [66]. The incidence in Southeastern Arizona is estimated to be between three and six times higher than that in Northern parts of the country [49]. Japanese studies also show a latitudinal difference however the numbers here are small [67]. Very convincing data from the United States in the 1970s show a latitudinal gradient of NMSC incidence

across the country [53]. The study involved ten USA metropolitan populations from 47.5°N (Seattle) to 30.0°N (New Orleans) with a statistically significant gradient of incidence rates inversely related to latitude across the cities studied.

NMSC is an immensely common and costly tumour to European-derived populations, and incidence is increasing internationally at an alarming rate. A better understanding the complex causes of these cancers should translate into valuable gains in the quality of life of millions of people worldwide.

1.4. Epidemiology and Risk Factors

Much has been learned about epidemiological risk factors for BCC in the last few decades. The main four epidemiological risk factors for the development of skin cancers (including basal cell carcinomas) are ultraviolet radiation exposure, increasing age, male sex, and inability to tan. Increasing age is one of the strongest risk factors for the development of BCC, and as with other cancers, the risk of developing the disease each year of life combines to give a rising cumulative risk. Increasing age could be considered an indicator of cumulative exposure to environmental risk factors [39, 68-71]. In addition to the the likely effect of cumulative UV exposure, increasing risk with age may reflect the normal decline in DNA-repair capacity [72] and the effect of age on the ability of immune surveillance to eliminate micro-tumours [73]. It is evident from international incidence data already described, that in most countries at most points in time measured, men have a greater incidence of BCC than women, but that in younger persons (< 40 years old) with BCC there may be a reversal of this sex ratio [1, 57, 74], a phenomenon also seen in measures of melanoma incidence [75]. It is possible that the reduction in incidence in the younger age groups has been influenced by public health campaigns on skin cancer prevention.

1.4.1. Ultraviolet radiation

1.4.1.1. Aetiological role in skin cancer

Exposure to ultraviolet radiation (UVR) is associated with the incidence of all three major types of skin cancer; BCC, SCC, and melanoma. As an aetiological factor in skin

cancer initiation, its role is inferred indirectly from a variety of sources. One of the first to associate BCC with UVR exposure was Molesworth [76], a Sydney Dermatologist at Royal Prince Alfred Hospital in 1927 who noticed that there was a much greater incidence of “rodent disease” (as BCC was also known) in Australia as compared with England. He noted that the Australian “loves the sunshine and glories in it and is very liable to regard any precaution against sunburn as womanish and ridiculous”. He suggested that sun exposure, not heat, dust, or trauma as previously hypothesized, was the primary reason for this difference in incidence. He also put forward the hypothesis that it is the ionizing radiation in sunlight that is responsible for causing these tumours.

There are abundant epidemiological associations between UVR and BCC linking them aetiologically. Genetic diseases involving a greater sensitivity to UVR and a concomitant risk of early onset NMSC include the autosomal recessive disorder xeroderma pigmentosum [77]. People who live in areas of greater UVR exposure have a much greater risk of developing skin cancers than those living in areas of lower UVR as can be seen in the change of incidence with latitudinal gradients. BCC is commonest on regularly exposed skin sites such as the head, neck and face, and there is a correlation between BCC and sun-exposure-related conditions including; presence of actinic keratoses, solar elastosis of the neck [78], solar lentigines [79] and degree of sun damage to the backs of the hands as measured by cutaneous microtopography [80]. Actinic keratoses are premalignant lesions occurring on chronically sun exposed sites that rarely may progress to SCC if left untreated. Solar elastosis is a degenerative change in the dermis on chronic sun exposed sites. It is characterized by thickened skin with yellow discolouration and well-defined furrows. Solar lentigines are permanent macular areas of brown pigmentation occurring after either acute or chronic sun exposure. There is frequently a history of acute sunburn followed by the sudden appearance of large numbers of these lesions. Childhood freckling is also associated with all three types of skin cancer, however as this phenotypic characteristic is both genetically and environmentally determined, it is difficult to determine whether one or both of these factors are responsible for the association [78, 81]. Freckles also appear on sun exposed sites but differ from lentigines by fading during periods without sun exposure.

1.4.1.2. Effects on DNA

While animal models exist for the capacity of UV radiation to cause SCC and melanoma, there are no comparable models for BCC, other than a knockout mouse model for Gorlin syndrome. Unfortunately, there is also no practical way that the action spectrum for skin cancer can be measured directly in humans. There is however, direct evidence that UVB wavelengths produce 'UV signature' mutations in certain genes in BCC lesions, providing biological evidence to support the epidemiological observations. These are C to T or CC to TT mutations at dipyrimidine sites and peak at around 300nm, suggesting that this wavelength of UV (in the UVB range) may be effective in causing skin cancers in humans [82]; this does not exclude a role for UVA light in causing BCC. The type of DNA damage induced by UVA seems to differ from UVB in the proportion of nondimer damage induced, such as DNA strand breaks which increase 100-1000 fold in UVA compared to UVB irradiated cells [83, 84]. This is likely to be due to the important role that reactive oxygen species play in UVA related damage to cells as opposed to the dimer induction in UVB damage that follows direct DNA absorption [85]. The latitude gradient seen in skin cancer incidence data around the world tends to be higher for SCC incidence than melanoma, with the latitudinal gradient of BCC incidence between the two. There is a correspondingly higher latitude gradient for UVB radiation than UVA radiation, an association suggesting that melanoma and BCC may be more influenced by UVA than is SCC [86]. Artificial sources of UV used for tanning primarily use UVA as this has been thought to be the safer part of the UV spectrum. These devices have recently been shown to be associated with increased risk of melanoma and SCC, but there is equivocal evidence regarding the risk of BCC [87-90]. Recent studies in mice have contradicted this earlier work with reports of UVA having no role in the initiation of melanoma [91].

1.4.1.3. Effects on immune system

UVR is likely to have an effect on the immune system that results in defective immune surveillance. Animal studies show that multiple exposures to UVR have systemic effects on the immune system with increased tolerance to transplanted UV-induced skin tumours [92]. This susceptibility to tumour growth was explained by the action of suppressor T-

cells which arose in UVR treated animals and prevented immunological rejection of the tumour [93, 94]. Localised, low-dose UVB radiation impairs the induction of contact hypersensitivity in humans and susceptibility to this immune impairment may be associated with skin cancer risk, as persons with a history of NMSC have been shown to be more likely to display this UV-associated impairment than normal controls [95]. UVR also has an effect on CD4/CD8 T-lymphocyte ratios in a study following high sun exposure in the previous 18 months, although this ratio had no predictive ability for development of BCC once sun exposure and previous BCC were accounted for [96]. The overall increased incidence of BCC and SCC seen in immunosuppressed transplant recipients is evidence for a role of the immune system in tumour surveillance and development [97]. In a follow up study in Queensland of renal transplant patients with no skin cancer prior to transplantation, over a 25 year period, 50% developed both BCCs and SCCs with a cumulative incidence of BCCs approximately 25% at 10 years and 54% at 20 years [98]. Studies showing that transplant recipients develop more BCCs on the trunk and arms than non-immunosuppressed patients supports suggestions that immune surveillance has a role in determining tumour site [20].

1.4.1.4. Pattern of UVR exposure

It had long been believed that risk of NMSC was solely related to total cumulative dose of UV. While the latitudinal gradient of risk would suggest this, the smaller gradient for BCC compared with SCC suggests a differing relationship with amount of UV exposure. Also, although BCC is commonest on the heavily exposed head and neck it also favours sites such as the trunk which are not regarded as continuously exposed. It has emerged that BCC has a complex non-linear relationship with sunlight exposure, the characteristics of which have only begun to be unravelled over the last two decades. It is apparent that the amount, timing and pattern of sun exposure causing BCC is quite different from that causing SCC and more similar to that associated with melanoma. Patterns of personal sun exposure are usually described with reference to total/cumulative lifetime exposure, occupational/continuous exposure, or recreational/intermittent exposure. Each of these may be estimated over the whole or part of life and new validated questionnaires have evolved to enable this. One such questionnaire links recall

of sun exposure to a lifetime residence and work history calendar that subjects complete [38, 80]. Creating a link with personal history has proven to be a useful tool, but does not necessarily completely prevent differential recall bias.

The timing of sun exposure appears to be particularly important in BCC and melanoma risk. In particular, childhood exposure to UVR seems to be more closely associated with adult risk of BCC and melanoma than adult exposure. The link between BCC and childhood exposure has been demonstrated with evidence that migration to Australia before ten years of age is associated with the same risk of BCC as Australian-born persons, and that this risk decreases three-fold with arrival to Australia at ten years or older [78]. Also, there is evidence that having had two or more painful sunburns in childhood or adolescence increases risk of BCC in later life more significantly than sunburns in adulthood [81, 99]. International studies confirm these observations [47, 100]. Furthermore, there is good evidence that occupational UVA exposure after the age of twenty years does not increase risk of BCC [81].

The pattern of personal sun exposure is also now known to be an important determinant of a person's risk to different types of skin cancer. Intermittent, recreational sun exposure has emerged as the most important pattern of exposure for risk of BCC as compared with continuous or occupational exposure, with a complex interaction of UV dose and response. This was first suggested in a report on NMSC in Maryland watermen by Strickland et al in 1989 [101] and subsequently by Hunter et al [102]. Both studies found little evidence for increasing risk of BCC with increasing cumulative UV exposure, suggesting a possible plateau in risk at higher total doses. This effect has been repeated in a number of studies since these seminal studies [81, 99, 103]. Instead, risk of BCC has been positively associated with intermittent sun exposure (especially in the teenage years), hours of reported sun exposure on holidays or during water sports, and painful sunburn, especially in childhood [81, 99, 103]. It has been speculated that the target epithelial cells maybe highly mitotic and may require a relatively low threshold of total solar radiation for malignant transformation [103]. BCCs tend to be of the superficial subtype on the less exposed trunk and upper limbs [19, 20, 104] and occur at a younger average age, suggesting that there also may also be a lower threshold for UV carcinogenesis of the superficial subtype. People that tan poorly may have an increased

risk of BCC that plateaus with increasing intermittency of sun exposure while people who tan well have a constant linearly increasing risk with increasing intermittent exposure [81, 103, 105]. This supports a hypothesis of a plateau in the dose-reponse curve for BCC in relation to UVR exposure. For those who tan well, the effect of tanning and epidermal thickening protects their basal cells so that the *effective* dose received at the basal cell level is on the rising part of the dose-response curve. Thus further exposure correlates with increasing risk. For those who tan poorly, their basal cells receive a higher effective dose with the same actual hours in the sun, placing them on the hypothesised plateau of the dose-response curve.

Public education 'skin cancer control' programs have been operating in Australia for over 25 years and have focused on the importance of reducing sunlight exposure. A fall in incidence of malignant melanoma and NMSC in younger people may be evidence that these programs are starting to take effect [1, 106]. Despite these probable gains, the prevalence of potentially risky amounts and patterns of sun exposure remains high in Australia, with studies showing 10-20% of the population having had multiple blistering sunburns, frequent sunbathing/intermittent exposure [99], or skin changes suggesting a high cumulative amount of exposure [78]. Armstrong and Kricker [14] performed a metanalysis of the studies published that have looked at different types of sun exposure and sunburn (at any age) in relation to risk for the three types of skin cancer. Relative risks (RR) for BCC with different types and amounts of sun exposure remained in the low range, but was highest with sunburn (RR 1.40; 95% CI 1.29-1.51) and intermittent sun exposure (RR 1.38; 95% CI 1.24- 1.54). Occupational exposure also had a low-range association with risk (RR 1.19; 95% CI 1.07-1.32). Cumulative exposure was not associated with any increased risk of BCC in this metaanalysis, supporting other evidence of the lack of importance of this factor. Melanoma showed a somewhat higher risk association with sun exposure than BCC but still in the low range: for intermittent exposure the RR is 1.71 (95% CI 1.54- 1.90) and for sunburn it approaches a doubling of risk (RR 1.91; 95% CI 1.69-2.17). SCC was the only type of skin cancer in this metanalysis that displayed a strong association with cumulative sun exposure and occupational exposure. Together these results suggest that a history of sunburn generally

reflects an intermittent pattern of sun exposure, and that intermittent exposure and sunburn are risk factors for BCC development.

1.4.1.5. Population differences in UVR and skin cancer risk

The difference in incidence of NMSC between white populations (see section 1.3.3.1) may intuitively be simply attributed to differences in UV exposure; however the reality is likely to be more complex. Models relating BCC risk to increases in UV exposure suggest that an increase of 1% of UV exposure increases the risk of BCC by 1.7% [107]. Hawaii has a climate similar to that of Australia, but the rates of BCC in white populations in Hawaii remain much lower than in genetically similar populations in Australia [44]. The differences in incidence seen between these varying populations internationally are likely to be due to a complex interplay of different factors including differences in UV exposure, genetic heterogeneity, skin types, and behaviour; in other words, different combinations of genotype, phenotype and environmental risk factors. This inconsistency in incidence underlies the urgency in further evaluating the genetic susceptibility to NMSC.

It has been suggested that continued depletion of stratospheric ozone and climate change may be involved in increased international skin cancer rates (see section 1.3.3.3) [108-110]. However, the increases in incidence are not uniform internationally and cannot be entirely explained by the depletion of the ozone layer. For instance, Norway has seen a steady increase in skin cancer incidence over the last five decades with no concomitant change in measured ozone levels [66]. The observed international increase in BCC incidence is likely to be due to a complex interplay of a variety of factors including ozone depletion, increased detection rates, change in type of clothing, increased mobility of populations between countries and climates, and increase in outdoor recreation and sports [111].

1.4.1.6. Sensitivity to UVR

Studies have found that the complex of inherited characteristics defining a person's 'sun sensitivity' (often measured by the ability to tan) is more important in mediating skin

cancer risk than the colour of the person's skin. Of the three types of skin cancer, the weakest evidence for an increase in risk with increasing fairness of the skin is seen for BCC and the strongest for melanoma. It is important to note however, that there have been difficulties in accurately measuring skin colour in an objective way, and that these problems with measurement bias should affect the interpretation of associations found through such studies [14]. Kricker et al [78] found that when sun sensitivity and skin colour were analysed in a single logistic regression model with hair colour and eye colour, only the ability to tan remained a significant predictor of risk for BCC. Most other studies that have found an association between skin colour and skin cancer have not controlled for the ability to tan [81, 112-114]. The seminal study by Kricker et al [78] shows that the relative risk for BCC in persons with no ability to tan is 3.7 (95% CI 1.9-7.3) times that of persons with the capacity for a deep tan, and that there is a gradient to increasing risk with increasing sensitivity; meaning that 'ability to tan' is a moderate-range risk factor, and more significant than that seen for sun exposure history. A gradient of risk with sun sensitivity also exists for SCC and melanoma [115, 116]. All of these sun sensitivity gradients were steeper than the corresponding gradients in risk with decreasing skin colour, supporting a greater importance of the former with respect to skin cancer risk. The poor association of risk of NMSC with density of pigmentation is also supported by the similarly low rate of skin cancer amongst USA blacks and the lighter-skinned Chinese people living in the same environment [14]. Many other studies confirm that skin that burns easily and tans poorly is a risk factor for BCC [42, 47, 102, 117, 118].

Early studies on pigmentary traits such as hair colour and eye colour were contradictory and suffered from methodological weaknesses relating to ascertainment, use of inappropriate controls, and subjective measurements. Blond or red hair and light eye colour were reported to increase the risks of skin cancer in several studies [47, 102, 117]; subsequently two Australian studies [112, 119] and an Irish study [120] found no associations of skin cancer with hair or eye colour. Studies that have controlled for other factors such as the ability to tan found that hair colour and eye colour were no longer independent risk factors for BCC [78, 81].

Evidence that Southern European ancestry is protective for BCC is seen in a study where any Southern European grandparent greatly reduces the risk of BCC (OR 0.05, 95% CI 0.01-0.42) [78], and this is likely to indicate the protection associated with the complex combination of genetic, pigmentary and sun sensitivity traits of people of this ethnic background. Celtic origin has been reported to be associated with skin cancer in limited studies [121, 122] and has been refuted by others [78] who found no evidence of this as a risk factor per se.

1.4.1.7. Personal sun protection

It is intuitively appealing to assume that personal sun protection measures such as the use of hats, clothing and sunscreens should decrease skin cancer risk. Personal sun protection is difficult to measure for several reasons; it is difficult to separate sun protection and sun sensitivity as those with more sensitive skin are more likely to employ greater sun protection measures, and in addition, it would be impossible to ethically support a placebo sun protective measure given the strong inferential data suggesting that appropriate sunscreen use protects the individual from skin cancer. Several randomised controlled trials (RCTs) confirm the ability of sunscreen use to reduce the risk of SCC precursors and probably also to reduce the risk of SCC [123-126]. The effect of sunscreen use on BCC risk is more contentious: daily sunscreen use has been shown in a randomized controlled trial to have no effect on the risk of BCC compared with the discretionary use of sunscreen [126]. One observational study indicates a probable effect of sun protection measures in adults in reducing risk of BCC in the short term [96]: patients were determined to be in either a high or low sun exposure group based on their personal protection measures, and this level of sun exposure over 18 months significantly predicted the number of new BCCs independently of the number of previous BCCs. Other large population-based prospective case-control studies have found that sunscreen use is associated with an *increased* risk of BCC; also that BCC risk is higher on the head and neck for those who had worn a hat compared with those who had not [99, 102, 121]. That sunscreen use in that study showed a similar within-study pattern of risk to hat use suggests that any increased risk found was not due to any specific harmful effect of sunscreens (such as chemical mutagenicity or increased exposure to longwave UV

radiation). These results should be interpreted with the awareness that protective behaviours may be adopted after high risk subjects become aware of their risk status, and that the risk of BCC may be associated with sun exposure behaviour prior to this awareness. Firm conclusions cannot be made with respect to personal sun protection measures.

The reported associations between melanoma and personal sun protection have also been controversial with several studies showing increased risks of melanoma associated with sunscreen use and ascribing this to the increased duration of UV exposure permitted by the use of sunscreen. A population based case-control study in Sweden reported a slightly increased risk of melanoma associatied with sunscreen use [127] and a retrospective study on sunscreen use and naevus counts in children concluded that sunscreen use increases the risk of melanoma [128]. A well designed RCT found that sunscreen use may reduce the incidence of new naevi in children [129]. Following much debate [130] [131-133] and criticism over the methodological weaknesses of the studies showing associations between sunscreen use and melanoma [132, 134], the answer is still unclear.

1.4.2. Phenotypic subtypes

One research group has looked at BCC risk with respect to measurable phenotypic differences within populations of individuals affected by BCC. They describe subgroups of patients with BCC that fall into two major phenotypic classifications; the multiple presentation phenotype (MPP) characterized by the presence of clusters of 2-10 new, primary BCC tumours at any presentation [135]; and the truncal phenotype characterized by an initial presentation with a BCC on the less sun exposed trunk as opposed to the more frequently affected head and neck region [136]. Initial presentation with a truncal tumour has been shown by this group to be associated with the development of significantly more BCC lesions (0.13 per year) on this site compared with patients who presented initially with head and neck lesions (0.03 per year) . The mean age of presentation in patients with initial truncal lesions is younger (59.6 years) than those first presenting with head and neck lesions (64.9 years), and these patients are also more likely to have multiple BCCs at presentation. Together these results could point towards a

predisposition to BCC, and the authors propose that this predisposition is likely to be due to a combination of genetic factors [137] and a reduction in immune surveillance [135].

1.4.3. Previous BCC

One of the strongest known risk factors for developing BCC is a history of one or more previous BCC [39, 96, 138-141]. The highest risk period for a second BCC is within one year of the previous BCC [141], and the three year cumulative risk is between 33 and 77% (depending on the number and type of lesions present), at least a 10-fold increase in incidence compared with the rate in a comparable general population [142].

1.4.4. BCC risk and other malignancies

The association of BCC with the risk of other malignancies remains unclear, with some studies showing no association and others showing inconsistent and small increased risks of several internal cancers eg lung, thyroid, breast, testicular, cervix, and non-Hodgkin's lymphoma [143-148]. Patients diagnosed with BCC at an earlier age (before 60 years) have been shown to be at greater risk of breast, testicular and non-Hodgkin's lymphoma [145]. An increased risk of malignant melanoma has been shown more consistently in patients with prior BCC, and varies from 2.2 to 17-fold [144, 146, 149]. Shared risk factors such as exposure to the same carcinogens eg UVR, and increased surveillance of patients with prior BCC may contribute to this overlap in risk. Overall cancer mortality of people with a history of NMSC may also be increased, with reported relative risks of 1.30 for men (95% CI 1.23- 1.36) and 1.26 for women (95% CI 1.17- 1.35) [150].

1.4.5. Other risk factors

Other well established risk factors for BCC include exposure to therapeutic ionizing radiation [151-153], and arsenic [154-156] and scars [157, 158]. Smoking has been identified as a risk factor for BCC however reports have been inconsistent and no firm conclusions can currently be made [88, 100, 159, 160]. Dietary factors such as antioxidant vitamin and fat intake may also play a role in risk [161, 162], although large prospective studies have refuted this evidence [163, 164].

1.4.6. Association with other UVR-associated lesions

Numbers of melanocytic naevi are unlikely to be associated with individual risk of BCC development. Melanocytic naevi are known to be caused by a combination of genetic factors and environmental UV exposure, especially in childhood [129, 165] and are not a good direct surrogate measure of the sun exposure required for BCC development. One study suggested that numbers of larger moles (greater than 5mm in diameter) is associated with an increased risk of BCC however the authors note that as subjects performed their own mole counts, that there may have been some problems with misclassification of solar lentigines as moles [78]. Studies from Australia and internationally have noted the poor concordance between patients' and doctors' mole counts [166, 167] further discounting the relevance of these results.

Seborrhoeic keratoses are probably related to sun exposure and so it is intuitive that their presence may be associated with risk of skin cancer. This question has not yet been adequately addressed and despite their frequency, very little is known about the nature or causes of these benign cutaneous tumours. What is known is that seborrhoeic keratoses may occur on any site of the body except palms and soles; occur more commonly in populations with lighter skin than more darkly pigmented populations [168]; seem to increase in frequency with age; and are more common on sun exposed areas of the body [169]. The incidence of these lesions may have increased in Australian populations over the last couple of decades, and seems to be greater in Australia than the UK [170, 171]. Together this data gives circumstantial evidence that UVR is involved in the aetiology of these lesions, and does not discount the possiblility of genetic predisposition to these lesions.

It is obvious that despite the growth in evidence of important epidemiological associations with development of SCC and BCC, studies have not looked at this evidence in the context of population-based family studies, and very little is known about the probable complex interaction of these epidemiological risk factors with genetic factors.

1.5. PTCH gene

1.5.1. PTCH as a tumour suppressor gene

Much of the current understanding of the molecular genetics of BCC comes from the identification of causative mutations in genes involved in familial cancer syndromes. Perhaps the most significant of these to our understanding of BCC is the discovery of the molecular basis of naevoid basal cell carcinoma syndrome (NBCCS), also known as Gorlin syndrome. In the last decade, disruptions to the hedgehog signalling pathway have been inextricably linked to human tumorigenesis, most notably in the pathogenesis of BCC. It has long been known that this pathway is pivotal to embryonic development [172, 173]. More recently it has been shown that this pathway also has a role in tumour suppression, a function that is not surprising considering that many developmental genes continue to function in regulation of cell growth and differentiation after embryogenesis.

Tumour suppressor genes are recessive oncogenes (or anti-oncogenes) whose homozygous inactivation is required for their carcinogenic expression. In NBCCS individuals are born with an inherited or germline mutation of one allele of the *PTCH* gene. This causes an autosomal dominant syndrome of cancer predisposition, because the remaining allele is likely to be lost in a proportion of susceptible cells through somatic events. This is explained by the Knudson 'two-hit hypothesis' [174] which suggests that the first 'hit', or mutation, is inherited; the second hit is the loss of the remaining allele (or loss of heterozygosity) due to random somatic events causing mitotic nondisjuction, deletion, or mitotic recombination. In NBCCS, each cell in the body carries the mutation, so that only the one additional hit (eg mutations following UV radiation) is needed. The same mutation is seen in sporadic BCCs, where both hits come from the somatic type of mutation [175-178]. Individuals with NBCCS get a head start on the mutation of the *PTCH* gene alleles so that BCCs occur earlier than in their non-syndromic counterparts.

1.5.2. The Hedgehog pathway

What is known about the Hedgehog pathway in vertebrates has largely been inferred from studies in *Drosophila melanogaster*. The importance of this pathway is displayed by its high degree of conservation through evolution [179]. Drosophila hedgehog works in concert with other molecules to lay down the basic framework of the embryo,

determining anterior-posterior relationships (segment polarity) in developing structures. A number of proteins are recognized to be important for the function of this pathway, and in most cases a single gene encoding one of these proteins in *Drosophila* corresponds to a family of related homologues in vertebrates.

The model for hedgehog signaling in vertebrates involves a receptor complex at the cell surface made up of two transmembrane proteins *PTCH* and *smoothened* (smo) [180, 181]. While in Drosophila there is one *hedgehog* protein, in vertebrates there are three homologues, named Sonic, Desert and Indian, although Sonic is the most commonly expressed. There are also several *PTCH* homologs in vertebrates, with *PTCH1 (PTCH)* likely to act as the major receptor molecule for all three forms of human hedgehog. *PTCH2* is a close homolog of *PTCH* [182] and its normal function is not known, although there is evidence for its involvement in a medulloblastoma and also a BCC tumour, and thus it may rarely act as a tumour suppressor gene in these tumours [183]. *PTCH* is a 12-pass protein that binds the protein hedgehog, whilst *smoothened* is responsible for transducing the hedgehog signal, promoting transcription of downstream target genes. In the absence of hedgehog binding, *PTCH* holds *smoothened* in an inactive state, thus inhibiting signalling to downstream genes. When *hedgehog* (hh) binds to the receptor complex (via *PTCH*), *PTCH* inhibition of *smoothened* is released and the signal is transduced. It is unclear how these two surface molecules interact with each other; directly (eg. via a conformational change), or indirectly (eg. via a catalytic mechanism [184]).

Downstream mediation of the hedgehog signal in *Drosophila* occurs via a zinc finger transcription factor, *cubitus interruptus* (ci). In the absence of a *hedgehog* signal, this molecule forms a tetrameric complex at the microtubules with three proteins; *fused*, *suppressor of fused*, and *costal-2*, causing cleavage of ci to generate a smaller fragment that enters the nucleus and prevents transcriptional activation. In the presence of a *hedgehog* signal, the tetrameric complex dissociates and inhibits the cleavage of ci causing a full length ci to enter the nucleus and activate target genes [185]. Once again the vertebrate has three homologues of the *Drosophila* ci, and these are named *Gli1, Gli2* and *Gli3* after their role in human gliomas [186].

1.5.3. Target genes

Studies have uncovered many possible target genes of the hedgehog pathway and these are mostly genes involved in cell cycling, cell adhesion, signal transduction and regulation of apoptosis [187, 188]. Hedgehog signaling has been shown to oppose cell cycle arrest and increase the replicative capacity of cultured epithelial cells [189]. Extrapolation from target genes of Drosophila *PTCH* implicates several possible target genes for humans: *PTCH* itself, signaling-protein members of the Wnt family (*wingless* in Drosophila) and members of the TGFβ family (*decapentaplegic* in Drosophila) encoding the bone morphogenetic proteins (BMPs) [190]. The upregulation of *PTCH* expression is thought to cause the sequestration of hedgehog within the cells that it is produced, thus limiting its further movement [191]. The Wnt and TGFβ family proteins may be the main mediators of the hedgehog effect by both autocrine effects and paracrine effects on surrounding tissues [192]. An expected consequence of *Wnt* overexpression in BCC is increased levels of intracellular β-catenin, a protein that interacts with E-cadherin and is important in cell adhesion [193, 194]. The TGFβ superfamily members have diverse functions with effects on cell proliferation, expression of extracellular matrix proteins, morphogenic movements, apoptosis, and differentiation [195]. BMPs in particular play an important role at sites of epithelial-mesenchymal interaction and may be important in mediating tumour invasion [196]. There is also some evidence that Gli1 and Gli2 may mediate the carcinogenic effect. The *Gli1* gene has been previously shown to act as an oncogene in brain tumours including medulloblastomas [186]. More specific to its role in the hedgehog pathway, mouse models overexpressing *Gli1* or *Gli2* in the epidermis develop skin tumours that resemble BCCs [197, 198].

1.5.4. Naevoid Basal Cell Carcinoma (Gorlin) Syndrome - NBCCS

NBCCS was first described in 1960 [199] and is a rare autosomal dominant disorder characterized by three ‘major’ features - multiple basal cell carcinomas, dyskeratotic palmar and plantar pitting, and odontogenic keratocysts. Many other developmental and skeletal anomalies constitute ‘minor’ features and include overgrowth, epidermal scysts, calcified falx cerebri, rib anomalies such as bifid ribs, cleft lip and palate, and spina

bifida occulta. Other tumours also occur with increased frequency in this disorder and these include medulloblastoma, ovarian fibromas, meningioma, and rhabdomyoma [200]. The most common and debilitating of all the effects of this syndrome are the BCCs that appear much earlier than in the normal population and can number more than 500 in a lifetime [201].

As with other cancer susceptibility genes, a proportion of those carrying the mutation do not actually develop the disease, and it is likely that the actual manifestation of tumours depends on the constellation of gene and environmental risk factors particular to the family and individual. For example, Australian sufferers develop BCCs at an earlier age than their English counterparts, which is likely to be due to genetic susceptibility interacting with risk due to ultraviolet light exposure in the different environments [201, 202]. In Australia, nearly 50% of Gorlin syndrome patients will have at least one BCC removed prior to the age of 20, a rate 900- fold higher than the Australian population of that age. By the age of 40, 95% of Australian Gorlin syndrome sufferers will have had a BCC, some 300- fold higher than the rate of the general population [1, 201]. In the UK Gorlin sufferers also have a 900- fold increased risk of developing BCC eventually, however they do so at an older age, with only 4% having had a BCC removed by the age of 20, a 50- fold increase compared with the population [54, 202, 203]. Some 15% of Gorlin syndrome sufferers internationally do not manifest basal cell carcinomas at all, and this figure is even higher for individuals from darker skinned races. The BCCs in NBCCS may appear as early as two years of age, especially on the nape and most often proliferate between puberty and 35 years of age [200]. The syndrome's prevalence is now agreed to be around 1 in 60,000 [204].

1.5.5. Discovery of PTCH mutations as cause of NBCCS

An interesting gene-hunting detective story unfolded over the last decade, finally culminating in the discovery of the *PTCH* gene as the gene responsible for NBCCS. Tumour suppressor genes are regulators of cell growth and differentiation, and murine knockout models support their role in normal development [205] [206, 207]. The clinical features and behaviour of neoplasms in NBCCS suggested that the underlying defect may

have been a tumour suppressor gene: tumours are multiple and occur relatively early compared with sporadic tumours of the same type, and the syndrome also features derangements in normal development. If the Gorlin syndrome gene functioned as a classical tumour suppressor, then it should be homozygously inactivated in BCCs and the other tumour types in this syndrome. Since we know that inactivation of a tumour suppressor gene often occurs through mutation of the first allele (the first hit) and loss of the second allele (the second hit) then we would expect to see loss of heterozygosity (LOH) for polymorphisms surrounding the tumour suppressor gene.

Linkage analysis placed the gene on chromosome 9q22-31 [176, 208-212]; [213]. As part of a collaborative positional candidate search, Hahn et al [213] then found as many genes as possible that mapped to that area and searched for submicroscopic rearrangements in patients. The *PTCH* gene was found to lie in the correct region of the chromosome and patients were screened for mutations in this gene using single strand conformation polymorphism analysis (SSCP) and DNA sequencing. Inactivating mutations within the *PTCH* gene were found in six unrelated NBCCS patients. At almost the same time, Johnson et al [214] postulated *PTCH* as a candidate for NBCCS and found two affected individuals with inactivating mutations by SSCP. Furthermore, both of these seminal studies also found the *PTCH* gene to be mutated in sporadic BCCs, implicating this gene as a possible aetiologal necessity in the development of non-syndromic BCCs as well.

There is wide phenotypic variation in NBCCS-affected families, and screening of the *PTCH* coding region also reveals a wide spectrum of mutations in NBCCS patients. The majority of the mutations found are predicted to result in premature protein truncation [175, 215], but the phenotypic variability does not seem to correlate with the nature or location of the mutations in *PTCH*. Interestingly, even kindreds with identical mutations differ markedly in their clinical features, suggesting that other genetic or environmental factors may be important modifiers of developmental and neoplastic traits [215].

1.5.6. PTCH inactivation in sporadic BCC

Further evidence has since been found for the inactivation of *PTCH* as a major factor in sporadic BCC formation. LOH has been found in over 50% of BCCs suggesting that in

many tumours one of the alleles is inactivated by deletion [176]. In some tumours without LOH, direct sequencing reveals inactivating mutations in *PTCH* that have not resulted in loss of the allele, suggesting that mutation of *PTCH* may be a necessary step for tumour formation of sporadic BCCs [216]. It is likely that in tumours not showing allelic loss, both copies of the gene have undergone point mutation. As with NBCCS *PTCH* mutations, where mutations are found, most lead to premature protein termination, and a number of them are C-to-T substitutions typical of ultraviolet B irradiation (UVB) involvement. UVB radiation mainly produces DNA lesions between adjacent pyrimidines (TT, CT, TC, CC) – the 'UVB signature' – and two types of lesions are produced; the cyclobutane pyrimidine dimers and the 6-4 photoproducts [217-219]. However, more than 50% of the *PTCH* mutations in sporadic BCCs do not have the UVB signature [216], suggesting that environmental factors other than UVB exposure may be important in the pathogenesis of BCCs (eg. UVA exposure). Mutations in *PTCH* have also been detected in BCCs associated with Xeroderma Pigmentosum (XP) and in contrast to sporadic tumours, XP tumours show a high rate of UVB signature mutations [77]. This is consistent with the mechanism of XP whereby sufferers are unable to repair DNA lesions such as mutations caused by UVB.

Despite an absence of an animal model for BCCs, tumours that closely resemble BCCs have been demonstrated in *PTCH1* +/- knockout mice, and exposure to UVR increases the size and number of these tumours and shifts their histological features so that they more closely resemble human BCCs [4]. Studies also show that sonic hedgehog signaling is a component of T-cell responses to mediate CD4+ T-cell effector function and that response varies in individuals, suggesting that *PTCH* alleles may be involved in the effectiveness of immune surveillance [220].

1.5.7. PTCH involvement in other tumorigenesis

A number of key members of the hedgehog signaling pathway are involved in tumorigenesis of a range of tumours. These include medulloblastoma, meningioma [221], squamous cell carcinomas of the oesophagus [222], transitional cell carcinomas of the bladder [223], and the benign skin lesions trichoepitheliomas [224]. Several of these

sporadic tumours are not involved in NBCCS therefore suggesting a wider role for *PTCH* mutation in cancer pathogenesis. Other members of the hedgehog pathway have been investigated for their role in tumorigenesis. Activating mutations of *smoothened* have the same downstream effects as inactivating mutations of *PTCH*, and a number of independent studies have detected activating mutations of *smoothened* in 10 to 20% of BCCs [225, 226]. Mice overexpressing downstream members of the hedgehog pathway, *Gli1* or *Gli2*, develop BCC-like lesions [197, 198]. The Hh gene has also been implicated, and overexpression in transgenic human and mouse skin leads to tumours that are morphologically indistinguishable from BCCs [227, 228]. The induction of BCC with the activation of this pathway alone in the absence of deliberate mutagenesis suggests that activation of the hedgehog signaling pathway initiates BCC formation. The exact mechanism of dysregulated hh signaling and increased risk of cancer development remains to be elucidated. Interestingly, hedgehog pathway target-gene overexpression is seen in all BCC subtypes suggesting that this dysregulation is an early rate-limiting step in BCC carcinogenesis [177].

1.5.8. PTCH pathway in future therapies

A better understanding of the molecular processes underlying the pathogenesis of BCCs may allow improvements in therapeutic success in its management. Therapies involving inhibition of hedgehog signaling might be expected to suppress tumour growth. Cyclopamine from the Veratrum lily species is know to inhibit hedgehog signaling and reverse the effects of oncogenic *smoothened* and *PTCH* mutations [229]. There appears to be no adverse effects of exposure of adults to this compound, and it is therefore interesting as a basis for a possible therapeutic agent for tumours resulting from dysregulation in hedgehog signaling.

1.5.9. PTCH polymorphisms and BCC

Some important preliminary work has been performed to investigate *PTCH* polymorphisms in relation to BCC susceptibility. The consequences of polymorphisms of exons of *PTCH* are unknown, and they may have functional implications. There is increasing evidence that even silent substitution of nucleotides (effecting no amino acid

change) could influence mRNA processes, including affecting splicing accuracy or efficiency [230]. This may be the case for the *PTCH* intronic SNP IVS 15+9 which may form part of an intronic enhancer region, as the adjacent sequence conforms to a splicing enhancer sequence identified in the growth hormone gene [231, 232]. Haplotypes may confer increased risk through encoding of a protein with less efficient function by the combination of the individual effects of the SNPs involved; alternatively they may be in linkage disequilibrium with other significant variants.

One research group have found significant associations between certain *PTCH* haplotypes and rate of development of further BCCs/year in English subjects who initially developed a head/neck BCC [233]. Subjects were genotyped for two exonic SNPs (exon 12: C>T 84 and exon 23: T>C 140) and one intronic SNP (exon 15: IVS 15+9). They found no association between rate of BCC accrual and these genotypes individually, but did find an association with the haplotype of the exon 12/exon 23 variant, giving a relative risk of increased BCC accrual (BCCs/year for one year following presentation) of 2.46 (95% CI 1.27- 3.97). The exon 23 variant causes a proline to leucine change (Pro>Leu), and has been linked with breast cancer risk in women using the oral contraceptive pill [234], suggesting that there is possibly an as-yet-uncharacterised functional significance to this SNP. The haplotype association with BCC accrual remained significant when controlled for UVR-exposure parameters, skin type, gender and age at first presentation. These researchers also found an association between the exon 15 variant/exon 23 wild type haplotype and decreased risk of BCC in a case control analysis (OR 0.44, p=0.009), with significance unaffected by adult UVR exposure. It should be noted that despite a claim to control for UVR-exposure parameters, childhood and adolescent exposure were not included in the UVR-exposure questionnaire for either study; and as it is evident that UVR exposure at these ages is likely to affect later risk of BCC [78] and omission of this may overestimate the effect of the genetic association in these analyses. Nevertheless, the importance of further investigation into the significance of *PTCH* polymorphisms with respect to risk of BCC is highlighted by these studies.

The exon 23 Pro>Leu SNP has been investigated further in a pilot study that examined the association with populations of differing pigmentary characteristics and risk of NMSC [235]. The Pro>Pro genotype was significantly less common in populations with characteristically lighter skin colour (eg. Swedes) compared with populations with darker skin colour (eg. African-Americans), suggesting a possible association between the eumelanin-to-phaeomelanin shift and a shift from the Pro>Pro genotype to Leu-containing genotypes. There was a non-significant trend for increasing Pro>Pro frequency with increasing BCC severity (early-onset or multiple tumours). The authors suggest that the failure to replace Pro in phaeomelanin-prevalent populations may be associated with an increased population risk for BCC, and an increased individual risk for multiple BCC.

No studies have investigated the involvement of other hedgehog pathway genes or indeed any other possible BCC susceptibility genes in the general population outside of the context of NBCCS.

1.6. Familial Cancer Syndromes

Several other familial cancer syndromes involve a predisposition to the development of multiple BCCs. Bazex-Dupre-Christol syndrome was first described in 1966 [236] and is a rare X-linked dominant syndrome characterized by congenital generalized hypotrichosis, follicular atrophoderma and multiple early-onset BCCs occurring in the second or third decade of life that have an aggressive course and are prone to relapse [237, 238].

Rombo syndrome was first described in 1981 and is a rare familial disorder for which inheritance is likely to be autosomal dominant [239-241]. This rare familial disorder has skin changes that become evident at seven to 10 years of age with cyanotic redness and follicular atrophy of the sun exposed skin, and later milia-like papules and telangectasias. The skin changes become more pronounced with age and lead to a “worm-eaten” appearance of the skin known as atrophoderma vermiculatum. BCCs develop around the age of 35, depending on the exposure to other risk factors such as UVR.

The recessively inherited diseases; xeroderma pigmentosum (XP), albinism and epidermo-dysplasia verruciformis, are also associated with the development of multiple BCCs in association with a predisposition to NMSC in general. Patients with XP are deficient in the repair of UV-induced DNA lesions and are characterized by their predisposition to early-onset NMSC (average age 10) on sun-exposed skin [77, 242]. XP cells are deficient in those gene-products required for catalyzing the incision step in nucleotide-excision repair of damaged DNA [243, 244]. Albinos also develop BCC at an increased rate compared with normally-pigmented persons, although SCCs are more common in this group [245]. Epidermo-dyplasia verruciformis is a rare multifactorial disorder involving genetic, actinic, and immunologic factors, with a susceptibility to NMSC (mostly SCC) associated with the development of multiple pityriasis versicolor-like lesions as well as flat wart-like lesions [246].

There have been several unrelated reports of non-syndromic hereditary BCC [3, 247] suggesting the possibility of a genetic predisposition to BCC in addition to the rarer familial syndromes described: these include cases exhibiting unilateral distribution suggesting mosaicism [248-250]. The *PTCH* gene is an obvious candidate to contribute to non-syndromic susceptibility to BCC, either via a different mutation spectrum or in the absence of modifiers that are essential for the full Gorlin phenotype. The reports of polymorphisms in this gene associated with number of BCCs per year and their possible association with pigmentary factors have given weight to this possibility [231, 233, 235].

1.7. MC1R

1.7.1. Melanin and pigmentation

Cutaneous pigmentation results from the synthesis and distribution of melanin in the skin. Melanin is a pigmented heteropolymer produced by melanocytes in the complex process of melanogenesis, the regulation of which involves more than 80 genetic loci [251]. Melanocytes are specialized dendritic cells that reside at the dermoepidermal junction and synthesise and package melanin within membrane bound organelles called melanosomes. The melanosomes are distributed to the keratinocytes and the growing hair shaft through the dendritic processes of the melanocytes. The keratinocytes then

differentiate, mature and migrate to the epithelial surface where the melanin creates light-absorbing and light-scattering effects depending on its quantity and chemical composition [252].

1.7.2. MC1R receptor

The MC1R receptor is one of five forms of melanocortin receptors (MC1R - MC5R) that have distinctive tissue distribution and physiologic roles. All are activated by the melanocortins, a family of structurally-related peptide hormones derived from one precursor protein, proopiomelanocortin (POMC). These include corticotropin (ACTH) and the α (alpha), β (beta), and γ (gamma) melanocyte stimulating hormones (MSHs) [253, 254]. The melanocortin-1 receptor (MC1R) is a G-protein coupled receptor with 7 transmembrane spanning domains. Although melanocytes have the highest density of these receptors, it is expressed in many other cell types in the skin including keratinocytes, fibroblasts, endothelial cells and antigen presenting cells [255]. Ligand binding activates the enzyme tyrosinase gene expression and activity, melanocyte proliferation and melanocyte dendricity [256-258].

1.7.3. Forms of Melanin

Melanin exists in two differing forms within human skin, brown-black eumelanin and yellow-red pheomelanin. These forms have differing unique biochemical and ultrastructural properties within melanosomes. The eumelanosomes are large and elliptical with a highly organized matrix, containing high molecular weight, poorly soluble melanin. The pheomelanosomes in contrast are small and spherical with an unstructured particulate matrix containing low molecular weight, soluble pheomelanin [259]. *MC1R* expression appears to be centrally important to the regulation of melanocytes, including induction of photoprotective melanisation in response to UV exposure.

The ratio of eumelanin to pheomelanin as well as total melanin content is higher in persons with more darkly pigmented skin compared with those of lighter pigmented skin [260]. Pheomelanin levels are generally highest in very red hair, while eumelanin

predominates in the hair of most other colours [261 review]. Eumelanin is thought to be more photoprotective than phaeomelanin because it is more resistant to degradation by UV and more efficient at scavenging reactive oxygen radicals produced by exposure to UV [262]. Eumelanosomes also form supranuclear caps that shield the nuclei of cells from UV radiation therefore conferring additional photoprotection [263]. Pheomelanin, in addition to absorbing a narrower range of wavelengths than eumelanin, is photolabile and generates oxidative stress on irradiation, leading to less efficient photoprotection and increased photosensitivity [251].

1.7.4. MC1R variants

Following the first report of an association between germline variant *MC1R* alleles and red hair [264], human *MC1R* variants have been found to occur in more than 50% of individuals in white populations [265-267]. There are over 30 variant alleles reported in European-derived populations [264, 266-273], with several of these found to be associated with red hair and fair skin. There is in fact, such a great deal of *MC1R* coding diversity that it is difficult to identify a wild type. Several groups have looked at a combination of gene sequences to deduce a consensus sequence [266, 268, 273], using phylogenetic analysis of *MC1R* gene sequences in geographically diverse populations [272].

1.7.5. Red Hair Colour variants of MC1R

The three most common red hair colour variants account for 60% of all cases of red hair- Arg151Cys, Arg160Trp, and Asp294His, with individuals carrying two of these alleles almost always having red hair [266]. The frequency of these variants differs according to the population; studies show the allele frequency of these three types together is approximately 20% in Australians [252, 274] and nearly 50% in an Irish cohort [266]. These three Red Hair Colour (RHC) alleles give rise to loss-of-function mutations although some studies suggest that they may retain some function and that red hair is not the null phenotype [275, 276]. The RHC alleles also contribute to fair skin and poor tanning response to UV, giving rise to the ‘RHC phenotype’. These phenotypic features are explained by increased phaeomelanin in the skin and hair and/or decreased capacity

to produce eumelanin, creating a red colour in the hair shaft and reduced skin photoprotectivity [264]. There is also evidence that RHC alleles impart a heterozygote effect: one study shows that presence of one allele increases the odds of having red hair nine- to 16-fold and fair skin five- to seven-fold [266]. The weaker association with skin colour in this study may have been due to the limitations imposed by the use of the Fitzpatrick classification of skin type used in its determination.

It is clear that *MC1R* variant-allele presence is necessary for the RHC phenotype but not sufficient. Individuals with identical MC1R variants may nevertheless display different hair colour (eg auburn, red or strawberry blonde): even monozygotic twins (sharing the same genotype) have been found to differ in their hair colour [266] [268, 277]. Similarly, the exact relationship between skin phototype and pheomelanin/eumelanin production in the human epidermis is not straightforward [260, 278], although it appears that activation of *MC1R* is involved in switching between eumelanogenesis and pheomelanogenesis [279]. Thus other loci must be involved in creating the RHC phenotype, perhaps by moderating the expressivity of *MC1R* variants or masking of the trait.

1.7.6. MC1R associations with skin cancer

The involvement of the *MC1R* locus in pigmentary traits and the cutaneous response to UVR makes it an intuitively relevant candidate gene for susceptibility to cutaneous malignant melanoma and nonmelanoma skin cancer. *MC1R* gene variants have been shown to be associated with melanoma independent of their association with pigmentation phenotype giving two- to four-fold increased risk [274, 280, 281]. The large number and low frequency of *MC1R* alleles make assessment of this gene in the pathogenesis of skin cancer difficult, and initial explorations of a possible link with NMSC yielded inconsistent results [266] [282]..

More convincing evidence for associations between *MC1R* genotype and NMSC has since been demonstrated. Box et al [283] demonstrated an association between higher NMSC risk individuals and the nine commonly reported *MC1R* variants, an association that persisted after adjusting for pigmentation phenotype. A larger case-control study [267] replicated these findings and extended them to include most of the other known

MC1R variants. Carriers of any two variant alleles in this study were at increased risk of BCC and SCC. Carriers of one variant allele had half the risk, confirming the heterozygote effect of this gene. Unfortunately, the *MC1R* studies involving NMSC to date have suffered from a lack of reliable and objective consensus measure of measuring constitutive skin colour [284, 285]. These results do however indicate that *MC1R* variant status may be an independent risk factor for the development of BCC: this needs to be further explored. Also, other genes and proteins may be involved in modifying the expression of *MC1R* variants. For example, it is possible that *PTCH* polymorphisms such as exon 23 Pro>Leu (see section 1.5.9) or mutations in this gene could interact with MC1R variant status to affect NMSC risk.

1.8. p53

The gene *P53* encodes the protein p53 which has been termed "guardian of the genome". The gene is located on chromosome 17 band p13, and the protein is a 53 kd nuclear phosphoprotein [286, 287]. Mutations of this gene occur in a wide variety of tumour types and are in fact the most commonly detected genetic abnormality in human cancer [288]. The protein is known to be associated with malignant melanoma susceptibility through its association with the protein $p14^{ARF}$ and the CDKN2A locus [289]. The p53 protein functions to sense genotoxic injury and arrest cell division in late G1 of the cell cycle, allowing DNA repair to occur before replication. In the case of extensive DNA damage, it induces apoptosis in an effort to eliminate defective and potentially malignant cells. Mutated p53 can act in one of two ways to contribute to malignancy; as a dominant negative oncogene by affecting normal p53 activity [290] or as a tumour suppressor gene by creating a nonfunctioning p53 protein in the presence of further mutational events [291].

A study by Rady et al was the first to identify *P53* mutations in BCC, finding 50% of 14 tumours carrying a P53 mutation, and was the first to give evidence that these are likely to be UV induced mutations [292]. Studies following this found mutations from between 44% and 100% of tumours [8, 84, 291, 293-296], and showed that the UVB portion of the spectrum is likely to be more important than UVA in p53 mutations [84]. Mutations in

NMSC occur at several hotspots that are much less frequently mutated in internal cancers, and most of them contain the dipyrimidine transition mutation that indicates UV induced origin [293, 297, 298]. Most of these hotspots are localized in an area of the gene that is evolutionarily conserved [299], suggesting that the mutations are likely to have functional significance and therefore act as causative mutations. Skin-cancer-specific hotspots have been seen to be repaired more slowly by nucleotide excision repair than those at surrounding positions on the same strand, supporting a role in tumour induction [300] [72]. There are however, likely to be other mechanisms at work in BCC to inactivate *P53*, some tumours without *P53* coding sequence mutations have been found to harbor aberrant p53 protein stability [293].

BCC case subjects have been reported as three times more likely to have a *P53* mutation in normal skin taken from the mirror-image site to the cancer site, suggesting that *P53* mutation is an early occurrence that may predict the risk of BCC development [301]. *P53* point mutations may occur in cells during sun exposure early in life, which then over many years acquire other genetic alterations required for BCC [293]. This multiple-mutation concept is supported by the finding that multiple samples from individual tumours have at least one *P53* mutation in common, suggesting that the tumour is derived form a single cell with a unique *P53* mutation as an early event.

It is also possible that there is a genetic interaction in tumour suppression between *PTCH* and P53, with evidence from *PTCH* +/- mice that concomitant loss of *P53* dramatically accelerated tumorigenesis in medulloblastomas [302]. A germ line polymorphism in *P53* has also been noted and its contribution to HPV induced skin cancer remains controversial: polymporphisms may lead to increased susceptibility of the p53 protein to HPV-mediated degradation [303, 304].

1.9. HPV

While the aetiologic role of HPV in anogenital cancer is well established, the epidemiological, molecular and functional data required to fulfill the World Health Organisation criteria for viral carcinogenesis have yet to be met in NMSC [305]. Fifteen epidemiologically defined high risk mucosal HPV types have been found to cause a

persistent infection of the cervix as a necessary risk factor for the development of cervical cancer [306]. An association between papillomaviruses and NMSC pathogenesis was first seen in an animal model whereby SCCs of the skin developed reproducibly following experimental infection from virus-induced warts in rabbits [307]. Unlike HPV involvement in cervical cancer, HPV may not be necessary for NMSC pathogenesis but simply act as a cofactor along with UV radiation exposure. Complicating all deductions made with regard to HPV in NMSCs is the fact that HPV is detected with varying prevalence in normal skin: the prevalence ranges from 4.7% [308] to 35% [309].

The earliest evidence for an association between HPV and NMSC in humans originated in studies on patients suffering from epidermo-dysplasia verruciformis (EV), a rare hereditary disease. The genetic basis of this disorder is not understood, however EV patients have increased susceptibility to a group of HPV types (differing from the mucosal types) and develop multiple skin warts; multifocal NMSC (mostly SCC) also occur on sun exposed skin at a young age [246]. EV HPV types have been detected in over 90% of skin cancers in EV patients. It is clear that genetic and immunologic factors combine with HPV infection and UVR exposure in EV patients to contribute to their NMSC susceptibility. The suggestion of an association between HPV and NMSC is also inferred from the susceptibility of renal transplant recipients (RTRs) to both viral warts and NMSC (mostly SCCs), suggesting a pathogenic role of HPV in tumour development [310] [310] [97]. The long term immunosuppression needed to support a renal transplant is associated with an increased risk for certain forms of malignancies including that of the skin [311]. HPV has been found in high prevalence and of a similar spectrum in both BCCs and SCCs of RTRs prompting some investigators to suggest a differing role of HPV in the different tumour types, leading to preferential development of SCCs [312].

Most studies of the association between HPV and NMSC are small case series without control groups. Early studies employed laboratory methods that caused variability in prevalence and spectrum of HPV types found. Despite increasingly sensitive laboratory methods, no specific subset of HPV types has been consistently associated with either BCC or SCC in immunosuppressed or immunocompetent populations [308, 312-316].

Studies on HPV prevalence in NMSC lesions of immunocompetent subjects from the general population report lower figures than those found in RTRs with lower potential for mixed HPV infection [308, 312, 315, 317]. With newer laboratory techniques however, studies have found HPV infection in over 25% of lesions (see table 1.3).

Table 1.3 **Studies reporting HPV prevalence in NMSC lesions of immunocompetent people**

NMSC type	Iftner et al. [308]	Harwood [312]	Shamanin [315]	Biliris [317]
BCC	27.8%	36.7%	60%[#]	30.5%
SCC	59.7%	27.2%	65%	13.0%

[#] Note: only 3 BCCs included in this study

Recently there has been one report of high risk genital type HPV infection of both BCC and SCC with significantly greater prevalence seen in malignant lesions compared with warts or precancers, supporting an aetiological role of the high risk genital types in skin cancer [308]. Patients who were DNA-positive for the high risk types HPV-16, 31, 35 and 51 were nearly sixty times more likely to have NMSC as compared with unaffected controls. This greatly increased risk was calculated after adjusting for age, gender, and sun exposure, and despite proving an association only, provokes important questions regarding the possible role of specific HPV subtypes in the pathogenesis of skin cancer. This evidence is supported by earlier studies that have found mucosal HPV types in skin cancers [315, 317-319]. A role for high risk genital types in the aetiology of NMSC makes biological sense considering their link already with skin cancer in EV patients and to cervical cancer. In addition, three of these types have also been shown to posses transforming activity in tissue culture [320, 321].

Papillomavirus has been detected in the long-living epithelial root sheath and bulb of hair follicles in rabbits, [322], which may be relevant to the development of NMSC, as the hair follicle plays an important role in this process. The bulge region of the hair follicle is considered an immune-privileged site that may contain the reservoir from which HPV infection spreads [323]. Recent studies have examined the prevalence of HPV in eyebrow

hairs and the association with NMSC, but no firm associations with NMSC have been found so far [324] [323, 325].

E6 and E7 gene products of mucosal HPVs bind to and degrade p53 via the ubiquitin pathway and the retinoblastoma protein (pRb) respectively [326]. It was recently discovered that certain EV HPV types may bind pRb as efficiently as the mucosal types, and that they show transforming properties in human keratinocytes [327]. Other cutaneous HPV types from RTRs may mediate p53 carcinogenesis following exposure to UVB [328]. Clearly further work is also needed in this area to delineate the involvement of HPV and UV light in NMSC carcinogenesis.

1.10. Other Genetic Influences On BCC Susceptibility And Development

A number of members the human herpes virus family contribute to human cancer pathogenesis: Epstein Barr Virus (EBV) has been implicated in nasopharyngeal carcinoma and African Burkitt's lymphoma [329]; and cytomegalovirus (CMV) has been associated with cervical carcinoma [330] and adenocarcinomas of the prostate [331] and colon [332]. Because of the ubiquity of CMV and the high seroconversion rates, it has been difficult to establish an association between this virus and human cancer, and has only been looked at recently with respect to NMSC. A recent study demonstrated a frequency of CMV infection in NMSC lesions that suggests a possible role for this herpes virus in both SCC and BCC development and this warrants further exploration [333].

UVR constitutes an oxidative stress on the skin, generating radical oxygen species such as hydroxyl and superoxide radicals, hydrogen peroxide and singlet oxygen, that lead to protein, lipid, DNA, and gene mutation [334, 335]. It is intuitive to assume that the way individuals deal with this oxidative stress may contribute to their susceptibility to cutaneous carcinogenesis. The glutathione-S-transferases (GSTs) are involved in the protection against this stress as they are critically involved in detoxification of electrophilic compounds such as carcinogens and cytotoxic drugs, and they act to protect DNA from damage and adduct formation through conjugation [336-338]. GSTs are a large family of isoenzymes that comprise five classes: alpha (GST A), mu (GST M), pi (GST P), theta (GST T) and zeta (GST Z) [339, 340]. Polymorphic loci have been

identified in all GST gene families, but the pathological consequences of these remain largely unknown [341]. The polymorphism displayed by these genes makes them obvious candidates for cancer susceptibility: reduced ability to remove potential carcinogens may result in mutations in key tumour suppressor genes. This has already been shown for GST polymorphisms in association with p53 in lung [342] and ovarian tumours [343].

In relation to skin carcinogenesis, interest has focused on polymorphisms in GST T1 and GST M1 as both are expressed in skin [341]. It should be noted however that only a proportion of the GST enzyme have been studied: it is possible that the genes with the greatest effects on skin carcinogenesis have not been investigated. GST polymorphisms appear to have a greater influence on outcome within different BCC subgroups rather than on susceptibility to BCC overall: variants may affect BCC tumour number and accrual. Both GST M1 and GST M3 in combination with skin type I appear to influence BCC number [344, 345]. Tumour accrual has been shown to be influenced by male gender, number of BCCs at presentation and presence of GST T1 null [345, 346]. GST T1 null also appears to be associated with the truncal phenotype compared with patients with no truncal lesions [347, 348]. An interesting and poorly understood interaction between the GST M1 A and B alleles has been found with GST M1 A/B protective against multiple BCC, but not the homozygous state of GST M1 AA or GST M1 BB [349]. GST polymorphisms have also been associated with MM [350] and SCC [351] susceptibility.

Cytochrome P450 (CYP) enzymes are also genetically polymorphic and have been studied with respect to BCC susceptibility and development. They are also involved in detoxification of numerous xenobiotics including carcinogenic components of tobacco smoke [352] [341]. Again, associations of polymorphisms of CYP loci have been found only with respect to subgroups of patient with BCC rather than overall risk for these cancers. Associations are seen with increased numbers of BCCs [344], with truncal tumours in association with GST T1 null and with increased no of primary lesions, increased accrual [344] and reduced time to next tumour presentation [347].

DNA repair systems are complex and include mismatch repair, photolyases, base excision and post-replication repair. The maximum rate of pyrimidine dimer repair in normal skin cells is reportedly barely sufficient to cope with the rate at which damage is imposed on skin in full sunlight [353]. DNA repair capcity (DRC) was looked at by a group who used a plasmid/host-cell reactivation assay in which a UV-damaged expression vector plasmid is transfected into peripheral blood T cells from a subject. The host cellular repair enzymes repair the photochemical damage in the plamid and it is possible to measure the end amount of repair [72]. DRC measured in this way in controls shows an age related decline from the age of 20 at a rate of about 0.61% per year, adding up to a 25% loss in repair ability over 40 years [72]. This is consistent with the age related decline in DNA repair seen in other studies although results have not been repeatable [354] [355-357]. This apparent decline in DNA repair activity of normal lymphocytes has also been seen in repair of UV [358], x-ray [359], and gamma irradiation [360]. Subjects with early onset of BCC (< 44 years) and those with a family history of BCC have been shown to have a significantly lower DRC than control subjects [72]; DRC has also been linked to MM with a lower DRC seen in those with MM on sun-exposed areas than unexposed [361]. It has been suggested by the authors of these studies that the normal decline in DRC with age may account for the increased risk of skin cancer that begins in middle age, and that skin cancer in the young may represent precocious ageing.

Several investigators have noted a weak association of multiple BCCs with the HLA immunoregulatory loci. An initial study investigated HLA class II antigens with respect to ethnic groups found an association between multiple BCCs and HLA-DR1 in non-Irish and non-Ashkenazi patients, however the sample size of this group (14 subjects) may have been too small to detect a real difference [362]. Other studies supported this association in other populations however they were no longer significant once corrected for multiple testing [363, 364] or were contradictory [365-368]. A small significant negative association between HLA-DR4 and multiple BCC has been seen in two studies [364, 365] however this same HLA type was later shown to be positively associated with the development of both multiple BCC and malignant melanoma in the same individual

[366]. Overall, the role of HLA types in the development of multiple BCCs appears likely to be small.

The *Ras* gene family has also been implicated in the development of skin cancer however appear to have greater links to the aetiology of malignant melanoma [369] and SCC [370, 371] than BCC. *Ras* mutations are relatively infrequently seen in BCCs [370, 372, 373], however there is evidence that PDGFRα is upregulated in BCC with associated activation of the Ras/MAPK pathway [374]. It may be that this pathway is activated in BCCs in an analogous way to breast cancer [375] due to a number of factors including overexpression of tyrosine kinases.

1.11. Family and Twin Studies

The discovery of familial aggregation is a "universal signal for geneticists to begin investigation of genetic causes" [376] alongside classical environmental causes that also may be shared in families. Systematic study of the patterns of clustering of both common and rare cancers has been successful in this aim. In some cases it has led to the identification of specific susceptibility genes of strong effect, for example, in the study of breast cancer [377, 378] through segregation and linkage analysis. Understanding of genetic susceptibility to prostate cancer [379], colon cancer [380], pancreatic cancer [381], endometrial cancer [382], and nasopharyngeal cancer [383], have also been enriched from this type of research. In the case of melanoma, for example, genetic causes of familial aggregation are now understood to include, in a small proportion of cases, rare high-penetrance (high lifetime risk) mutations in the genes *CDKN2A* and *CDK4*, and much more prevalent low-penetrance (mildly elevated lifetime risk) variants in the pigment control gene *MC1R [384]*.

Twin studies enable the contributions of genetic and environmental factors to the aetiology of a disease to be directly examined by comparing the similarity of monozygotic and dizygotic twins. Finland has a nationwide Central Population Register that includes data on twins, and also a nationwide cancer registry with compulsory reporting of all cancers including non-melanoma skin cancers. Case reports of BCC in monozygotic twins and in twins of unknown zygosity emerged from this Finnish twin

cohort in the 1970s [385]. A more recent study looked at the difference in prevalence associated with zygosity by identifying 335 twin pairs of known zygosity in which at least one twin had BCC diagnosed between 1953 and 1996 [386]. The co-twin of a twin with BCC was found to have an increased risk of BCC (RR 7.0: 95% CI 3.7-1.3), suggesting familial aggregation of this cancer. With only 11 concordant pairs for BCC in the data from 1976 to 1996, and a total of 16 concordant pairs if the retrospective (1953-1975) data was included, there was very little power with which to make conclusions regarding the contributions of genetic and environmental effects. The proportion of phenotypic variance in susceptibility due to genetic factors was estimated at 8%, however the 95% confidence limits were 0-56%.

There have been very few other studies of familial clustering of BCC outside the context of Gorlin syndrome. One Australian study [3] described 1108 consecutive cases of histologically confirmed nonmelanoma skin cancers in a single Melbourne clinic over an 18 month period in the early 1990s. Among these patients, 12 families were identified in which more than one member had a nonmelanoma skin cancer and in which the known inherited predisposition syndromes were excluded. Because of potentially biased ascertainment of cases with a positive family history of skin cancer to the clinic it is not possible to conclude whether or not this proportion of family history positive cases is greater than would have been expected by chance. However the patterns of family history seen are instructive. In 11 of these families skin cancer had occurred in more than one generation, and in nine multiple skin cancers had developed in two or more generations. Most of the affected members developed BCCs only; there were some with both BCCs and SCCs, and a few with only SCCs. This suggests that familial forms of NMSC predisposition may exist, that they may genetic and cancer type-specific. The vertical patterns of ‘inheritance’ seen indicate that they may be an autosomal dominant trait.

Case reports also support the existence of familial aggregation of BCC with case reports of multiple superficial BCC in twins [387] or in two generations, including instances of male-to-male transmission [247, 388]. There have also been three reports of unilateral manifestations of superficial BCC [250], in which familial cancer syndromes were excluded by examination and in one case also by radiography. The phenomenon of

lateralisation is difficult to explain without the assumption that non-syndromic hereditary multiple BCC can occur as a distinct Mendelian trait, with the apparent mosaicism likely to have occurred as an early postzygotic mutational event. Thus, a separate phenotype of hereditary non-syndromic mulitiple BCC may exist, characterized by the presence of multiple superficial BCC and by the absence of other anomalies (as per the MPP described in section 1.4.2). One author [250] suggests that this possibly polygenic trait has a clinical appearance that differs from those observed in Gorlin, Bazex-Dupre-Christol and Rombo syndromes because the BCCs are stereotypically of a superficial type and preponderantly involve the trunk. There is already preliminary evidence that BCC has differential genetic susceptibility to SCC and MM, as suggested by the different HLA associations for these tumours [366, 389].

In summary, there is preliminary evidence for specific familial aggregation of BCC, but no knowledge of its causes. Based on the sum of research on other common cancers, it would be expected that a small proportion of such aggregation might be due to rare, high-penetrance alleles such as *PTCH*, and the majority due to common, low- to medium-penetrance factors such as pigmentation genotypes (e.g. *MC1R*), and sharing of the major environmental risk factor, sun exposure. However, it is clear that much further research is needed to understand the genetic epidemiology of this common and burdensome cancer.

1.12. Aims and scope of this thesis

Although much work has been done on rare genodermatoses involving BCC, very little is known about genetic susceptibility to BCC outside these settings. A small Australian study looking at familial clustering of NMSC that noted apparent differences in susceptibility to BCC versus SCC between NMSC pedigrees, suggesting differing genetic influences [3]. However, there have been no larger-scale studies of this phenomenon. Gorlin syndrome is one of the rare familial syndromes that has received research attention, and involves autosomal dominant inheritance of marked susceptibility to BCC and a range of developmental defects. Mutations in the cell growth regulatory gene *patched* (*PTCH*), when present in the germline are responsible for this disorder. They have also been found in a high percentage of sporadic tumours, suggesting a critical

involvement of this pathway in BCC tumour development generally. Furthermore, mice with Gorlin syndrome-like knockouts of one copy of *PTCH* develop BCCs if exposed to UVR, mimicking the human situation [4]. No studies have yet investigated whether germline mutations or less highly penetrant variants of this gene might account for part of non-syndromic BCC susceptibility in the general population.

Chapter 2: Methods

2.1. Patients and recruitment

2.1.1. Ascertainment

The subjects of this analysis are part of a larger study of familial aggregation of non melanoma skin cancer, which involves cases of early-onset of both BCC and SCC occurring in the greater Sydney metropolitan area, and their relatives. This geographical area is defined by the borders of Wollongong to the South, Gosford to the North, and the Blue Mountains to the West. These cases were identified through the database of the Dermatopathology Division of the Skin & Cancer Foundation Australia (SCFA). This academically affiliated service receives pathology from a significant proportion of dermatologists practising in New South Wales (NSW), as well as from those working at the SCFA's two large Sydney clinics in Darlinghurst and Westmead. Patients are referred to these dermatologists from their general practitioners for assessment and treatment of skin conditions including skin cancer, and to make use of the dermatopathology service of the SCFA. Referrals to these services are unlikely to have been biased towards people with a family history of NMSC. Neither BCC nor SCC are registrable in Australia, but their combined incidence has been estimated by population-based surveys to be 2200 per 100 000 in 1997 in Australia [390]. This figure is likely to also represent the incidence in Sydney because NSW has melanoma rates (a surrogate for sun-related skin cancer) that are close to the national average, and greater Sydney includes more than two thirds of the State's population. The incidence of melanoma in NSW in the year 2001 was 42.4 and 31.6 per 100,000 for males and females respectively, age standardised to the 2001 world population. The national average was 53.7 and 38.0 per 100,000 for males and females respectively in the year 2000, age standardised to the 2001 world population (from the Australian Bureau of Statistics, www.abs.gov.au). Preliminary searches of the SCFA database for BCC and SCC showed that it had captured 8200 individuals histologically-verified as having NMSC from 1994- 1999, of whom 80% were residents of the Sydney metropolitan area. This equates to 200 per 100,000 cases per year in Sydney (ie 8200/4,000,000 x 100,000), which is 10% of the total estimated incidence. The SCFA database can thus be considered a quasi-population-based register of biopsy proven NMSC in the Sydney metropolitan area.

2.1.2. Recruitment

Eligible subjects (probands) were those of either sex, with histologically verified primary cutaneous BCC, diagnosed at forty years of age or younger during the 12 month period from January to December 2000, or SCC diagnosed at fifty years or younger, during the 24 month period from January 1999 to December 2000. These age thresholds were selected so that the youngest 7-8% of the total number of BCC and SCC-affected individuals per year were captured, and the longer period of SCC eligibility was required because SCC is significantly less common than BCC in these age groups [1]. Only the BCC arm of the study will be reported here.

A letter was sent to the referring doctors of 257 BCC cases asking permission to contact their patients regarding this project. Once consent was given, and information pack was sent from SCFA to the patient, containing information on the research project, consent forms to participate in the study and a form to release this consent from SCFA to the research team. . If there was no response to this information, SCFA attempted to contact the subjects by telephone for a response. For subjects that were unable to be contacted by these methods, a 'Yes-No' response form was sent out asking them to simply tick a box and return the form, to let us know that they were actively refusing participation. If there was still no response a second Yes-No form was sent. For all correspondence that was 'returned to sender' by the postal service, the referring doctor was asked for updated contact details. For all subjects whose current address or telephone numbers were unable to be discovered by these methods, 'Marketing Pro' software was used to attempt to obtain current details. If new details were discovered by any of these means, the process began again with a new information pack sent to the new address and/or a telephone call from SCFA to the new telephone number to follow up on information packs sent. The team only contacted the subjects once their consent was returned, which included 56 BCC affected individuals, and 48 SCC affected individuals.

Twenty-three of 201 eligible subjects were denied consent by their treating doctor. Of the remaining 178 early-onset BCC cases approached, 56 consented, 50 actively or passively refused, 13 were not contactable after exhausting the methods detailed above, and for 59 it was unclear whether or not the patient had received the information or not. Thus the actual percentage of eligible *contactable* subjects consented is between: 56/165 (34%) and 56/106 (53%), depending on how many of the not-contactable subjects were actually passive refusals.

2.2. Interviews and histological confirmation of cancers

Each family stucture was determined by interviewing probands about their first and second degree relatives. Each first degree relative was then approached, and if they consented to participate in the study, they were interviewed about their cancer history. Cancer history information on deceased first degree family members was also obtained where possible through proxy interviews with a consented family member. Attempts were made to verify all reports of cancer with histopathological records if possible or alternatively through medical records. This was done through contacting the treating doctor or relevant pathology service for BCCs and SCCs, and through the cancer registry for registrable cancers. If the registry could not verify the reported cancer then the treating doctor was contacted for these records. The earliest reported cancer of each type was the priority for verification. Where multiple reports were received, they were all followed up until at least one confirmation of NMSC was received. Where more than one NMSC was confirmed, the earliest dates of BCC and SCC diagnoses were used for subsequent analysis. The information was coded according to the level of confirmation (self-report alone/ clinical diagnosis alone/ histological confirmation). In situ SCC was accepted as equivalent to invasive SCC because recent data indicate that the prospective risk of further SCC is similar in these two conditions. Relatives were only labelled 'unaffected' if they never had any potential NMSC lesions treated, and if they had no NMSC or premalignant lesions present on examination. The oldest unaffected sibling for each proband (if available) was designated as an intra-family control.

In all subsequent analyses, the word 'relatives' refers to the consented first-degree relatives of probands in this study.

2.3. Phenotyping

2.3.1. Skin examination

All probands and their sibling controls were invited to attend skin examinations at SCFA in Westmead or Darlinghurst, at Westmead hospital, or at outer metropolitan or rural hospitals for a few subjects who could not attend a city venue. The same measurement equipment was used at each site, and the same lights were used to enable standardized illumination. The candidate, a medical graduate, was trained to assess premalignant and malignant lesions and the markers of sun damage on subjects, according to a standard protocol as, described below.

Height and weight were recorded. Subjects were asked to identify the hair colour that was closest to their natural colour at age 18, using synthetic hair swatches (Clairol, USA) designed

for wig-making. Colours used in this study included: High-Lift golden blonde HL-G, Sable Brown 48D, Dusk Blonde 93D-N, Nightfall Brown 95D-N, Sunberry 72R, Reddest Fire Red 206RR, Golden Apricot 41G, Black Azure 52D. Skin colour was recorded using a reflectance spectrophotometer, as described in 2.3.3.

All subjects were tested for features of Gorlin Syndrome, including mandibular keratocysts (through the questionnaire) and examination of the palms for pitting. Each of these features would be expected to be seen in over 80% of patients with Gorlin syndrome [200]**.**

Markers of sun exposure and damage were then assessed on a semi-quantitative scale and included: ephelides (freckles), solar lentigines, actinic keratoses, actinic damage/elastosis, seborrhoeic keratoses, and presence of any new skin cancers noted. If a lesion was suspicious of malignancy, the subject was referred to their local GP or dermatologist. Results were recorded on a patient data sheet and entered into a password-protected database in secure facilities. Data for analysis were deidentified.

All subjects undergoing skin examination also filled out a questionnaire (see section 7.1 for clinical protocol) regarding their skin type, hair colour, eye colour, childhood and adult freckling, and history of dental cysts.

The candidate undertook an informal audit of examining technique with an experienced dermatologist after the examinations were complete. Her technique was considered adequate for all variables except seborrhoeic keratoses, where fainter lesions were missed. Absolute values for this variable should be regarded as underestimates and were not interpreted, whereas categorisation of individuals with respect to number of these lesions was regarded as suitable for analysis.

2.3.2. Skin examination recoding variables

All skin phenotype variables, from examination and questionnaire, were subjected to an initial analysis to determine their distribution and informativeness. The number of categories was reduced by recoding in many cases in order to maximise the number of individuals in each recoded category. All recoded variables are described here.

Only one subject (1.1%) reported having black hair when asked to name their hair colour at age 21, and this subject chose a brown hair swatch when asked to select a representative hair

swatch colour visually: the category of ‘black’ was therefore combined with the category ‘dark brown’ for the analysis.

Few people (3.4% or 3/88) reported that they ‘go brown without any sunburn’ when asked “which statement best describes what would happen if your skin were exposed to bright sunlight for the first time in summer for one hour in the middle of the day without any protection?” (see section 7.1). Therefore the answers for the ‘no sunburn/tanning’ were combined with the ‘mild sunburn’ category for analysis.

No subject out of 88 reported that they ‘never burn’ as a response to the question “in general, how does your skin react to the sun?” and therefore this phototype category was removed from the analysis.

Examination for presence of actinic keratoses (AKs) revealed that most subjects did not have many of these lesions on the regions of the body included (see section 4.2.4). The categories of ‘sparse’ and ‘moderate’ were therefore collapsed to the single category of ‘present’ or ‘absent’ for presence of any AK on the regions head/neck, chest, dorsum arms and dorsum hands examined. A large proportion of subjects had a high amount of solar elastosis, skewing the results. Therefore the score for each body region (Head/neck/anterior chest, periorbital, lips, dorsa forearms, and dorsa hands) were added up with a score of 0 for ‘absent’, 1 for ‘mild’ and 2 for ‘severe’. This was added up across all categories and each subject was given a score of ‘low’for a total body score between 0 and 9, and a score of ‘high’ for a total body score of 10.

Semi-quantitative counts of solar lentigines were skewed across the 88 subjects examined with a disproportionate number falling into a low or high count category (see table 7.8 for raw scores). Therefore the median count category for each body region was determined and subjects re-categorised into ‘low’ or ‘high’ solar lentigines groups. For the face this meant that raw scores of 0 – 2 were recoded as ‘low’ with the remainder of scores 3 – 10 recoded as ‘high’. Similarly, the raw scores for solar lentigines on the forehead were recoded 0 – 1 ‘low’ and 2 – 10 ‘high’. For the upper limb raw scores, 0 – 4 became ‘low’ and 5 – 9 became ‘high’. For the upper back and shoulders, 0 – 6 became ‘low’ and 7 – 9 became ‘high’.

Reporting of freckling in childhood and adulthood was skewed to include a greater proportion in the category ‘very few’ than the other positive freckling categories (see table 7.9 for raw scores). ‘Very few’ was therefore renamed ‘some’, and the ‘few’, ‘some’, and ‘many’

categories were collapsed into the single category of ‘many’. As with the analysis of semi-quantitative examination results for solar lentigines, subjects’ ephiledes (freckling) scores were skewed or biphasic (see table 7.9) and so the median (if skewed) or the score between the two peaks (if biphasic) for each region was taken, and subjects reclassified into high and low numbers of freckles based on this median division. For ephilides on the face, raw scores of 0 – 3 were recoded as ‘low’ and 4 – 10 as ‘high’. For raw scores of ephilides on the forehead, 0 – 3 were re-classified as ‘low’ and 4 – 10 as ‘high’. For the upper limbs, raw scores of 0 – 7 were scored as ‘low’ and 8 – 10 as ‘high’. For the upper back and shoulders, 0 – 6 raw scores were recoded as ‘low’ and 7 – 10 as ‘high’.

Examination results for numbers of seborrhoeic keratoses were skewed with many subjects scoring ‘nil’ on this variable for several of the body regions (see table 7.10). The results were reclassified into an absent/present dichotomy for each region examined to allow any real associations to be seen. The score for all the body regions examined (head/neck, upper limbs, chest, abdomen, back, lower limbs) were then summed to give a total body score, and this was also divided into ‘low’ (score of 0 – 1) and ‘high’ (score of 2 – 8) categories based on the median score.

2.3.3. Skin colour assessment

The apparent colour of a person’s skin is due to the aggregate of reflected light, the wavelengths of which depend on four biochromes: melanin, carotenoids, oxyhaemoglobin and reduced haemoglobin. Melanin and the carotenoids are brown and yellow respectively, and are found in the epidermis, the uppermost layer of the skin. Oxyhaemoglobin is bright red and found in the arterioles and capillaries of the upper dermis of the skin, just deep to the epidermis. Reduced haemoglobin is bluish-red and is found in the venous plexus of the dermis, just deep to the arterioles and capillaries [21].

Skin colour of each subject examined was assessed with a BYK Gardner spectrophotometer; a portable instrument that measures reflected colour and the colour difference using the Commission International de l’Eclairage (CIE; 1976) *L*a*b** standard colour system. The *L** value (luminance) represents the relative lightness ranging from total black to total white; the *a** value represents the balance between red and green; and the *b** value the balance between yellow and blue. This instrument uses the same wavelengths of light (400 to 700nm at 20nm intervals) to measure skin reflectance as the Minolta 508, a spectrophotometer with

measurement of skin colour that correlates highly (r = 0.68) with melanin density seen histologically on biopsied skin from the site measured [391]. The Minolta does not separate the reflected light into the *L*, *a* and *b* axes, giving a single reading of reflected light for each 20nm interval of reflected light. Based on best correlation with melanin density over the spectrum of light used, researchers using the Minolta generally use the difference between the reflectance measurements at 400nm and 420nm of incident light as a single parameter.

The Minolta and BYK Gardner instruments are regarded as equivalent in commercial colour measurement applications. However, there are no published data on correlates between 420-400nm reflectance and L**a*b** measurements, or between *L*a*b** measurements and melanisation. In this thesis, the raw *L*a*b** measurements were used without further manipulation in order that all available information on skin colour should be used.

The spectrophotometer was recalibrated prior to each skin examination session to ensure comparable readings between subjects, and the mean of 6 consecutive readings was recorded. The measurements were taken on the medial axillary wall (constitutional skin colour) and upper outer arm (sun exposed skin colour) of each subject examined. The mean *L*a*b** values was recorded on the subject's data sheet.

2.4. DNA extraction

Blood (20 ml EDTA) was collected from the probands, sibling controls and parents of probands by standard venipuncture. DNA was extracted from whole blood as described in appendix 7.2. The total concentration of the DNA was checked by spectrophotometry at 260 nm and 320 nm (Genequant Pro; Amersham Pharmacia Biotech, England). The DNA quality was estimated by running the DNA samples on a 1% agarose gel, stained with ethidium bromide (final concentration of 0.5µg/ml). Gels were photographed under UV light (NovaLine Gel Documentation System; Sweden).

2.5. Genotyping of PTCH

2.5.1. Exon and primer selection

The exons chosen for amplification and analysis were exons 2, 3, 5 to 18, and 20 to 23. Exon 23 was split into 23a and 23b to allow for easier amplification and dHPLC (exon numbering of the PTCH exons is as described by Johnson et al [214]. The priorities for exon choice were based on those for which PTCH mutations have been documented in Gorlin subjects. The

genbank accession number for the PTCH gene is U59464. Exons 1 and 4 were excluded because no mutations have been seen in these exons in previous studies, and exon 19 was excluded due to repeated difficulties with PCR amplification. Primer pairs used are according to Hahn et al (exons 6, 7, 8, 9, 10, 11, 15, 17 and 18) [213], Fujii et al (exon 2) [392] and Xie et al (exons 12, 13, 14, and 21) [221]. Primer pairs for exons 3, 5, 16, 20, 22, 23a, and 23b were provided by BJ Wainwright (personal communication). Primer sequences for all exons amplified are listed in table 2.1 and have been used extensively for dHPLC analysis of Gorlin syndrome patients and somatic mutations in non-familial BCC in the laboratory of Prof BJ Wainwright, University of Queensland; Brisbane, Australia.

Table 2.1 Primer sequences to amplify PTCH exons

Exon	Primer sequence
2	F 5'-ACTCCTCCCTTCTGCTTCGT-3'
	R 5'-GCGCTGGCGAATATCTCTAT-3'
3	F 5'-CTATTGTGTATCCTATGGCAGGTAGTCAGATAACAGAT-3'
	R 5'-ATTAGTAGGTGGACGCGGCGGGCCT-3'
5	F 5'-GCAAAAATTTCTC AGGAACACC-3'
	R 5'-GGAACAAACAATGATAAGCAA
6	F 5'-CCTACAAGGTGGATGCAGTG-3'
	R 5'-TTTGCTCTCCACCCTTCTGA-3'
7	F 5'-GTGACCTGCCTACTAATTCCC-3'
	R 5'- GGCTAGCGAGGATAACGGTTTA- 3'
8	F 5'- GAGGCAGTGGAAACTGCTTC -3'
	R 5'- TTGCATAACCAGCGAGTCTG-3'
9	F 5'-GTGCTGTCGAGGCTTGTG-3'
	R 5'-AGAAGCAGGAGCAGTCATGG-3'
10	F 5'-TTCGGCTTTTGTTCTGTGC-3'
	R 5'-CCGGTGGCATTTGTCAAC-3'
11	F 5'-CTGTTAGGTGCTGGTGGCA-3'
	R 5'-CTTAGGAACAGAGGAAGCTG-3'
12	F 5'- GACCATGTCCAGTGCAGCTC-3'
	R 5'- CGTTCAGGATCACCACAGCC-3'
13	F 5'- AGTCCTCTGATTGGGCGGAG-3'
	R 5'- CCATTCTGCACCCAATCAAAAG-3'
14	F 5'-AAAATGGCAGAATGAAAGCACC-3'
	R 5'-CTGATGAACTCCAAAGGTTCTG-3'
15	F 5'-GACAGCTTCTCTTTGTCCAG-3'
	R 5'-ACGCAAAAGACCGAAAGGACGA-3'
16	F 5'-CGCTAGGACCAGGGTCCTTCTGGCTGCGAGTTATA-3'
	R 5'-TCAGTGCCCAGCAGCTGGA-3'
17	F 5'-AACCCCATTCTCAAAGGCCTCTGTTC-3'
	R 5'-CACCTCTGTAAGTTCCCAGACCT-3'
18	F 5'-AACTGTGATGCTCTTCTACCCTGG-3'
	R 5'-AAACTTCCCGGCTGCAGAAAGA-3'
20	F 5'- CATTTAGGACAGAGCTGAGCA-3'
	R 5'- GGCCCAATCACAATGATTTC-3'
21	F 5'-TGTTCCCGTTTCCTCTTG-3'
	R 5'-GCACAGGAAACACAGCATTC-3'
22	F 5'-AGTGTGGCCAGCAGGTAAAT-3'
	R 5'-CTCCAGGCCCACTACCAC-3'
23a	F 5'-AAACCCAAGGAGGGAAGTGT-3'
	R 5'-CCGAGGGTTGTGAGAACG-3'
23b	F 5'-GCATTCTGGCCCTAGCAATA-3'
	R 5'- TCTTTGCCTGGCTCTAGGTC-3'

Forward (F) and reverse (R) primer sequences of exons 2 to 4 and 5 to 23b of the PTCH gene. The primer pairs of exons 15, 17 and 18 correspond to primer pairs 14, 16 and 17 respectively as described by Hahn et al. [213]

2.5.2. PCR protocol for amplification of PTCH exons

PCR reactions were performed in a total volume of 52.8µl. The reaction contained 5µl of PCR buffer (1.5 mM $MgCl_2$; 10mM Tris-HCl, pH 8.5; 50mM KCl), 1 µl DMSO, 1µl dNTP 10mM (Fisher Biotec, Australia), 1µl of each primer 50µM (forward and reverse), 0.3µ of Ampli*Taq* Gold 5U/µl (Roche Applied Biosystems, USA), 3µl DNA 25ng/µl, and 40.5 µl dH_2O (table 2.2). The same PCR protocol suited the amplification of exons 2, 5- 20, 22, and 23a (and 19?). For exons 3 and 21, 2.5 mM $MgCl_2$ was used, and for exon 23b, 2.0 $MgCl_2$ was used. Each reaction contained a negative control that contained all the stock ingredients with water, to ensure that the reagents were not contaminated. A positive control was not available. All PCR reactions were done using sterile techniques, working in a laminar flow hood.

The annealing temperature for exon 2, 3, 5, 7, 8, 9, 10, 12, 13, 15, 17, 18, and 20 was 55ºC, for exons 6, 11, and 14 it was 57ºC, for exons 16, 21, 22, and 23a it was 59ºC and for exon 23b it was 60ºC. The thermocycling parameters included an initial denaturing step at 95º for 30 minutes, followed by 95º for 45 seconds, the specific annealing temperature for 50 seconds, 72º for 50 seconds for 35 cycles, and a final elongation cycle of 72º for 7 minutes (Palm-Cycler version 2.2; Corbett Research, Australia). The PCR tubes were stored at -20ºC until required for denaturing High Performance Liquid Chromatography (dHPLC) or sequence analysis.

Table 2.2 PCR Cocktail (52.8 µl total)

Reagent (conc)	Volume (µl)	Final concentration
dH_2O	40.5	-
PCR Buffer ($MgCl_2$, Tris-HCl, KCl)	5.0	$MgCl_2$: 1.5mM; Tris-HCl: 10mM (pH 8.5); KCl: 50mM
DMSO	1.0	-
dNTP's (10 mM)	1.0	0.19 mM
PTCH F primer (50 µM)	1.0	0.95 µM
PTCH R primer (50 µM)	1.0	0.95 µM
Ampli*Taq* Gold (5U/µl)	0.3	1.5 U
DNA (25 ng/µl)	3.0	75 ng
Total	49.8 + 3.0 µl	

2.5.3. Agarose gel electrophoresis

All PCR products were checked for purity and correct sizing on a 1.5% agarose gel (1.5g agarose in 100 ml 0.5x TBE), stained with ethidium bromide (final concentration of 0.5

µg/ml). For all samples, 3 µl of each PCR product was mixed with 2 µl of loading dye and pipetted into successive wells of the gel, running them next to 2µl of low DNA mass ladder (4µl/appl; Invitrogen Life Technologies, Australia) and the negative control. The gels were electrophoresed at 80V for approximately 30 minutes at room temperature and subsequently photographed under UV light (NovaLine Gel documentation system; Sweden). It was always ascertained that the negative control did not show any bands on the gel. If the negative control did show a band in a particular experiment, the resultant samples were discarded, the reagents replaced, and the reaction repeated until the negative control did not show a band on the gel.

2.6. dHPLC analysis of the PTCH gene

The PCR products were denatured at 95ºC for 5 minutes, followed by slow renaturing at -1ºC per minute, from 94º to 60ºC (Palm-Cycler version 2.2; Corbett Research, Australia) and subsequently stored at -20ºC until dHPLC analysis. Denatured PCR products (5-20µl) were analyzed for heteroduplexes by dHPLC (WAVE® DNA Fragment Analysis System; Transgenomic Inc, Omaha, Nebraska, USA) at the dHPLC Fragment Analysis Facility, Westmead Millennium Institute, Sydney, Australia. The PCR product was eluted from a temperature-equilibrated DNASep® analytical column (Transgenomic Inc, Omaha, Nebraska, USA) using a gradient of 0.1 M triethylammonium acetate buffer (TEAA) (A) and 0.1 M TEAA/25% acetonitrile (B), pH 7.0, at a constant flow rate of 0.9 ml/min. The gradient was predicted by the WAVEMaker™ software (Transgenomic) (table 2.4). Eluted DNA fragments were detected with ultraviolet absorption at wavelength 260 nm. Temperatures used for dHPLC were based on temperatures used by the University of Queensland, Brisbane (kindly provided by Wainwright BJ; personal communication) and predictions determined by the WAVEMaker™ software (table 2.4). The sequence of the wild type PTCH amplicon used for predictions is Genbank sequence U59464 (see section 7.3).

For the eight exons most likely to harbour mutations based on previous research (exons 2, 3, 6, 8, 14, 15, 17 and 18), two temperatures were used to screen each exon, in an attempt to detect all variations in the majority of the exon. These eight exons have been found to have the highest mutation rate of all the coding regions of the PTCH gene, and mutations in these exons account for approximately 60% of the total mutations cited in the literature. Unfortunately, exons 6 and 8 were able to be screened at one temperature only, due to procedural difficulties and time constraints. Thus mutation in the exons screened at two temperatures accounted for approximately 50% of the total mutations cited in the literature.

The two temperatures were used to screen 19 of the 58 probands, including all of those probands for whom there was histopathological confirmation of a family history of BCC at the time that laboratory work was practicable. This included 17 probands from the BCC arm of the study and 2 from the SCC arm. See table 2.3 for a summary of family cancer prevalence in the 19 probands chosen to have extra screening for higher risk exons. For the remainder of the 39 probands for these seven exons and the remaining exons screened, one dHPLC temperature only was used. The melting profiles for the temperatures used as predicted by the WAVEMaker ™ software can be found in appendix 7.4; one example is given in figure 2.1.

Table 2.3 Family history of BCC, SCC, and MM (proband and first degree relatives) for high risk subjects included for two temperatures of dHPLC screening

Proband ID	*Total number of family members recruited*	*Number of BCC-affected family members*	*Age of onset of BCC*[#]	*Number of SCC-affected family members*	*Age of onset of SCC*	*Number of NMSC-affected family members*	*Number of MM-affected family members*	*Age of onset of MM*
BCC probands								
8755	5	2	31, 34*	1	55	3		
8761	4	2	34*, 62	1	65	2		
8763	8	2	36*, 43,	2	41, 71	4	1	42
8768	4	2	20*, 48	0	-	2		
8770	5	3	39*, 69, 70	1	75	3	2	33, 78
8773	6	2	39*, 52	0	-	2		
8781	3	2	35*, 51	1	51	2		
8797	9	2	35*, 53	2	53, 42	3		
8800	10	4	26, 38*, 43, 69	1	70	4		
8815	5	2	19*, 58	0	-	2		
8816	9	6	39*, 44, 50, 50, 53, 76	2	53, 76	6	2	38, 49
8840	6	6	23, 23, 25, 28*, 49, 54	2	42, 46	6	3	23, 37, 47
8846	4	3	26*, 55, 56	0	-	3		
8857	4	2	35*, 63	0	-	2		
9022	9	6	35*, 36, 44, 54, 58, 74	0	-	6		
9125	6	3	25, 37, 38*	2	43, 67	4		
9194	4	2	33*, 62	1	64	2		
9242[a]	7	2	30*, 50	0	-	2		
SCC probands								
8701	7	6	29*, 30, 39, 54, 69, 75	3	40*, 51, 84	6	1	69
8001	6	3	37, 37, 53	1	41*	4		
8196[a]	7	3	34,43, 45	3	34, 41*, 53	4		

[a]DNA unavailable at time of laboratory work for screening at two dHPLC temperatures

[#] Underlined age indicates that the subject is deceased

*Indicates proband age of diagnosis

Table 2.4 Amplicon size and DHPLC conditions for PTCH fragments

Exon	Size (bp)	dHPLC gradient	dHPLC temp (ºC)
2	294	54-63%B in 4.5 min 54-63%B in 4.5 min	59.9 62.5
3	313	55-64%B in 4.5 min 55-64%B in 4.5 min	56.5 58
5	248	52-60% in 4.5 min	57
6	335	57-66% in 3.5 min	59.2
7	294	54-63% in 4.5 min	57
8	256	53-62%B in 4.5 min	61.8
9	281	54-63%B in 4.5 min	60.8
10	242	54-63%B in 3.5 min	60.2
11	253	53-62%B in 4.5 min	58.3
12	211	53-62%B in 3.5 min	62.6
13	222	51-60%B in 4.5 min	59.2
14	540	59-68%B in 4.5 min 56-65%B in 4.5 min	61.5 63.8
15	425	57-66%B in 4.5 min 57-66%B in 4.5 min	58 60.5
16	219	51-60%B in 4.5 min	59.3
17	268	53-62%B in 4.5 min 51-60%B in 4.5 min	59.8 64.5
18	410	57-66%B in 4.5 min 57-66%B in 4.5 min	61.2 62.8
20	267	54-63%B in 4.0 min	61.3
21	186	49-58%B in 4.5 min	61.7
22	371	56-65%B in 4.5 min	62.8
23a	331	55-64%B in 4.5 min	63.9
23b	407	57-66%B in 4.5 min	63.1

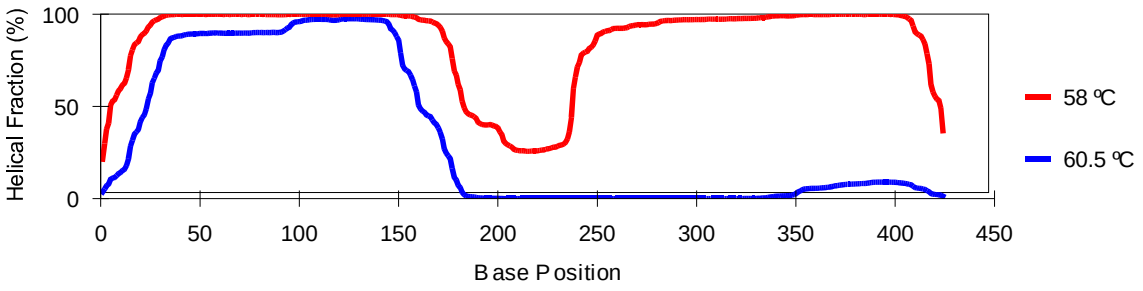


Figure 2.1 Predicted dHPLC melting curve for PTCH exon 15, wild type.
Helical fraction (%) of the DNA (Y-axis) versus base position in the amplicon (X-axis). The helical fraction ranges from 0% (completely denatured DNA; single stranded DNA) to 100% (double stranded DNA). dHPLC can detect mutations in partially denatured DNA, optimally 30-80% helical fraction (Leary J, Manager dHPLC Fragment Analysis Facility, Westmead Millennium Institute; Personal Communication).

2.7. DNA sequencing of PTCH exons

Samples with detectable dHPLC variants were sequenced, together with a sample of the same exon that displayed a normal dHPLC melting curve, for comparison. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen; Australia. Cat. No. 28106), following the manufacturer's instruction manual (see appendix 7.5). The quantity of the purified PCR product was estimated using gel electrophoresis (see above), loading 2 µl of each sample mixed with 2 µl of loading dye, comparing it to 2 µl of low DNA mass ladder.

Sequencing was carried out in both the forward and reverse direction or, in some cases, only in forward direction. Each sequencing reaction comprised template DNA, 3.2 pmol of the respective primer and 1µl of DMSO. Water was added to make up to a total volume of 12 µl. For all exons except exon 15, 10ng of template DNA was included, and for exon 15, 20ng of template DNA was included due to its larger size (see appendix 7.3 for exon sizes).

Sequencing was carried out in the DNA Sequencing Facility at the Westmead Millennium Institute, Sydney, Australia. The facility uses an ABI PRISM 3100 Genetic Analyser (Applied Biosystems; Australia); using POP-6 polymer with a 50 cm capillary array.

2.8. PTCH gene SNP identification numbers

Table 2.5 SNPs seen in probands that have previously been identified in SNP databases with their identification numbers

Exon	SNP	SNP identification number
2	C>T 116	rs1805153
6	IVS6-55T>C	rs2297087
8	IVS8+23G>A	rs2066840
11	IVS11-50G>C	rs574688
12	T>C 63	rs16909910
12	C>T 84	rs2066836
15	IVS15+9 G>C	rs2066829
23a	140 T>C	rs357564

2.9. Estimating Australian population cumulative risk (prevalence of a prior diagnosis) of NMSC

2.9.1. Estimating cumulative risk from incidence

NMSC are not notifiable to cancer registries in most of Australia, so verification of self-reported past diagnoses is not as feasible as it is for other cancers. Cumulative risk (prevalence of a prior diagnosis) of SCC and BCC was estimated from the most recent Australian general population incidence data available [1]. Five-year incidence data was obtained for men and women separately from survey data collected in 1995. Although lifetime prevalence of skin cancer was surveyed in this study, only the previous 12 months of skin cancer incidence data had been validated, and therefore only this data was suitable for analysis. Although the numbers of non-melanoma skin cancers were provided for five year age groups beyond 69 years (ie. 70-74, 75-79, 80-84, 85+), the number of people at risk for these age groups was only provided as a single group (70+). Therefore, incidence and prevalence was able to be estimated only for individuals up to 70 years of age.

Estimating prevalence and cumulative risk from incidence data has been discussed by other researchers [393] [394]. Most approaches require registry data which are not available in this instance. These models allow for the effects of competing risks. Statistician collaborators, Dr Karen Byth and Ms Peta Forder constructed estimates by making simplifying assumptions, and adapting existing epidemiological methods to incidence and cumulative risk estimation for BCC and SCC. The general population cumulative risk was estimated via a suitable model for the incidence. This novel approach is described below. All references to "prevalence" after this point mean prevalence of a prior diagnosis of skin cancer (ie cumulative risk).

Assume for the moment that the incidence of the particular cancer of interest at age *i* is independent of year (period) and of year of birth (cohort). Let the incidence of this cancer at age *i* be denoted by λ_i and note that $\lambda_i << 1$. Suppose that there are N_i individuals of

age *i*. Assume that the probability distribution of the number of individuals who develop the cancer of interest at age *i* can be approximated by a Poisson distribution with parameter $N_i\lambda_i$. Inspection of the observed incidences for ages <70 years in the Staples *et al* (1998) data suggested a log-linear model for λ_i of the form

$$\ln(\lambda_i) = \beta_0 + \left(\beta_1 \times age_i\right)$$

so that

$$\lambda_i = e^{\beta_0 + (\beta_1 \times age_i)}.$$

Assume further that any immigration or emigration processes operating on the general population occur independently of the cancer incidence process. Since we are interested in non-melanoma skin cancers, also assume that the death process operating on the general population is independent of the cancer incidence process. These assumptions and their implications are discussed in detail later.

Let P_i denote the probability that an individual has a first cancer of the prescribed type diagnosed at age *i*. Using conditional probability arguments, this is the product of the probabilities that no cancer of that type was diagnosed at any earlier age multiplied by the probability of this cancer diagnosis at age *i*. Therefore,

$$\begin{aligned} P_i &= \left\{ \prod_{j=0}^{i-1} e^{-\lambda_j} \right\} \times e^{-\lambda_i} \lambda_i \\ &= \lambda_i \left\{ \prod_{j=0}^{i} e^{-\lambda_j} \right\} \\ &= \lambda_i e^{-\sum_{j=0}^{i} \lambda_j}. \end{aligned}$$

The 'prevalence' of first cancer by age *i* is then :

$$\begin{aligned}\text{Prev}_i &= \sum_{j=0}^{i} P_j \\ &= \sum_{j=0}^{i} \lambda_j e^{-\sum_{k=0}^{j}\lambda_k} \\ &\approx \sum_{j=0}^{i} \lambda_j (1-\sum_{k=0}^{j}\lambda_k), \text{ since } e^{-x} = 1-x+\frac{x^2}{2!}-\frac{x^3}{3!}+\ldots \approx 1-x \text{ for } |x| << 1.\end{aligned}$$

Substituting $\lambda_i = e^{\beta_0+(\beta_1\times age_i)}$ into the above equation and denoting $\beta_1 \times age_i$ as $\beta_1(i)$ gives

$$\begin{aligned}\text{Prev}_i &\approx \sum_{j=0}^{i}\left[e^{\beta_0+\beta_1(j)}\left(1-\sum_{k=0}^{j} e^{\beta_0+\beta_1(k)}\right)\right] \\ &= \sum_{j=0}^{i}\left[e^{\beta_0+\beta_1(j)} - e^{\beta_0+\beta_1(j)}\sum_{k=0}^{j} e^{\beta_0+\beta_1(k)}\right] \\ &= \sum_{j=0}^{i}\left[e^{\beta_0+\beta_1(j)} - \sum_{k=0}^{j} e^{2\beta_0+\beta_1(j+k)}\right] \\ &= \sum_{j=0}^{i} e^{\beta_0+\beta_1(j)} - \sum_{j=0}^{i}\sum_{k=0}^{j} e^{2\beta_0+\beta_1(j+k)}\end{aligned}$$

The prevalence at age i ($\hat{\text{Prev}}_i$) can therefore be estimated by 'plugging in' the ML estimates $\hat{\beta}_0$ and $\hat{\beta}_1(i)$. The delta method (Armitage *et al*, 2002), which is a first order approximation based on a Taylor series expansion, can then be used to provide an estimate of the variance of $\hat{\text{Prev}}_i$.

If $D_0 = \dfrac{\partial \hat{\text{Prev}}_i}{\partial \hat{\beta}_0}$ and $D_1 = \dfrac{\partial \hat{\text{Prev}}_i}{\partial \hat{\beta}_1}$ then the delta method gives

$$\begin{aligned}\hat{\text{var}}(\hat{\text{Prev}}_i) &= \begin{bmatrix} D_0 & D_1 \end{bmatrix}\begin{bmatrix} \hat{\sigma}^2_{\beta_0} & \hat{\sigma}_{\beta_0\beta_1} \\ \hat{\sigma}_{\beta_0\beta_1} & \hat{\sigma}^2_{\beta_1}\end{bmatrix}\begin{bmatrix} D_0 \\ D_1\end{bmatrix} \\ &= D_0^2\hat{\sigma}^2_{\beta_0} + 2D_0D_1\hat{\sigma}_{\beta_0\beta_1} + D_1^2\hat{\sigma}^2_{\beta_1}\end{aligned}$$

Under the log-linear model,

$$\frac{\partial \text{Prev}_i}{\partial \beta_0} \approx \sum_{j=0}^{i} \left\{ e^{\beta_0+\beta_1(j)} - 2\sum_{k=0}^{j} e^{2\beta_0+\beta_1(j+k)} \right\}$$
$$= \sum_{j=0}^{i} e^{\beta_0+\beta_1(j)} - 2\sum_{j=0}^{i}\sum_{k=0}^{j} e^{2\beta_0+\beta_1(j+k)}$$
$$= \sum_{j=0}^{i} e^{\beta_0+\beta_1(j)} \left(1 - 2\sum_{k=0}^{j} e^{\beta_0+\beta_1(k)} \right)$$

and

$$\frac{\partial \text{Prev}_i}{\partial \beta_1} \approx \sum_{j=0}^{i} \left\{ j e^{\beta_0+\beta_1(j)} - \sum_{k=0}^{j} (j+k) e^{2\beta_0+\beta_1(j+k)} \right\}$$
$$= \sum_{j=0}^{i} j e^{\beta_0+\beta_1(j)} - \sum_{j=0}^{i}\sum_{k=0}^{j} (j+k) e^{2\beta_0+\beta_1(j+k)}$$

The 95% confidence bounds for prevalence at each age *i* can be estimated as

$$\hat{\text{Prev}}_i \pm \left(1.96 \times \sqrt{\hat{\text{var}}(\hat{\text{Prev}}_i)} \right).$$

2.9.2. Comparison of the observed and expected prevalences within a sample

Finally, we must estimate the expected number of cancers amongst the proband relatives under the null hypothesis H_0 that the relatives are a random sample of particular ages and genders from the general population. Consider a single individual and suppose that the prevalence in the general population for that individual's age and gender is π.

$$\text{Let X} = \begin{cases} 0, \text{ if the individual has no cancer} \\ 1, \text{ if the individual has cancer.} \end{cases}$$

Then, under H_0, X has a Bernoulli distribution with parameter π. In particular, under H_0,

$$\text{E(X)} = \pi,$$
and
$$\text{var(X)} = \pi(1-\pi).$$

Suppose that π is estimated by $\hat{\pi}$, calculated from a random sample of the general population. (In our study, $\hat{\pi} = \text{Prêv}$, the prevalence estimate for the individual based on the random 1995 National Survey). Using a variance decomposition result based on the conditional variance of X (see, for example, Lindgren, 1976), we know that

$$\begin{aligned} \text{var}(\text{X}) &= \text{E}\left[\text{var}(\text{X}|\Pi=\hat{\pi})\right] + \text{var}(\text{E}\left[\text{X}|\Pi=\hat{\pi}\right]) \\ &= \text{E}\left[\hat{\pi}(1-\hat{\pi})\right] + \text{var}(\hat{\pi}) \\ &\approx \text{Prêv}(1\text{-Prêv}) + \text{var}(\text{Prêv}) \end{aligned}$$

For a sample of individuals, the observed number of cancers is simply $O = \sum_{i=1}^{n} \text{X}_i$.

The expected number of cancers (E) under H_0 can therefore be estimated by

$$\begin{aligned} E &= \text{E}_{\text{H}_\text{O}}\left(\sum_{i=1}^{n} \text{X}_i\right) \\ &= \sum_{i=1}^{n} \text{E}_{\text{H}_\text{O}}(\text{X}_i) \\ &\approx \sum_{i=1}^{n} \text{Prêv}_i \end{aligned}$$

which is the sum of the estimated gender and age-specific prevalences for each individual in the sample.

Furthermore,

$$\begin{aligned} \text{var}_{\text{H}_\text{O}}\left(\sum_{i=1}^{n} \text{X}_i\right) &= \sum_{i=1}^{n} \text{var}_{\text{H}_\text{O}}\left(\text{X}_i\right) \text{ because } \text{X}_i \text{ are independent under } \text{H}_\text{O} \\ &\approx \sum_{i=1}^{n} \left\{\text{Prêv}_i(1\text{-Prêv}_i) + \text{var}(\text{Prêv}_i)\right\} \\ &= \text{V} \end{aligned}$$

It is therefore possible to estimate an approximate 95% confidence interval for the expected number of cancers in the sample under H_0 by calculating

$$\sum_{i=1}^{n} \hat{\text{Prev}}_i \pm \left(1.96 \times \sqrt{\text{V}}\right)$$

This allowed the observed prevalences of NMSC from the proband relatives to be compared with the 95% confidence intervals of the estimated Australian population prevalence.

The difference between observed and expected prevalences for each cancer was deemed to be significant if the observed prevalence fell outside of the 95% confidence intervals of the expected prevalence for that cancer.

2.10. Estimating expected prevalence of malignant melanoma

Malignant Melanoma is notifiable and as such we have cancer registry records of the incidence and prevalence of this disease in the Australian general population. Cancer registry prevalence of MM was calculated from cancer registry data from 1996, to enable it to be comparable to the data used in the calculations for BCC and SCC expected prevalences. Statistician collaborators determined 95% confidence limits for the 1996 Australian registry incidence data using world standardised population data from that year [395]. The number of expected MMs with 95% confidence intervals was then estimated from this data for each gender and relative type. For MM, calculations could be made on all live subjects, as the registry included data on all agre groups by five years to 80 – 84 years. This allowed the observed prevalences of MM from the proband relatives to be compared with the 95% confidence intervals of the estimated Australian population prevalence of MM.

Chapter 3: Incidence of skin cancer in families of BCC probands

3.1. Introduction

Despite being the most common of all cancers, almost nothing is known about the patterns and causes of familial aggregation of basal cell carcinoma in the population. Studies of susceptibility to this disease to date have either focussed on the specific, rare familial syndrome NBCCS (see section 1.5.4), in which features other than BCC must be present, or have assessed risk factors without making use of the information that family history can provide (section 1.4).

Systematic study of the patterns of clustering of other common tumour types has given completely new insights into their causes. They have led to successful mapping of new susceptibility loci and the development of genetic models incorporating rare high-penetrance gene mutations and common medium- and low-penetrance genetic variants. This complex genetic aetiology, coupled with the influence of the environment has furthermore led to the recognition that risks caused by even classic susceptibility gene mutations are highly dependent on an individual's and a family's overall risk profile (section 1.11). There is no reason why BCC ought not to be subject to the same complexities.

One major BCC susceptibility gene, *PTCH*, is well known to cause familial BCC susceptibility in the context of Gorlin syndrome; its alleles are probably very rare, but confer risks of up to 900- fold, depending on the baseline incidence in the population (see section 1.5.4). It is not known to what extent familial BCC susceptibility occurs other than in association with Gorlin syndrome, and if it does, whether it could be accounted for in part by high-penetrance mutations in *PTCH* or other genes. No genome-wide linkage studies have been performed on familial clusters of non-Gorlin-associated BCC; indeed no such cohorts have been reported.

One critically important low-medium genetic risk factor for both BCC and SCC has been identified: the red hair-associated variant alleles of the pigmentation control gene MC1R (see section 1.7). In Australian populations these variants have a frequency of around 20% and relative risks of two to four, and are likely to contribute strongly to familial aggregation of BCC and SCC in European-derived populations. Finally, high-risk patterns of sun exposure are a ubiquitous risk factor for all forms of skin cancer, with frequency in the 15-25% range and relative risks of 1.2 -1.9, i.e. in the low range (see section 1.4.1.4).

The high prevalence of susceptible skin phototypes ie MC1R variants, and of high-risk sun exposures in Australia suggests that a large proportion of cases of BCC and SCC in the population will be due to these two risk factors alone. They are both commonly shared in families and are risk factors for SCC and melanoma, so it would be predicted that these two skin cancers would also co-aggregate strongly with BCC in families.

The present study sought to test this hypothesis by focussing on a cohort of individuals that had developed BCC much earlier than average, and therefore might be enriched for BCC risk factors; examining the incidence of skin cancer among their relatives, and conducting a preliminary study of the factors associated with BCC by comparing these cases with unaffected siblings. The incidence of BCC and SCC was confirmed separately in relatives of probands, and this may establish whether there is a differential risk in relatives of people with early-onset BCC with respect to these two forms of NMSC. A greater risk of BCC than SCC in relatives may suggest the influence of BCC-specific risk factors on this group. In this chapter the patterns of aggregation and incidence of skin cancers are described in a series of 56 individuals who developed BCC under the age of 40, and in their close relatives.

3.2. Results

3.2.1. Subject selection and characteristics

3.2.1.1.Probands

The primary subjects ascertained for the study were individuals diagnosed with histologically-confirmed BCC at the Skin and Cancer Foundation in the year 2000, who were under the age of 40 at the time of diagnosis (see section 2.1.2). After obtaining treating doctors' consent, subjects were approached first by mail and then by telephone with information about the study and an invitation to participate. 56 of the 201 eligible subjects consented to the study. 23 of 201 were refused contact by their treating doctor, 50 actively or passively refused, 13 were not contactable after multiple attempts, and for 59 it was unclear whether or not the patient had received the information or not. The percentage of eligible, *contactable* subjects consented is between 56/165 (34%) and 56/103 (53%) depending on how many 'not contactable' subjects actually received the invitation.

The probands were 18 men and 38 women aged 22 to 44 years (at recruitment) who together had a median age of 40 years. Both the female and the male probands had a median age of 40 years at recruitment.

3.2.1.2.Family members

The family structures for the 56 families were determined by interviewing probands about their first degree relatives; permission was sought to contact all of these individuals which included 260 parents and siblings. Of these 260 eligible first-degree relatives, 17 were denied contact by probands including 12 siblings and five parents. For two of these relatives the reason given by probands was that it was due to mental or medical illness, for six it was due to intra-family discord, for four it was because it was felt that they would not be interested in participating, and for five no reason was given. Interviewers contacted all of those relatives for whom permission was granted for contact by the proband. Overall, 82% (212/260) of the eligible first-degree relatives consented to participate in the study. Excluding those for whom no proband consent for contact was given, there was an 87% (243/260) overall participation rate. This included 86% (89/103) of the available parents and 88% (123/140) of the siblings. Two probands were adopted and had no knowledge of their biological relatives; this meant that approximately four

(212/54) first-degree relatives were recruited for participation per proband where biological relatives were available to approach.

Overall, 212 first-degree relatives of probands participated in the study, of which 50% (107/212) were men and 50% (105/212) were women. Of the 123 recruited siblings of probands, 53% (65/123) were men and 47% (58/123) were women. Included in this group were two half-siblings (sharing one biological parent), and one who was deceased. Of the 89 parents recruited, 47% (42/123) were men and 53% (47/89) were women, including 17 who were deceased. Consent and cancer histories for deceased relatives were obtained by proxy interview with another recruited family member.

3.2.2. Skin cancer prevalence

Each subject was asked whether they had been diagnosed with any form of cancer, and all self-reports were followed up with an attempt at histopathological verification (see section 2.2). In the case of NMSC reports, the treating doctor was approached, and if this contact was unsuccessful then the local pathology service was contacted. Where multiple reports were received, they were all followed up until at least one confirmation of NMSC was received. Where more than one NMSC was confirmed, the earliest dates of BCC and SCC diagnoses were used for subsequent analysis.

Table 3.1 describes for each proband the total number of subjects that were recruited in each family; the number of them who have had histologically confirmed BCC, SCC, and melanoma (MM), and the age of onset for each affected family member.

Table 3.1 Number of family members with BCC, SCC, and MM (proband and first degree relatives) for each proband

Proband ID	Total number of family members affected	Number of BCC-affected family members	Age of onset of BCC[#]	Number of SCC-affected family members	Age of onset of SCC	Number of NMSC-affected family members	Number of MM-affected family members	Age of onset of MM
8755	5	2	31, 34*	1	55	3		
8756	2	1	38*	0	-	1		
8759	4	1	37*	2	38, 60	3		
8761	4	2	34*, 62	1	65	2		
8762	6	1	31*	0	-	1		
8763	8	2	36*, 43	2	41, 71	4	1	42
8764	6	1	36*	0	-	1		
8766	4	1	39*	0	-	1		
8767	4	1	40*	0	-	1		
8768	4	2	20*, 48	0	-	2		
8769	5	1	27*	0	-	1		
8770	5	3	39*, 69, 70	1	75	3	2	33, 78
8771	5	1	36*	1	71	2		
8772	3	1	37*	0	-	1		
8773	6	2	39*, 52	0	-	2		
8774	3	1	22*	0	-	1		
8775	7	1	39*	1	81	2		
8776	5	1	29*	0	-	1		
8778	2	1	37*	0	-	1		
8779	5	1	33*	0	-	1		
8781	3	2	35*, 51	1	51	2		
8794	6	1	39*	0	-	1		
8797	9	2	35*, 53	2	53, 42	3		
8800	10	4	26, 38*, 43, 69	1	70	4		
8810	4	1	32*	0	-	1		
8815	5	2	19*, 58	0	-	2		
8816	9	6	39*, 44, 50, 50, 53, 76	2	53, 76	6	2	38, 49
8818	7	2	37*, 44	0	-	2		
8825	3	2	34*, 73	1	67	3	1	60
8826	2	1	35*	0	-	1		
8832	2	1	35*	0	-	1		
8840	6	6	23, 23, 25, 28*, 49, 54	2	42, 46	6	3	23, 37, 47
8846	4	3	26*, 55, 56	0	-	3		
8857	4	2	35*, 63	0	-	2		
8928	4	1	27*	0	-	1		
9022	9	6	35*, 36,	0	-	6		

			44, 54, 58, 74					
9094	5	1	39*	1	65	2		
9125^	6	3	25, 37, 38*	2	43, 67	4		
9167	4	1	32*	0	-	1		
9168	5	1	38*	0	-	1		
9194	4	2	33*, 62	1	64	2		
9207	2	1	36*	0	-	1		
9208	1	1	38*	0	-	1		
9240	3	1	33*	0	-	1		
9241	7	1	28*	0	-	1		
9242	7	2	30*, 50	0	-	2		
9273	1	1	31*	0	-	1		
9274	4	3	34*, 38, 59	1	59	4	1	59
9275	5	3	30*, 45, 45	0	-	3		
9334	1	1	36*	0	-	1		
9445	5	5	38*, 50, 51, 68, 75	1	78	5	1	53
9446	9	1	35*	1	83	2		
9459	5	1	37*	1	70	2		
9465	6	1	31*	0	-	1		
9491	4	3	30, 31*, 52	0	-	3		
9527	4	1	39*	0	-	1		

*indicates the age of onset for the proband, the person first entering the study and the person screened for mutations in the PTCH gene

[#] Underlined age indicates that the subject is deceased

[^] Gorlin syndrome reported in family

Table 3.2 Number of skin cancers in first degree relatives of early-onset BCC probands

Number Cancers in 1st Degree Relatives	**BCC Frequency***	**SCC Frequency**	**NMSC Frequency**	**MM Frequency**
1	13	14	14	4
2	6	6	9	2
3	1	-	3	1
4	1	-	1	-
5	3	-	3	-

*Indicates number of families out of 56 with the specified number of that cancer among first degree relatives

Table 3.3 Number of BCCs in families affected by BCC alone (ie without any members affected by MM or SCC)

Number cancers in 1st degree relatives	**Frequency of families with BCC alone (no MM or SCC history)***
2	6
3	3
6	1

*Indicates number of families with the specified number of BCC among first degree relatives with no members affected by MM or SCC

The 56 probands had developed BCC at a median age of 36. None of them had developed either SCC or MM.

Overall, 28% (60/212) of the first-degree relatives had been affected by NMSC, including 20% (25/123) of the siblings and 40% (36/89) of the parents. Taking the pool of relatives as a whole, the earliest confirmed age of onset of NMSC for each affected individual ranged from 23.0 to 83.0 years, with a median age of 53.0 years. As is expected from ascertainment bias in family studies, ages of onset for siblings (median 42.0, range 23.0 – 54.0) were earlier than for parents (median 62.0, range 46.0 – 84.0) because, as a generation, the siblings have lived through fewer years at risk. The median age of diagnosis for mothers (58.5 years) was non-significantly earlier than for fathers (65.0 years)(X^2 = 56, p=0.229), and the median age of diagnosis for brothers (41.0 years) was earlier than for sisters (43.0 years) (X^2 = 20, p=0.220).

More than half the probands (30/56, 54%) had at least one first-degree relative with NMSC, more than one quarter (16/56, 29%) had two or more, and in three cases (3/56, 5%) five relatives were affected by NMSC. These affected relatives included 23 siblings and 24 parents affected by BCC, and five siblings and 22 parents affected with SCC. Three siblings and ten parents had been affected by both cancer types.

Overall, 22% (47/212) of the first-degree relatives had been affected by BCC, including 19% (23/123) of the siblings and 27% (24/89) of the parents. When considering the pool of relatives as a whole, the earliest confirmed age of onset of BCC for each affected individual ranged from 23.0 to 76.0 years, with a median age of 51.0 years. The earliest confirmed age of onset of BCC among the siblings ranged from 23.0 to 54.0 years, with a median age of 43.0. The earliest confirmed age of onset of BCC among the parents ranged from 48.0 to 76.0 years with a median age of 58.5 years. The median age of diagnosis for mothers (57.0 years) was earlier than for fathers (62.0 years) (X^2 = 6, p=0.199), whereas the median age of diagnosis for brothers (50.0 years) was earlier than for sisters (57.0 years) (X^2 = 12, p=0.213).

Nearly one half of the probands (24/56, 43%) had at least one first-degree relative with BCC, one fifth (11/56, 20%) had two or more, and in three cases (3/56, 5%) five relatives were affected by BCC.

Overall, 13% (27/212) of first-degree relatives had been confirmed to be affected by SCC, including 4% (5/123) of the siblings and 25% (22/89) of the parents. When considering the pool of relatives as a whole, the earliest confirmed age of onset of SCC for each affected individual ranged from 38.0 to 83.0 years, with a median age of 64.5 years. The earliest confirmed age of onset of SCC among the siblings ranged from 38.0 to 53.0 years, with a median age of 42.0 years. The earliest confirmed age of onset of SCC among the parents ranged from 42.0 to 83.0 years with a median age of 67.0 years. The median age of diagnosis of SCC for fathers (66.0 years) was slightly earlier than for mothers (67.0 years), and the median age for brothers (41.0 years) was slightly earlier than for sisters (42.5 years).

More than one third of the probands (20/56, 36%) had at least one first-degree relative with SCC, and in six cases (6/56, 11%) two relatives were affected by SCC. There were no cases where more than two relatives within a family were affected by SCC. In first-degree relatives of probands, there were nearly twice as many BCC-affected relatives as SCC-affected ones, with a BCC-affected: SCC-affected ratio of 1.7:1.

Overall, 5% (11/56) of first-degree relatives had been confirmed to be affected by MM, including 4% (5/123) of the siblings and 7% (6/89) of the parents. When considering the pool of relatives as a whole, the earliest confirmed age of onset of MM for each affected individual ranged form 23.0 to 78.0 years, with a median age of 47.0 years. The earliest confirmed age of onset of MM among the siblings ranged from 23.0 to 53.0 years with a median age of 38.0 years. The earliest confirmed age of onset of MM among the parents ranged from 37.0 to 78.0 years, with a median age of 53.0 years.

More than one tenth (7/56, 12.5%) of the probands had at least one first-degree relative with MM; in two cases (2/56, 4%) two relatives were affected by MM and for one case (1/56, 2%) there were three relatives affected by MM.

It can be hypothesised that increased susceptibility to BCC primarily reflects a general increase in risk of skin cancer, as might be related to high sun exposure or a sun-vulnerable skin type. This hypothesis predicts that BCC, SCC and possibly melanoma would co-aggregate in families. The incidence of NMSC in the cohort of relatives is formally compared with incidence data from the general population in the next section. However a survey of the patterns of aggregation gives evidence both for and against this hypothesis. Among 30 probands with a positive family history of NMSC in first-degree relatives, 20 had at least one relative affected by SCC; so the majority of family clusters included both BCC and SCC. However, of the other ten probands there were four who had at least two first-degree relatives affected by BCC and none affected by SCC, one being part of a six-case cluster of BCC (see table 3.3). This amounts to nearly one fifth of families affected by BCC alone, and suggests that a subset of the probands shares a specific susceptibility to BCC, rather than to NMSC generally, with their relatives.

One proband (ID 9125) out of 56 (2%) reported a personal history of clinically diagnosed Gorlin syndrome. Two of her three siblings had histologically confirmed BCC, with both of these affected siblings having had an early-onset of their tumours; her sister at 37 years and her brother at 25 years of age. Two of her first-degree relatives also had a history of SCC with her father having been affected at 67 years of age and her sister at 43 years of age (see appendix 7.7 family ID 29125 for pedigree). Clinically, her mother did not have Gorlin syndrome and her father was deceased at the time of her diagnosis. From a review of his records a diagnosis could not be made conclusively. Mutation analysis of the *PTCH* gene in several family members at the Queensland Institute of Medical Research had revealed no *PTCH* mutation in this family (participant's medical record).

3.3. Comparison of NMSC prevalence in relatives with that in the Australian population

As described in section 2.9.1, the term "prevalence" used here refers to prevalence of a prior diagnosis of NMSC. In order to address whether the prevalence of previous NMSC in this cohort of relatives differed from that expected for this group, it was necessary to make use of data from the most recent Australian survey of the population incidence of

NMSC (note that the word 'relatives' refers to consented first-degree relatives of probands as per section 2.2). This survey of 60,000 individuals Australia-wide was judged appropriate for this purpose, even though the cohort of the present study was concentrated in the Sydney basin. The incidence of melanoma (a surrogate for all sun-related skin cancer) in NSW is close to the national (see section 2.1.1 for exact data). This presumably reflects two facts: that a large proportion of the Australian population resides in NSW, and that greater Sydney lies about mid-way on the gradient of population incidence of melanoma in Australia. The overall Australia-wide NMSC incidence is noted to be most similar to the central (including NSW) zone than the northern or southern zones, and therefore can be assumed to be a fairly good estimate of the incidence of these cancers for the Sydney metropolitan area. We hypothesised that NMSC incidence in Sydney would therefore also be similar to the national average. Incidence data from state-based cancer registries are available for melanoma but not for NMSC, as these cancers were not registrable in NSW at the time of writing.

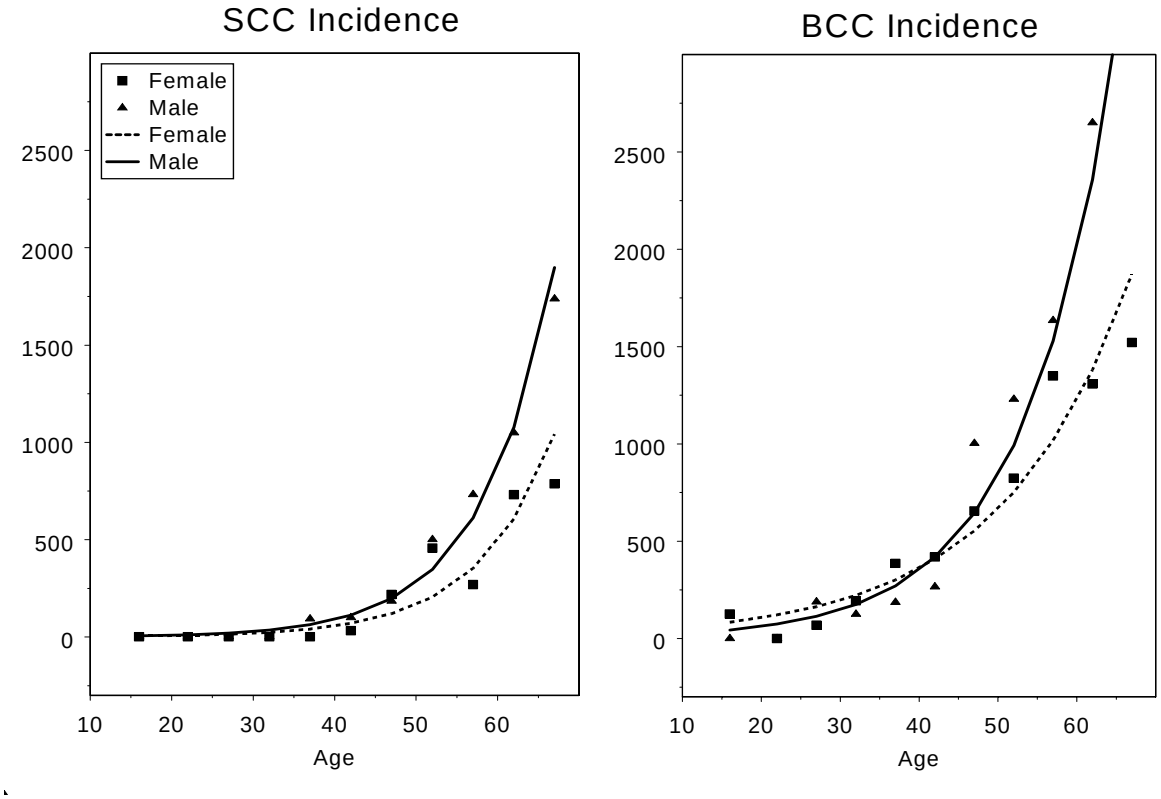
Raw data were obtained from the authors of the Australia-wide survey, and used by statistician collaborators Dr Karen Byth and Ms Peta Forder to re-estimate age-specific incidence rates of BCC and SCC, together with their 95% confidence limits (see methods section 2.9.1). This was necessary because the original publication only included confidence limits for the observed crude incidence rates in each age/sex group, but not for the curve the authors had fitted to their data. First, a curve was fitted to the raw data from this survey by maximum likelihood estimation using a Poisson regression routine in the statistical package STATA. The estimated regression parameters and their associated variances are presented in Table 3.4.

Table 3.4 Maximum likelihood parameters and associated variances for incidence of BCC and SCC in data from Staples et al [1] using a Poisson regression routine

Cancer	Group	$\hat{\beta}_0$	$\hat{\beta}_1$	Var($\hat{\beta}_0$)	Var($\hat{\beta}_1$)	Cov($\hat{\beta}_0$, $\hat{\beta}_1$)
SCC	Male	-11.5525	0.113261	0.468784	0.000129	-0.00768
	Female	-11.8153	0.1082343	0.722557	0.000203	-0.011942
BCC	Male	-9.112422	0.0865304	0.122610	0.000037	-0.00208
	Female	-8.058947	0.0608958	0.109614	0.000037	-0.001963

The incidence rates for BCC and SCC are shown in Figure 3.1, from Staples *et al.* (1) together with the curve newly fitted to the data.

Figure 3.1 Fitted curves for incidence rates for BCC and SCC in men and women in Australia in 1996 (re-analysis of data provided from Staples et al [1])



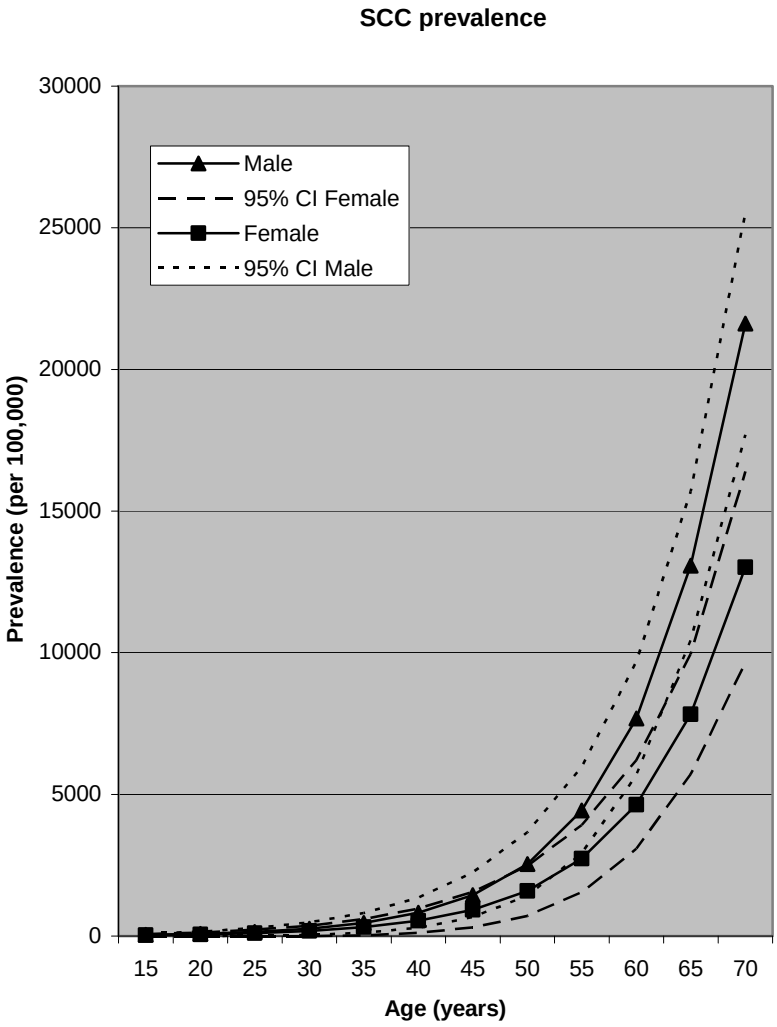
Cumulative risk (prevalence of a prior diagnosis) of BCC and SCC in Australia was then calculated for men and women of various ages, together with their associated 95% confidence limits, from the fitted data. These results are summarized in tables 3.5 and 3.6 and shown graphically in Figure 3.2.

Table 3.5 Estimated prevalence (with 95% CI) of SCC and BCC in Australian men in 1996 (re-analysis of data provided from Staples et al [1])

Age	**Estimated Prevalence (cases per 100,000 people)**			
	SCC	**(95% CI)**	**BCC**	**(95% CI)**
15	41	(0,87)	364	(154,575)
20	78	(0,160)	627	(293,961)
25	144	(8,280)	1030	(529,1531)
30	260	(40,480)	1648	(927,2368)
35	464	(118,810)	2592	(1589,3594)
40	821	(293,1350)	4028	(2680,5375)
45	1448	(665,2232)	6196	(4448,7944)
50	2542	(1423,3662)	9431	(7259,11603)
55	4438	(2908,5967)	14160	(11593,16728)
60	7677	(5678,9675)	20844	(17961,23727)
65	13074	(10446,15703)	29703	(26577,32829)
70	21622	(17700, 25546)	39931	(36771, 43091)

Figure 3.2 Estimated prevalence (with 95% CI) of SCC (A) and BCC (B) in Australian men and women in 1996 (re-analysis of data provided from Staples et al [1])

A.



B.

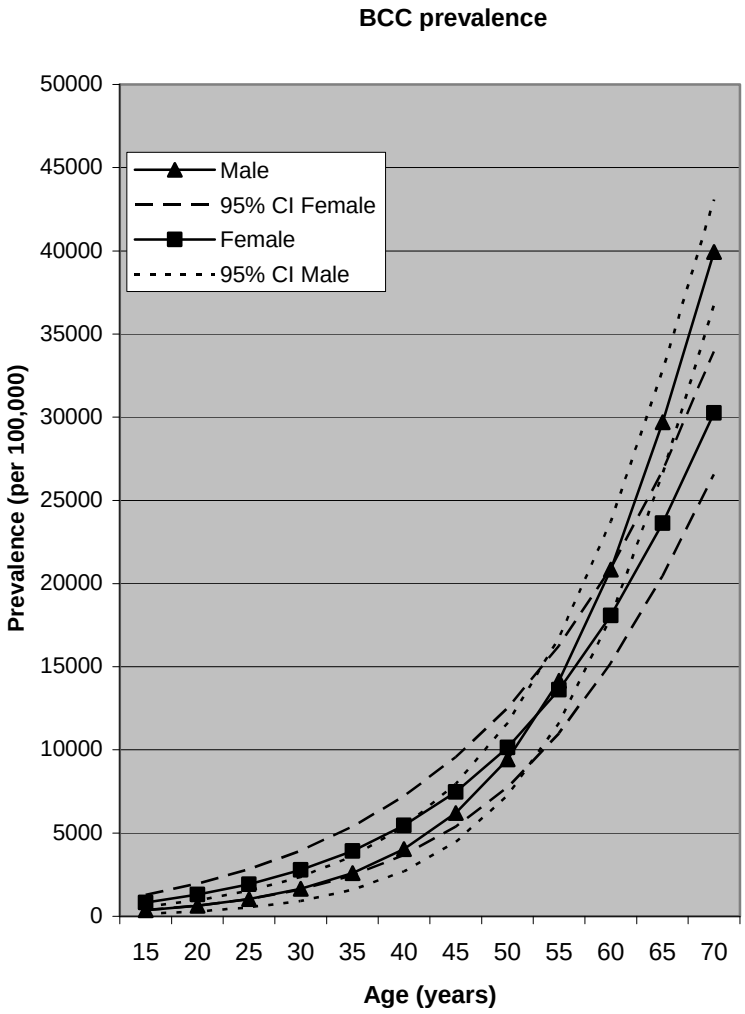

BCC prevalence
Prevalence (per 100,000)
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Male
95% CI Female
Female
95% CI Male
15
20
25
30
35
40
45
50
55
60
65
70
Age (years)

Table 3.6 Estimated prevalence (with 95% CI) for SCC and BCC in Australian women in 1996 (re-analysis of data provided from Staples et al [1])

Age	Estimated Prevalence (cases per 100,000 people)			
	SCC	**(95% CI)**	**BCC**	**(95% CI)**
15	30	(0,72)	827	(377,1278)
20	56	(0,129)	1297	(642,1951)
25	101	(0,220)	1930	(1037,2822)
30	179	(0,366)	2782	(1618,3945)
35	311	(23,600)	3924	(2462,5386)
40	539	(107,970)	5449	(3673,7226)
45	928	(302,1555)	7476	(5386,9565)
50	1594	(714,2474)	10145	(7767,12523)
55	2726	(1537,3915)	13621	(10995,16247)
60	4638	(3078,6198)	18071	(15216,20925)
65	7825	(5706,9944)	23619	(20458,26781)
70	13012	(9632,16393)	30252	(26562,33942)

3.3.1. Comparison of observed and expected prevalence of NMSC in first degree relatives

The population data from Staples et al. were only age-stratified to the age of 70 yr, so analysis of the cohort of relatives in the present study was restricted to data from the siblings and parents of the BCC proband cases who were alive and aged less than 70 years. Of the 123 siblings for whom data was obtained, one deceased subject was removed from analysis, leaving 122 sibling individuals (63 men, 59 women). Of the 89 parents for whom data was available, 17 subjects were deceased and 29 subjects were removed because they were aged 70 years or older. Thus, the parental group for analysis included only 43 subjects (18 men, 25 women). The subjects whose data was removed because they were deceased are identified in table 3.1 by underlining of the age of onset of their cancers.

For each group, the observed prevalence of a prior diagnosis of BCC and SCC was calculated. The method outlined above was then used to calculate the expected number of BCC and SCC in that group of relatives, taking into account their current age, and their 95% CIs, under the null hypothesis that the subjects had the same prevalence distribution as the general population. The observed and expected prevalences are presented in table 3.7 below.

Table 3.7 Observed and expected prevalence of skin cancers in siblings and parents of proband subjects by gender

Group	Gender	Cancer Type	Observed prevalence	Expected prevalence (95% C.I.)		Relative Risk
Siblings	Men	SCC	1	0.70	(0.00, 2.32)	1.43
		BCC	15	2.87	(0.00, 6.05)	5.23*
	Women	SCC	3	0.55	(0.00, 1.99)	5.45*
		BCC	8	4.05	(0.27, 7.82)	1.98*
Parents	Men	SCC	7	1.69	(0.00, 4.09)	4.14*
		BCC	5	4.16	(0.70, 7.63)	1.20
	Women	SCC	1	1.18	(0.00, 3.24)	0.85
		BCC	9	4.22	(0.61, 7.84)	2.13*

*indicates result was significant at 0.05 level (non-overlapping 95% confidence limits of expected and observed prevalences, as per section 2.9.2)

Significantly more BCC cases were observed among the siblings, both brothers and sisters (relative risks 5.23 and 1.98 respectively), and in the mothers of probands (RR 2.13), than expected. Significantly more SCC cases were observed among the sisters (RR 5.45) and fathers (RR 4.14) of probands than expected.

The degree of familial aggregation of NMSC therefore differed by sex, relative and cancer type. In brothers, only BCC risk was elevated, whereas sisters had increased risks of both BCC and SCC. Among mothers only BCC risk was elevated, and among fathers only SCC risk was increased. Taken together, these data show that being a first-degree relative of a case of early-onset BCC was associated with marked increases in risk of NMSC, both BCC and SCC.

3.3.2. Comparison of observed and expected MM in first degree relatives

The expected number of malignant melanomas for the group of first degree relatives was calculated directly from the 1996 Australian Cancer Registry data (see methods section

2.10). Of the 123 siblings for whom data was obtained, one deceased subject was removed from analysis, leaving 122 sibling individuals (63 men, 59 women). Of the 89 parents for whom data was available, 17 subjects were deceased, leaving 72 subjects (eight men, nine women) for the analysis. A comparison of the observed and expected numbers of this cancer is seen in table 3.8.

Table 3.8 Relative risk of MM in first degree relatives of early-onset BCC probands

Group	Gender	Observed Prevalence	Expected Prevalence (95% C.I.)		Relative Risk
Siblings	Men	3	0.289	(0, 1.339)	10.3*
	Women	1	0.323	(0, 1.433)	3.1
Parents	Men	3	0.987	(0, 2.901)	3.0*
	Women	2	0.74	(0, 2.437)	2.7

*indicates result was significant at 0.05 level (non-overlapping 95% confidence limits of expected and observed prevalences, as per section 2.10)

All relatives had an increased risk of MM compared with the general population, and this was significant for male relatives. Brothers of the early-onset probands had a significant ten-fold increased risk of MM compared with the Australian population, and fathers had a three-fold increased risk.

3.4. Other cancer diagnoses

No proband reported a personal history of any cancer other than BCC. It can be seen in Table 3.9 that 17 first-degree relatives reported cancer diagnoses other than skin cancer, most of which could be histologically verified. Seventeen first degree relatives exhibited a variety of cancer diagnoses including colorectal (six relatives), breast (three), renal cell (two), laryngeal SCC (two), ovarian (one), prostate (one) and squamous cell lung (one) cancers, and malignant fibrous histiocytoma (one). The six relatives with colorectal carcinoma had an average age of onset of 66 years, which is close to the population average. Three first-degree relatives had breast carcinoma, two with invasive ductal histological types and the other not specified, and an average age of onset of 52 years. None of these data suggest abnormal aggregation of non-skin cancers in these families.

Table 3.9 All cancer diagnoses (excluding skin cancers) in first degree relatives of probands

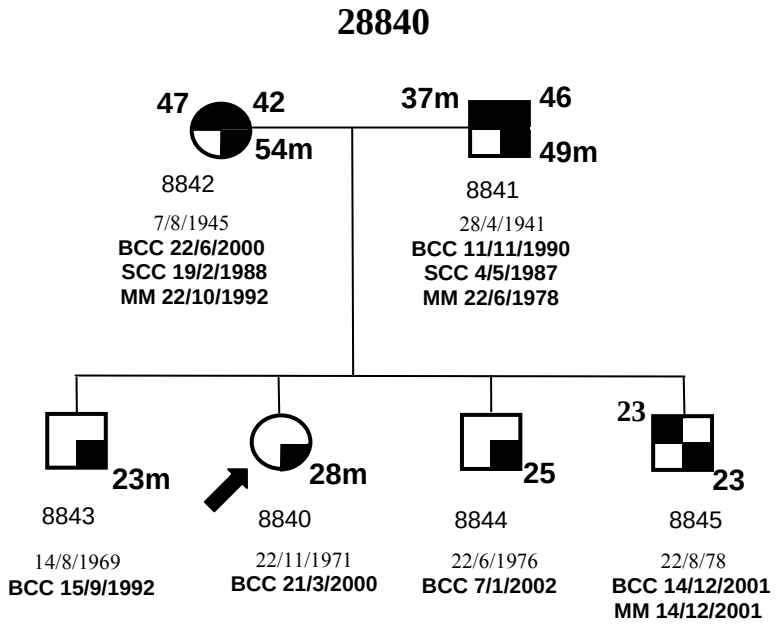
Family ID	Relationship to Proband	Gender	Cancer diagnosis	Age of Onset
28775	Sibling	Female	Breast (invasive ductal)	47
28770	Parent	Male	Laryngeal SCC	77
29779	Parent	Female	Ovarian	45
28781	Parent	Male	Colorectal	65
28797	Parent	Female	Colorectal	65
28816	Parent	Male	Renal Cell Carcinoma	68
28818	Parent	Female	Breast[a]	49
28825	Parent	Male	Colorectal	69
28857	Parent	Male	Laryngeal SCC	55
29094	Parent	Male	Colorectal	58
29168	Parent	Female	Colorectal	73
29168	Parent	Male	Squamous Cell Lung	69
29240	Parent	Male	Malignant fibrous histiocytoma (sarcoma)	62
29275	Parent	Female	Breast (invasive ductal)	60
28767	Parent	Male	Renal Cell Carcinoma	61
28762	Parent	Female	Colorectal	67
28762	Parent	Male	Prostate	70

[a]Clinical information only for this patient (not histologically verified)

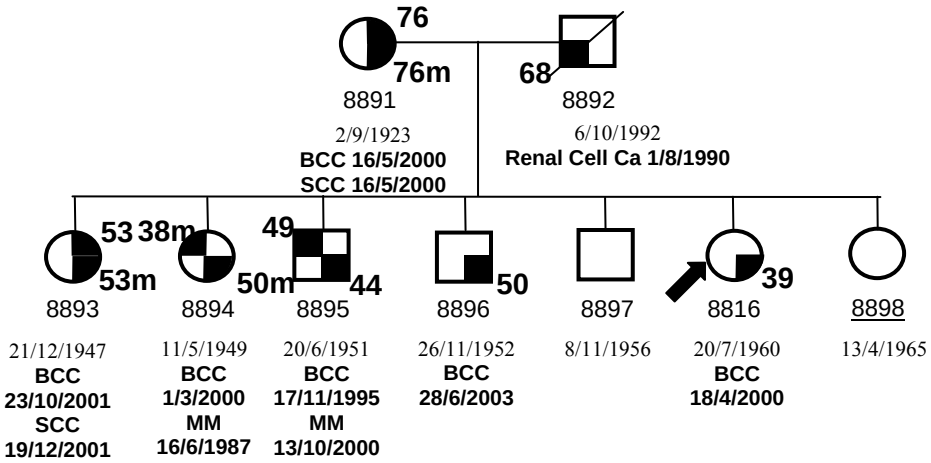
3.5. Pedigrees of affected families

Of the 56 probands included in this study, three had histopathologically-verified clustering of 6 cases of BCC among first-degree relatives. As is implied by the analysis in section 3.3, and shown in Figure 3.3, most families with more than one NMSC exhibited a combination of both types of skin cancer. However some families' cancer histories included only BCC (e.g. see pedigree for family ID 29022).

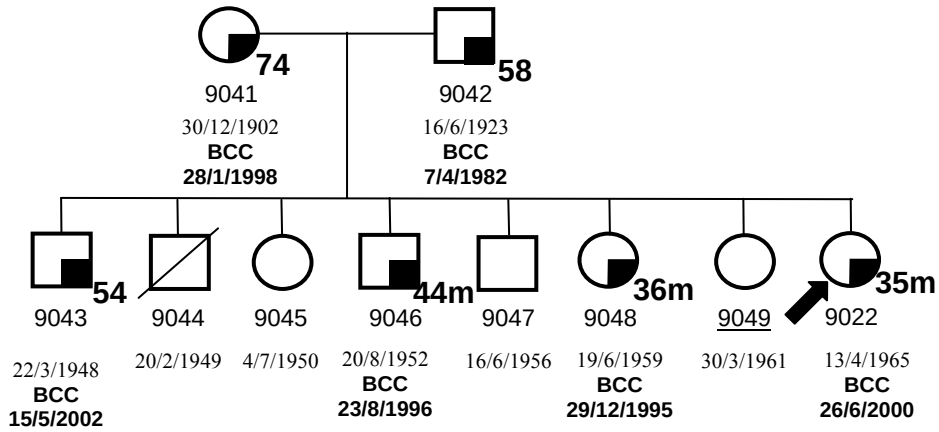
Figure 3.3. Pedigrees for families 28840, 28816, and 29022



28816


76
76m
68
8891
8892
2/9/1923
BCC 16/5/2000
SCC 16/5/2000
6/10/1992
Renal Cell Ca 1/8/1990
53
38m
53m
49
50m
44
50
39
8893
8894
8895
8896
8897
8816
8898
21/12/1947
BCC
23/10/2001
SCC
19/12/2001
11/5/1949
BCC
1/3/2000
MM
16/6/1987
20/6/1951
BCC
17/11/1995
MM
13/10/2000
26/11/1952
BCC
28/6/2003
8/11/1956
20/7/1960
BCC
18/4/2000
13/4/1965

29022


74
58
9041
9042
30/12/1902
BCC
28/1/1998
16/6/1923
BCC
7/4/1982
54
44m
36m
35m
9043
9044
9045
9046
9047
9048
9049
9022
22/3/1948
BCC
15/5/2002
20/2/1949
4/7/1950
20/8/1952
BCC
23/8/1996
16/6/1956
19/6/1959
BCC
29/12/1995
30/3/1961
13/4/1965
BCC
26/6/2000

Legend forFigure 3.3:


BCC

36 **Age first affected; m: multiple cancers**


SCC


proband


Malignant Melanoma


Other Cancer

xxxx **Control sibling**

Text: Date of Birth
Type cancer
Date of diagnosis

3.6. Discussion

As with other malignancies, the causes of BCC are likely to be due to a complex interplay of environmental and genetic factors. A better understanding of the way that BCC aggregates in families may help us to start to unravel these aetiological factors. In this chapter we see an examination of familial aggregation for BCC and SCC, a study that has no parallel in the literature to date.

The participation rate of eligible subjects for this study was 34%, however the *effective* participation rate of 53% may be a better assessment of participation: there were a relatively large number of non-contactable subjects due to the young age and mobility of the population sample compounded by the fact that the SCFA database contact information on these subjects was three years old. The population from which the subjects were ascertained needed to be from this database to allow comparison with the information gathered for the SCC arm of the study (see section 2.1.2). Despite this, there may be some ascertainment bias in that consenting subjects may be those in whom there is a family history of cancer, giving an overestimate of the cancer prevalence in relatives of early-onset BCC subjects. A family history of melanoma, the skin cancer of highest mortality, may be thought to produce a larger ascertainment bias than other types of skin cancer, due to the nature of the prognosis and likelihood of discussion among family members. If all eligible subjects who did not participate were to have no family history of

cancer, then our overestimates would be between 1.9-fold and 2.9-fold at worst (based on the inverse of the participation rates calculated above).

The participation rate of eligible relatives of this study was very high at over 85% in both parents and siblings. There was a female preponderance of proband subjects with a F:M ratio of 2.1:1. As this does not affect the sex distribution of their relatives, and as the sex ratio of relatives was equal, the bias towards female sex in probands should not affect the interpretation of cancer prevalence in relatives.

From examination of histopathologically-verified prevalence of NMSC in these 56 families of people with early-onset BCC, it is evident that familial aggregation of NMSCs is occurring. Over half of the families studied display aggregation of either BCC or SCC with two thirds of these including a history of both types of NMSC. Significantly, the other one-third of these families include family members with a history of BCC but not SCC, and in nearly half of these, clusters of three first-degree relatives or more affected relatives exist. There are also three families in whom two or more first-degree relatives were affected by melanoma. Together these results indicate that families of people with early-onset BCC may be at increased risk for skin cancer in general, and also for BCC in particular.

Here for the first time, relative risks of BCC, SCC, and MM for first-degree relatives of early-onset BCC cases have been determined. The computed relative risks confirm suspicions that arise when looking at the aggregation seen in the pedigrees in these families: first-degree relatives of people with early-onset BCC have an increased risk of NMSC. The expected prevalence of NMSCs in the subjects was calculated from data on the Australian population, and a significantly increased risk of BCC, SCC and melanoma occurred for first-degree relatives, with differential risks for different relative, sex and cancer type. Sisters and mothers of probands had a two-fold increased risk of BCC, and brothers had a 5-fold increased risk of BCC compared with that estimated for the general population. Sisters and fathers of people with early-onset BCC had over four times the prevalence of SCC than expected. For melanoma, the increased risk was significant for male relatives only, with a 10-fold increased risk for brothers of people with early-onset

BCC and three-fold for fathers. In general, the relative risks of cancers were greater in siblings than parents, which is not surprising given the larger baseline risk of parents due to their more advanced age.

These estimates should be interpreted with the understanding that they are based on some strong underlying assumptions. Cumulative risk of NMSC was estimated from validated Australian population annual incidence data (see section 2.9.1). This annual incidence data was used despite unvalidated lifetime data being available from this survey, as only validated data was deemed suitable for such an analysis. It was assumed that the period- and cohort-specific incidence rates for a given age and gender were constant. This assumption may not be correct especially given the recorded increase in incidence seen in Australia and internationally over the last few decades (see section 1.3). The national survey from which the incidence figures were obtained for the estimation of prevalence suggest that SCC age-specific incidence rates have increased between 1985 and 1995, while BCC rates have fallen for those aged under 50 years and have risen in those aged older than 50 years during this same 10-year period [1]. Thus it is possible that estimates of expected prevalence for SCC are in fact overestimates, such that the risk ratio based on observed prevalences could be slightly underestimated. Thus the SCC relative risks calculated are likely to be conservative estimates. If BCC incidence has actually declined by 10% in people under 50 years of age from 1985 to 1995 as suggested by the latest national survey, then the BCC relative risk in siblings may be a slight overestimate. Changes of this magnitude in the younger age group, even if real, cannot fully account for the two- to five-fold excess of observed to expected BCCs seen in siblings and mothers of the probands.

Empirical evidence supporting the Poisson model assumptions is illustrated in the close agreement between the observed and fitted incidence rates provided in figure 3.1. Death from NMSC is known to be extremely rare in Australia [396]. Although the occurrence of malignant melanoma is known to be associated with the presence of NMSC, death from malignant melanoma as an overall cause of death in Australia is also rare. Therefore, it is reasonable to approximate the incidence processes for SCC and BCC as acting independently of the overall death process on the Australian population. If

probands' relatives are more prone to death from malignant melanoma which in turn is associated with increased rates of NMSC, then those relatives with NMSC who died of melanoma have not been counted amongst the observed cases. This is because only those relatives who were alive contributed to this observed total. There is, therefore, a conservative effect on risk assessment due to any association between NMSC incidence and melanoma death in the probands' relatives.

It has been observed that the risk of NMSC is lower for immigrants (arriving after the age of 10 years) than native-born Australians [78]. Consequently, the SCC and BCC incidence processes do not act independently of the immigration process, as assumed in our mathematical derivation of estimated prevalences. It is not clear whether the emigration process acts independently of the SCC and BCC incidence processes. Unfortunately, using the available data, it is impossible to quantify these effects and those of other related variables, such as ethnicity and lifetime latitude of residence.

The mean age of diagnosis of BCC in the Australian population has been estimated at 63 for men and 59 for women, and for SCC it is 66 years for men and 64 years for women [1]. Although these figures cannot be directly compared with those from the subjects of this study due to differences in ascertainment and measurement; it is evident that mean ages of onset in the family members of this study were younger for the siblings (as expected due to ascertainment bias), and of a similar magnitude for parents.

Since the majority of families with aggregation of skin cancer have a combination of SCC and BCC, and it is likely that general risks for skin cancer are increased in relatives of people with early-onset BCC. These general risk factors are likely to include environmental, genetic, and phenotypic risks that are common to UVR-related skin cancers in general. Environmental risk factors that may to be common to all three types of skin cancer include sun exposure levels, sun protection measures employed, HPV or other viral infection, and increased surveillance in family members. Inherited, genetic characteristics that may cause increased risk to all three types of skin cancer may include those which relate to pigmentary traits such as MC1R, HPV susceptibility eg through polymorphism p53Arg, and probably multiple as-yet unidentified genetic susceptibilities.

Phenotypic risk factors for all three types of skin cancer include sun sensitivity, pigmentary characteristics, signs of sun exposure such as actinic keratoses, and phenotypic results of a combination of higher risk skin pigmentation and sun exposure such as freckling.

Given that nearly one fifth of families in this study had aggregation of BCC to the exclusion of SCC or MM, BCC-specific risk factors are also likely to be at work. BCC-specific factors probably include environmental risks such as the pattern of sun exposure, with childhood and intermittent exposure affecting BCC risk to a greater degree than SCC risk [47, 78, 81]. Toxins such as arsenic are also specific risk factors for BCC. Genetic factors such as *PTCH* polymorphisms or mutations within the germlines of these families could also affect risk of BCC. This tumour-suppressor gene causes increased risk of BCC to those with the rare genodermatosis Gorlin syndrome, but beyond some preliminary haplotyping studies, has not been examined as a potential contributor to BCC susceptibility in the general population (considered in chapter 5). Presence of Gorlin Syndrome would also cause an increased risk of BCC differentially compared with SCC, and one proband reported a prior clinical diagnois of this syndrome.

Further information about the origins of skin cancer risk may be obtained through phenotypic examination of probands and their unaffected siblings who can act as intra-family controls. Environmental risk factors are assumed to be similar for siblings within a family, meaning that observed phenotypic differences are likely to be attributable to genetic causes. Questionnaires to ascertain exposure to environmental risk factors would be useful and could be employed in future studies. These issues will be explored further in the next chapter.

Sources of error in comparing this study's subject prevalence with population prevalence include the differences in latitude of the people involved, and differences in sibling numbers of the people measured. However, as discussed in section 2.1.1, the Sydney basin is likely to have a similar incidence of skin cancer to the general population.

Chapter 4: Case-control analysis: phenotypic risk factors for early-onset BCC

4.1. Introduction

The subjects of this study are 56 people who have developed BCC under 40 yr, i.e. earlier than 93% of Australians who are affected by this type of skin cancer. It was hypothesised that they would exhibit increased risk factors for BCC. In the previous chapter their relatives were shown to have increased prevalence of not only BCC, but also SCC and melanoma. Importantly, in a proportion of these families there were multiple cases affected by BCC in the absence of SCC, in one family this included six first-degree affected relatives. Exploration of underlying causative factors therefore needs to address both susceptibility to skin cancer in general and also susceptibility factors that might be unique to BCC.

Particular skin phenotypes are known to be associated with skin cancer risk. The complex of inherited characteristics defining a person's 'sun sensitivity' has a moderate association with BCC. Pigmentary characteristics such as skin colour and tanning ability are key features of this complex and may play a causative role, with eye colour and hair colour surrogate markers (see section 1.4). Genetic variation in MC1R is largely responsible for these individual differences in pigmentary characteristics, in combination with other as yet unknown genes, and acts as an independent risk factor for BCC (see section 1.7).

Risk of BCC is increased by sun exposure, with acute intermittent exposure rather than cumulative total dose the more important pattern of exposure for determining risk of this tumour (see section 1.4). Signs of high total cumulative sun exposure such as presence of pterygium on the sclera of the eye, and solar elastosis or actinic keratoses on the chronically sun exposed regions of the body have been associated with SCC, and to a lesser extent BCC. However it is difficult to disentangle these phenotypes from varying individual susceptibility to sun-related skin damage: they are not uncontaminated markers of solar radiation dose. Some commonly occurring skin lesions that are the result of

combinations of pigmentary traits and sun exposure include solar lentigines and freckling. More poorly understood are the presence of seborrhoeic keratoses; these benign lesions may be associated with sun exposure and are very prevalent in Australian populations. They do not have any known associations with skin cancer. In this chapter, the causes of BCC in the probands are explored by comparing their skin phenotype with that of their unaffected siblings. Siblings are similar genetically and are even more likely to have shared a common early environment. Differences seen between affected-unaffected sibling pairs can therefore not be confounded by population admixture and, if not due to chance, are more likely to have a genetic than an environmental basis.

Several skin characteristics were measured, using both self-reports and physical examination by a trained examiner under standardised conditions (see section 2.3). Pigmentary characteristics of the skin were measured along with sun sensitivity factors, characteristics relating to previous sun exposure, and characteristics due to a combination of sun sensitivity and sun exposure. All tests of association described in this chapter were carried out using logistic regression, either unconditionally or conditioning on family membership (i.e. taking into account their membership of a proband-sibling pair). Differences in skin colour, skin phototype, signs of sun sensitivity and sun exposure were seen between the sibling pairs, indicating identifiable phenotypic risk factors for early-onset BCC.

The following chapter (section 5) describes analysis of genetic factors that may explain the clustering of skin cancer within these families.

4.2. Results

4.2.1. General characteristics

A nested case-control analysis was performed by selecting the oldest NMSC-unaffected sibling as a control for each proband where available. Examinations were performed on 88 subjects, including assessments of pigmentary characteristics and semi-quantitative analysis of various sun exposure-related lesions on their skin. Of these 88 subjects, 73%

(64/88) were part of proband-sibling case-control pairs, with the 27% (24/88) remaining subjects undergoing examination unpaired.

Table 4.1. Age, Height, Weight paired t-tests

Measurement	Mean Case-Control	SD	Sig (2-tailed)
Age (years)	-1.8	6.0	.101
Height (cm)	-1.3	9.3	.449
Weight (kg)	-2.9	14.7	.266

There were no significant differences between probands and siblings with respect to age, height, or weight (see table 4.1). The siblings chosen as a control for the probands were selected to be the oldest NMSC-unaffected sibling. The average age of the siblings was 1.8 years older than the probands, or one year and 10 months (SD 6.0 years). The sibling controls were also on average slightly taller than their proband pair by 1.3 cm (SD: 9.3 cm) and heavier by 2.9 kg (SD: 14.7 kg).

All subjects were investigated for evidence of Gorlin syndrome, through history of mandibular keratocysts and presence of palmar pitting (see section 2.3.1). These features are both pathognomonic of Gorlin syndrome, and presence of both features occurred in one patient only (proband ID 9125) who had previously been clinically diagnosed with this syndrome.

4.2.2. Pigmentary factors

4.2.2.1.Hair Colour

Table 4.2 Case control comparison of hair colour by self report and by identification of a swatch of hair colour (from subjects' answers to questionnaire)

Variable	n (%)	Unconditional Logistic Regression	Odds Ratio	(95% CI)	p-value	Conditional Logistic Regression	Odds Ratio	(95% CI)	p-value
Hair colour self report	88(100.0)	As continuous variable	1.112	(.803-1.543)	.521	As continuous variable	1.094	(.675-1.773)	.715
	32(36.3)	Brown/black	1	-	-	Brown/black	1	-	-
	33(37.5)	Light brown	1.789	(.646-4.957)	.263	Light brown	2.916	(.611-13.925)	.180
	17(19.3)	Fair	1.111	(.337-3.659)	.862	Fair	.821	(.116-5.800)	.843
	6(6.8)	Red	1.556	(.248-9.750)	.637	Red	1.708	(.087-33.488)	.725
Identification of hair colour by swatch	88(100.0)	As continuous variable	1.147	(.616-2.137)	.665	As continuous variable	1.000	(.464-2.155)	1.000
	36(40.9)	brown/black	1	-	-	brown/black	1	-	-
	39(44.3)	blonde/fair	1.429	(.558-3.655)	.457	blonde/fair	1.000	(.277-3.608)	1.000
	13(14.)	red	1.143	(.312-4.189)	.840	red	1.000	(.191-5.241)	1.000

Subjects most commonly reported a light or mouse brown hair colour with dark brown the next most common hair colour, then blonde/fair, then red. Only one subject reported having black hair (see table 4.2 and appendix table 7.2). Dark brown was the second most common hair colour reported with 31/88 (35.2%) in this category. There were 17/88 (19.3%) who reported blonde/fair hair with 6/88 (6.8%) reporting red hair and 1/88 (1.1%) reporting black hair. Black and brown hair results were aggregated for the analysis (see table 4.2).

Subjects were asked to self-identify their hair colour in two ways: by answering the question "Which colour best describes your natural hair colour at age 21- red, fair/blonde, light/mouse brown, grey, dark brown, or black?", and by matching their recalled hair colour at age 21 by a standard wigmaker's hair swatch (see section 2.3.1). Hair colour observed at time of examination is not a relevant variable in studies of human pigmentation because of age-related and cosmetic changes. Identification of hair swatch

is a more objective measure because it removes the subjectivity of naming the colour and is therefore probably a more objective measure of the subjects' true hair colour.

The subjects showed reasonably high correlation between these two self-reported measures, with Spearman's rank correlation of 0.71 (significant correlation at the 0.01 level), indicating 71% agreement between the measures (see appendix table 7.2). All subjects who self-reported red hair also identified their hair as red visually by hair swatch. Only one person reported having black hair, and this individual identified their hair colour as brown by selection of hair swatch. Nearly all (94.1%, 16/17) self-reported blondes also identified this colour by hair swatch, with one (5.9%) selecting a red hair swatch. Most (87.1%, 27/31) self-reported brunettes identified a brown swatch, with three (9.7%) selecting a fair/blonde swatch and one (3.2%) a red hair swatch. A 'light or mouse brown' self-report was less specific, with most selecting a fair/blonde swatch (20/33 or 60.6%), and the remainder either a brown swatch (8/33, 24.2%) or red (5/33, 15.2%) swatch. If the 'light or mouse brown' named category is combined with the 'dark brown' named category to allow an analysis of agreement with the self-report by swatch, the agreement between the two measures is 0.43 (Kronbach's Kappa). If instead, the 'light or mouse brown' named category is combined with the 'fair/blonde' named category for a comparison with the swatch self-reports, agreement improves to a measure of 0.65 (Kronbach's Kappa).

No consistent or significant trend between hair colour and history of BCC was seen in the subjects examined, whether determined by self report or identification of hair swatch colour (see table 4.1). Subjects with self-reported lighter pigmented hair than dark brown/black were more likely to have had BCC; however the odds did not increase consistently with increasing fairness. When the groups were compared as matched pairs, the odds of BCC were still raised for light-brown (OR= 2.92, 95% CI 0.61 -13.93) or red-heads by self report (OR = 1.71, 95% CI 0.09 – 33.49), but was the relationship was completely lost for the more reliable measure of hair colour by hair swatch. Overall, there is little evidence for an association of hair colour and BCC risk in this group.

4.2.2.2.Eye Colour

Table 4.3 Case control comparison of eye colour (from subjects' answers to questionnaire)

Variable	n (%)	Unconditional Logistic Regression	Odds Ratio	(95% CI)	p-value	Conditional Logistic Regression	Odds Ratio	(95% CI)	p-value
Eye colour	88(100)	As continuous variable	1.700	(.759-2.404)	.306	As continuous variable	1.155	(.547-2.433)	.706
	17(19.3)	brown/black	1	-	-	brown/black	1	-	-
	34(38.6)	green/hazel	.887	(.273-2.884)	.842	green/hazel	.825	(.211-3.225)	.782
	37(42.0)	blue	1.655	(.500-5.470)	.409	blue	1.337	(.298-6.001)	.704

Blue (37/88 or 42.0%) or green eyes (34/88 or 38.6%) were more commonly reported by subjects with only 19.3% (17/88) reporting brown/black eyes (see table 4.3).

No significant relationship between eye colour and history of BCC was evident, with slightly increased odds of BCC in subjects with lighter eye colour, a relationship that was weakened by comparing matched proband-sibling pairs (OR = 1.16, 95% CI 0.55- 2.43), reducing the likelihood of a real association. There was also no trend for consistently increasing risk with decreasing eye pigmentation, as the odds of BCC were decreased for green/hazel eyes (OR = 0.83, 95% CI 0.21- 3.23) and increased for blue eyes (OR = 1.33, 95% CI 0.30- 6.00). Together, these results provide no evidence for an association between eye colour and BCC risk in these subjects.

4.2.2.3.Skin Colour

The commonest reported skin colour among all subjects was fair (47/88 or 53.4%) with 39.8% (35/88) reporting very fair skin, and only 6.8% subjects overall (6/88) reporting olive or brown skin colour (see table 4.4).

The odds of BCC was higher for people reporting a lighter skin colour, and this relationship strengthened in the case-control analysis (OR = 2.04, 95% CI 0.67- 6.29) but remained non-significant. There was a trend for increasing risk of BCC with progressively increased fairness of skin, with ORs of 1.47 (95% CI 0.27- 8.10) for fair skin and 2.18 (95% CI 0.38- 12.58) for very fair skin compared with the whole unpaired group of control siblings. Given that there were so few subjects reporting olive/brown

skin, odds ratios were unable to be determined for categories of increasing fairness of the skin for case-control pairs.

People with BCC had very slightly lighter overall constitutive skin colour as measured by luminance (see 2.3.3 for detailed explanation of skin colour parameters as determined by reflectance spectrophotometry), which also strengthened with case-control matching but did not reach significance (OR = 1.20, 95% CI 0.93- 1.55; see table 4.6). Inner arm (constitutive, non-sun exposed) skin colour was slightly less red on a red-green reflectance scale in probands compared with the group of siblings (OR = 0.91, 95% CI 0.76- 1.08), with cases on average having nearly one unit lower readings on reflectance spectrophotometry on this variable. This association became significant when case-control pairs were matched (OR = 0.59, 95% CI 0.36 – 0.98, with paired t-test 2-tailed significance =0.019), suggesting that constitutive red-green reflectance of the skin is associated with a lower risk of BCC compared with those whose skin is made up of less of this pigment. People with BCC also had less yellow in their skin pigment as measured by the yellow-blue reflectance parameter, and although this relationship strengthened with matching of probands with their siblings in the analysis (OR = 0.83, 95% CI 0.64-1.07), it remained small and not significant.

None of the outer arm (a sun-exposed skin area) measurements of skin colour were significantly different between cases and controls. There was very little difference in exposed skin colour, with essentially no difference between exposed skin in overall fairness (OR = 1.08, 95% CI 0.94- 1.24), and little difference seen before case-control pairing in red-green reflectance or yellow-blue reflectance. In an analysis with case-control pairing, there was a trend for a small association with outer arm red-green reflectance (OR = 0.83, 95% CI 0.62- 1.11) and yellow-blue reflectance (OR = 0.85, 95% CI 0.69- 1.07) with BCC, with an increasing degree of either measure decreasing the odds of BCC slightly.

The measures *L* (‘luminance’), *a* (‘red-green reflectance’) and *b* (‘yellow-blue reflectance’) are independent dimensions of colour, but skin types are likely to be expressed as recurring combinations of these measures, due to the effect of a small

number of skin structures contributing to colour in a limited number of ways (see section 2.3.3 for a discussion on the main contributors to skin colour). Correlations between the measures were examined using linear regression; Spearman's correlation coefficient for the outer arm measure *L* was R^2=0.81, i.e. 81% of the variance in the measure *L* is explained by the outer arm measures *a* and *b*. Inner arm measure *L* had a Spearman's correlation coefficient of R^2=0.72, suggesting that 72% of the variance in inner arm *L* is explained by the inner arm *a* and *b* measures. When the *a* and *b* measures for the outer arm and the inner arm are combined in a linear regression analysis, inner arm red-green reflectance was the only independent predictor of BCC risk.

Table 4.5 Case control comparison of self- reported skin pigmentation (from subjects' answers to questionnaire)

Variable	n (%)	Unconditional Logistic Regression	Odds Ratio	(95% CI)	p-value	Conditional Logistic Regression	Odds Ratio	(95% CI)	p-value
Self reported skin pigmentation	88(100)	As continuous variable	1.479	(.714-3.058)	.292	As continuous variable	2.044	(.665-6.289)	.212
	6(6.8)	Olive/Brown	1	-	-	Olive/Brown	1	-	-
	47(53.4)	Fair	1.474	(.268-8.091)	.655	Fair	2085.670	?	.899
	35(39.8)	Very Fair	2.182	(.378-12.583)	.383	Very Fair	3649.923	?	.892

Table 4.6 Case control comparison of spectrophotometric measurements of outer and inner arm skin colour

Variable	Paired t test	Mean difference case - control	(95% CI)	Sig (2-tailed)	Unconditional Logistic Regression	Odds Ratio	(95% CI)	p-value	Conditional Logistic Regression	Odds Ratio	(95% CI)	p-value
Observed exposed skin colour	Outer arm luminance	1.02452	(-.97464-3.02367)	.304	Continuous variable	1.019	(.955-1.088)	.563	Continuous variable	1.076	(.935-1.238)	.309
	Outer arm red-green reflectance	-.66129	(-1.66190-.33932)	.187	Continuous variable	.963	(.842-1.101)	.582	Continuous variable	.828	(.621-1.105)	.200
	Outer arm yellow-blue reflectance	-.89097	(-2.13435-.35242)	.154	Continuous variable	.975	(.886-1.073)	.607	Continuous variable	.854	(.685-1.065)	.162
Observed constitutive skin colour	Inner arm luminance	1.06194	(-.34603-2.46990)	.134	Continuous variable	1.041	(.947-1.144)	.404	Continuous variable	1.197	(.925-1.548)	.171
	Inner arm red-green reflectance	-.99484	(-1.81555--.17413)	.019*	Continuous variable	.906	(.757-1.083)	.278	Continuous variable	.591	(.356-.980)	.041*
	Inner arm yellow-blue reflectance	-.88742	(-2.04297-.26813)	.127	Continuous variable	.952	(.855-1.060)	.372	Continuous variable	.826	(.639-1.068)	.145

4.2.3. Propensity to burn, ability to tan, and phototype

Overall, only nine (10.2%, 9/88) subjects reported severe sunburn with blistering following a single exposure to bright sunlight for one hour for the first time in summer. Most subjects reported mild or no sunburn (29.5%, 26/88) or painful (60.2%, 53/88) sunburn (see section 2.3.2 for recoding of variable information and appendix table 7.3 for raw data).

There was a trend for progressively increased risk of BCC for people with increasing propensity to burn, and this effect was magnified by analysis by case-control pairs. A painful burn increased risk of BCC slightly (OR = 1.21, 95% CI 0.47 – 3.15) and a severe burn increased the risk more than two-fold (OR = 2.57, 95% CI 0.44 – 14.82). Analysis by case-control pairs increased this relationship, with a greater than three-fold risk of BCC for a painful burn after their first summer exposure to sunlight (OR = 3.50, 95% CI 0.73- 16.85), and a seven-fold risk if reporting severe sunburn with blistering (OR = 7.00, 95% CI 0.70- 70.74); this trend did not quite reach significance (p for trend = 0.08).

However, only 6.8% (6/88) of persons reported a response of deep tanning following repeated exposure to the sun (‘ability to tan’), with 18.2% (16/88) of people at the other end of the spectrum, reporting no tanning/freckling only. The remainder fell nearly equally into the groups of ‘moderately tanned’ (38.6% or 34/88) or ‘mildly/occasionally tanned’ (36.4% or 32/88) (see appendix table 7.3).

There was an effect of increased risk of BCC in people with reduced ability to tan although significance was not achieved: there was a small increased risk of OR 1.61 (95% CI 0.28- 9.23) for moderate tanning, OR 1.46 (95% CI 0.25- 8.40) for mild tanning and a larger effect of OR 3.00 (95% CI 0.42- 21.30) for no tan or freckling only (p for trend = 0.33). This effect was increased by case-control analysis, with the odds of BCC 2.15 (95% CI 0.19- 24.93) for moderate tanning, 1.74 (95% CI 0.13- 22.75) for mild tanning, and six-fold increased odds of BCC (OR = 5.97; 95% CI 0.33- 107.26) for those reporting no tan or freckling only (p for trend = 0.25). These results are consistent with a protective effect of the ability to tan on risk of BCC.

Only 9.1% (8/88) reported ‘always burning/never tanning’, with the remainder reporting ‘usually burning/sometimes tanning’ (52.3%, 46/88), or ‘sometimes burning/usually tanning’ (38.6%, 34/88) (see appendix table 7.4).

The trend for risk of BCC with lower skin phototype was present but small (OR = 1.72, 95% CI 0.84 – 3.56) but became larger and statistically significant (p for trend = 0.045) in the case-control analysis, with a more than three-fold increased risk of BCC with decreasing phototype (OR = 3.56; 95% CI 1.03 – 12.35); the risk of BCC was greater than four-fold for phototype II (usually burn/sometimes tan; OR = 4.24; 95% CI 0.91 – 19.81), and nearly ten-fold for people with phototype I as compared with phototype III (always burn/never tan; OR = 9.79; 95% CI 0.668 – 143.42), although remained non-significant.

In summary, effects of these measures were only weakly discernible in these data due to limited power of the study, and the intrinsic limitation of a sibling-control design in which many risk factors will be shared by cases and controls. However the directions of the trend data are all consistent with effects observed in population-based studies of BCC. The trend was for increased risk of BCC with increased propensity to burn, decreased ability to tan, and lower skin phototype, with this latter variable the only one to reach significance (p = 0.045.)

Table 4.7 Case- control comparison of skin reactions to single, repeated, and general exposure to the sun (from subjects' answers to questionnaire)

Variable	**n (%)**	**Unconditional Logistic Regression**	**Odds Ratio**	**(95% CI)**	**p-value**	**Conditional Logistic Regression**	**Odds Ratio**	**(95% CI)**	**p-value**
Single exposure to sun (propensity to burn)	88(100.0)	As continuous variable	1.429	(.688-2.967)	.338	As continuous variable	2.747	(.876-8.621)	.083
	26(29.5)	mild/no sunburn	1	-	-	mild/no sunburn	1	-	-
	53(60.2)	painful sunburn/peeling	1.210	(.465-3.147)	.696	painful sunburn/peeling	3.500	(.727-16.848)	.118
	9(10.2)	severe sunburn/blistering	2.567	(.444-14.822)	.292	severe sunburn/blistering	7.000	(.693-70.743)	.099
Repeated exposure to sun (ability to tan)	88(100.0)	As continuous variable	1.288	(.770- 2.155)	.334	As continuous variable	1.518	(.749- 3.077)	.247
	6(6.8)	deeply tanned	1	-	-	deeply tanned	1	-	-
	34(38.6)	moderately tanned	1.615	(.283- 9.235)	.590	moderately tanned	2.148	(.185-24.933)	.541
	32(36.4)	mildly tanned	1.462	(.254- 8.401)	.671	mildly tanned	1.742	(.133-22.746)	.672
	16(18.2)	no tan/freckle only	3.000	(.423- 21.297)	.272	no tan/freckle only	5.973	(.333-107.257)	.225
General reaction to sun (phototype)	88(100)	As continuous variable	1.727	(.839-3.559)	.138	As continuous variable	3.559	(1.029-12.346)	.045*
	88(100)	As continuous variable recoded	1.934	(.799-4.695)	.144	As continuous variable recoded	4.500	(.972-20.827)	.054
	34(38.6)	sometimes burn/usually tan (phototype III)	1	-	-	sometimes burn/usually tan (phototype III)	1	-	-

	46(52.3)	usually burn/sometimes tan (phototype II)	1.837	(.737- 4.577)	.192	usually burn/sometimes tan (phototype II)	4.243	(.909-19.811)	.066
	8(9.1)	always burn/never tan (phototype I)	2.667	(.470-15.136)	.268	always burn/never tan (phototype I)	9.787	(.668-143.415)	.096

4.2.4. Sun exposure

Table 4.8 Case-control comparison of presence of actinic keratoses, solar elastosis and pterygium (as measured by skin examination)

Variable	n (%)	Unconditional Logistic Regression	Odds Ratio	(95% CI)	p-value	Conditional Logistic Regression	Odds Ratio	(95% CI)	p-value
Presence of actinic keratoses	44 (50.0)/ 44 (50.0)	Presence/ Absence	3.000	(1.216-7.399)	.017*	Presence/ Absence	3.667	(1.023-13.143)	.046*
Total solar elastosis	27 (30.7)/ 60 (68.2)	Low/high categories	1.016	(.396-2.604)	.974	Low/high categories	1.222	(.723-2.064)	.454
Presence of pterygium	56 (63.6)/ 31 (35.2)	Presence/ Absence	.917	(.368-2.285)	.852	Presence/ Absence	.750	(.260-2.162)	.594

Half of the subjects had at least one actinic keratosis at examination (see table 4.8 and appendix table 7.6). One subject was not examined for total solar elastosis or pterygium and so the analysis for these two factors included 87 subjects only. A large proportion of subjects had a high amount of solar elastosis (see section 2.4.2 and appendix table 7.7), and nearly two-thirds of subjects had the sun-exposure-related lesion 'pterygium' visible on the sclera of their eye at examination.

The presence of any actinic keratoses were significantly associated with history of BCC with an OR of 3.00 (95% CI 1.22- 7.40; p = 0.017) for this variable, an association that was increased marginally in the case-control analysis (OR = 3.67; 95% CI 1.02- 13.14; p = 0.046). The other signs of sun exposure showed smaller associations and required case-control analysis to become evident; total solar elastosis was associated with a slightly increased risk of BCC in the case-control analysis (OR = 1.22, 95% CI 0.72- 2.06); and risk of BCC was paradoxically slightly reduced in people with a pterygium when cases were analysed with respect to their matched siblings (OR = 0.75; 95% CI 0.26- 2.16).

In summary, the large amount of sun-exposure-related skin changes in this group made any potential associations with BCC risk difficult to determine. The direction of the trend data for actinic keratoses and solar elastosis was consistent with population-based studies on BCC risk, with actinic keratoses presence significantly associated with BCC risk (p = 0.017).

4.2.5. Skin characteristics that are influenced by sun exposure

Solar lentigines and freckling are pigmentary lesions with complex aetiology (see 1.4.1.1). Lentigines have their strongest associations with prior sunburn and occur in sites of previous burning, freckles are influenced by sun exposure more broadly. Freckling is strongly geneticly determined whereas the genetics of solar lentigo formation, if any, are not understood.

4.2.5.1.Solar lentigines

Table 4.9 Case-control comparison of numbers of solar lentigines on the face, forehead, upper limbs, and back and shoulders (as measured by skin examination)

Variable	n (%)	Unconditional Logistic Regression	Odds Ratio	(95% CI)	p-value	Conditional Logistic Regression	Odds Ratio	(95% CI)	p-value
Solar lentigines face	88 (100)	Continuous variable	1.088	(.856-1.382)	.491	Continuous variable	1.146	(.873-1.503)	.326
	43 (48.9)/ 45 (51.1)	low/high categories	1.185	(.500-2.811)	.700	low/high categories	1.667	(.606-4.586)	.323
Solar lentigines forehead	88 (100)	Continuous variable	.949	(.757-1.190)	.653	Continuous variable	1.010	(.771-1.322)	.945
	47 (53.4)/ 41 (46.6)	low/high categories	.729	(.306-1.732)	.474	low/high categories	1.167	(.392-3.471)	.782
Solar lentigines dorsal upper limbs	88 (100)	Continuous variable	1.254	(1.026-1.532)	.027*	Continuous variable	1.424	(1.075-1.886)	.014*
	46 (52.3)/ 42 (47.7)	low/high categories	2.583	(1.052-6.346)	.038*	low/high categories	3.250	(1.060-9.967)	.039*
Solar lentigines upper back/shoulders	88 (100)	Continuous variable	1.074	(.878-1.313)	.487	Continuous variable	1.384	(.958-2.000)	.084
	43 (48.9)/ 45 (51.1)	low/high categories	1.440	(.605-3.426)	.410	low/high categories	2.000	(.602-6.642)	.258

Table 4.9 shows a trend for increased risk of BCC for people who have had a high number of solar lentigines (see section 2.3.2 for recoding and appendix table 7.8 for raw data). Having a high number of solar lentigines on the upper arms increased risk of BCC significantly (OR = 2.58; 95% CI 1.05- 6.35), and this association was strengthened to a 3-fold risk of BCC in the case-control analysis (OR = 3.25, 95% CI 1.06- 9.97). There was a smaller, non-significant risk with high numbers of solar lentigines on the face (OR = 1.19, 95% CI 0.50- 2.81) and this increased marginally in the case-control analysis (OR = 1.67, 95% CI 0.61- 4.59). Solar lentigines on the forehead had essentially no effect on risk, with an odds ratio approaching unity. High numbers of

solar lentigines on the upper back and shoulders was associated with a small increased BCC risk, and this increased to a two-fold risk (OR = 2.00; 95% CI 0.60 – 6.64) in the paired case-control analysis.

4.2.5.2.Freckling

Table 4.10 Case-control comparison of freckling (from questionnaire answers and from skin examination)

Variable	n (%)	Unconditional Logistic Regression	Odds Ratio	(95% CI)	p-value	Conditional Logistic Regression	Odds Ratio	(95% CI)	p-value
Reported freckling in childhood	88 (100.0)	As continuous variable	1.455	(.757-2.798)	.261	As continuous variable	1.752	(.749-4.097)	.196
	8 (9.1)	None	1	-	-	None	1	-	-
	31 (35.2)	Some	1.385	(.291-6.581)	.682	Some	.250	(.016-3.997)	.327
	49 (55.6)	Many	2.062	(.456-9.328)	.347	Many	1.500	(.251-8.977)	.657
Reported freckling in adulthood	88 (100.0)	As continuous variable	1.177	(.632-2.191)	.607	As continuous variable	1.472	(1.472-3.038)	.296
	15 (17.0)	None	1	-	-	None	1	-	-
	43 (48.9)	Some	1.633	(.496-5.383)	.420	Some	3.000	(.555-16.208)	.202
	30 (34.0)	Many	1.511	(.430-5.313)	.520	Many	3.000	(.555-16.208)	.202
Clinical freckling face	88 (100.0)	Continuous variable	.975	(.802-1.186)	.803	Continuous variable	1.012	(.746-1.374)	.938
	60 (68.2)/ 28 (31.8)	low/high categories	1.000	(.420-2.379)	1.000	low/high categories	1.333	(.298-5.957)	.706
Clinical freckling forehead	88 (100.0)	Continuous variable	1.099	(.906-1.334)	.338	Continuous variable	1.249	(.932-1.674)	.137
	66 (75.0)/ 22 (25.0)	low/high categories	.975	(.407-2.337)	.956	low/high categories	1.750	(.512-5.978)	.372
Clinical freckling dorsal upper limbs	88 (100.0)	Continuous variable	1.160	(.909-1.480)	.233	Continuous variable	1.903	(1.068-3.391)	.029*
	78 (88.6)/ 10 (11.4)	low/high categories	1.815	(.749-4.396)	.187	low/high categories	4.000	(.849-18.836)	.080
Clinical freckling upper back/shoulders	88 (100.0)	Continuous variable	1.112	(.884-1.398)	.365	Continuous variable	1.221	(.883-1.688)	.228
	43 (48.9)/ 45 (51.1)	low/high categories	1.105	(.460-2.654)	.823	low/high categories	1.400	(.444-4.411)	.566

Reported freckling in childhood was not consistently associated with BCC risk, as seen in table 4.10 (see section 2.3.2 for recoding of variables and appendix table 7.9 for raw scores). Reporting of some freckles in childhood was associated with a small increased risk of BCC (OR = 1.39, 95% CI 0.29- 6.58); many freckles were associated with a higher risk (OR = 2.06, 95% CI 0.46- 9.32); neither association was significant. The validity of these predictions are questioned when considering the marked decrease in predicted risk resulting from the case-control analysis (some freckles; OR = 0.25, 95% CI 0.02- 4.00, many freckles; OR = 1.50, 95% CI 0.25- 8.98).

Freckling in adulthood showed a more consistent but still non-significant association with more evidence for a trend, with a slightly elevated risk that increased to 3-fold in the case-control analysis (OR = 3.00; 95% CI 0.55- 16.21 for both).

As with examination of solar lentigines, the dorsal upper limbs were the only region of the body examined to show a significant association of freckling with BCC risk. Here, there was a four-fold risk (OR = 4.00, 95% CI 0.85- 18.84) of BCC with high numbers of freckles seen on the upper limbs, with a p value approaching significance at 0.08. The p value drops to significance at the 0.05 level when all categories on the semi-quantitative scale are included in the analysis, giving a 2-fold risk of BCC associated with upper arm freckling (OR = 1.90, 95% CI 1.07- 3.39, p = 0.029). Observed freckling on the face and forehead both had no effect on BCC risk without the case-control analysis, but was associated with slightly increased odds of BCC with the case-control analysis (face freckling; OR = 1.33; 95% CI 0.30- 5.96; forehead freckling; OR = 1.75; 95% CI 0.51- 5.96). Upper back and shoulder freckling also showed a small non-significant association with BCC risk with odds of 1.40 (95% CI 0.44- 4.41) with case-control matching.

In summary, the trends seen in this case-control data reflect those seen in population studies on BCC. Risk of BCC was increased by the presence of sun-exposure-related lesions including solar lentigines and freckling. The most significant associations were seen for both variables on the chronically sun-exposed region of the arm (p = 0.014 and p = 0.029 respectively). The amounts of solar lentigines were more consistently associated with BCC risk than freckling, which may reflect their relatively simple relationship to sun-exposure. The measures of freckling included both self-reported variables and clinically measured ones. The self-reported measure of childhood freckling showed the most inconsistent associations with risk of BCC, which may reflect the difficulties associated with reliance on subjects' memory of the variable.

4.2.6. Seborrhoeic keratoses

Table 4.11 Case-control comparison of numbers of seborrhoeic keratoses (from skin examination)

Variable	n (%)	Unconditional Logistic Regression	Odds Ratio	(95% CI)	p-value	Conditional Logistic Regression	Odds Ratio	(95% CI)	p-value
Seborrhoeic Keratoses head and neck	88 (100.0)	Continuous variable	.456	(.148-1.402)	.170	Continuous variable	.429	(.111-1.657)	.220
	15 (17.0)/ 73 (83.0)	Present/Absent categories	.456	(.148-1.402)	.170	Present/Absent categories	.429	(.111-1.657)	.220
Seborrhoeic Keratoses upper limbs	88 (100.0)	Continuous variable	1.283	(.530-3.108)	.581	Continuous variable	1.000	(.351-2.851)	1.000
	29 (33.0)/ 59 (67.0)	Present/Absent categories	1.214	(.480-3.069)	.682	Present/Absent categories	1.000	(.351-2.851)	1.000
Seborrhoeic Keratoses chest	88 (100.0)	Continuous variable	.500	(.188-1.332)	.166	Continuous variable	.286	(.059-1.375)	.118
	22 (25.0)/ 66 (75.0)	Present/Absent categories	.500	(.188-1.332)	.166	Present/Absent categories	.286	(.059-1.375)	.118
Seborrhoeic Keratoses abdomen	88 (100.0)	Continuous variable	.919	(.412-2.051)	.837	Continuous variable	.667	(.188-2.362)	.530
	25 (28.4)/ 63 (71.6)	Present/Absent categories	.683	(.266-1.756)	.429	Present/Absent categories	.500	(.125-1.999)	.327
Seborrhoeic Keratoses back	88 (100.0)	Continuous variable	.778	(.350-1.730)	.538	Continuous variable	.625	(.204-1.910)	.410
	34 (38.6)/ 54(61.4)	Present/Absent categories	.776	(.321-1.874)	.572	Present/Absent categories	.714	(.227-2.251)	.566
Seborrhoeic Keratoses lower limbs	88 (100.0)	Continuous variable	.841	(.373-1.896)	.676	Continuous variable	.857	(.288-2.550)	.782
	30 (34.1)/ 58 (65.9)	Present/Absent categories	.688	(.279-1.697)	.417	Present/Absent categories	.857	(.288-2.550)	.782
Seborrhoeic keratoses total body (sum)	88 (100.0)	Continuous variable	.884	(.680-1.150)	.358	Continuous variable	.717	(.461-1.117)	.141
	40 (45.5)/ 48 (54.5)	Low/high categories	.551	(.228-1.335)	.187	Low/high categories	.500	(.171-1.463)	.206

Overall, scores for seborrhoeic keratoses were low and found to be absent for many subjects on different regions of their bodies (see section 2.3.2 for recoding and appendix table 7.10 for raw scores). There were no significant associations between BCC and seborrhoeic keratoses. The overall summed score of total body seborrhoeic keratoses for proband-sibling pairs gave an odds ratio of 0.72 (95% CI 0.46 – 1.12) when assessed as a continuous variable, and 0.50 (95% CI 0.17 – 1.46) when divided into high and low categories; these results did not quite reach significance. In addition, odds ratios of association between seborrhoeic keratoses in the various body sites were less than 1.0, suggesting that presence of seborrhoeic keratoses may be associated with a reduced risk of BCC.

The decreased risk of BCC for presence of seborrhoeic keratoses on the chest approached significance for the case-control paired analysis, with an OR of 0.27 (95% CI 0.06- 1.38, p = 0.118).

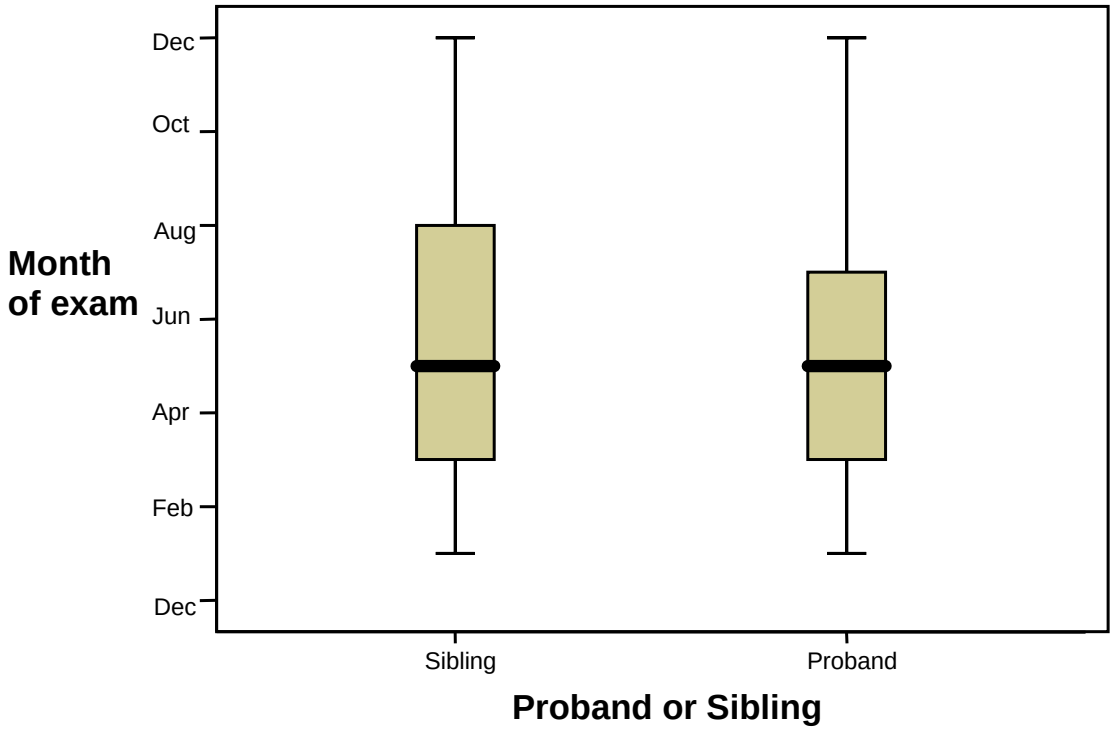
In summary, the data trend is for lower BCC risk with increased seborrhoeic keratoses. Little literature exists on the risks associated with these benign lesions (see section 1.4.6) although these trends contradict the suggested relationship of seborrhoiec keratoses to sun exposure.

4.3. Effect of date of examination on measurements

Sun exposure-related variables and skin pigmentary measurements are likely to be affected by the time of year that examination took place, due to variation in the amount of UVR exposure according to season. The dates of examination for all probands and siblings were compared (see figure 4.1 and 4.2). It is evident that the date of examination was not significantly different between the proband and control groups for both the unconditional and conditional logistic regression analyses and it is therefore unlikely that measurements were systematically biased by this variable.

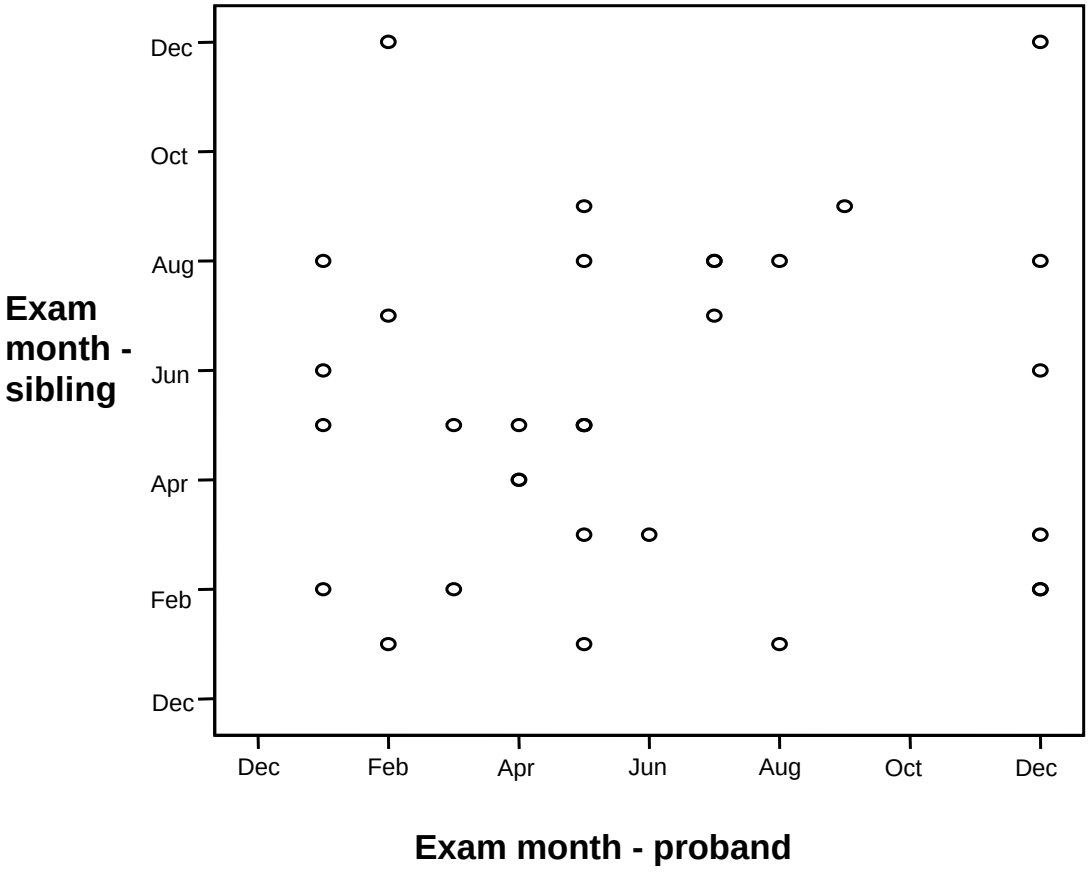
The number of subjects from the whole group of 88 probands and siblings is sufficient to allow analysis by month of examination (see figures 4.1 and appendix table 7.11). As seen in figure 4.1, there is no significant difference between the month of examination in the proband compared with the sibling groups (Fisher's Exact Test p= 0.860).

Figure 4.1 Proband and sibling groups' date of skin examination by month



Case-control pairs were also compared by month of skin examination in a scatterplot that shows no correlation between the two measures (see figure 4.2), meaning that there is no systematic difference between the two groups on this variable.

Figure 4.2 Date of skin examination by month for case-control pairs



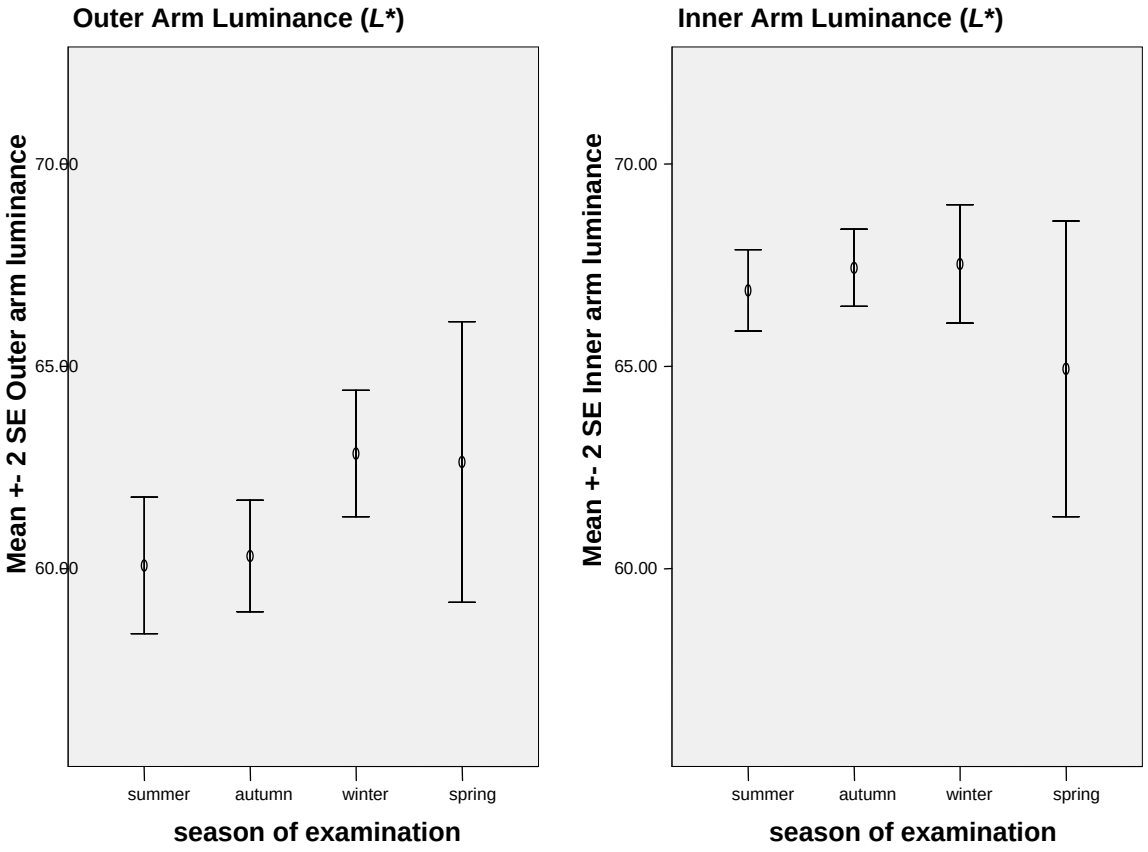
The 66 subjects in the case-control sample were best compared for significance by the season of examination, as there were too few subjects to allow enough power for a comparison by month (see appendix table 7.12). There was also no significant difference between the season of examination of the probands compared with their matched sibling pairs (McNemar Test p = 0.267).

Among all of the variables measured in the skin examinations, the '*L* a* b**' spectrophotometric measurements of the skin (see section 2.3.3) are the parameters most likely to have been affected by the date of examination. Other sun exposure variables measured reflect the cumulative effects of sun exposure as well as acute effects, while pigment of skin in general, and the outer aspect of the arm in particular, is affected more acutely by changes in melanin density and distribution due to recent UVR exposure. Outer arm measures of luminance (*L*) were smaller than on the inner arm, reflecting the constitutive lightness of pigment overall on the less exposed skin. Outer arm red-green reflectance (*a*) and yellow-blue reflectance (*b*) was greater than inner arm, reflecting greater

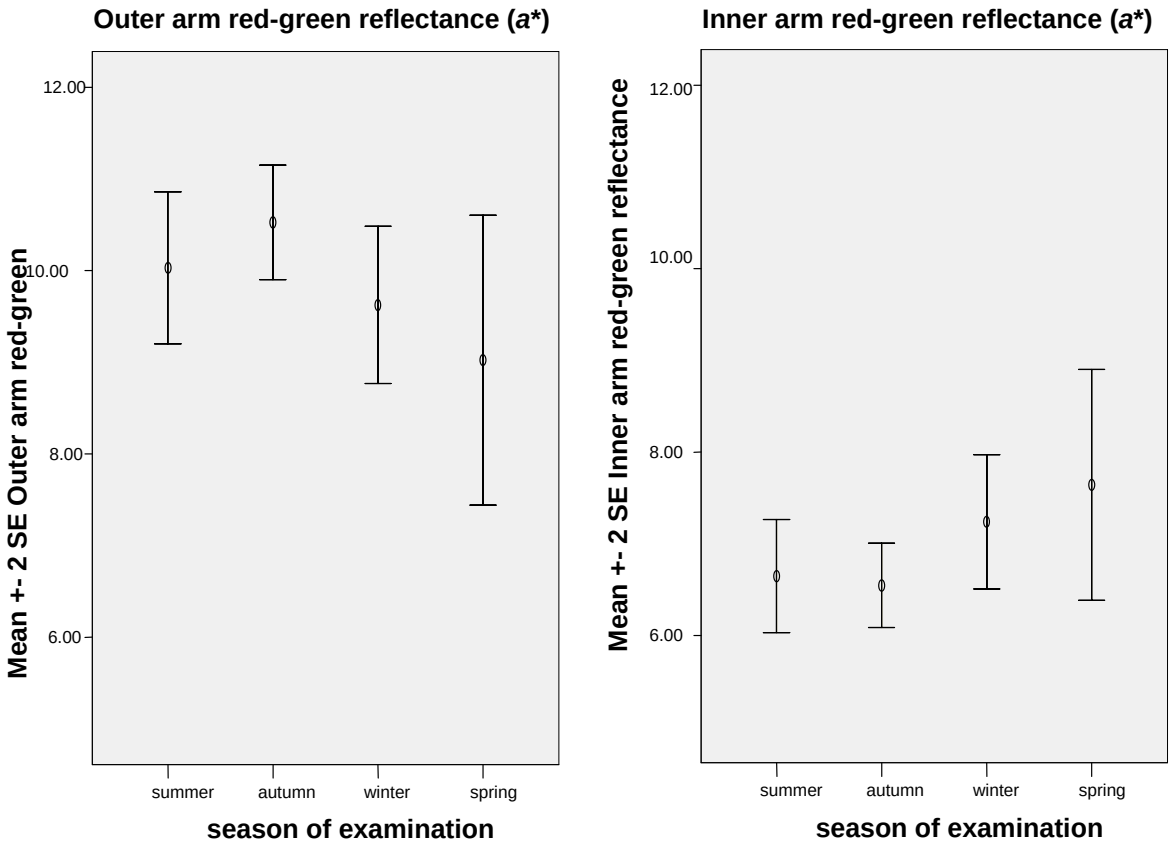
amounts of pigment on the exposed area compared with the less exposed area. *L* can be seen as a measure of whiteness of the skin and an overall average increase in this measure was seen among all subjects on the outer arm in winter as compared with summer, when UVR exposure is assumed to be reduced. Inner arm (constitutive) *L* varied slightly also, but predictably not as much as the outer arm measure. Overall, outer arm *a* in subjects showed an increase over the warmest season, with inner arm *a* decreasing slightly over this same period. Outer and inner arm *b* both increased in the warmest months (see figure 4.3). In figure 4.3, the variance of results for spring are larger than results for the other seasons due to the smaller number of subjects examined in this season (n = 4 examined in spring).

Figure 4.3 Outer and inner arm luminance (A), red-green reflectance (B) and yellow-blue reflectance (C) as measured by spectrophotometry, by season of examination

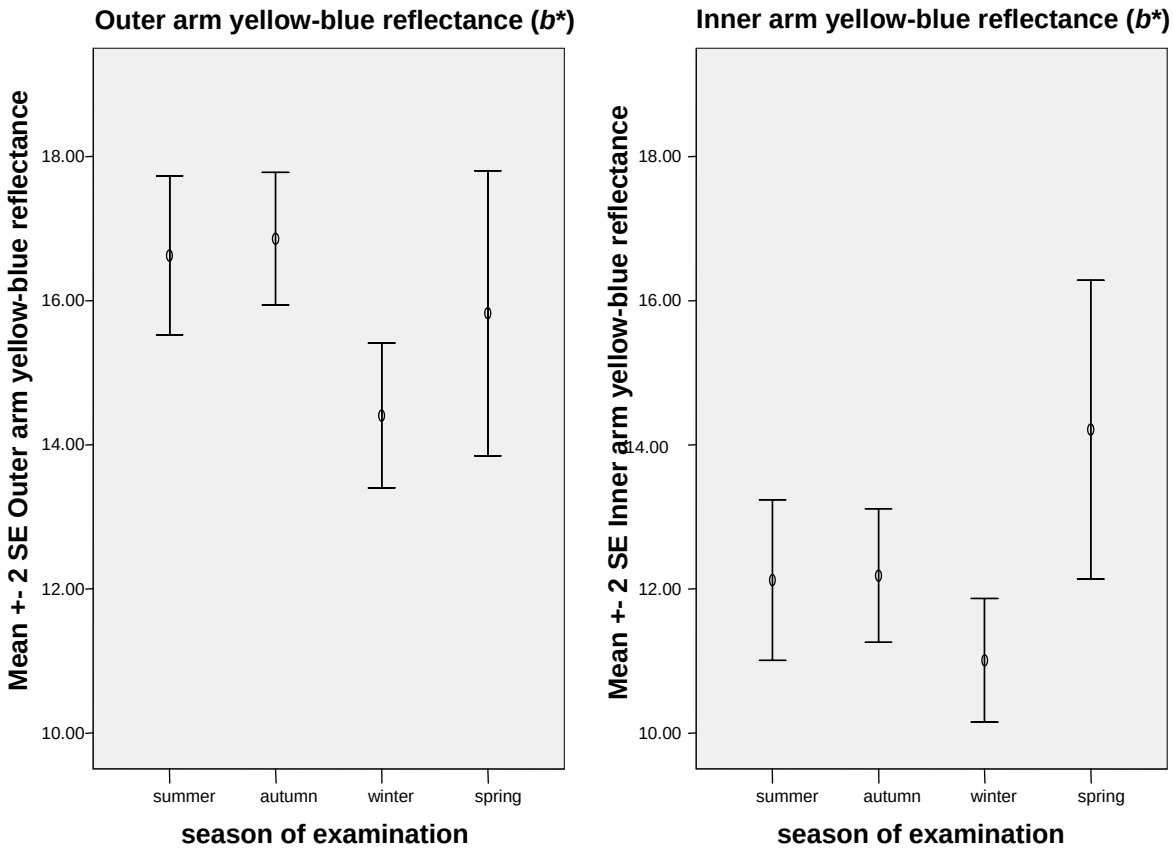
A.



B.


Outer arm red-green reflectance (a*)
Mean +- 2 SE Outer arm red-green
12.00
10.00
8.00
6.00
summer
autumn
winter
spring
season of examination
Inner arm red-green reflectance (a*)
Mean +- 2 SE Inner arm red-green reflectance
12.00
10.00
8.00
6.00
summer
autumn
winter
spring
season of examination

C.



4.4. Discussion

Probands and their unaffected siblings were examined for phenotypic differences in skin, eye, and hair pigmentation; as well as differences in sensitivity to the sun and signs of the amount of previous sun exposure. Of the pigmentary variables, only constitutive red-green reflectance was significantly associated with BCC risk, and this occurred in the direction opposite to that expected, in that red-green reflectance of the skin associated with a decreased risk of early-onset BCC. Of the sun sensitivity factors, decreasing skin phototype was significantly associated with an increasing risk of BCC. A marker of cumulative sun exposure - presence of actinic keratoses - was associated with significantly increased risk of BCC. Signs reflecting the combination of pigmentary characteristics

and sun exposure - in the form of arm freckling and solar lentigines - also gave subjects a significantly increased risk of this tumour.

There were no significant differences detected in height or weight of the two groups, and no systematic differences in the date of examination that may have skewed results that depend on effects of recent ambient UVR. The tendency towards older age in the sibling group is not surprising given that the oldest unaffected sibling was selected where available. Since the age difference was only 1.8 years on average, the difference did not reach significance. An older sibling was chosen as the control to ensure that the sibling would have attained at least the same age as the probands, as age is such a strong predictor of skin cancer. This ensured that the sibling pair was as discordant as possible with respect to skin cancer phenotype.

This group of subjects was fairly 'sun sensitive' in that most (96.6%) reported some burning in response to their first exposure to summer sun, and very few (6.8%) reported an ability to build up a 'deep tan'. In addition, nearly a fifth of the subjects overall (18.2%) reported a total inability to tan. There was a trend for an association of the propensity to burn with risk of BCC, with non-significant odds ratios reaching the moderate to high range (highest OR for affected-unaffected pairs 7.0) for people with a high propensity to burn. It is likely that with a larger sample size a significant association of BCC risk with propensity to burn may be seen. The ability to tan displayed a relationship with BCC risk that could be interpreted as due to a complex interplay of pigmentary factors and sun exposure behaviours. Despite no significant associations, the risk of BCC showed a tendency toward an association with moderate tanning ability and also with the total inability to tan; the risk was still positive in those with a mild ability to tan but lower than for the other two categories. The literature shows that ability to tan interacts with exposure so that if a person tans well, there is a linearly increased risk with intermittent exposure; whereas for those that tan poorly, risk plateaus with increasing intermittent exposure [81, 103]. Measurement of patterns and amount of sun exposure by interview would be necessary to help disentangle these effects but were beyond the scope of the present study.

It is unsurprising that there were no probands or siblings of the darker skinned skin phototypes IV, V or VI. Most people self categorised themselves as skin phototype II (usually burn/sometimes tan) or III (sometimes burn/usually tan). For the more sensitive measure of analysis by case-control pairs, a significant relationship was seen for risk of BCC with decreasing phototype, with an overall OR of 3.6. When broken down to assess the contributors to the risk in this figure, phototype II was

associated with a more than 4-fold risk, and phototype I a nearly 10-fold risk compared with people of phototype III. When looked at separately, these categories did not quite reach significance although given the significance of the relationship when looked at together, this relationship is likely to hold up with greater sample numbers. There is extensive evidence in the literature that skin that burns easily and tans poorly (ie skin of lower phototype) is at increased skin cancer risk, and this finding is replicated in the present study [42, 102, 117].

Actinic keratoses can be viewed as a surrogate measure of cumulative exposure and are premalignant lesions. Between 1:100 and 1:1000 will develop into an SCC and for this reason they are always treated or removed. Half of the subjects had at least one of these premalignant lesions, and there was a significant association of the presence of any actinic keratosis with a 3-fold increased risk of BCC, reflecting previous findings [78]. Solar elastosis was so common among the subjects that there was little power to detect an association: more specific measures of this variable, if developed, may be of more value. Presence of a pterygium was also common among the subjects with nearly two-thirds of them displaying this surrogate marker cumulative of sun exposure. Paradoxically, the presence of a pterygium was associated with a decreased risk for BCC, although the effect was small and not significant.

Both childhood and adult freckling have been associated with BCC and SCC risk and this effect is replicated here [78]. Pigmentary characteristics and sun exposure combine to cause freckling (ephelides) and solar lentigines (larger freckles that don't disappear in winter). All subjects had evidence of some of these lesions. Most subjects (91.9%) reported having freckles in childhood. Although 17% of subjects reported a lack of freckles in adulthood (on the face), most had some freckles present at the time of examination. Risk of BCC was moderately increased for people with increased numbers of both freckles and solar lentigines on their arms. The arms are a commonly sun exposed area and accessible to the clinician as a quick and easy addition to the assessment of a person's risk of skin cancer. For these lesions elsewhere on the body risks of BCC were mostly also elevated, with the highest risks (although still in the low range of OR 2.0 or less) seen in the most sensitive case-control analysis analysis ie people with a low versus a high number of the lesion. These trends of increased risk of BCC with freckling and solar lentigines on other parts of the body require confirmation in a larger study of early-onset BCC.

More than half the subjects had no evidence of seborrhoeic keratoses on each body site examined. These poorly-studied benign lesions are also known as 'senile warts' and have been associated with

sun exposure, but not with skin cancer to date (see section 1.4). No significant associations with BCC risk were found, and there was a consistent and paradoxical reduced risk of BCC with presence of seborrhoeic keratoses: risk was halved with higher overall body numbers of these lesions. Similarly, lesions on the chest, abdomen, head and neck, back and lower limbs were all associated with small but not significantly decreased risks. The arms were the only body site where risk of BCC was not reduced, although it showed no evidence of giving an increased risk. Numbers of seborrhoeic keratoses found in this study were far lower than those seen in a comparable Australian population [169] (see section 2.3.1). Fainter lesions that would be assessed as seborrhoeic keratoses by an experienced examiner may have been missed in the proband and sibling examinations. If undercounting of these lesions occurred, it is likely that they would have occurred across all body areas and for all subjects examined, and therefore does not account for the paradoxical reduced risk of BCC seen. The possibility that any genetic predisposition leading to BCCs in these subjects also suppresses the development of seborrhoeic keratoses needs further exploration. The trends of decreased risk of BCC with these lesions require confirmation in a larger study of early-onset BCC.

Hair colour and eye colour reflected the general fairness of skin pigmentation with few subjects reporting dark hair or brown eyes. It was evident by the two measures employed to estimate constitutive hair pigmentation that reporting by naming of hair colour is subjective and probably unreliable. Self-reported hair colour by naming differed from self-report by identification of hair swatch, with the term ‘light or mouse brown’ associated with the most variation in selection of swatch; more of these individuals selected a fair/blonde hair swatch than a brown one. Neither eye colour nor hair colour emerged as convincing predictors of BCC risk: green eye colour was associated with a small non-significantly decreased risk and blue with a small non-significantly increased risk compare with darker eyes. Thus there was no clear trend of an effect with for this variable on the risk of BCC. Similarly, hair colour associations were inconsistent although mostly tended towards increased risk of BCC with fairer hair. The most reliable measure to detect a difference in risk with hair colour - if one was indeed present - was from the hair swatch selection by case-control pairs. No effect of hair colour was seen in this study. This is not a surprising finding given the variability in predictive value of these traits in the literature [47, 112]. They may act as surrogate markers for pigmentary characteristics or sun sensitivity, but seem to be relatively unreliable ones.

Overall, these early-onset BCC probands and their siblings were relatively fair-skinned, with 93.2% describing themselves as fair- or very fair-skinned, and the remainder olive/brown. Significant differences in skin pigmentation were detected between the two groups as measured by spectrophotometer, suggesting that constitutive red-green reflectance of the skin decreases the risk of BCC (OR 0.6). Constitutive luminance was associated with a slightly (but non-significantly) increased risk of BCC, however much of the variance of this measure was explained by the measures of yellow-blue reflectance and red-green reflectance. Constitutive yellow-blue reflectance tended towards a decreased risk of BCC but was also not significant. Red-green reflectance was the only independent predictor of BCC risk.

The red-yellow pigment phaeomelanin prevalent in lighter skinned persons probably causes decreased skin photoprotectivity [251, 264] compared with the darker, more photoprotective eumelanin [262]. Although lighter, easy-burning skin is likely to have more phaeomelanin and less eumelanin than darker skin, the exact relationship between skin phototype and pheomelanin:eumelanin production in the epidermis is not straightforward or directly predictable [260, 278]. MC1R variants are thought to contribute to individual differences in pigmentation, and in particular, to eumelanogenesis and pheomelanogenesis; MC1R variants also contribute to risk of BCC, independently of their effect on pigmentation [283]. Individuals with the same MC1R variants may display differences in hair pigmentation; for example, auburn or strawberry blonde [266]. Thus other loci are likely to be involved in the creation of pigmentary characteristics. It has been hypothesised that MC1R variants may be involved in the switching between eumalanogenesis and pheomelanogenesis [279].

Skin colour has shown less association with risk of BCC in the literature than sun sensitivity has; however the lack of access to or use of objective measurement techniques for skin colour may have contributed to this. Skin colour is not only dependent on melanin pigmentation, but is also influenced by the presence of superficial capillaries (increases redness), collagen content (increases yellowness), body hair, thickness, and moisture [397]. The BYK Gardner spectrophotometry readings are not calibrated to melanin content of the skin or a suitable surrogate such as MC1R variant status, and this analysis is urgently necessary to allow better interpretation of spectrophotometric measures. Nevertheless, it is evident from the changes in pigmentation on the outer arm seen over the seasons (see figure 4.3) that yellow-blue reflectance and red-green reflectance are greater on the outer than the inner arm, suggesting a relationship with sun exposure

that is likely to be related to melanin in the skin. Yellow-blue reflectance and red-green reflectance also increase slightly in the hotter months (summer) compared with the cooler months (winter), suggesting that these two variables do increase concomitantly with the tanning response; of the possible contributors to skin colour, melanin pigmentation is the only one that is likely to change skin colour acutely in this way. Constitutive skin colour also changes with the season although not as markedly as the outer arm, with a small increase of yellow-blue reflectance and decrease of red-green reflectance with summer as compared with winter.

Constitutive red-green reflectance was associated with a significantly decreased risk of BCC in our subjects. It was seen in these data that outer arm *a** (red-green) values increased in the summer, so it is a reasonable inference that high *a** values are positively correlated with skin melanisation. The simplest interpretation of these data is that high constitutive pigmentation, as seen in the inner arm measurements, is associated with reduced risk of early-onset BCC. However further studies are required to establish the relationship of the *L*a*b** parameters with biological parameters such as skin melanin content and changes due to tanning.

We used the BYK Gardner spectrophotometer to measure skin pigmentation (see section 2.3.3), whereas the few other studies that have used full-spectrum spectrophotometry for objective measurement of skin colour have used other insturments such as the Minolta 508 spectrophotometer, and recorded the measurements differently. Using the Minolta, one group found that risk of BCC in men and women had a small positive association with spectrophotometric readings [398]. These two instruments use the same wavelengths of incident light to measure reflected light which may be recorded in different ways: using the *L*a*b** system or through a measure at each wavelength in 20nm increments across all or part of the spectrum measured (400nm to 700nm). Both machines were tested on the upper arm of three human subjects and the *L*a*b** readings were found to be almost identical (see appendix 4.23). Researchers using the Minolta have chosen to use the difference between readings at 400nm and 420nm for measurements of skin pigmentation; some of the information on the spectrum of reflected light is lost by the use of only two wavelengths of light. We have chosen to use the *L*a*b** measure as it may capture more information about the reflected light across wavelengths of relevance to the perception of the human eye, and therefore probably provides more information about the skin's colour than a difference between two wavelengths. This difference in recording affects the ability to compare the results of research using both instruments. A study has shown that presence of hair may overestimate pigmentation readings, although this was

seen using a different measure of reflected light, and on a different part of the body: it is difficult to know how this would apply to our results [399]. It may be worthwhile to test this on the outer arm in future to determine whether subjects should be shaved at the test site.

MC1R variant analysis was not carried out in this project and will be essential to elucidate the relationships between the *L*a*b* reflectance measures and risk. A first step would be analysis of associations between the MC1R red hair-associated (RHC) variants and *L*a*b** measures in a large cohort of controls, examined at different times of the year, in both sun-exposed and non-sun-exposed sites.

Together these results suggest that skin colour, sun sensitivity, and sun exposure are associated with an increased risk of BCC.

Chapter 5: Analysis of *patched* for germline mutations in clusters of BCC cases

5.1. Introduction

BCC, SCC and MM all showed significant increases among the first-degree relatives of cases of early-onset BCC, as shown in chapter three. Sun sensitivity, skin pigmentary characteristics, and evidence of sun exposure all showed associations with BCC when the cases were compared with unaffected sibling controls. These data are most consistent with the increase in BCC risk to these probands being largely due to causes that increase the risk of all forms of skin cancer.

Nevertheless, some striking family clusters of only BCC, to the exclusion of SCC and MM, were observed. The hypothesis therefore remained that at least some proportion of the observed clusters of BCC cases, or at least some part of their aggregation, was due to a genetic factor or factors specific to BCC risk.

Inactivating germline mutations of *PTCH* cause Gorlin Syndrome, a rare and usually familial genodermatosis comprising early-onset BCC and developmental abnormalities (section 1.5.4). Mutation of this gene is associated with a 900-fold increased risk of BCC in Australia. Recently, a common single nucleotide polymorphism (SNP) in *PTCH* exon 23 (Pro>Leu) has been associated with degree of skin pigmentation [235], and combinations of polymorphisms (ie certain haplotypes) in this gene have been associated with rate of BCC accrual [233].

The melanocortin-1 receptor (MC1R) is involved in individual variation in the type of melanin synthesized and the way it is distributed in the skin, and variations in this receptor contribute to individual differences in pigmentary characteristics. MC1R variants are very common in light skinned populations including Australians of European origin, and red hair-associated variant alleles of this gene have been associated with a two- to four-fold risk of BCC [267, 283]. No other germline mutations have been consistently associated with BCC risk, although p53(Arg72) has been linked with NMSC risk. This chapter addresses the question of whether mutations in *PTCH* are contributing significantly to the higher risk of BCC in these individuals. Probands from 56 families with early-onset BCC were included for genetic analysis. In addition, two probands from a related cohort of early-onset SCC cases was analysed; these had three or more BCC-affected first degree family members (see section 2.6 and table 2.3).

DNA from peripheral blood from all 58 probands was screened for mutations of the *PTCH* gene using PCR, dHPLC and direct sequencing. Twenty of the 23 exons of the *PTCH* gene were amplified by PCR and screened in this way. Two dHPLC temperatures were used to screen the subjects with the highest probability of carrying a *PTCH* mutation, for the eight exons that most commonly bear *PTCH* mutations in Gorlin syndrome (see section 2.6). The remaining subjects were screened at a single dHPLC temperature for all 20 exons.

No mutations were found in the exons screened for these 58 subjects with personal or family history of high risk BCC. The SNPs observed were present at frequencies similar to those in the literature. Where population frequency data for a SNPs were lacking, they were determined in a comparable Australian cohort without BCC; the frequencies for these SNPs were also similar to the probands'. Therefore no evidence was found to implicate either *PTCH* inactivating mutations or SNPs in risk of BCC in these early-onset cases.

5.2. Results

5.2.1. dHPLC and sequencing analysis of the PTCH gene

Twenty of the 23 *PTCH* exons were screened for mutations by dHPLC as described in Section 2.5.1 and 2.6. These covered 97% of the coding sequence of *PTCH*, in which also 97% of all Gorlin syndrome-associated mutations have been found. The equipment and protocols used were identical to those used by a collaborating reference laboratory that has been screening Gorlin syndrome subjects for germline *PTCH* mutations by this method for several years. In six of the eight exons (2, 3, 14, 15, 17, 18) that most commonly bear *PTCH* mutations in Gorlin syndrome two dHPLC temperatures were used to screen the 19 subjects with the highest probability of carrying a patched mutation. A single temperature was used for the other 14 exons and for screening the other 39 subjects. All products were run with sequence proven wild-type controls and any test sample that gave a profile different from the control was submitted to bi-directional DNA sequencing. One variant-negative test sample at random from each exon analysed was sequenced in the forward direction to confirm presence of the wild type sequence.

Exons 3, 7, 9, 13, 16, 17, 18, 20, 21, 22, and 23b did not show any variant elution profiles in any of the 58 subjects screened. In the other nine exons (2, 5, 6, 8, 11, 12, 14, 15 and 23a), dHPLC variants were seen in some of the samples. All these samples were confirmed to be heterozygous for

previously described single nucleotide polymorphisms (SNPs) as shown in table 5.1. dHPLC and sequence appearances of each SNP may be seen in appendix 7.9 and 7.10.

Table 5.1 PTCH SNPs observed and comparison of relative frequencies with database population data

Exon	**SNP[a]**	**Amino acid change**	**Proband heterozygote relative frequency (n=58)**	**Population heterozygote relative frequency**	**Source of population data[c]**
2	116C>T	Leu>Leu	0.034	No data	n/a
5	80A>G	Thr>Thr	0.103	No data	n/a
6	IVS6-55T>C[b]	N/A	0.450	0.267	HapMap project: USA European descent n=30
				0.500	CCR[d]: USA European descent n=24
				0.125	CCR: USA Han Chinese n=24
				0.261	CCR: USA African American n=23
8	IVS8+23G>A	N/A	0.017	0.029	NIHPDR[e]: USA mixed descent n=450
11	IVS11-50G>C[b]	N/A	0.431	0.468	CCR: USA Caucasians n=95
				0.250	CCR: USA European descent n=24
				0.267	NIHPDR: USA mixed descent n=450
				0.167	CCR: USA Han Chinese n=24
				0.087	CCR: USA African American n=23
12	63T>C[b]	Asn>Asn	0.224	0.167	CCR: USA European descent n=24
				0.250	CCR: USA Han Chinese n=24
				0.304	CCR: USA African American n=23
	84C>T	Ala>Ala	0.362	0.458	CCR: USA European descent n=24

				0.100	NIHPDR: USA mixed descent n=450
				0.167	CCR: USA Han Chinese n=24
				0.087	CCR: USA African American n=23
14	351G>A	Ser>Ser	0.017	No data	n/a
15	IVS15+9G>C[b]	N/A	0.483	0.500	CCR: USA European descent n=24
				0.375	NIHPDR: USA mixed descent n=450
				0.458	CCR: USA Han Chinese n=24
				0.565	CCR: USA African American n=23
23a	140C>T	Pro>Leu	0.552	0.214 - 0.436 (two reports on same population)	WICVAR[f]: USA mixed descent

[a]All SNPs are referred to in the forward direction
[b]Refers to forward sequence; SNP database refers to complement reverse sequence
[c]Using *ensembl human geneview* (www.ensembl.org) and *CHIP bioinformatics* (http://snpper.chip.org) SNP databases
[d]CCR= Coriell Cell Repository
[e]NIHPDR= National Institute of Health Polymorphism Discovery Resource
[f]WICVAR is a population submitted by the laboratory of the Centre for Genome Resarch at the Whitehead Institute, Cambridge MA

In summary, the only variants observed had been previously described in various populations of normals and can be classified as single nucleotide polymorphisms (SNPs). None of the variants had known associations with Gorlin syndrome or any other disease.

Each of the SNPs will be described in turn and their frequencies in the test probands compared with available data from the *Ensembl Human Geneview* (www.ensembl.org) and *Children's Hospital Informatics Program (CHIP) bioinformatics* (http://snpper.chip.org) SNP databases. Table 5.1 shows all population data available to date from these two databases and compares these with the dHPLC variant frequency observed in the test cohort. In brief, for the six of these variants where published data was available, dHPLC-detected SNP heterozygotes were seen at frequencies similar to those expected from the population frequencies observed in samples of similar ethnic background.

In the exon 2 assay, a C>T SNP was observed at position 116, synonymous Leu>Leu at the amino acid level. The heterozygote relative frequency in the early-onset BCC probands was 2/58 (0.034) and no population data were available for comparison.

In the exon 5 assay, a A>G SNP was observed at position 80, synonymous Thr>Thr at the amino acid level. The heterozygote relative frequency in the early-onset BCC probands was 6/58 (0.103) and no population data were available for comparison.

In the exon 6 assay, a SNP was observed at intron 6, position -55 (IVS6-55 T>C) in 26/58 probands (frequency of heterozygosity 0.45). A similar frequency of this SNP (12/24, 0.50) in healthy adults was seen among North Americans of European descent sampled in the Coriell Cell Repository. Population heterozygosity varies between samples of assumed similar genetic background, as the frequency of heterozygosity among another group of European descent was 0.267 (see table 5.1). Frequencies of this SNP in Chinese and African American groups have been lower than in the European samples.

In the exon 8 assay, a G>A SNP was observed at intron 8, position 23 (IVS8+23 G>A), in 1/58 probands (frequency of heterozygosity 0.017). This frequency was similar to that found in a population from North America of mixed descent (0.029).

In the exon 11 assay, a G to C base substitution was found at intron 11, position -50 (IVS11-50 G>C) in 25/58 probands (frequency of heterozygosity 0.431). The frequency of this SNP varied widely in other populations, ranging from 0.087 to 0.468. The frequency found in a North American population sample of 95 ‘Caucasians’ was 0.468, which differed little from our proband group who are mostly also of European descent.

Two SNPs were seen in the exon 12 assays. A base substitution of T to C occurred at position 63 (63 T>C) in 13/58 probands (frequency 0.224) and a C to T substitution occurred at position 84 (84 C>T) in 21/58 probands (frequency 0.362). Neither of these SNPs changes the amino acid sequence. Five probands harboured both variants (see appendix 7.10 for sequence). SNP 63 T>C occurred at similar heterozygote frequencies of between 0.167 and 0.304 in mixed populations from the USA, with the most similar frequency seen in a group of 24 Han Chinese from North America. The SNP 84 C>T was seen in varying rates of between 0.087 and 0.458 in populations of differing genetic backgrounds from the US. The most similar frequency of heterozygosity to that seen in our group of probands was seen in 24 North Americans of European descent.

In the exon 14 assay, a G>A SNP was observed at position 351, synonymous Ser>Ser at the amino acid level. The relative frequency of heterozygosity in the early-onset BCC probands was 1/58 (0.017) and no population data were available for comparison.

In the exon 15 assays a G>C SNP was commonly seen at intron 15, position 9 (IVS15+9 G>C), at a rate of 0.483. A similar frequency of heterozygosity was evident among populations previously studied (ranging from 0.375 to 0.565) from varied ethnic backgrounds.

In exon 23a, a SNP was observed at position 140 (140 C>T) occurred commonly, with a base substitution of C for T in 32/58 probands (frequency of heterozygosity 0.552). This SNP alters proline to leucine at amino acid position 1315 of the protein. It has been reported to occur at a similar frequency in North American populations of mixed racial background, albeit at two different heterozygosity frequencies, 0.214 and 0.436.

In summary, none of the SNPs observed in these exons, for which published population data existed, showed frequencies in this cohort that deviated from published values. Where there was no relevant data from other populations, control data was obtained from an Australian sample.

5.2.2. Comparison with Australian population SNP frequency

For the three exonic, synonymous SNPs observed in exons 2, 5, and 14, there was no population data available with which to compare the frequencies seen in the probands. In order to seek preliminary evidence as to whether these SNPs were associated with early-onset BCC, a 'convenience' control group was sought. The set of control siblings would only have been suitable for this purpose in a much larger study, given the low frequencies observed in the probands.

Age- and sex-matched controls (n=110) were selected from subjects in an Australian population-based study of melanoma [410]. They were relatives of the probands (who were cases of early-onset melanoma in Sydney, Melbourne or Brisbane). The protocol of that study does not include collection of data from relatives as to previous diagnoses of NMSC, so their status with respect to BCC is unknown. However they are known to have 91% Anglo-Celtic ethnicity, a frequency similar to that expected in this cohort of BCC-affected probands.

One hundred and ten control samples were screened for the exon 2, 5 and 14 SNPs and the results are shown in table 5.2. In addition, data are shown for the exon 15 SNP, which was screened (in an earlier, preliminary project) in 30 controls from this group age- and sex-matched to the 19 initial

probands that had been screened at two dHPLC temperatures for exon 15. Published data existed for this SNP, but this comparison was to rule out any possible large differences in frequency of *PTCH* SNPs between these Australian controls and other populations previously tested.

Table 5.2 PTCH SNP frequency data comparison with Australian population sample

Exon	**SNP**	**Amino acid change**	**Relative frequency early-onset BCC probands**	**Relative frequency Australian population sample**	**p value: Fisher's exact test (2-sided)**
2	116C>T	Leu/Leu	0.034 (2/58)	0.028 (3/105)	**1.000**
5	80A>G	Thr/Thr	0.103 (6/58)	0.047 (5/106)	**0.197**
14	351G>A	Ser/Ser	0.017 (1/58)	0.020 (2/102)	**1.000**
15	IVS15+9 G>C	N/A	0.483 (28/58)	0.519 (14/27)*	**0.818**

*population frequency range 0.375 – 0.565 (see table 5.1)

None of these SNPs were novel, but no data on their prevalence in the population was available. The purpose of the comparison of heterozygote frequencies in the BCC probands and a comparable, though not strictly controlled, Australian population sample was to rule out the possibility that these were otherwise rare SNPs with a potential association with BCC. This would have remained a possibility if they had not been observed in the 'control' sample, however all three were observed at similar frequencies in both samples (see table 5.2).

The exon 2 116C>T SNP was observed with a relative frequency of 3.4% in the Australian controls (p=1.000, Fisher's Exact Test for comparison with BCC proband sample). The exon 5 80A>G substitution (A>G 80) was observed with a relative frequency of 4.7% of the Australian controls (p= 0.197, Fisher's Exact Test). The exon 14 351G>A SNP was observed in 1.9% of controls (p=1.000, Fisher's Exact Test). For the exon 15, IVS15+9G>C SNP, its frequency in the Australian population sample (51.9%), was similar to that in the BCC proband group (p=0.818, Fisher's Exact Test), as well as within the range of published values (population frequency range 0.375 – 0.565, table 5.1).

5.3. Limits on PTCH mutation frequency in early-onset BCC probands

Given that no mutations were found in this group of 56 early-onset probands, what can we infer about the general population from which this sample was drawn?

We assume a Poisson distribution because *PTCH* mutation is considered to be a rare event and calculate the upper 95% confidence limit:

$$P(X) = \frac{e^{-\lambda}\lambda^{x}}{X!}$$

Where P = 95%, X = 0, λ= rate of mutation

$$0.95 = \frac{e^{-\lambda}\lambda^{0}}{0!}$$

$$0.95 = e^{-\lambda}$$

$$-\lambda = \ln(0.95)$$

$$\lambda = - 0.051$$

The upper 95% confidence rate of the mutation frequency is 5.1%, meaning there is a 97.5% chance that *PTCH* mutations occur in fewer than 5.1% of Australians with early-onset BCC.

5.4. Discussion

Nine of the 20 exons screened were found to harbour single nucleotide polymorphisms (SNPs), seen on dHPLC as variant melting curves and confirmed on direct sequencing. In eight exons, only one SNP was seen, and in one, two SNPs were apparent. All SNPs produced non-coding variants except for one, the T>C 140 SNP on exon 23a causing a proline to leucine change, seen in 46% of the subjects screened. This SNP was seen in a similar frequency (43.6%) in a SNP database (CHIP) comprised of persons of European background who were likely to be of similar racial mix to this sample. Five more of the 9 exons harbouring SNPs had population SNP data for comparison, and in each, the proband SNP frequency was similar to the population SNP frequency. For the remaining three exons harbouring SNPs there were no population SNP data available. For these three exons, a control group was chosen from the Australian population for comparison of these exons in the *PTCH* gene. The frequency of SNPs in this group for each of the three exons lacking comparable population SNP frequency data was not significantly different to that found in the proband group

(see table 5.2). A subset of these controls was compared with the SNP frequency of one of the exons for which population SNP data was available (exon 15). This confirmed that Australian SNP frequency for *PTCH* is likely to be similar to that from international data on European-derived populations.

The familial aggregation of BCC in nearly a fifth of all the families screened is suggestive of genetic susceptibility as a contributing factor to its cause. The tumour suppressor gene *PTCH* is a candidate as a contributor to genetic susceptibility. Gorlin syndrome is caused by the autosomal dominant inheritance of a gene that leads to marked susceptibility to BCC and a range of developmental defects. *PTCH* is the cell growth regulatory gene responsible for this disorder, and mice with knockouts of one copy of *PTCH* develop BCCs when exposed to UVR [4]. In addition, *PTCH* mutations are found very commonly within sporadic tumours suggesting a possible necessary involvement of this pathway in tumours outside of Gorlin syndrome. Importantly, it appears that no studies have previously looked at possible germline mutations of this gene in non-Gorlin families with susceptibility to BCC. Thus it is obligatory that this gene be examined when investigating possible genetic causes of familial aggregation of this tumour.

Twenty *PTCH* exons (exons 2, 3, 5 to 18, and 20 to 23) were screened for mutations in this study. Together these exons account for 4196 coding base pairs (97% of the total of the 4345 coding base pairs) and for 97% (199/206) of the published mutations in *PTCH*. The only exon with published mutations not to be screened was exon 19, and this was excluded due to technical problems with amplification of the exon by PCR. The primers used amplified the full coding segment and the splice junctions next to each exon. dHPLC screening found variants in the introns adjacent to exons 6, 8, 11 and 15, which confirms that those assays were effective.

dHPLC identifies DNA variants mainly through detection of the altered affinity and mobility of any heteroduplexes formed between mismatched nucleotides in double stranded DNA during the reannealing phase at the end of PCR. The sensitivity of dHPLC to detect these heteroduplexes in amplicons sized 100 to 732 base pairs is estimated to be between 92-100% [400-402] and is increased by performing dHPLC at multiple temperatures. [402]. Thus for single temperature screening, the sensitivity is 92% at worst. Generally, dHPLC is a reliable technique to screen for single nucleotide substitutions, deletions and insertions [403].

Screening of the *PTCH* gene was nearly complete in these patients. 97% of the coding regions were screened, and although this accounts for 97% of the Gorlin syndrome-associated mutations as reported in the literature, there is still a small part of the gene left that could harbour DNA variants. It is also possible that *PTCH* mutations causing non-Gorlin familial BCC might have a different mutation spectrum to those which cause Gorlin syndrome. In the interests of efficiency, those probands of highest risk were screened more thoroughly by the use of two dHPLC temperatures for the subjects with the highest probability of carrying a mutation (ie those with greatest familial aggregation). Taking into account the sensitivity of dHPLC and the coverage of the assays performed, the completeness of *PTCH* gene screening in this study is at least 95% (0.92 x 0.97) for known Gorlin mutations and 95% (0.92 x 0.97) for the coding sequence of PTCH. Therefore, based on the findings of this study it can not be completely excluded that some probands have pathogenic DNA variants in the *PTCH* gene, although given the completeness of the screen it is unlikely.

Given that no mutations were found in any of the exons screened in any subject, no evidence was found to implicate either *PTCH* inactivating mutations or SNPs in risk of BCC in these early-onset cases. Combinations of polymorphisms (haplotypes) have been associated in the literature with particular phenotypes such as skin pigmentation and rate of accrual of tumours. Even silent substitution of nucleotides (effecting no amino acid change) could influence mRNA processes, including affecting splicing accuracy or efficiency [230] eg through effect on enhancer regions or a combination effect on protein function. They could also act as markers for other significant gene mutations/variants through linkage disequilibrium. The SNPs found here are classified as polymorphisms based on their frequency in controls, not on their possible functional significances. Given that the frequencies are similar in our subjects compared with controls, it is unlikely that these SNPs have any functional significance, and there is no evidence at present that they are associated with familial BCC. The potential role of uncommon SNPs as low penetrance alleles for BCC should be specifically tested in larger case-control studies.

Assuming a Poisson distribution, and having observed no mutations in a sample of 56, we can be 97.5% confident that if there are any *PTCH* mutations contributing to early-onset BCC in the Australian population, then their prevalence is less than 5.1%. Thus high penetrance germline mutations in this gene may still be responsible for a small proportion of the increased risk seen in this family study. This suggests that screening *PTCH* will be unlikely to prove relevant to clinical geneticists, given that it is a large and expensive gene to test for mutations [404]. Larger family

studies may be able to determine if this gene does contribute at all to increased risk of BCC, and multiple large studies showing a lack of association will be needed to exclude *PTCH* as a contributory factor to BCC.

One proband (9125) was affected with Gorlin syndrome, although the clinical features of this family were atypical for Gorlin's syndrome: the proband had pathognomonic Gorlin syndrome features (multiple early-onset BCC, palmar pitting, characteristic facies and calcification of the falx cerebri), but there were no typically affected first degree relatives. Clinical records show that the proband's father had falx cerebri calcification, but no BCC. *PTCH* gene screening for this subject was also done in a clinical setting and this showed that no DNA variants were present in the *PTCH* gene of either individual. This confirms our finding (of no mutation in *PTCH* for this proband), and suggests a contribution to the disease of other genes in this family, most likely genes coding for proteins involved in the hedgehog signalling pathway (see section 1.5.2). To thoroughly exclude other members of the hedgehog pathway from involvement in population risk of BCC, other genes in this pathway should be screened for high risk families in the same way (eg *smoothened, Gli1, Gli2,* and *Gli3*).

Functional studies of the SNP variants found could be performed to determine whether or not they are truly benign polymorphisms, although this is very unlikely as no associations with BCC susceptibility were seen here. Since all of the SNPs seen here except one are intronic or synonymous variants, the protein sequence will not be affected. To rule out an effect on the function of the protein however, a bioinformatics analysis of the relevant sequence should be undertaken. The involved regions of the gene would be examined to determine whether they are in evolutionarily conserved domains indicating a possible important role in the function of RNA processing or trafficking, for example altering splice junctions to cause a false splice site. If the involved domains are highly conserved, a study of mRNA splicing should be undertaken in cell lines from carriers and non-carriers of a similar genetic background.

Examination of MC1R variant frequency is the next priority when considering genes that could be contributing to the BCC-specific aggregation occurring in this family study. This polymorphic gene codes for a receptor that is involved in individual differences in pigmentary characteristics such as skin, hair and eye colour. Studies have shown that variants of MC1R are also associated with BCC risk, and that this occurs independently of pigmentation phenotype (see section 1.7.6): it would be interesting to see the correlation between variants of this gene and phenotypes of patients in family

studies. As discussed in the previous chapter, correlation of MC1R genotype with skin colour parameters recorded by the BYK Gardner, or other full-spectrum reflectance, spectrophotometer will be necessary for their full interpretation (see sections 2.3.3 and 4.2.2.3).

Chapter 6: Discussion

6.1. Introduction

This family study aims to investigate the causes of BCC. People who had BCC before the age of 40 were recruited in order to study people who are enriched for risk factors: these are the youngest 7% of the population to have this disease. A study of 56 families of people with early-onset BCC has enabled an investigation into the causes of BCC and has confirmed the hypothesis that first degree relatives of people with early-onset BCC are at greater risk of NMSC and MM than the general Australian population. Despite no differential overall risk to relatives of BCC over SCC, there were nevertheless a subgroup of families with aggregation of BCC alone (to the exclusion of SCC), suggesting the existence of BCC-specific risk in some families.

Examination of the probands and their unaffected siblings confirmed the hypothesis that identifiable phenotypic risk factors for early-onset BCC exist. These were found to be a mixture of pigmentary and sun-exposure-related risk factors and suggest that combinations of genetic and environmental factors are involved in BCC susceptibility. A study of the *PTCH* gene failed to find any mutations and does not support the hypothesis that mutations in this gene are responsible for the increased risk of BCC seen in the probands. Polymorphisms in this gene were found in a similar proportion to the general population, the significance of which to BCC risk is unclear.

6.1.1. Context

This family study is the first of its kind to look at familial aggregation of NMSC in any general non-syndromic population, and shows that this kind of research is an important addition to current cancer research in NMSC. Given the enormous burden of disease that BCC and SCC present to the Australian and international communities, it is surprising that this is the first time familial aggregation of NMSC has been examined in this way. NMSC does aggregate in families of probands with early-onset BCC, showing that some families exist that are enriched for risk factors to these tumours. These risk factors are made up of genetic and environmental factors, both of which have been examined to

some extent here, and that should be examined in further detail in future studies. A bank of DNA from these multiple-case BCC-affected families has been created for the first time by this project, and may be used and expanded upon for further studies on the genetics of skin cancer.

Important additions to the understanding of the aetiology, prevention and treatment of other cancers such as breast cancer and colon cancer have been gathered from this type of research; in some cases the identification of high and medium-penetrance susceptibility genes. Despite its limited scale, this project has demonstrated the feasibility and efficiency of a strategy of recruitment of early-onset BCC cases. Further work with larger samples need to be done; this will allow for segregation and linkage analysis for further characterisation of the way this disease may be inherited.

The current study also uses a novel approach to determining prevalence from recent nationwide Australian incidence data on NMSC, in lieu of cancer registry data on these cancers. The resulting estimate is not perfect, but is the best approximation to calculations performed using cancer registry data in other cancer studies. The success of this approach leads to several important conclusions: that such incidence studies on the Australian population are important for family studies in NMSC; that regular collection of this rapidly changing data will be important for genetic epidemiological studies involving NMSC in the future; and that prevalence may be estimated from incidence data for cancers not on cancer registries to allow comparison of sample populations with the general population.

6.2. Hypothesis 1: First degree relatives of people with BCC are at increased risk of NMSC compared with the general population, with a differentially greater risk of BCC than SCC

We have shown that first-degree relatives of people with early-onset BCC are at increased risk of BCC, SCC and malignant melanoma. The exact risk seen in this study varies by cancer, gender, and relative type. The risk of BCC was elevated in all relatives, but significantly elevated in brothers, sisters, and mothers of people with early-onset BCC, with the greatest risk seen to brothers who had a greater than five-fold increased

risk of also having this tumour. The risk of SCC to relatives of people with early-onset BCC was elevated in brothers, sisters and fathers of people with early-onset BCC, and significantly elevated in sisters and fathers. The greatest risk seen was in sisters of probands who had a greater than five-fold risk of having this tumour. The risk of malignant melanoma was also raised in all first-degree relatives, but significantly elevated for male relatives, the greatest risk seen to brothers who had over ten-fold increased risk of having this tumour.

These figures should be interpreted with the knowledge that the cases were ascertained from the SCFA database which is not a statewide registry (see section 2.1.1) and as with all family cancer studies, there may be an ascertainment bias whereby people who have a history of cancer in the family may be more likely to consent to participate in cancer studies. As calculated in section 3.6, any overestimation due to this potential bias is likely to be less than two- to three-fold, and therefore the observed increased risk to relatives of all three types of skin cancer is likely to be true of early-onset BCC cases as a whole. However, given these limitations the results cannot be generalised to an estimation of prevalence of NMSC in the families of people in the general population with early-onset BCC.

The hypothesis of a differentially increased overall risk of BCC specifically as compared with SCC in relatives of people with early-onset BCC was not supported by this study. Instead, risks for both types of NMSC were increased. Nevertheless, in one third of families affected by NMSC, clusters of BCC alone (to the exclusion of other skin cancer types) did occur. This suggests the possibility of BCC-specific risk factors operating in some families, despite the absence of a differentially increased risk for BCC overall in the whole sample of 56 families. This concept needs to be explored further with larger family studies that are likely to identify more families with clusters of BCC. Further genotypic and phenotypic analysis could be done on identified BCC-specific families.

6.2.1. Estimation of population prevalence

To determine how the subjects' risk of skin cancer compares to the Australian population, we would ideally compare the incidence per year of each cancer in each subject with that

of the incidence of cancer in the same age and sex of the Australian population at the same period in time. This data is not available and instead we have estimated the risks for the Australian population based on a national incidence survey for NMSC from the year 1995 [1] and cancer registry data for melanoma data, from the year 1996.

The incidence figures from the nationwide survey used as a representative of Australian population cancer figures may not truly represent the current population incidence, as discussed in section 3.6. The Poisson model used to fit a curve to the incidence figures from the survey suggest that it provides a reasonable approximation to the whole population incidence. We have used the *incidence* figures from the year 1996 to estimate the expected *prevalence* of disease in our subjects and there are inherent problems in doing this.

The estimated incidences relate to the risk of skin cancer in the population in 1996, and may differ from the risk in other years. It is clear that the incidence of both melanoma and nonmelanoma skin cancers in Australia have been increasing over the last several decades, at least in older persons, meaning that this "snapshot" of risk for skin cancers from 1996 may be an overestimate of the total risk of individual subjects in this study who have lived through many years where the risk was likely to have been significantly smaller. Consequently it is also likely that the overestimate will be smaller for younger persons as there has been less time in which to expect a change in the population's incidence. In addition, incidence of BCC may actually be decreasing in younger people (under the age of 50 years), meaning that for siblings of probands, our population estimates could be slight underestimates [1].

Inferring individual risk of skin cancer from this data also assumes that the data is taken from a homogeneous population, that is, one where each member of the population' risk is equal. The Australian population is obviously heterogenous with risks likely to be associated with skin phototype, other genetic influences, and environmental exposures of the individual. This data is likely to include individuals with multiple skin cancers who will represent a larger proportion of this risk than others. It will also include persons of skin phototype III or IV who are likely to contribute relatively little to the overall risk to

the population. As the subjects of the current study are largely of European background and born in Australia, the estimates of population incidence could provide an underestimate of the actual risk to the subjects of this study.

Despite these possible biases, our approach to the estimation of population prevalence of NMSC provided a reasonable basis with which to compare subject cancer prevalence, and could be used as a model for determining population prevalence in future studies.

6.3. Increased cancer risk in these families: possible causes?

Overall, susceptibility to NMSC and MM is increased in these 56 families compared with the general population: this fact needs some explanation and warrants further investment into family studies in the area of NMSC. The fact that a proportion of families have a cluster of multiple BCC also suggests a susceptibility to this cancer specifically, separately to NMSC susceptibility. The root of all this increased susceptibility is to be found in a combination of environmental and genetic risks, some of which may be reflected in the phenotypic risk factors identified in probands. Family members usually share very similar environments and therefore environmental risks; they also share very common genetic backgrounds making differences in either easier to identify than simply among unrelated members of the same cohort in the general population.

The possible causes of the familial aggregation of skin cancer seen in this study are due to a complex interaction of environmental and genetic risk factors. By looking at phenotypic differences between sibling pairs, the variance in environmental influences seen between members of the general population is reduced as siblings generally share similar environmental influences. This means that any differences between the individuals in the pairs are more likely to have a genetic basis. We looked at the phenotypic differences between affected probands and their oldest unaffected siblings (where available) in order to find out more about the possible basis for their increased risk of cancer.

6.3.1. Hypothesis 2: Identifiable pigmentary and sun exposure related risk factors are present in people with early-onset BCC compared with their unaffected siblings

Differences seen on examination between people with early-onset BCC and their unaffected siblings suggest that genetic differences in combination with high risk sun exposure behaviours have contributed to the increased risk of BCC in probands. It is very difficult to disentangle the environmental from the genetic causes of these differences, as these risk factors are interactive. The term 'sun sensitivity' encompasses a mixture of inherited characteristics that make up a person's skin colour, propensity to burn and ability to tan. Skin 'phototype' predicts BCC risk in our subjects and this confirms previous research: people with very fair skin (phototype I = always burn/never tan) have almost 10 times the risk of BCC compared with people with darker skin (phototype III = sometimes burn/usually tan). Phototype is relatively uncontaminated by environmental exposure as a risk and suggests a difference in genetic susceptibility. However, phototype is likely to affect sun exposure behaviour to some degree, although a lower phototype individual is likely to reduce their sun exposure and therefore cause an underestimation of risk at best.

A similar effect with increased BCC risk for a decreased 'ability to tan' and increased 'propensity to burn' was seen although significance was not achieved with this group. As seen in section 1.7, pigmentary characteristics are influenced by, but not directly related to an individual's MC1R variant status: MC1R expression may be modified by other as-yet-unknown genes and proteins to result in phototype. It is also possible that *PTCH* polymorphisms interact with MC1R variant status to affect NMSC risk as suggested by one research group [235]. MC1R variant status would be the next priority in genetic candidates to examine in such a group, and correlation with sun sensitivity factors and PTCH haplotype would be of interest as potential predictors of risk.

Actinic keratoses are related to cumulative sun exposure and are both precancerous and a marker of SCC risk. Our study confirms other work [78] showing that they may also be considered a marker for BCC risk, with a three-fold risk for BCC seen with presence of

any actinic keratoses. As BCC seems to be associated more with intermittent exposure than cumulative, and SCC more to cumulative than intermittent [14], we would expect actinic keratoses to be better markers for SCC than BCC. Freckling and solar lentigines were also associated with small increased risks, and although significant only on the arms, given the trends seen here are likely to act as markers for BCC risk elsewhere on the body in larger samples. Presence of these lesions probably reflects a high risk level of intermittent or cumulative sun exposure in combination with high risk skin pigmentary types. Questionnaires indexed to lifetime residential and work history used by other research groups [38, 80] would be valuable in this context, and we would expect to see increased intermittent sun exposure in this group, and perhaps, but not necessarily, increased cumulative exposure compared with unaffected relatives. Administration of such questionnaires should take high priority in sufficiently-funded future family studies on NMSC, for the information on interaction of environmental, genetic, and phenotypic risk factors that this would provide.

Red-green reflectance in constitutive skin colour was associated with decreased BCC risk, and this may be interpreted as a decreased risk of BCC with increased skin melanisation. Skin colour has shown less association with BCC risk than sun sensitivity in the literature, although firm conclusions have been difficult due to the lack of consistent and reliable measures of skin colour used to date. Few research groups have used the BYK Gardner spectrophotometer, and studies using similar instruments have reported single readings only, making comparisons with the present study's results difficult [398].

The BYK Gardner spectrophotometer needs to be calibrated to melanin content and perhaps a suitable marker such as MC1R variant status, and this correlation would be relatively simple in future studies where both measures are performed. This would allow important interpretation of spectrophotometer measurements with respect to genotype and phenotype. The influence of hair on the readings of this instrument should also be examined to ensure that systematic bias is not being introduced by the hairiness of the test site, as seen with the Minolta spectrophotometer [399].

Hair and eye colour were not good measures of BCC risk in this study, reflecting previous trends [103, 112]. Importantly, it is noted that self-report of hair colour by naming the colour may be an unreliable and subjective measure, and that the use of hair swatches may allow a more objective assessment of this variable. Seborrhoeic keratoses were paradoxically associated with a trend towards decreased risk of BCC. Many probands and siblings were scored as having no seborrhoeic keratoses, and this is an unlikely result in an Australian population based on previous studies. This indicates that faint lesions were probably missed by the examiner. The validity of the examination for these lesions is questioned, and highlights the need for adequate training of the examiner in identification of all lesions. It is unclear whether faint seborrhoeic keratosis lesions and pigmented seborrhoeic keratosis lesions are in a relatively constant proportion to each other between body sites and also between patients. Any undercounting is likely to have occurred systematically for all subjects and all body regions and therefore the unexpected paradoxical association with decreased risk of BCC would still require explanation. It should be considered that any genetic predisposition to early onset BCC in these subjects also in some way suppresses the development of these benign lesions. Solar elastosis and pterygium were so common as to lose sensitivity as markers, and broader spectra may be required in coding these variables in future.

6.3.2. Possible environmental causes

Environmental risks that are likely to increase risks of all three types of skin cancer include general sun exposure, with intermittent sun exposure probably predisposing more to BCC [101, 102] and MM, and cumulative exposure predisposing more to SCC [14]. Studies show that childhood and adolescent exposure may also contribute more to BCC and MM risk than later exposure, whereas for SCC risk the cumulative dose may be more important [81, 99, 103]. Questionnaires regarding sun exposure over the individuals' lifetime may help to support or refute these hypotheses. Despite the known risk of UVR, other factors must be operating to give the 5- to 10-fold increased skin cancer risks seen in these family members, as sun exposure contributes relative risks in the low 1.0 to 2.0 range, and cannot account entirely for the figures seen here.

Other environmental risk factors that may increase risks of NMSC include HPV infection or possibly other as-yet-unidentified viral agents. Infectious agents such as viruses may cluster in families. HPV is a common viral infection, and high risk genital types have recently been identified as possible risk factors for both BCC and SCC [308]. Several studies have shown that hair follicle reservoirs of HPV infection exist [323, 324, 405], and eyebrow hairs are a convenient source of these [324]. It is unclear if eyebrow hairs are an adequate marker of HPV infections elsewhere on the body or of the HPV-type within epidermal lesions. Collection of eyebrow hairs during examination of subjects is a reasonably non-invasive way of measuring sub-clinical HPV infection and conducting investigations into the possible association with skin cancer susceptibility. Several members the human herpes virus family contribute to human cancer pathogenesis, and CMV needs further investigation as a possible co-factor for NMSC [333].

Exposure to therapeutic ionising radiation also increases the risk of NMSC [151-153]. Surveillance of individuals and their families for all forms of skin cancer may also increase following the diagnosis of one member with any type of skin cancer. Arsenic exposure and sites of scars from physical trauma have also been reported to increase risk of BCC specifically. Theoretically these environmental predispositions could be shared by family members and contribute to their increased susceptibility.

6.3.3. Hypothesis 3: Mutation in PTCH is responsible for some of the increased risk in early-onset non-syndromic BCC probands

Candidate cancer susceptibility genes for NMSC and MM are likely to include *MC1R*: preliminary studies show that variants in this gene may increase risk of skin cancers independently of their effect on pigmentary characteristics [267, 274, 283]. It is possible that certain variant alleles negate the UVR protection usually afforded to people of higher phototype (darker skin) by their pigmentation.

The tumour suppressor gene *PTCH* is an obvious candidate for BCC-specific cancer susceptibility given its role in Gorlin syndrome, a rare familial genodermatosis involving predisposition to multiple early-onset BCC (see section 1.5.4). The current study is the first to examine this gene in the context of non-syndromic population susceptibility to

BCC. We found no mutations in the *PTCH* gene in 58 early-onset probands with BCC, indicating that if *PTCH* mutations are causing population susceptibility to this tumour, then they are not a major contributing factor. Almost all of the coding sequence of *PTCH* in which Gorlin syndrome-associated mutations have been found was thoroughly screened and found to harbour no mutations. Given the numbers of subjects involved and the completeness of screening of this gene, we can be sure that if *PTCH* mutations are causing population susceptibility to BCC, that this is affecting less than 5% of the high risk non-syndromic early-onset cases in the community. This important result needs to be confirmed in larger studies. It does give preliminary evidence that *PTCH* is unlikely to be a major susceptibility gene to BCC outside the context of Gorlin syndrome. As the prevalence of any mutations causing general population BCC is likely to be less than 5%, this result also indicates that screening *PTCH* will be unlikely to prove relevant to clinical geneticists, given that it is a large and expensive gene to test for mutations of such a low yield.

Single nucleotide polymorphisms in *PTCH* in these 58 subjects were seen at a similar frequency to that reported in other studies in SNP databases (see section 5.4). Most of the other studies are from small groups in the USA and all have different ethnic mixes to that seen in Australia, and as they have obviously been ascertained in different ways, are not an ideal comparison for the Australian sample examined here. The current polymorphism heterozygosity results for PTCH will add useful data to this bank of polymorphism data for comparison in the future for other studies on this gene in Australia and internationally. Given their similar frequency to that seen in controls, these SNPs are unlikely to be contributing to the BCC susceptibility seen in these families. However, functional studies of these SNPs would be required to rule out a role in susceptibility to BCC.

6.3.4. Other possible genetic causes

Other genes in the Hedgehog signalling pathway are still candidates for susceptibility genes for BCC, and should be further investigated in susceptible people. Activating *smoothened* mutations have been found in human BCC tumours themselves, and mice

overexpressing *Gli1* or *Gli2* develop BCC-like tumours. Overexpression of the hedgehog gene itself in human and mouse skin leads to BCC-like tumours also. Potential interactions of *PTCH* polymorphisms with other gene variants (eg MC1R) mutations would also be of interest before ruling out possible *PTCH* involvement in susceptibility to BCC.

Germline mutations or variants that may be involved in NMSC susceptibility also include p53Arg, whereby a polymorphism at codon 72 of P53 replaces proline with arginine and results in a protein with increased susceptibility to HPV-mediated degradation. Since UVR-mediated mutation of p53 is known to occur commonly in BCC tumours themselves [292], this variant of p53 may be contributing to population susceptibility to BCC and SCC through infection with HPV. Conflicting evidence has been provided regarding this possibility [406-409]. The genetically polymorphic glutathione s-transferase genes and cytochrome P450 enzyme genes are attractive candidates for cancer susceptibility because of the roles they play in metabolizing toxins. They have been studied with respect to NMSC susceptibility and again, no firm conclusions can be made.

Further studies are needed to map and identify BCC susceptibility genes. These should include genome-wide linkage studies of dense family clusters in which PTCH mutations have been excluded, in order to exclude the presence of as yet undetected hign-penetrance susceptibility gene mutations. It is likely that these will be more efficient if restricted to clusters with early median age of onset. In addition, genome-wide association studies are needed on large cohorts of cases and controls, and again it would seem prudent to restrict these to early age of onset in order to increase their sensitivity to genetic as opposed to environmental causes..

6.4. Clinical relevance

We can now be more certain that when an individual in Australia is treated for BCC at an early age, their close relatives are more likely to get all three types of skin cancer than others in their community. This could have implications for general practitioners and dermatologists who may be able to advise patients and their relatives of a need for increased preventative measures and surveillance. Patients who have the phenotypic

features that are associated with an increased risk of BCC may also be identified by their treating doctor: large degrees of freckling or solar lentigines on the arms could indicate the need for a closer examination for early tumours, or reduce the threshold to investigate or biopsy suspicious lesions in younger people.

6.5. Future research

The probability of familial aggregation of BCC and other skin cancers seen in this study suggest that large-scale familial aggregation studies are needed on BCC affecting the general population (outside of rare hereditary syndromes): to enable an understanding of gene/environment interactions, and to allow genome-wide linkage studies to identify as-yet unknown markers that may cosegregate with the disease. This could be followed by segregation analysis and linkage studies which could lead to the development of complex genetic models incorporating rare high-penetrance gene mutations, and common medium- and low- penetrance genetic variants.

The design of future familial aggregation studies could mirror the current study with larger numbers of probands with early-onset BCC and their first-degree relatives. Subject questionnaires could include history of lifetime sun exposure linked to work history to better quantify the amount of sun exposure in probands and their relatives. Skin examinations for pigmentary and sun exposure characteristics could be repeated in a similar manner to the current study. The MC1R gene would be the next priority when screening genes likely to be responsible for any familial aggregation seen. As this is a relatively small gene, screening could be done with direct sequencing rather than dHPLC and sequencing. Eyebrow hairs could be plucked from subjects for examination of HPV presence, subtyping, and correlation with BCC risk.

BCC is a very common human cancer that is likely to have complex interacting genetic and environmental causes that are as-yet poorly understood. Further investigation into these interactions are desperately needed to improve future monitoring, treatment and prevention of this incredibly burdensome disease.

Chapter 7: Appendices

7.1. Appendix: Clinical Protocol for Questionnaire and Examination

The University of Sydney

Westmead Institute for Cancer Research
Westmead Hospital, Westmead NSW 2145 Australia

Basal Cell Carcinoma Genetics Project

Surname _____

Given Name _____

Date of Birth _____

Identification no _____

Date of Examination _____

Genotype/Phenotype studies in Non-melanoma skin cancer

Clinical Protocol

SECTION A

Demographic Data

i. Sex: M ☐ F ☐

ii. Age: ____ years

Body Surface Area = 71.84 x $(h)^{0.725}$ x $(w)^{0.425}$

iii. Height: _____ cm (h)

iv. Weight: _____ kg (w)

v. Skin Reflectance

	L	a	b
Outer upper arm			
Lateral wall axilla (inner upper arm)			

SECTION B

COLOURING, SKIN TYPE.

Please circle one answer for each question below.

1. Which colour best describes your natural hair colour at age 21?

Red (including Auburn) 1
Fair or Blonde (including White) 2
Light or mouse brown 3
Grey 4
Dark brown 5
Black 6

2. Which colour best describes the colour of your eyes?

Blue or Grey 1
Green or Hazel 2
Brown or Black 3

3. Which colour type best describes your skin before tanning or on areas never exposed to the sun, such as the inside of your upper arm?

Very fair 1
Fair 2
Olive or Brown 3
Asian 4
Black 5
Other **(specify)** 6

Which statement best describes what would happen if your skin were exposed to bright sunlight for the first time in summer for one hour in the middle of the day without any protection?

Get a severe sunburn with blistering? 1
Have a painful sunburn for a few days followed by peeling? 2
Get mildly burnt followed by some tanning? 3
Go brown without any sunburn? 4

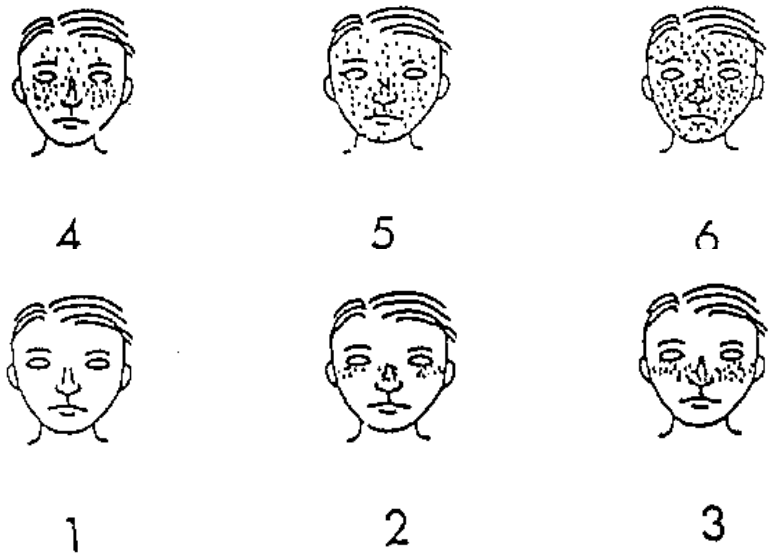
Which of the following best describes what would happen to your skin if it were repeatedly exposed to bright sunlight in summer without any protection?

Go very brown and deeply tanned 1
Get moderately tanned 2
Get mildly or occasionally tanned 3
Get no suntan at all or only get freckled 4

<u>In general</u>, how does your skin react to the sun ?

Always burns, never tans 1
Usually burns, sometimes tans 2
Sometimes burns, usually tans 3
Never burns, always tans 4

Please look at the faces below. Each of the faces shows some degree of freckling, from none to many.



Which of these faces best describes how many freckles you would have had at the end of summer <u>during childhood</u>?

None 1
Very few 2
Few 3
Some 4
Many 5
Very many 6

Which one of these faces best describes how many freckles you would have at the end of summer <u>as an adult</u>?

None 1
Very few 2
Few 3
Some 4
Many 5
Very many 6

Have you ever had dental surgery for a cyst (or cysts) in your upper or lower jaw?

Yes ☐

No ☐

C. Clinical Phenotype

1. Ephelides

(with respect to freckling quantification chart)

	0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100
E1 Face										
E2 Forehead										
E3 Dorsal Upper Limbs										
E4 Upper Back/ Shoulders										

2. Solar Lentigines

	0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100
SL1 Face										
SL2 Forehead										
SL3 Dorsal Upper Limbs										

SL4 Upper Back/ Shoulders										

3. Actinic Keratoses

	Nil	**Sparse (0-10)**	**Moderate (10-60)**	**Extensive/confluent >60**
AK1 Head/Neck				
AK2 Chest				
AK3 Dorsum Arms				
AK4 Dorsum Hands				

4. Actinic Damage/ Elastosis

	Absent	**Mild**	**Severe**	**Present**
AD1 Head, neck, Anterior Chest				
AD2 Periorbital				

AD3 Lips				
AD4 Dorsa Forearms				
AD5 Dorsa hands				
P1 Pterygium				

5. Seborrhoeic Keratoses

	Nil	**Sparse (0-10)**	**Moderate (10-60)**	**Confluent (>60)**
SK1 Head & Neck				
SK2 Upper Limbs				
SK3 Chest				
SK4 Abdomen				
SK5 Back				
SK6 Lower limbs				

6. Skin Cancers

	Body Site	Number
SCC		
BCC		
Melanoma		

7. Palmar Pitting

	Absent	**Equivocal**	**Present**
PP1 Palmar pitting			

8. Hair Colour

Hair colour at 21 years use swatches provided

Red ……..1

Blonde/fair.....2

Brown ……3

Black ……....4

9. Notes

7.2. Appendix: Protocol for DNA extraction from whole blood

Take ~3-5 mL blood and add 5x the volume of QIAGEN Buffer EL. Vortex to mix.
Place on ice for 15 min, vortexing after 10 min then again after a further 5 min.
Centrifuge at 1400 rpm for 10 min at room temperature. Discard supernatant appropriately.
Wash pellet with 2x the original volume of blood with Buffer EL and place on ice for 5 min.
Centrifuge at 1400 rpm for 10 min at room temperature. Discard supernatant appropriately.
Repeat step 4 and 5 if the samples have been frozen.
Rinse pellet with Buffer EL to remove all remaining red blood cells, using a transfer pipette.
Resuspend the pellet in 5 mL Enzyme solution (after aliquoting the desired amount under sterile conditions) and add 50 μL Proteinase K (150 U/mg).
Incubate at 55°C for at least 3 h, or 37°C overnight.
Transfer digest to a 15 mL Falcon tube and add 3.5 mL 3M NaCl (1.2M final concentration) and shake vigorously 15 times.
Centrifuge at 2500 rpm for 15 min at room temperature.
Inverting only once, remove supernatant to a second 15 mL tube and repeat centrifugation.
Again, inverting only once, remove the supernatant to a new tube and precipitate the DNA with 2 volumes of absolute EtOH.
If no precipitate is present, spin the sample in a high-speed centrifuge at ~10 000rpm (_g) for 10-15 min. Remove the supernatant and reconstitute in 1 mL 70% ethanol (EtOH).
Continue from step 15.
Spool the DNA from the EtOH using a 1 mL pipette tip and carefully transfer to an eppendorf tube containing 70% EtOH and mix.
Briefly centrifuge the sample to pellet the DNA and remove the EtOH by inverting only once then remove the remaining EtOH carefully with a suction line.
Briefly air dry and resuspend in 200-600 μL sterile $T_{10}E_1$ (for 2 x 10^7 cells initial dilution should be ~300 μL) and place in a heating block at 50°C for 15 min to aid help dissolve the pellet.
Leave at 4°C for at least one week to dissolve before quantitation and electrophoresis.

(Adapted from: kCkonFab; Agha-Hamilton C, Biospecimen Manager at the Westmead Millennium Institute, Sydney; Personal communication)

7.3. Appendix: Nucleotide sequences of PTCH exons

Nucleotide sequence of wild type PCR product of exons 2 to 4, and 5 to 23 of the PTCH gene. Forward and reverse primers are underlined. Exonic sequences are in capital letters, intronic sequences in small letters. The size of the amplicon is given between ( ). Sequence is from GenBank accession number U59464.

Exon 2 PTCH (294 basepairs)

actcctcccttctgcttcgtccccagGGGAAGGCTACTGGCCGGAAAGCGCCGCTGTGGCTGA
GAGCGAAGTTTCAGAGACTCTTATTTAAACTGGGTTGTTACATTCAAAAAAAC
TGCGGCAAGTTCTTGGTTGTGGGCCTCCTCATATTTGGGGCCTTCGCGGTGGGA
TTAAAAGCAGCGAACCTCGAGACCAACGTGGAGGAGCTGTGGGTGGAAGgtaag
agcgcccgccgcggcgcccgcgccccggcccggtccctcctcgcggttgatagagatattcgccagcgc

Exon 3 PTCH (313 basepairs)

ctattgtgtatcctatggcaggtagtcagataacagataaaacatgagtttgcagtgattttgctattctaattaaacctgtacatatttgt
cagTTGGAGGACGAGTAAGTCGTGAATTAAATTATACTCGCCAGAAGATTGGAG
AAGAGGCTATGTTTAATCCTCAACTCATGATACAGACCCCTAAAGAAGAAGGT
GCTAATGTCCTGACCACAGAAGCGCTCCTACAACACCTGGACTCGGCACTCCA
GGCCAGCCGTGTCCATGTATACATGTACAACAGgtaaggcccgccgcgtccacctactaat

Exon 5 PTCH (248 basepairs)

gcaaaaatttctcaggaacaccccagtagtgtgccttaacctaacgcatggcctcttctttttaactttgacagATAATAGAA
TATCTTTACCCTTGTTTGATTATTACACCTTTGGACTGCTTCTGGGAAGGGGCG
AAATTACAGTCTGGGACAGCATACCTCCTgtaagtgtgtgatcatgctttctgatgtctgtgacttctctag
gactcagtgtttctaatgttgcttatcattgtttgttcc

Exon 6 PTCH (335 basepairs)

cctacaaggtggatgcagtgggcgcagccgtgttactttacgatgcgtttagaaggctcttttcatggtctcgtctcctaatttcttttgc
agAGGTAAACCTCCTTTGCGGTGGACAAACTTCGACCCTTTGGAATTCCTGGAA
GAGTTAAAGAAAATAAACTATCAAGTGGACAGCTGGGAGGAAATGCTGAATA
AGGCTGAGGTTGGTCATGGTTACATGGACCGCCCCTGCCTCAATCCGGCCGAT
CCAGACTGCCCCGCCACAGCCCCCAACAAAAATTCAACCAAAgtgagtaccagcagtga
gcgctctcagaagggtggagagcaaa

Exon 7 PTCH (294 basepairs)

gtgacctgcctactaattcccataattatgtggctgtctttttattttagCCTCTTGATATGGCCCTTGTTTTGAA
TGGTGGATGTCATGGCTTATCCAGAAAGTATATGCACTGGCAGGAGGAGTTGA
TTGTGGGTGGCACAGTCAAGAACAGCACTGGAAAACTCGTCAGgtaagccagctcccg
aggacaatctatgccctgtagtcttcttcctttcctcaaaagccacttccctcctgacatcggcaagtgtatttgtattaatacttaaacc
gttatcctcgctagcc

Exon 8 PTCH (256 basepairs)

gaggcagtggaaactgcttcctgggaatactgatgatgtgccttcccttggactgtgctgcagCGCCCATGCCCTGCA
GACCATGTTCCAGTTAATGACTCCCAAGCAAATGTACGAGCACTTCAAGGGGT
ACGAGTATGTCTCACACATCAACTGGAACGAGGACAAAGCGGCAGCCATCCT
GGAGGCCTGGCAGAGGACATATGTGGAGgtaaacccaccttcgaatcggcgtgcagactcgctggtta
tgcaa

Exon 9 PTCH (245 basepairs)

gtgctgtcgaggcttgtggaagtgttcattgcatttgggcatttcgcattctgttgtgaccacagGTGGTTCATCAGAGT
GTCGCACAGAACTCCACTCAAAAGGTGCTTTCCTTCACCACCACGACCCTGGA
CGACATCCTGAAATCCTTCTCTGACGTCAGTGTCATCCGCGTGGCCAGCGGCT
ACTTACTCATGgtaacgctcgatgccatgctcctgggggctggagtttggtttggttgttttagtctttacttttccatgactg
ctcctgcttct

Exon 10 PTCH (242 basepairs)

ttcggcttttgttctgtgccccccattgttctgcttgcagCTCGCCTATGCCTGTCTAACCATGCTGCGCTG
GGACTGCTCCAAGTCCCAGGGTGCCGTGGGGCTGGCTGGCGTCCTGCTGGTTG
CACTGTCAGTGGCTGCAGGACTGGGCCTGTGCTCATTGATCGGAATTTCCTTTA
ACGCTGCAACAACTCAGgtactaaaggagccatttatctgctgtccgttgacaaatgccaccgg

Exon 11PTCH (253 basepairs)

ctgttaggtgctggtggcagagtcctaactagctttagaatcatctgaattgcatctcgcatgtctaatgccaccatcctctgtttttgct
gtagGTTTTGCCATTTCTCGCTCTTGGTGTTGGTGTGGATGATGTTTTTCTTCTGG
CCCACGCCTTCAGTGAAACAGGACAGAATAAAAGAATCCCTTTTGAGgtaatgcaa
aaacaaaagaagagagctttggggacatcacagcttcctctgttcctaag

Exon 12 PTCH (211 basepairs)

gaccatgtccagtgcagctctcagcgctgtgtttttttattcccagGACAGGACCGGGGAGTGCCTGAAGC
GCACAGGAGCCAGCGTGGCCCTCACGTCCATCAGCAATGTCACAGCCTTCTTC
ATGGCCGCGTTAATCCCAATTCCCGCTCTGCGGGCGTTCTCCCTCCAGgtgagcttct
ggtgatgaaggctgtggtgatcctgaacg

Exon 13 PTCH (222 basepairs)

agtcctctgattgggcggaggcatgttggtgacctctgaatttttttctgctcccagGCAGCGGTAGTAGTGGTGT
TCAATTTTGCCATGGTTCTGCTCATTTTTCCTGCAATTCTCAGCATGGATTTATA
TCGACGCGAGGACAGGAGACTGGATATTTTCTGCTGTTTTACAAGgtacattttcagact
gctgtggcctttgattgggtgcagaatgg

Exon 14 PTCH (540 basepairs)

aaaatggcagaatgaaagcaccatttccctgtttcagcataacattgcaacatgtttcccctcctttcagCCCCTGCGTCAG
CAGAGTGATTCAGGTTGAACCTCAGGCCTACACCGACACACACGACAATACCC
GCTACAGCCCCCCACCTCCCTACAGCAGCCACAGCTTTGCCCATGAAACGCAG
ATTACCATGCAGTCCACTGTCCAGCTCCGCACGGAGTACGACCCCCACACGCA
CGTGTACTACACCACCGCTGAGCCGCGCTCCGAGATCTCTGTGCAGCCCGTCA
CCGTGACACAGGACACCCTCAGCTGCCAGAGCCCAGAGAGCACCAGCTCCAC
AAGGGACCTGCTCTCCCAGTTCTCCGACTCCAGCCTCCACTGCCTCGAGCCCCC
CTGTACGAAGTGGACACTCTCATCTTTTGCTGAGAAGCACTATGCTCCTTTCCT
CTTGAAACCAAAAGCCAAGgtaatctgccacaacttaaggctcttcctgtcttcaaaaaaataacagaacctttgg
agttcatcag

Exon 15 PTCH (425 basepairs)

gacagcttctctttgtccaggaagagtcagtggtgctccctggggtctgaccttgtgcctcttctgttccagGTAGTGGTGA
TCTTCCTTTTTCTGGGCTTGCTGGGGGTCAGCCTTTATGGCACCACCCGAGTGA
GAGACGGGCTGGACCTTACGGACATTGTACCTCGGGAAACCAGAGAATATGA
CTTTATTGCTGCACAATTCAAATACTTTTCTTTCTACAACATGTATATAGTCAC
CCAGAAAGCAGACTACCCGAATATCCAGCACTTACTTTACGACCTACACAGGA
GTTTCAGTAACGTGAAGTATGTCATGTTGGAAGAAAACAAACAGCTTCCCAAA
ATGTGGCTGCACTACTTCAGAGACTGGCTTCAGGgtaagacggcgtgggaggctctcgtcctttc
ggtcttttgcgt

Exon 16 PTCH (219 basepairs)

agggtccttctggctgcgagttataatgtgttacaatcatttgccatttctagGACTTCAGGATGCATTTGACAG
TGACTGGGAAACCGGGAAAATCATGCCAAACAATTACAAGAATGGATCAGAC
GATGGAGTCCTTGCCTACAAACTCCTGGTGCAAACCGGCAGCCGCGATAAGCC
CATCGACATCAGCCAGgtactccagctgctgggcactga

Exon 17 PTCH (268 basepairs)

aaccccattctcaaaggcctctgttcttcccgtttttgtagTTGACTAAACAGCGTCTGGTGGATGCAGA
TGGCATCATTAATCCCAGCGCTTTCTACATCTACCTGACGGCTTGGGTCAGCAA
CGACCCCGTCGCGTATGCTGCCTCCCAGGCCAACATCCGGCCACACCGACCAG

AATGGGTCCACGACAAAGCCGACTACATGCCTGAAACAAGGCTGAGAAgtaagta
gcgttttatcgggaggtctgggaacttacagaggtg

Exon 18 PTCH (410 basepairs)

aactgtgatgctcttctaccctgggctgctcctaacctgtgcccttctctgtccagTCCCGGCAGCAGAGCCCATC
GAGTATGCCCAGTTCCCTTTCTACCTCAACGGCTTGCGGGACACCTCAGACTTT
GTGGAGGCAATTGAAAAAGTAAGGACCATCTGCAGCAACTATACGAGCCTGG
GGCTGTCCAGTTACCCCAACGGCTACCCCTTCCTCTTCTGGGAGCAGTACATCG
GCCTCCGCCACTGGCTGCTGCTGTTCATCAGCGTGGTGTTGGCCTGCACATTCC
TCGTGTGCGCTGTCTTCCTTCTGAACCCCTGGACGGCCGGGATCATTgtgagtgtatta
taaggggctttgtggaagtcaaattcctttcagcatagctctttctgcagccgggaagttt

Exon 19 PTCH (305 basepairs)

tttgatctgaaccgaggacacccttagccctctggcgacctcccttgtgggcagccccagagggtgacctgcccgcccactgacca
ctgtgtgccctgctccagGTGATGGTCCTGGCGCTGATGACGGTCGAGCTGGCATAGGAG
TGGAGTTCACCGTTCACGTTGCTTTGTTCGGCATGATGGGCCTCATCGGAATCA
AGCTCAGTGCCGTGCCCGTGGTCATCCTGATCGCTTCTGTTGgtatgggagacatttgaag
caaaaactttgtgaaaaaacaaccccatttcctctggctctgtttg

Exon 20 PTCH (214 basepairs)

catttaggacagagctgagcatttaccaggtgaagtccagcaacctgatcttgtgaacatcctcattgcacagGCCTTTCTG
ACGGCCATCGGCGACAAGACCGCAGGGCTGTGCTTGCCCTGGAGCACATGTTT
GCACCCGTCCTGGATGGCGCCGTGTCCACTCTGCTGGGAGTGCTGATGCTGGC
GGGATCTGAGTTCGACTTCATTGTCAGGTAAGCAGGCGTGTGCAAGGAGACAT
GTTTTAGAAATCATTGTGATTGGGCC

Exon 21 PTCH (187 basepairs)

tgttcccgtttcctcttgatctcccagGTATTTCTTTGCTGTGCTGGCGATCCTCACCATCCTCGG
CGTTCTCAATGGGCTGGTTTTGCTTCCCGTGCTTTTGTCTTTCTTTGGACCATAC
CTGAGgtcagtagtgacacggggatgtcccacgtgtaggccggctgaatgctgtgtttcctgtgc

Exon 22 PTCH (371 basepairs)

agtgtggccagcaggtaaatggacaagaacacttttaacatggaatccccttaaatagGTGTCTCCAGCCAACGG
CTTGAACCGCCTGCCCACACCCTCCCCTGAGCCACCCCCCAGCGTGGTCCGCTT
CGCCATGCCGCCCGGCCACACGCACAGCGGGTCTGATTCCTCCGACTCGGAGT
ATAGTTCCCAGACGACAGTGTCAGGCCTCAGCGAGGAGCTTCGGCACTACGAG
GCCCAGCAGGCGCGGGAGGCCCTGCCCACCAAGTGATCGTGGAAGCCACAGA
AAACCCCGTCTTCGCCCACTCCACTgtaagtgactctgcagaaccaggggagggtcttcccaccgtgg
tagtgggcctggag

Exon 23a PTCH (331 basepairs)

aaacccaaggagggaagtgtgggagctgcggggaccatgcccagcctgggataccacccttctaacccaccctcaccctctgc
agGTGGTCCATCCCGAATCCAGGCATCACCCACCCTCGAACCCGAGACAGCAG
CCCCACCTGGACTCAGGGTCCCTGCCTCCCGGACGGCAAGGCCAGCAGCCCCG
CAGGGACCCCCCCAGAGAAGGCTTGTGGCCACCCCTCTACAGACCGCGCAGA
GACGCTTTTGAAATTTCTACTGAAGGGCATTCTGGCCCTAGCAATAGGGCCCG
CTGGGGCCCTCGCGGGGCC**C**GTTCTCACAACCCTCGG

Exon 23b PTCH (406 basepairs)

GCATTCTGGCCCTAGCAATAGGGCCCGCTGGGGCCCTCGCGGGGCC**C**GTTCTC
ACAACCCTCGGAACCCAACGTCCACTGCCATGGGCAGCTCCGTGCCCGGCTAC
TGCCAGCCCATCACCACTGTGACGGCTTCTGCCTCCGTGACTGTCGCCGTGCAC
CCGCCGCCTGTCCCTGGGCCTGGGCGGAACCCCCGAGGGGGACTCTGCCCAGG
CTACCCTGAGACTGACCACGGCCTGTTTGAGGACCCCCACGTGCCTTTCCACG
TCCGGTGTGAGAGGAGGGATTCGAAGGTGGAAGTCATTGAGCTGCAGGACGT
GGAATGCGAGGAGAGGCCCCGGGGAAGCAGCTCCAACTGAGgtgagtgccactgacaa
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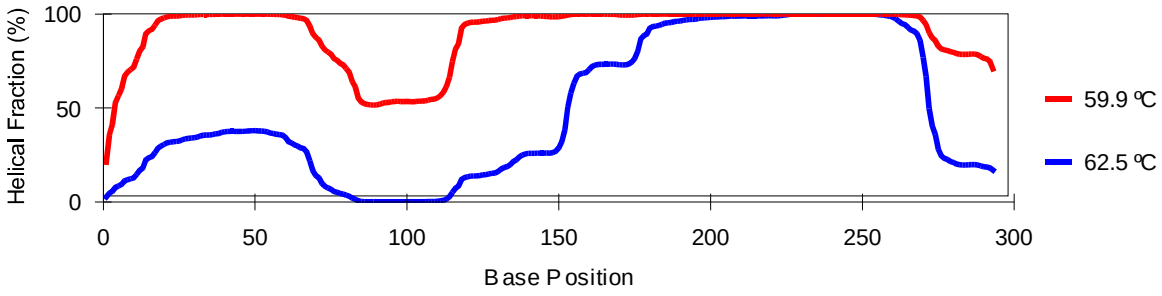
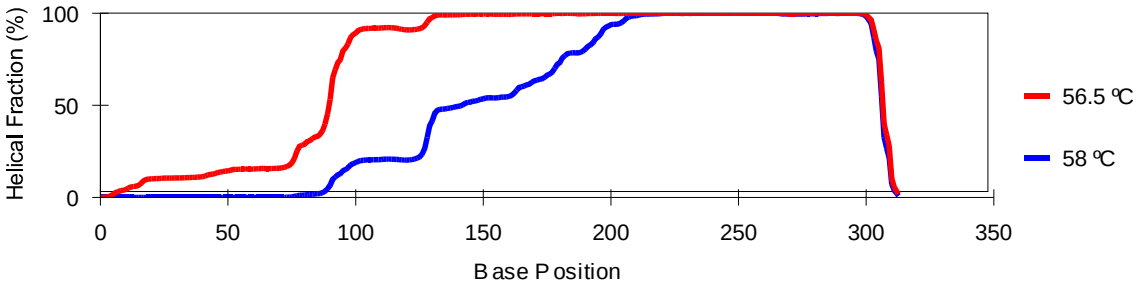
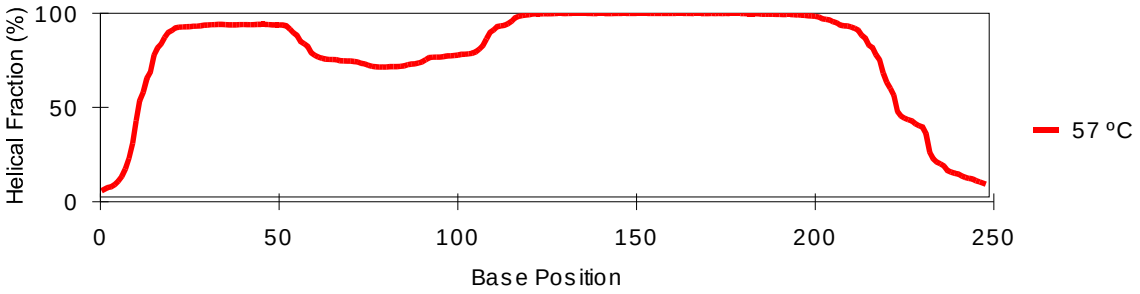
7.4. Appendix: DHPLC melting profiles



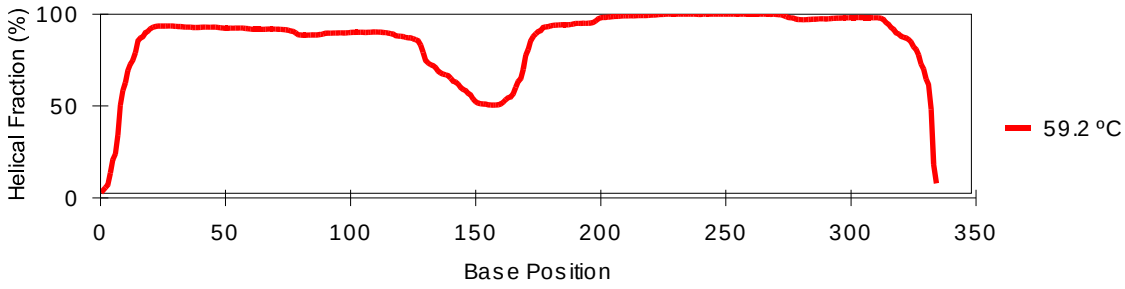
Predicted dHPLC melting profile for PTCH exon 2, wild type



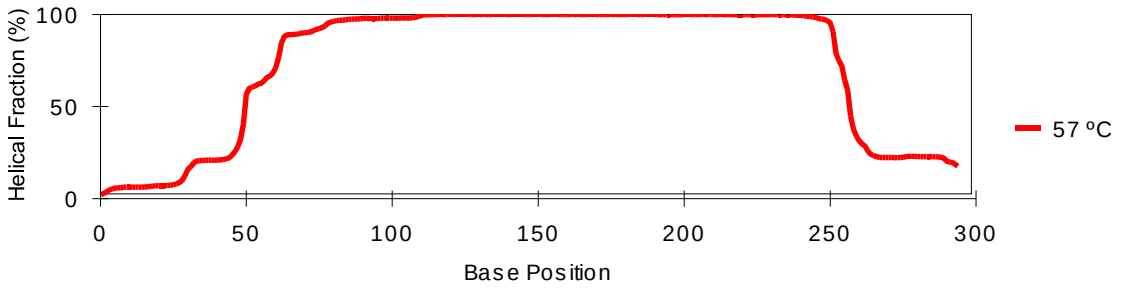
Predicted dHPLC melting profile for PTCH exon 3, wild type



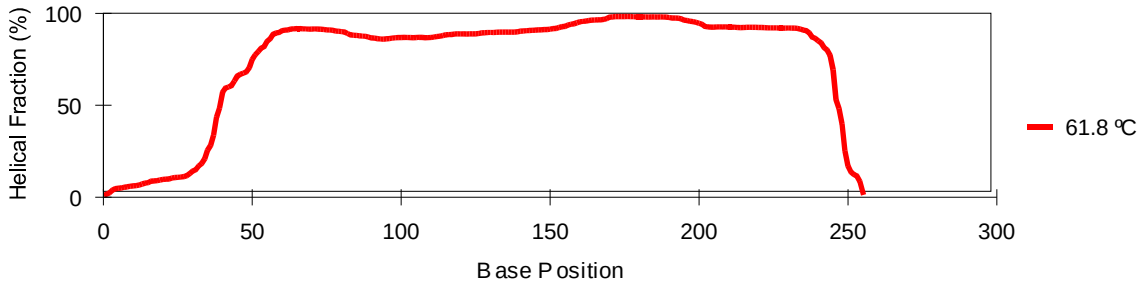
Predicted dHPLC melting profile for PTCH exon 5, wild type



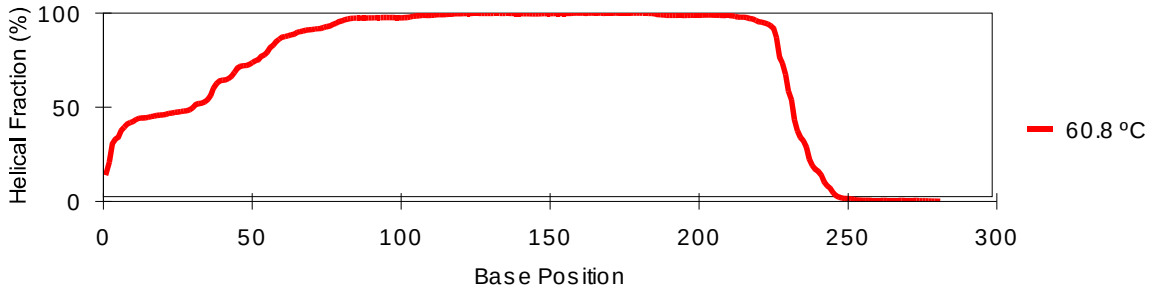
Predicted dHPLC melting profile for PTCH exon 6, wild type



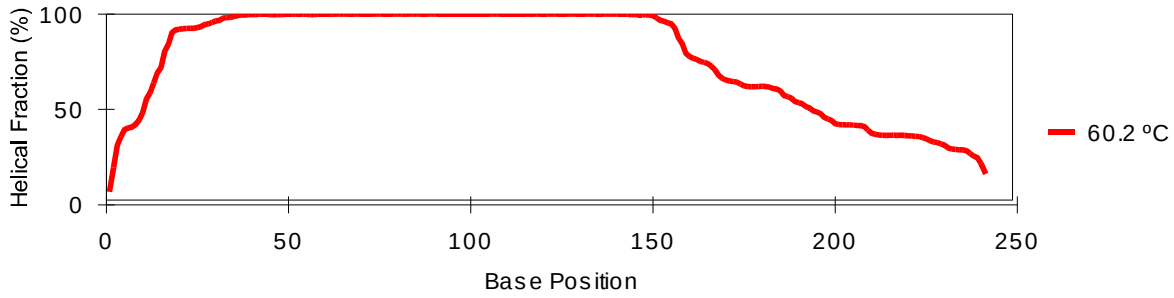
Predicted dHPLC melting profile for PTCH exon 7, wild type



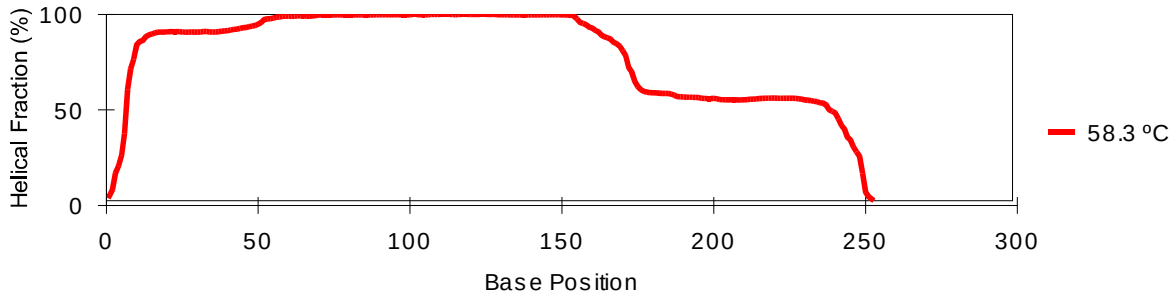
Predicted dHPLC melting profile for PTCH exon 8, wild type



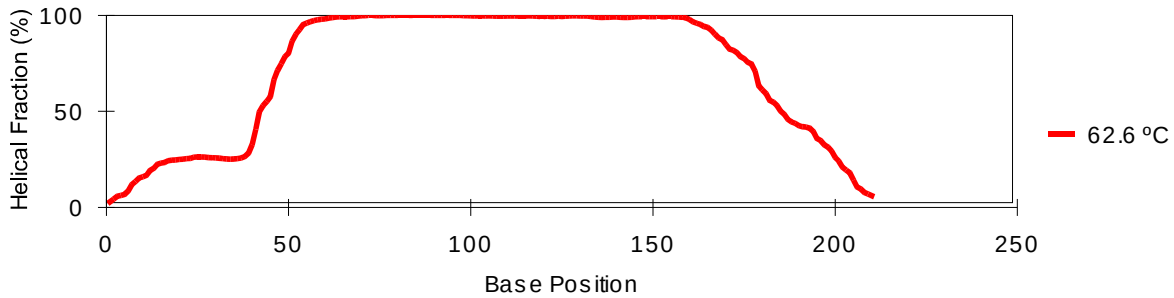
Predicted dHPLC melting profile for PTCH exon 9, wild type



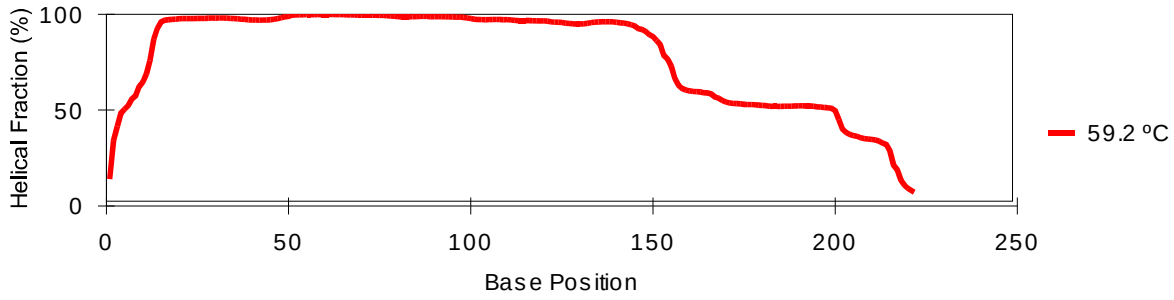
Predicted dHPLC melting profile for PTCH exon 10, wild type



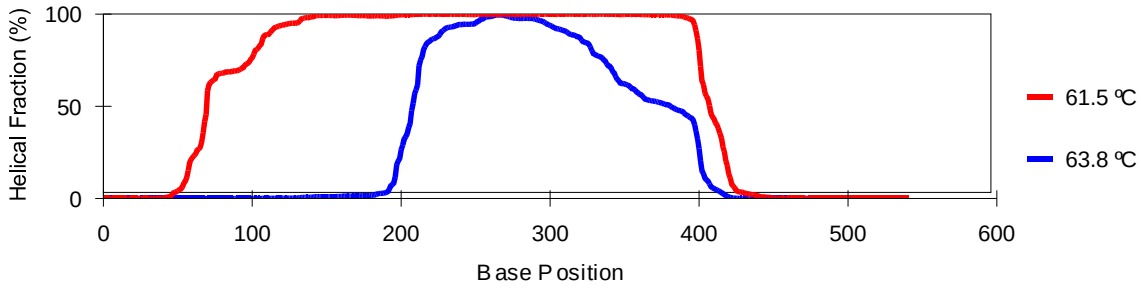
Predicted dHPLC melting profile for PTCH exon 11, wild type



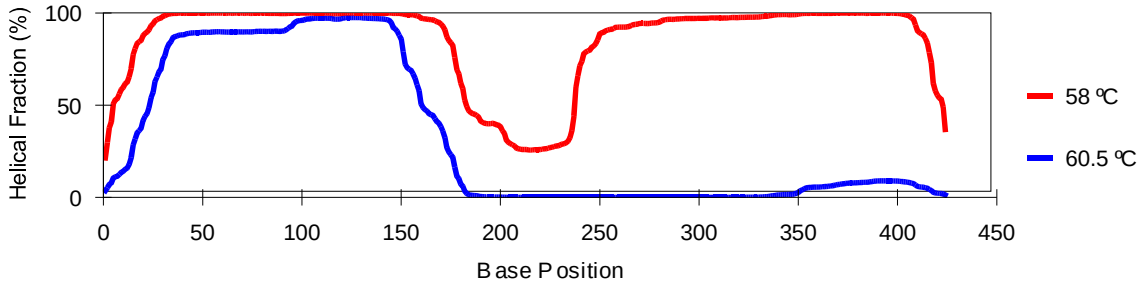
Predicted dHPLC melting profile for PTCH exon 12, wild type



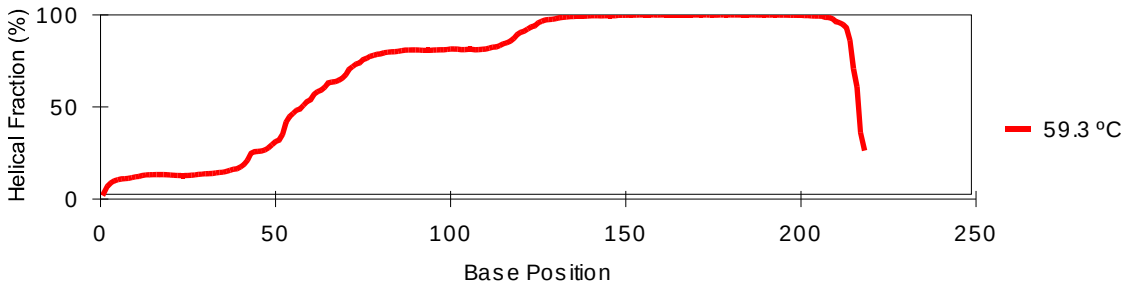
Predicted dHPLC melting profile for PTCH exon 13, wild type



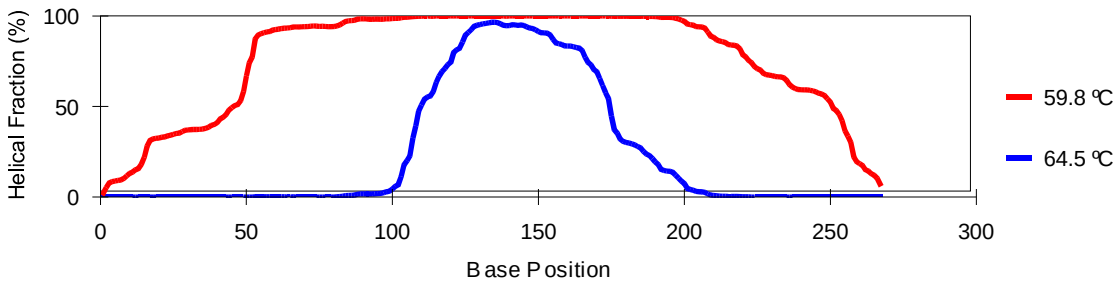
Predicted dHPLC melting profile for PTCH exon 14, wild type



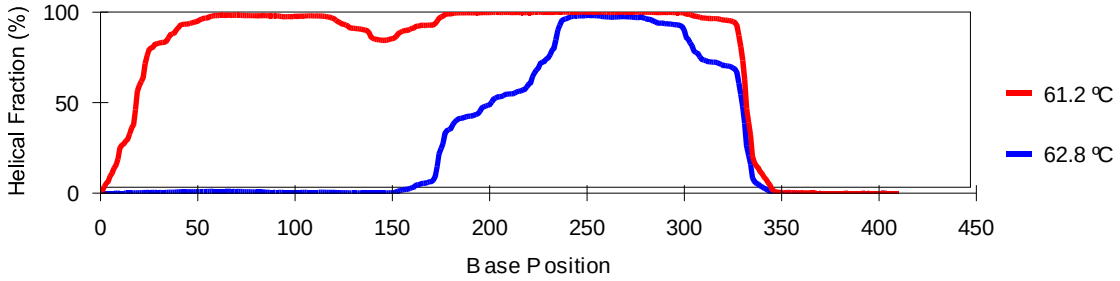
Predicted dHPLC melting curve for PTCH exon 15, wild type



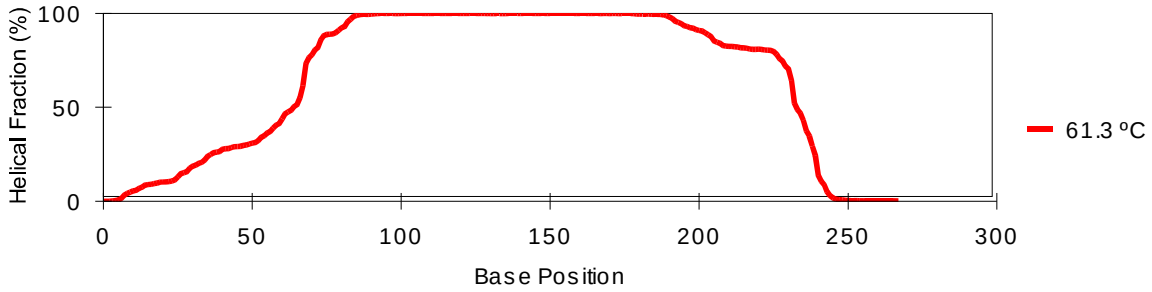
Predicted dHPLC melting curve for PTCH exon 16, wild type



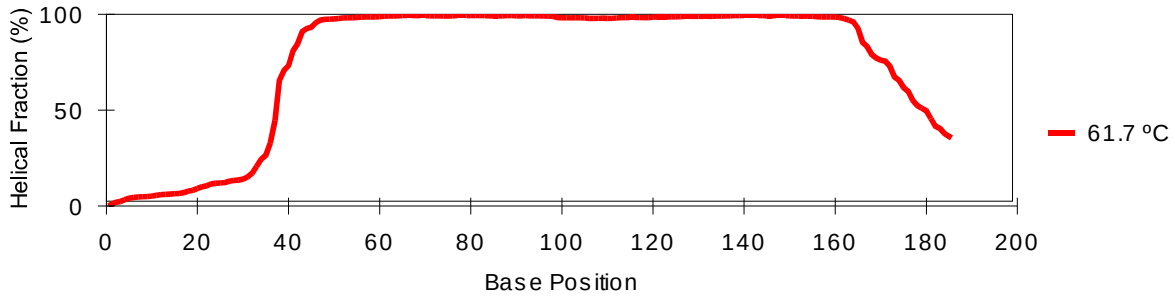
Predicted dHPLC melting curve for PTCH exon 17, wild type



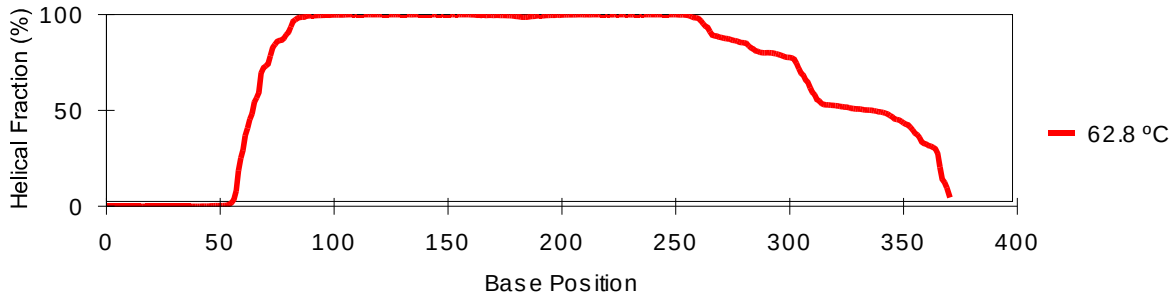
Predicted dHPLC melting curve for PTCH exon 18, wild type



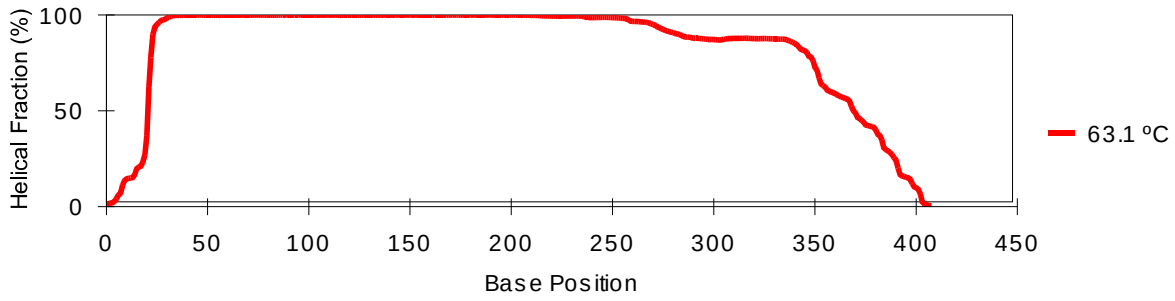
Predicted dHPLC melting curve for PTCH exon 20, wild type



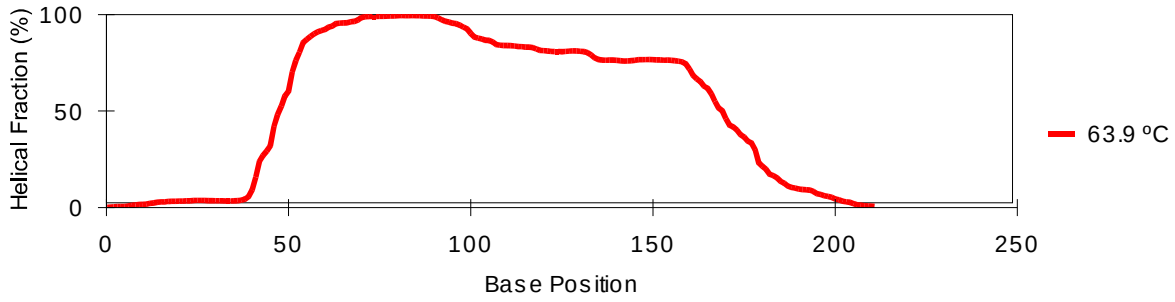
Predicted dHPLC melting curve for PTCH exon 21, wild type



Predicted dHPLC melting curve for PTCH exon 22, wild type



Predicted dHPLC melting curve for PTCH exon 23, wild type



Predicted dHPLC melting profile for PTCH exon 23a, wild type

7.5. Appendix: Protocol for PCR-product purification

Using QIAquick PCR Purification Kit and a conventional tabletop microcentrifuge, at 13,000 rounds per minute:

Suck up the total volume of PCR product using a pipette.
Add 5 volumes of Buffer PB to 1 volume of PCR sample and mix.
Place a QIAquick spin column in a provided 2 ml collection tube.
To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60 sec.
Discard the flow-through. Place the QIAquick column back into the same tube.
To wash, add 0.75 ml Buffer PE (with added ethanol) to the QIAquick column and centrifuge for 30-60 sec.
Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed.
Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
To elute DNA from the column, add 50 µl elution buffer (EB) (10 mM Tris-CL, pH 8.5) or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 min.
Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.
Store DNA at -20ºC until use, as DNA may degrade otherwise.

(Adapted from: Qiagen. QIAquick Spin Handbook. USA: Instructions manual; 07/2002. p11-8)

7.6. Appendix: subjects excluded from analysis

Table 7.1. Subjects excluded from analysis of comparison with population prevalence (due to death or age>70 years)

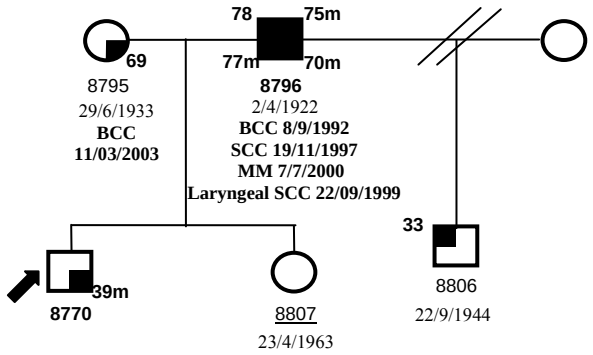
ID	Family ID	Cancers
9129	29125	-
8838	28762	Colorectal
8827	28763	MM
8930	28767	Renal Cell
9331	28775	-
9332	28775	SCC
9411	28779	Ovarian
9115	28797	Colorectal, SCC, BCC
8892	28816	Renal Cell
8823	28818	Breast
8824	28818	-
8859	28857	Laryngeal SCC
9042	29022	BCC
9128	29125	SCC
9169	29168	Colorectal
9170	29168	Squamous Cell Lung
9430	29240	Malignant fibrous histiocytoma (sarcoma)
9289	29275	Breast
8817	28762	Prostate
8801	28763	SCC
8811	29764	-
8765	28764	-
8867	28776	-
8929	28767	-
8796	28770	BCC, SCC, MM
8793	28771	-
8792	28771	SCC
8985	28773	BCC
8986	28773	-
9066	28794	-
9067	28794	-
9116	28797	-
9303	28800	-
9304	28800	BCC, SCC
8891	28816	BCC, SCC
8829	28825	SCC, MM
8830	28825	Colorectal, BCC

9041	29022	BCC
9096	29094	SCC
9127	29125	-
9429	29240	-
9455	29445	BCC
9456	29445	BCC, SCC
9447	29446	-
9448	29446	SCC
9461	29459	SCC
9530	29527	-

Note: all deceased parents except two had a cancer diagnosis (likely to be bias of inclusion in study of people with family history of cancer?)

7.7. Appendix: Pedigrees for all families with cancer affected first degree relatives

28770



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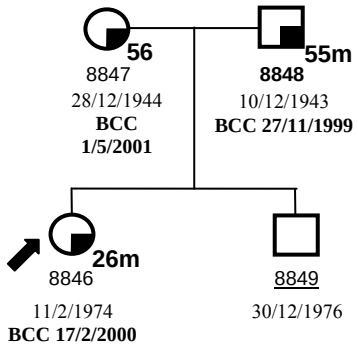
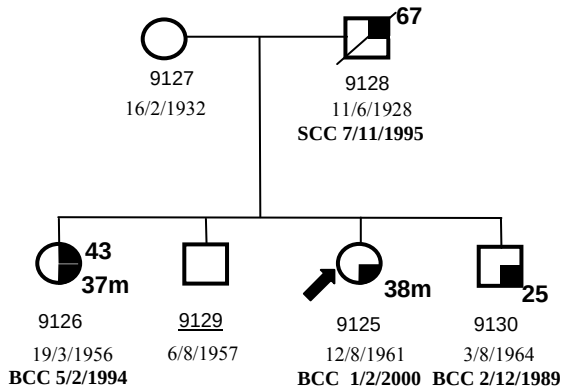
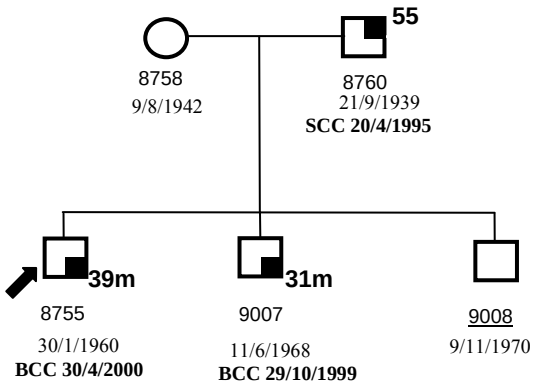


29125



SCC24/8/1999

28755



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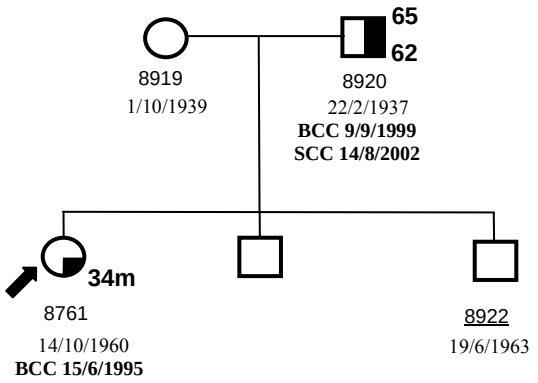

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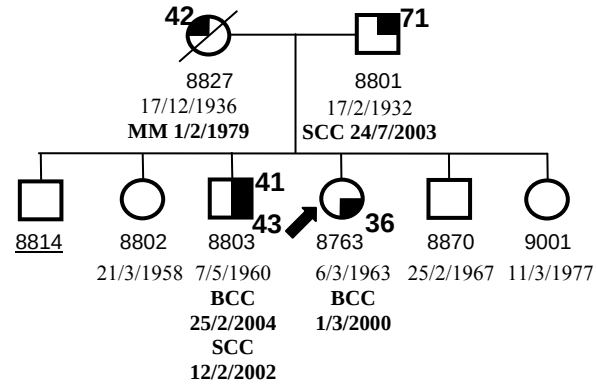
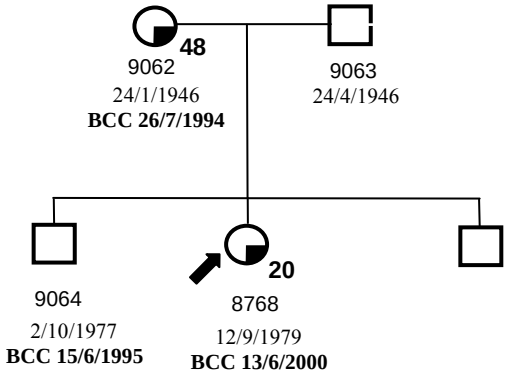


28768



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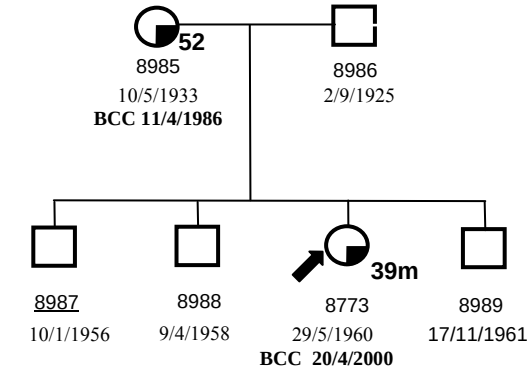

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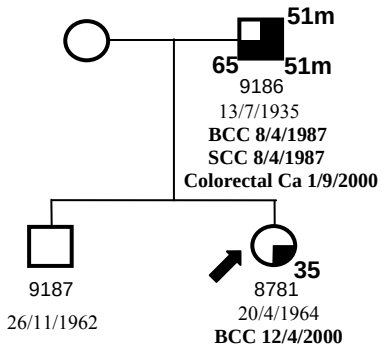

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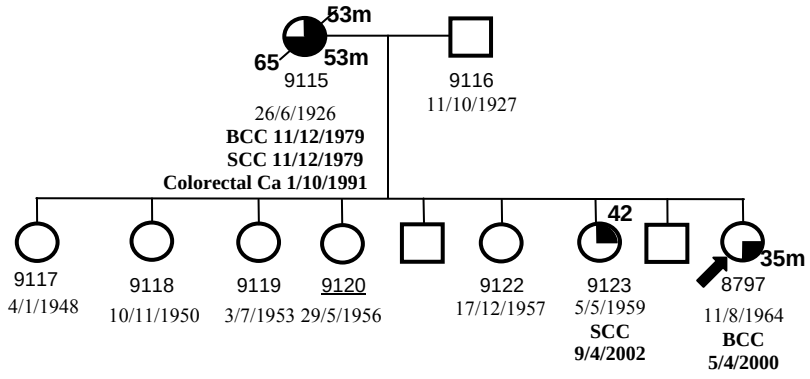

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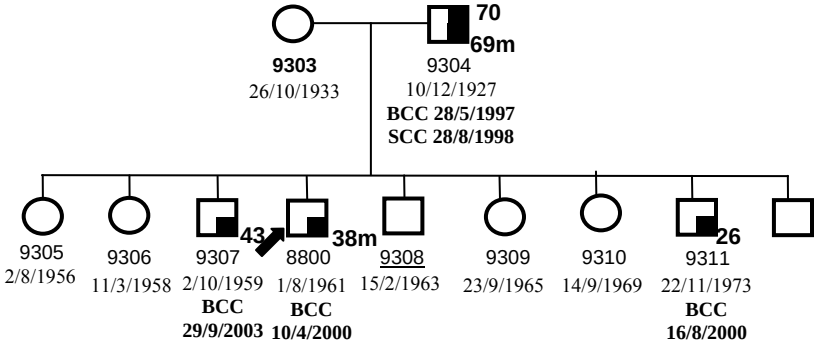

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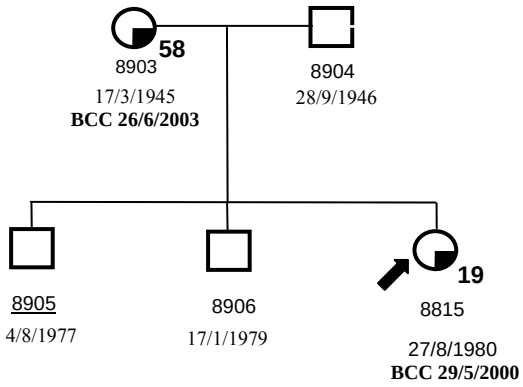
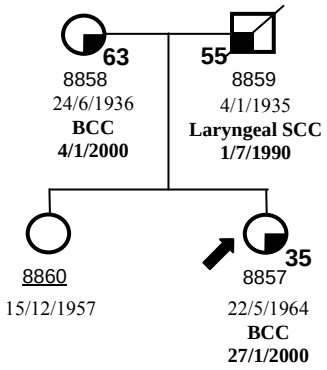


28857



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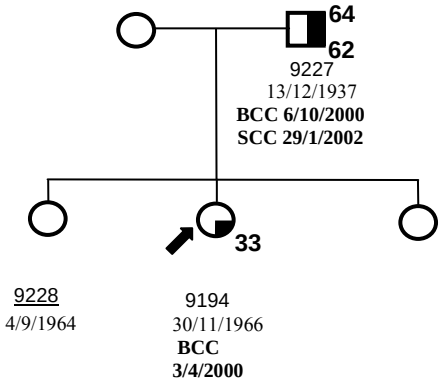

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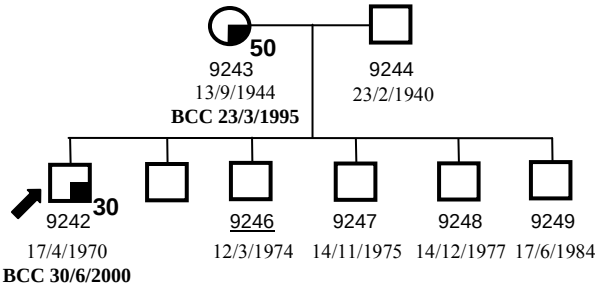

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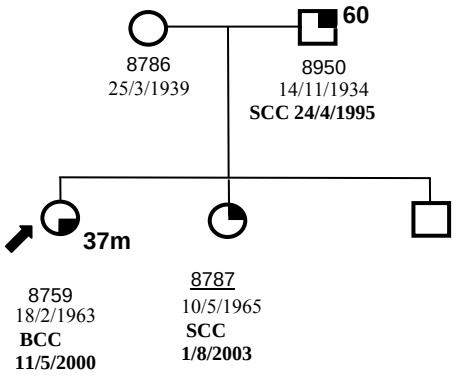

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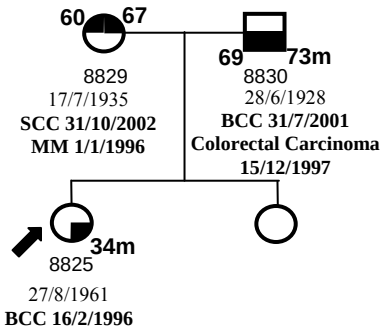

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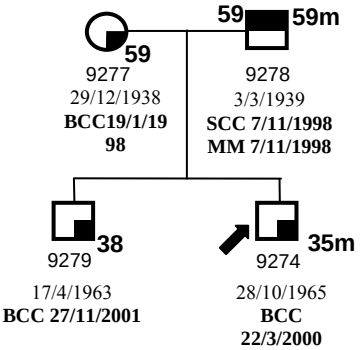

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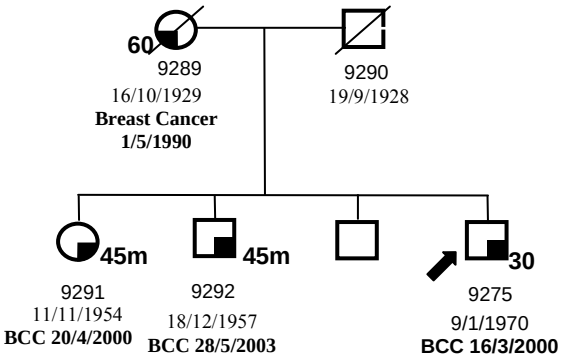
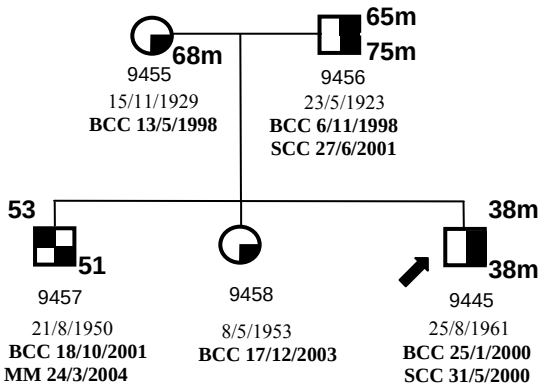
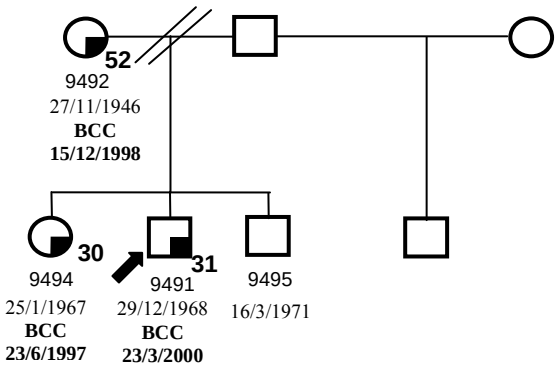

60
9289
16/10/1929
Breast Cancer
1/5/1990
9290
19/9/1928
45m
9291
11/11/1954
BCC 20/4/2000
45m
9292
18/12/1957
BCC 28/5/2003
30
9275
9/1/1970
BCC 16/3/2000

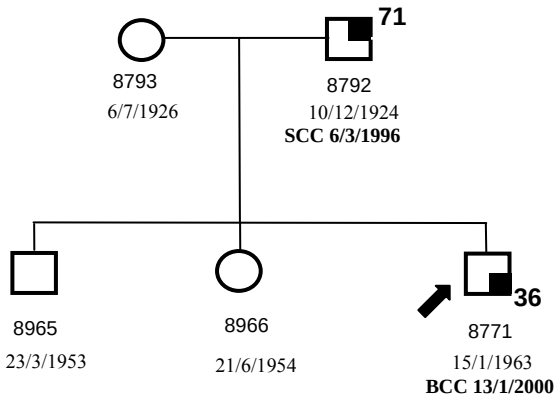
29445


68m
9455
15/11/1929
BCC 13/5/1998
65m
75m
9456
23/5/1923
BCC 6/11/1998
SCC 27/6/2001
53
51
9457
21/8/1950
BCC 18/10/2001
MM 24/3/2004
9458
8/5/1953
BCC 17/12/2003
38m
38m
9445
25/8/1961
BCC 25/1/2000
SCC 31/5/2000

29491



28771



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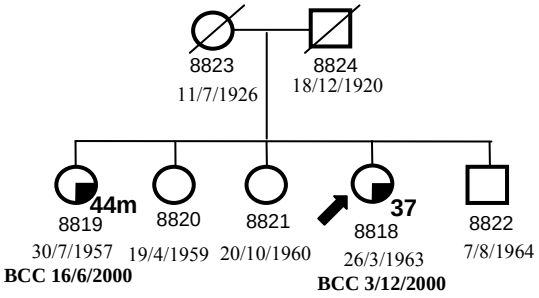


7.8. Appendix: Raw data

Raw, semi-quantitative data collected on each variable is displayed below as referred to in the text.

Table 7.2 Self reported hair colour crosstabulation: by questionnaire and hair swatch selection

		Hair colour by swatch			
		Red	Fair/Blonde	Brown	Total
Hair colour by questionnaire	Red	6	0	0	6
	Fair/Blonde	1	16	0	17
	Light or mouse brown	5	20	8	33
	Dark Brown	1	3	27	31
	Black	0	0	1	1
Total		13	39	36	88

Table 7.3 Single exposure to the sun ("sun sensitivity") raw data

Self-reported category	**n (%)**
Severe sunburn/blistering	9 (10.2)
Painful sunburn/peeling	53 (60.2)
Mild sunburn/tanning	23 (26.1)
No sunburn/tanning	3 (3.4%)
Total	88 (100)

Table 7.4 Repeated exposure to the sun ("ability to tan") raw data

Self-reported category	**n (%)**
Very brown/deeply tanned	6 (6.8)
Moderately tanned	34 (38.6)
Mildly/occasionally tanned	32 (36.4)
No tan/only freckled	16 (18.2)
Total	88 (100)

Table 7.5 General reaction to the sun ("phototype") raw data

Self-reported category	**n (%)**
Always burns, never tans	8 (9.1)
Usually burns, sometimes tans	46 (52.3)
Sometimes burns, usually tans	34 (38.6)
Total	88 (100)

Table 7.6 Actinic Keratoses (AK's) raw data

Number lesions	**Head/Neck n (%)**	**Chest n (%)**	**Dorsum Arms n (%)**	**Dorsum Hands n (%)**
Nil	59 (67)	84 (95.5)	70 (79.5)	73 (83)
Sparse (0-10)	27 (30.7)	3 (3.4)	16 (18.2)	13 (14.8)
Moderate (10- 60)	2 (2.3)	1 (1.1)	2 (2.3)	2 (2.3)
Total	88	88	88	88

Table 7.7. Solar elastosis raw data

Actinic Damage/Elastosis Score	**Head/Neck/ Anterior Chest n (%)**	**Periorbital n (%)**	**Lips n (%)**	**Dorsa Forearms n (%)**	**Dorsa Hands n (%)**
Absent	-	-	-	-	-
Mild - moderate	6 (6.9)	6 (6.9)	18 (20.7)	17 (19.5)	15 (17.2)
Severe	81 (93.1)	81 (93.1)	69 (79.3)	70 (80.5)	72 (82.8)
Total	87 (100)	87 (100)	87 (100)	87 (100)	87 (100)

Table 7.8 Solar lentigines raw data

Solar lentigines semi-quantitative score	**Face n (%)**	**Forehead n (%)**	**Dorsal Upper Limbs n (%)**	**Upper back/shoulders n (%)**
0-10	10 (11.4)	16 (18.2)	5 (5.7)	-
10-20	19 (21.6)	31 (35.2)	5 (5.7)	3 (3.4)
20-30	14 (15.9)	9 (10.2)	9 (10.2)	4 (4.5)
30-40	17 (19.3)	10 (11.4)	11 (12.5)	5 (5.7)
40-50	14 (15.9)	10 (11.4)	16 (18.2)	8 (9.1)
50-60	6 (6.8)	7 (8.0)	11 (12.5)	5 (5.7)
60-70	7 (8.0)	3 (3.4)	12 (13.6)	18 (20.5)
70-80	-	1 (1.1)	9 (10.2)	14 (15.9)
80-90	1 (1.1)	1 (1.1)	9 (10.2)	22 (25.0)
90-100	-	-	1 (1.1)	9 (10.2)
Total	88 (100)	88 (100)	88 (100)	88 (100)

Table 7.9 Freckling raw data

Self-reported *childhood* freckling	**Proband n (%)**	**Sibling n (%)**	Total n (%)
None	4 (12.1)	4 (7.3)	**8 (9.1)**
Very Few	13 (39.4)	18 (32.7)	**31 (35.2)**
Few	8 (24.2)	10 (18.2)	**18 (20.5)**
Some	2 (6.1)	13 (23.6)	**15 (17.0)**
Many	6 (18.2)	10 (18.2)	**16 (18.2)**
Total	**33 (100.0)**	**55 (100.0)**	**88 (100.0)**

Self-reported *adult* freckling	**Proband n (%)**	**Sibling n (%)**	Total n (%)
None	7 (21.2)	8 (14.5)	**15 (17.0)**
Very Few	15 (45.5)	28 (50.9)	**43 (48.9)**
Few	6 18.2)	7 (12.7)	**13 (14.8)**
Some	3 (9.1)	8 (14.5)	**11 (12.5)**
Many	2 (6.1)	4 (7.3)	**6 (6.8)**
Total	**33 (100.0)**	**55 (100.0)**	**88 (100.0)**

Freckling semi-quantitative score	**Face n (%)**	**Forehead n (%)**	**Dorsal Upper Limbs n (%)**	**Upper back/shoulders n (%)**
0-10	9 (10.2)	8 (9.1)	-	-
10-20	17 (19.3)	16 (18.2)	1 (1.1)	3 (3.4)
20-30	11 (12.5)	15 (17.0)	2 (2.3)	1 (1.1)
30-40	11 (12.5)	12 (13.6)	3 (3.4)	2 (2.3)
40-50	12 (13.6)	10 (11.4)	7 (8.0)	6 (6.8)
50-60	15 (17.0)	12 (13.6)	6 (6.8)	7 (8.0)
60-70	5 (5.7)	5 (5.7)	15 (17.0)	17 (19.3)
70-80	5 (5.7)	4 (4.5)	14 (15.9)	19 (21.6)
80-90	3 (3.4)	6 (6.8)	37 (42.0)	24 (27.3)
90-100	-	-	3 (3.4)	9 (10.2)
Total	88 (100)	88 (100)	88 (100)	88 (100)

Table 7.10 Seborrhoeic keratoses raw data

Seborrhoeic Keratoses semi-quantitative score	**Head/Neck n (%)**	**Upper Limbs n (%)**	**Chest n (%)**	**Abdomen n (%)**	**Back n (%)**	**Lower Limbs n (%)**
Nil	73 (83.0)	59 (67.0)	66 (75.0)	63 (71.6)	54 (61.4)	58 (65.9)
Sparse (0 – 10)	15 (17.0)	28 (31.8)	22 (25.0)	22 (25.0)	32 (36.4)	28 (31.8)
Moderate (10 – 60)	-	1 (1.1)	-	3 (3.4)	2 (2.3)	2 (2.3)
Confluent	-	-	-	-	-	-
Total	88 (100.0)	88 (100.0)	88 (100.0)	88 (100.0)	88 (100.0)	88 (100.0)

Table 7.11 Month of examination crosstabulation (all subjects)

Month	**No. siblings examined**	**No. probands examined**	Total
January	4	6	**10**
February	4	4	**8**
March	3	6	**9**
April	2	4	**6**
May	6	12	**18**
June	2	4	**6**
July	2	7	**9**
August	6	4	**10**
September	2	2	**4**
October	-	-	**-**
November	-	-	**-**
December	2	6	**8**
Total	**33**	**55**	**88**

Table 7.12 Season of examination of case-control pairs

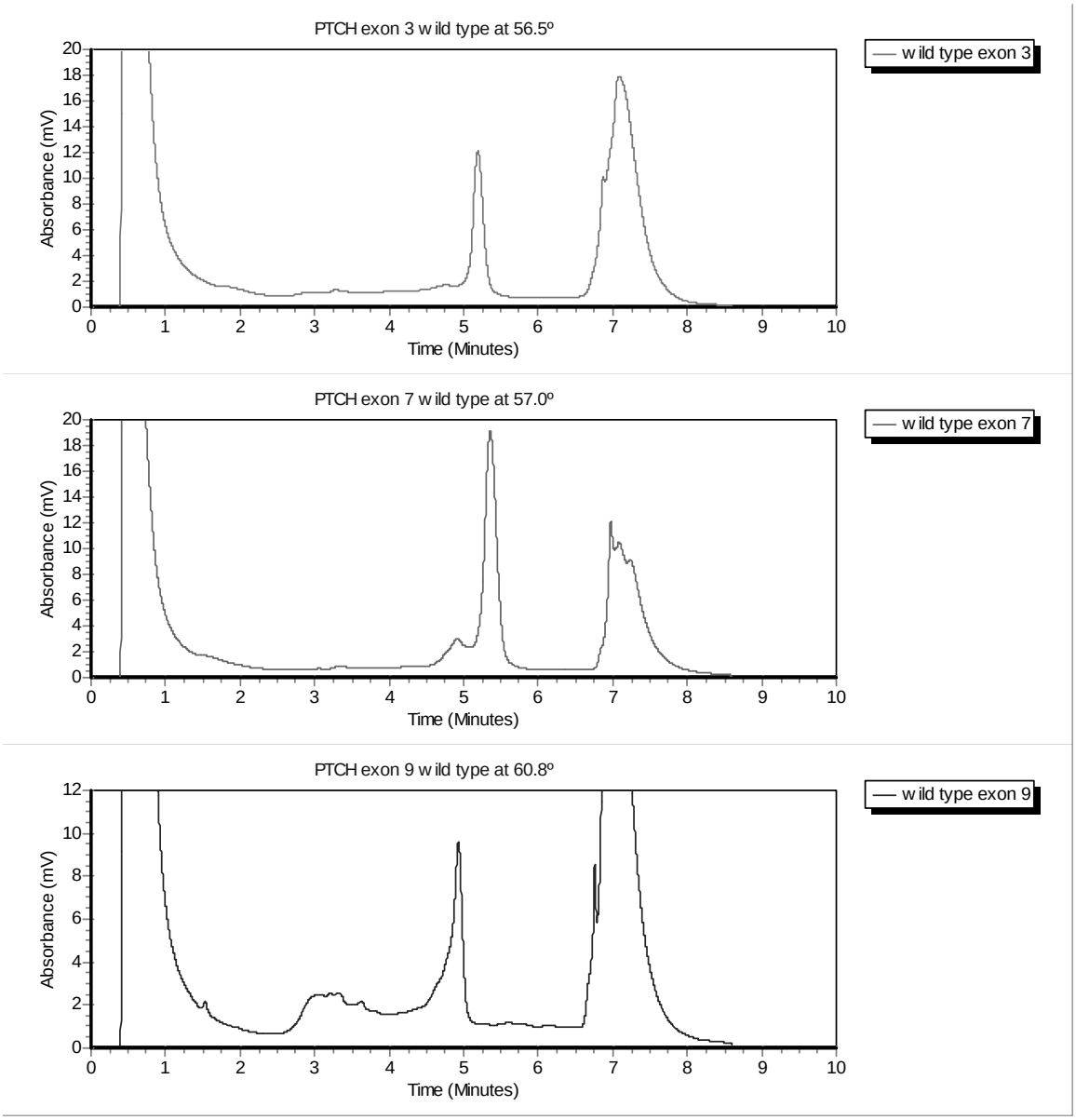
		Examination season proband				Total
		Summer	Autumn	Winter	Spring	
Examination season sibling	Summer	6	2	1	0	**9**
	Autumn	2	8	1	0	**11**
	Winter	5	1	4	0	**10**
	Spring	0	1	0	1	**2**
Total		**13**	**12**	**6**	**1**	**32**

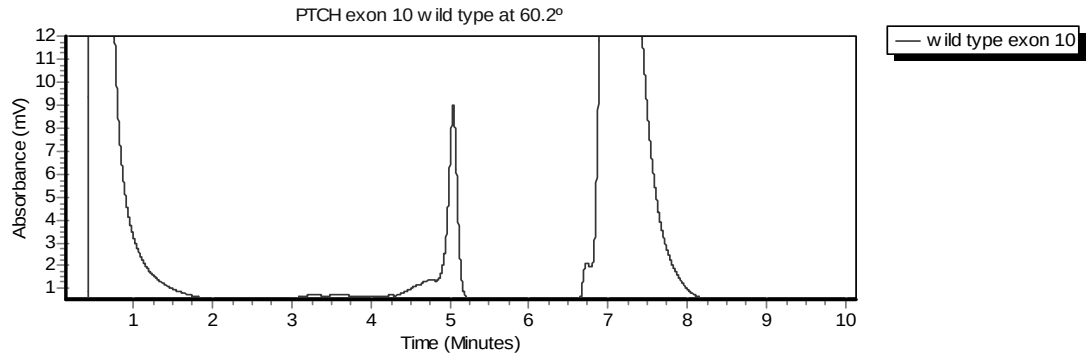
Note: number of pairs examined in different seasons compared by taking number pairs below and above the diagonal (where season matched) and comparing these two numbers (4 above, 9 below) by McNemar Test.

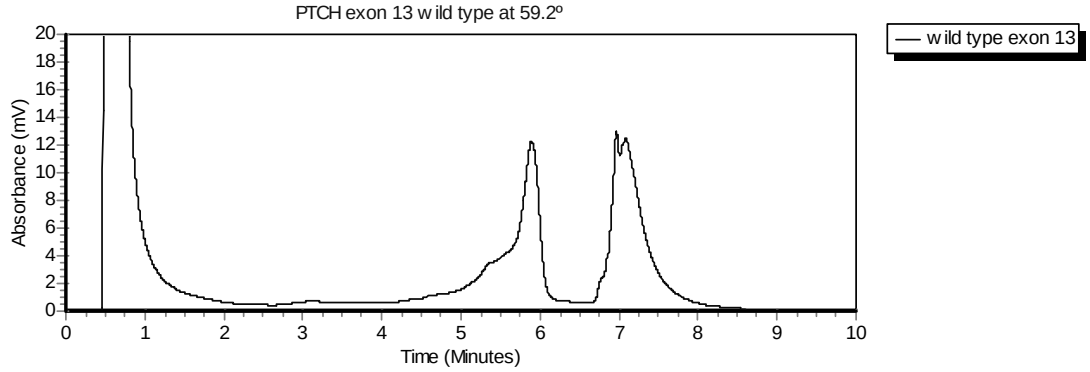
Table 7.13 Comparison of Minolta Spectrophotometer and BYK Gardner spectrophotometer L*a*b* readings on three subjects

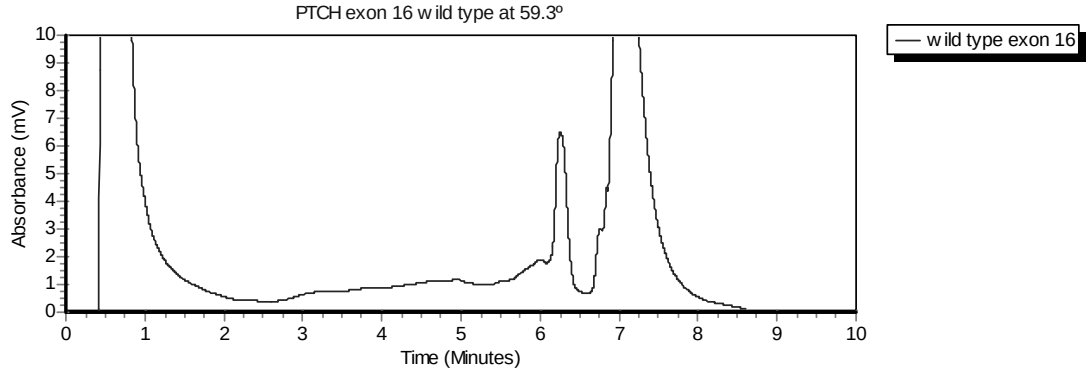
Subject		**Minolta CM-508d**		**BYK Gardner CGSS**	
	n=10	Mean	SD	Mean	SD
A	L*	60.099	1.284	60.931	0.263
	a*	10.473	0.718	11.026	0.297
	b*	18.719	0.96	19.43	0.158
B	L*	61.192	0.205	62.516	0.261
	a*	9.451	0.087	9.123	0.093
	b*	15.966	0.241	16.105	0.357
C	L*	62.504	0.211	64.354	0.26
	a*	9.408	0.45	8.834	0.349
	b*	15.102	0.263	15.659	0.148

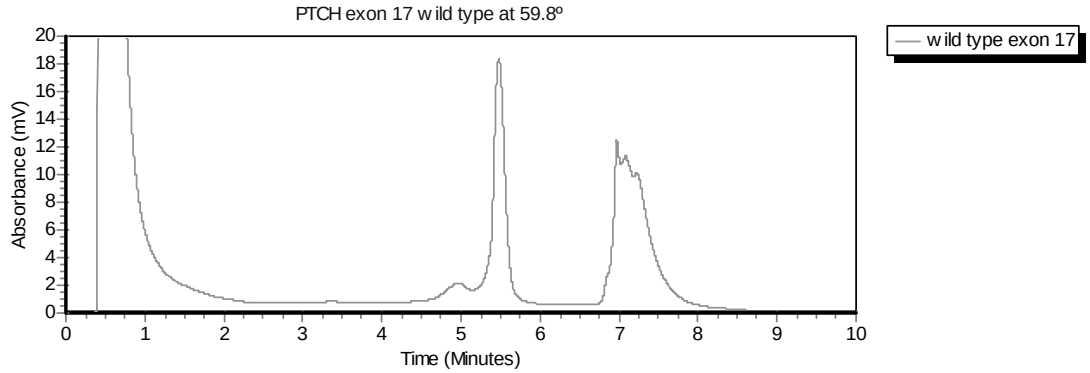
7.9. Elution profiles PTCH exons without variants (exons 3, 7, 9, 20, 13, 16, 17, 18, 20, 21, 22, 23b)

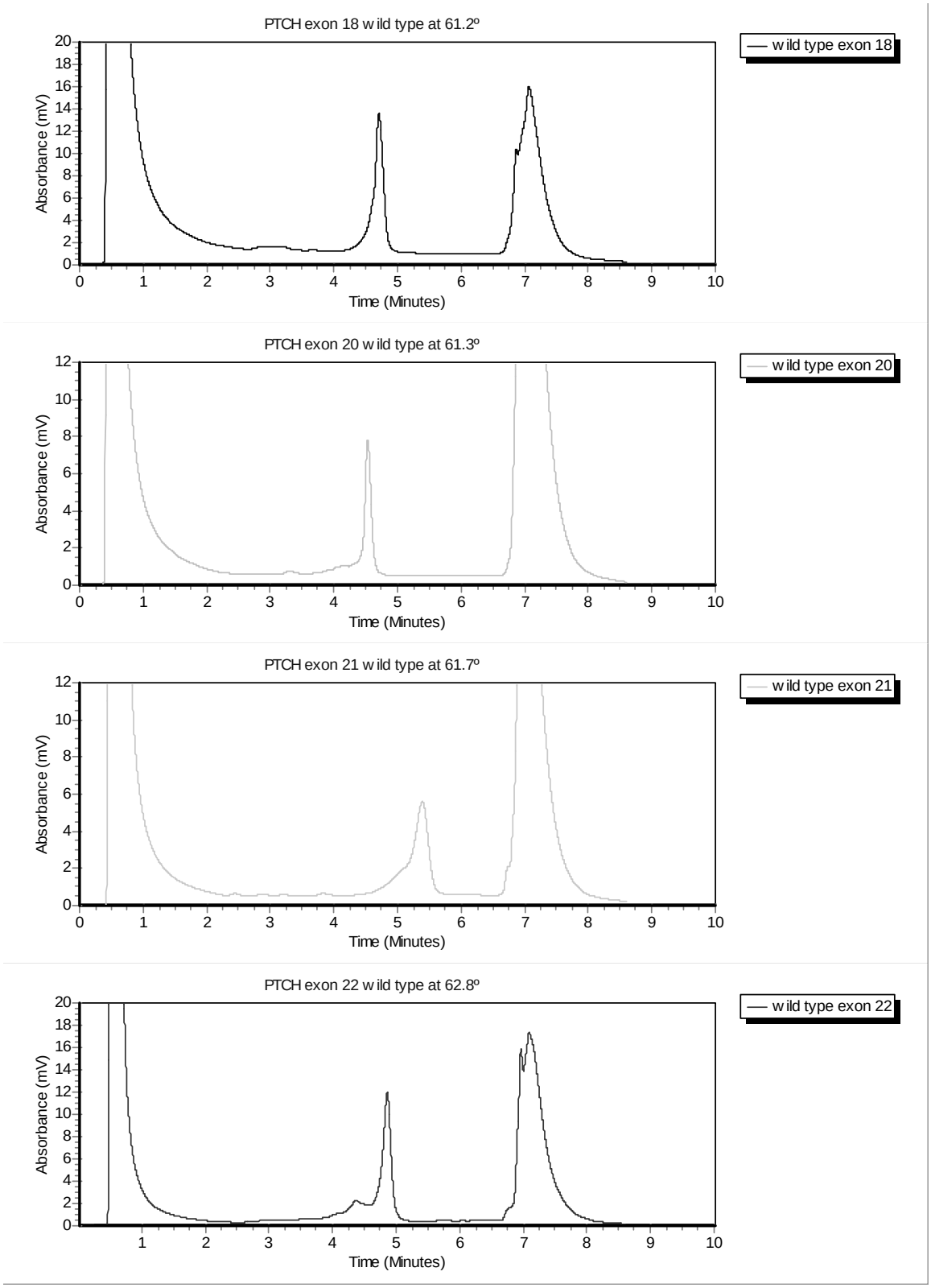



PTCH exon 10 wild type at 60.2º
Absorbance (mV)
Time (Minutes)
wild type exon 10


PTCH exon 13 wild type at 59.2º
Absorbance (mV)
Time (Minutes)
wild type exon 13


PTCH exon 16 wild type at 59.3º
Absorbance (mV)
Time (Minutes)
wild type exon 16


PTCH exon 17 wild type at 59.8º
Absorbance (mV)
Time (Minutes)
wild type exon 17


PTCH exon 18 wild type at 61.2º
wild type exon 18
Absorbance (mV)
Time (Minutes)
PTCH exon 20 wild type at 61.3º
wild type exon 20
Absorbance (mV)
Time (Minutes)
PTCH exon 21 wild type at 61.7º
wild type exon 21
Absorbance (mV)
Time (Minutes)
PTCH exon 22 wild type at 62.8º
wild type exon 22
Absorbance (mV)
Time (Minutes)

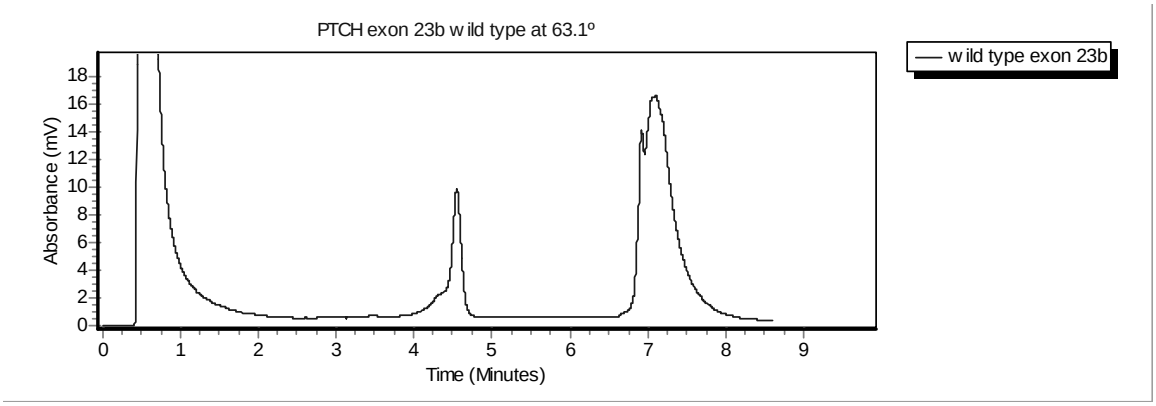
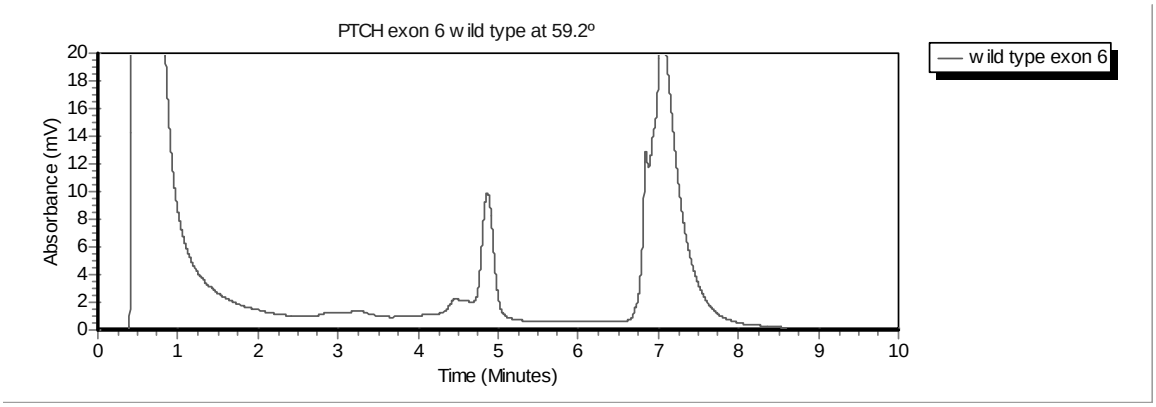


Figure 7.1 dHPLC elution profiles for PTCH exons 3, 7, 9, 13, 16, 17, 18, 20, 21, 22, and 23b s.
Elution time in minutes (X-axis) versus absorbance of the eluate in mV (Y-axis). The first peak is the sample injection peak; the second peak represents the PCR product elution peak, ideally found between 4-6 minutes; the third peak is an acetonitrile wash-peak. Some of the elution peaks appear to have a 'shoulder'; this is usually caused by the presence of AmpliTaq Gold. The small irregularities between the sample injection peak and the sample elution peak are primer-dimer artefacts [403] .

7.10. Elution profiles and sequences for PTCH exons with variants

Exon 6

A



B

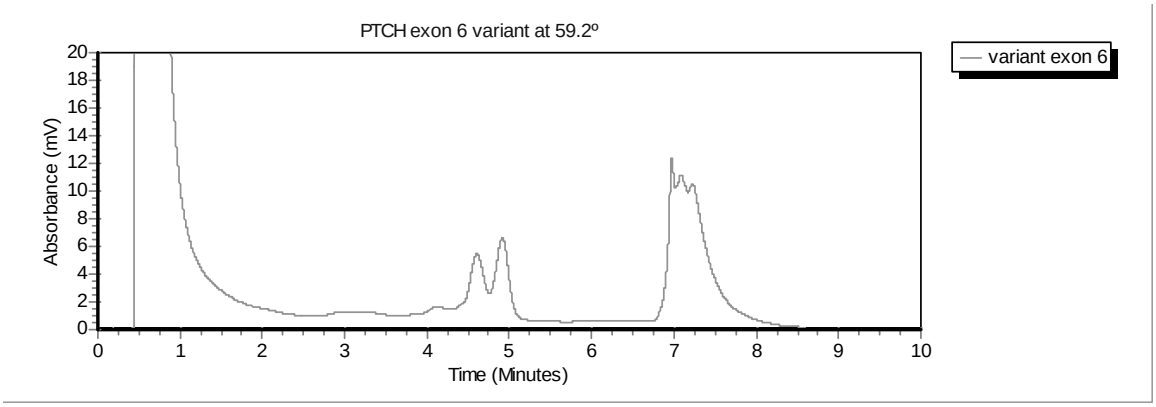


Figure 7.2 A-B dHPLC elution profile for PTCH exon 6, wild type (A) and variant (B).

B. Similar elution profiles to this were obtained for 26 samples

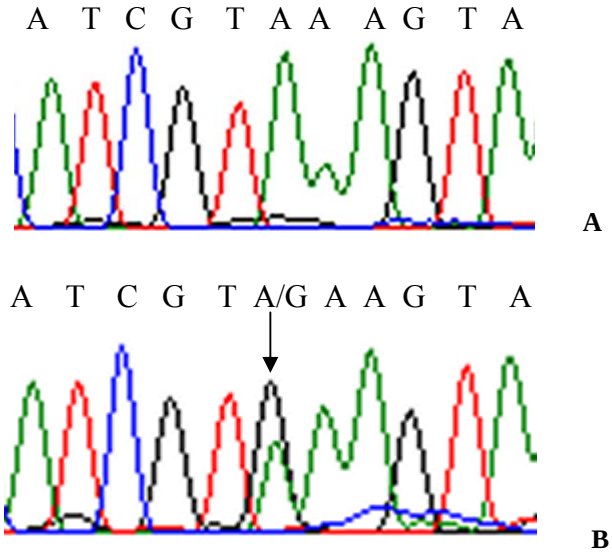
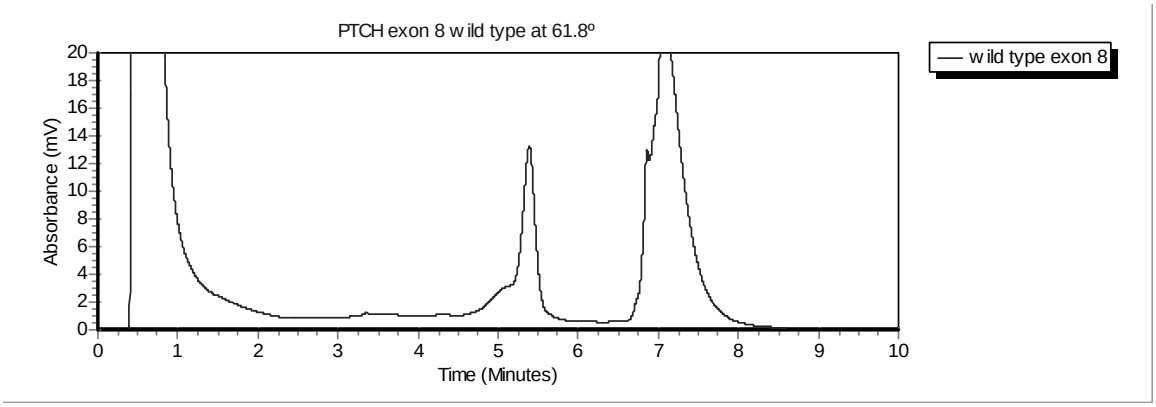


Figure 7.3 A-B Reverse sequence exon 6 wild type (A) and variant (B)
B The arrow indicates the IVS6-55 A>G variant.
NOTE: This section of forward sequence not visualized on sequencing therefore reverse shown

Exon 8



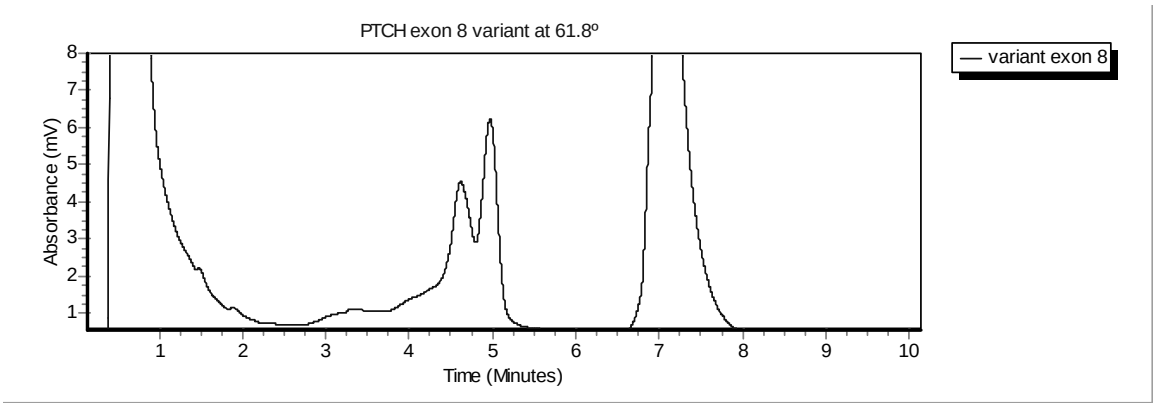


Figure 7.4 A-B dHPLC elution profile for PTCH exon 8, wild type (A) and variant (B).
Heteroduplexes have different retention properties to the dHPLC column than homoduplexes, creating an aberrant sample elution peak. The heteroduplexes are eluted from the colomn prior to the homoduplexes because of their reduced melting temperatures [403].

Exon 11

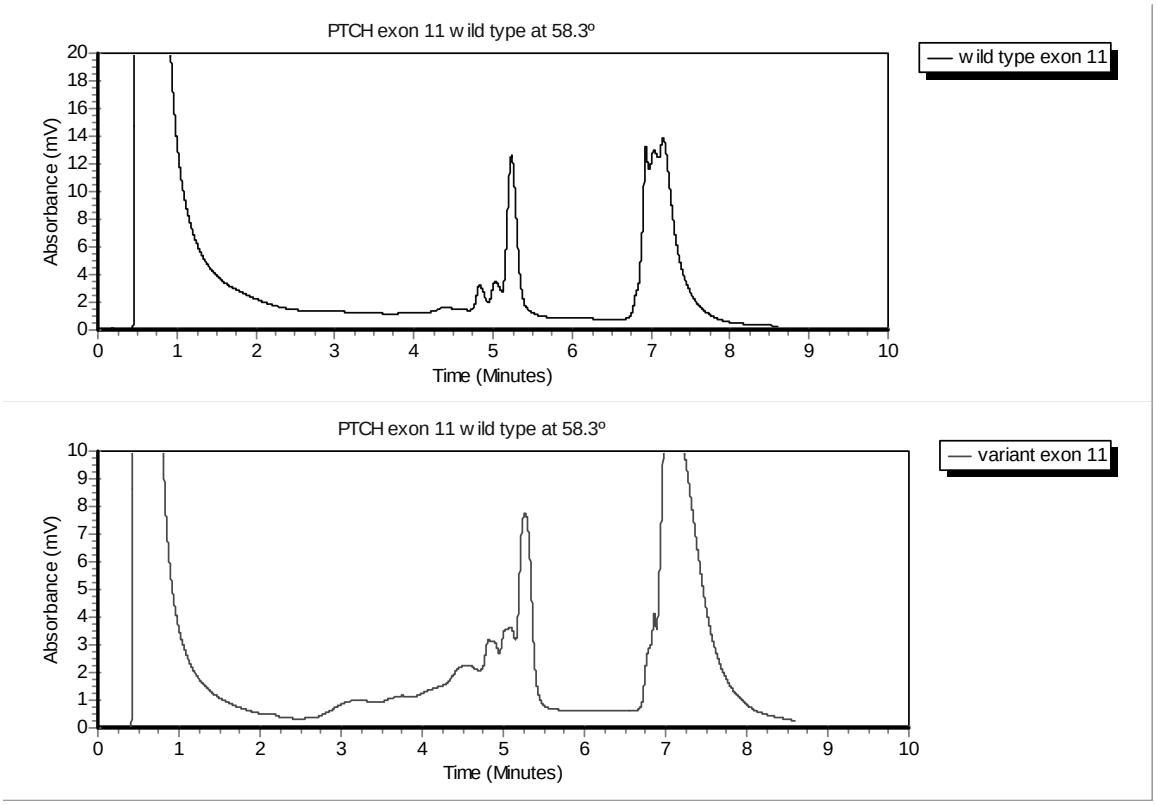


Figure 7.5 A-B dHPLC elution profile for PTCH exon 11, wild type (A) and variant (B)
B Similar elution profiles to this were obtained for 25 samples

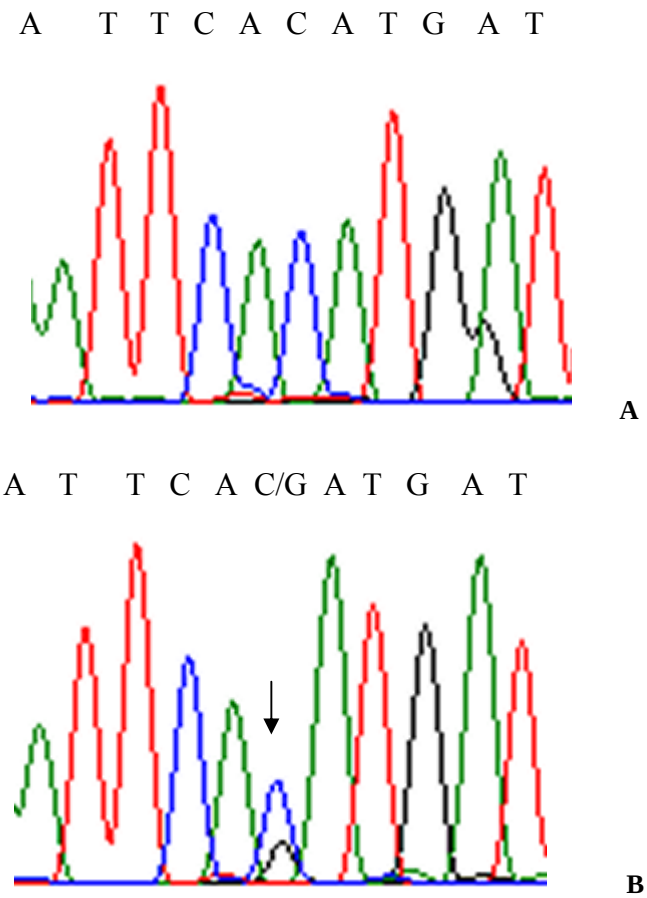
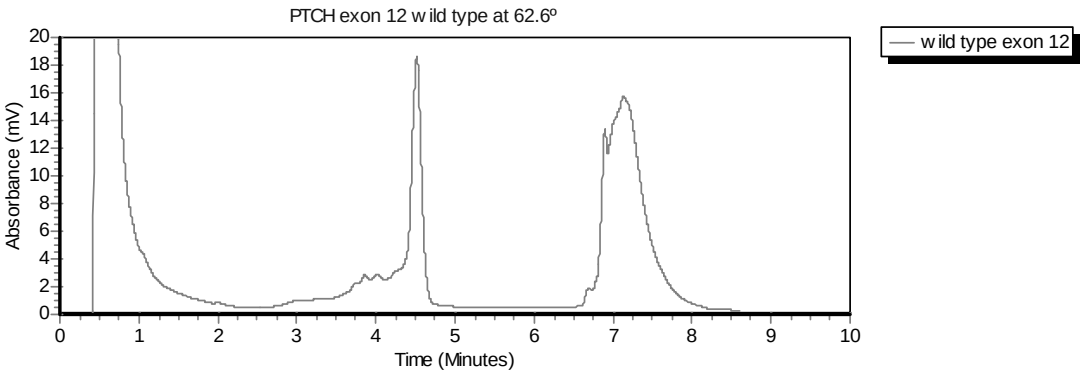


Figure 7.6 A-B Reverse sequence exon 11 wild type (A) and variant (B)
B The arrow indicates the IVS11-50 C>G variant.

Exon 12



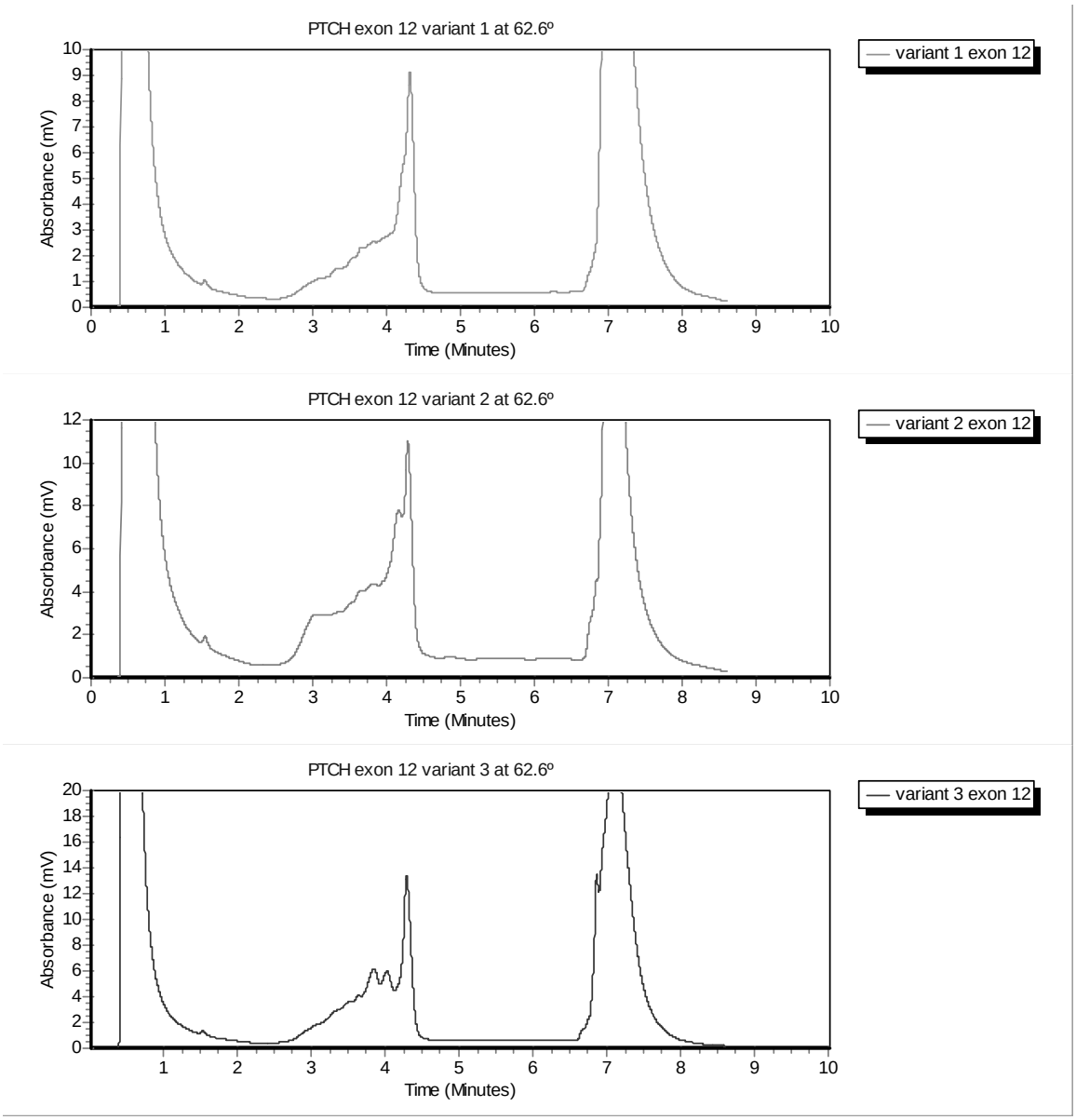
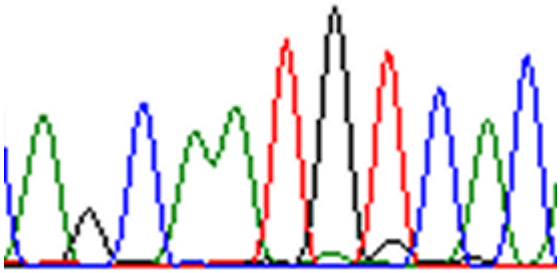


Figure 7.7 A-D dHPLC elution profiles for PTCH exon 12, wild type (A), variant 1(B), variant2 (C), and variant 3 (D)

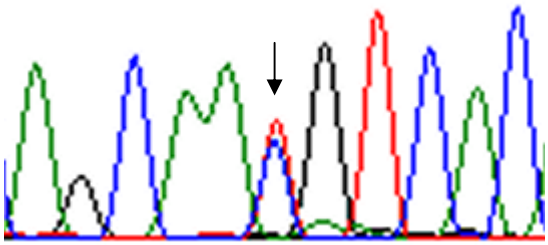
B Similar elution profiles to this were obtained for 8 samples [give all ID numbers?]
C Similar elution profiles to this were obtained for 16 samples
D Similar elution profiles to this were obtained for 5 samples

A G C A A T G T C A C



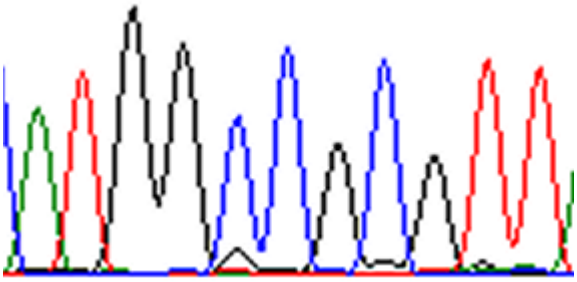
A

A G C A A T/C G T C A C



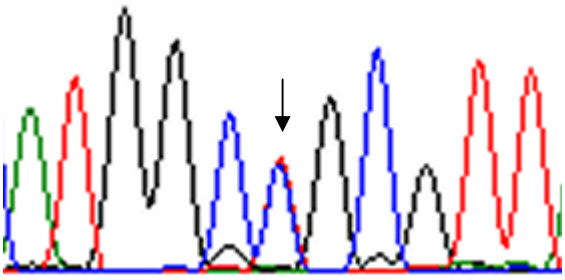
B

A T G G C C G C G T T

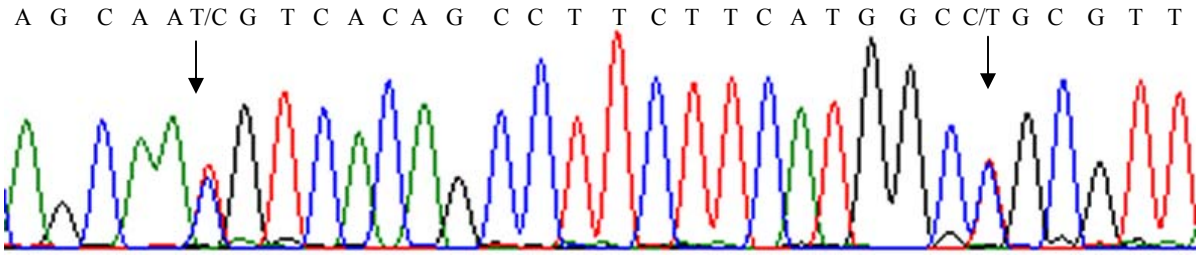


C

A T G G C C/T G C G T T



D



E

Figure 7.8 A-E Forward sequence exon 12 wild type (A, C) and variants (B,D,E)

B The arrow indicates T>C 63.

D The arrow indicates C>T 84

E The arrows indicate a combination of T>C 63 and C>T 84 within the same sequence. This occurred in 5 proband samples

Exon 23a

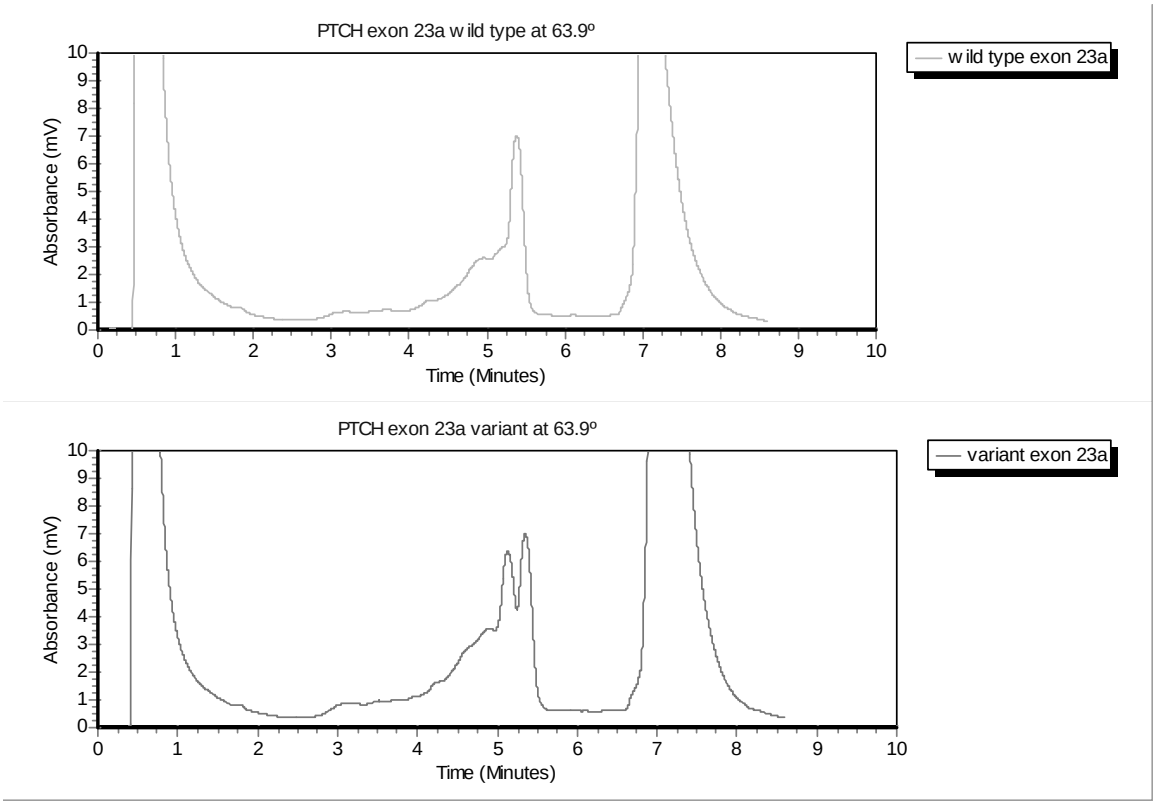
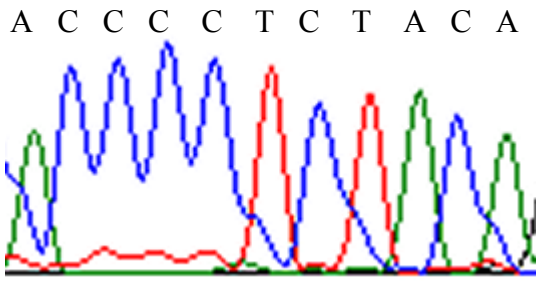
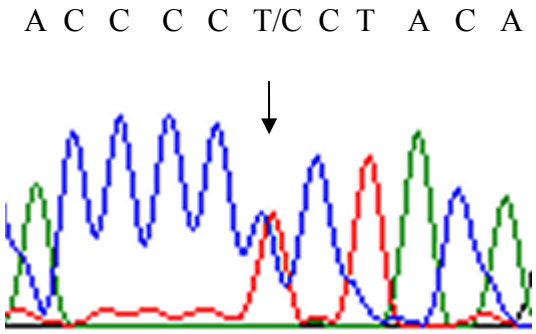


Figure 7.9 A-B dHPLC elution profile for PTCH exon 23a, wild type (A) and variant (B)

B Similar elution profiles to this were obtained for 26 samples



A



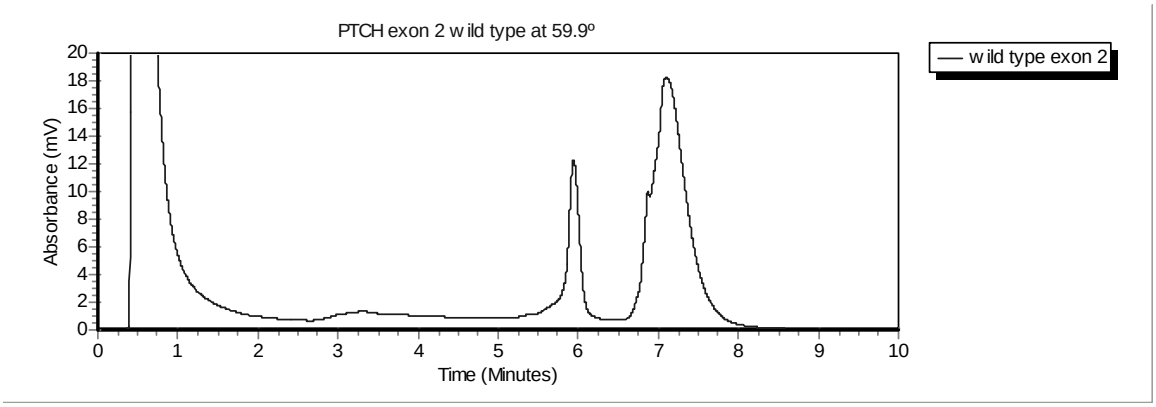
B

Figure 7.10 A-B. Forward sequence exon 23a, wild type (A) and variant (B)
B The arrow indicates the T>C 140 variant

7.10.1. Elution profiles and sequences for exons with Australian control data

Exon 2

A



B

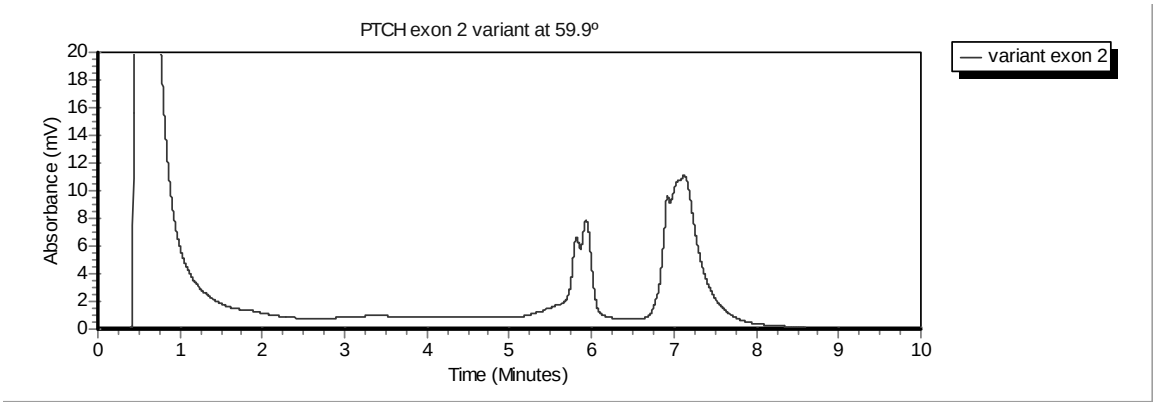


Figure 7.11 A-B dHPLC profile for PTCH exon 2, wild type (A) and variant (B)
B Similar elution profiles to this were obtained for both 8784 and 9208

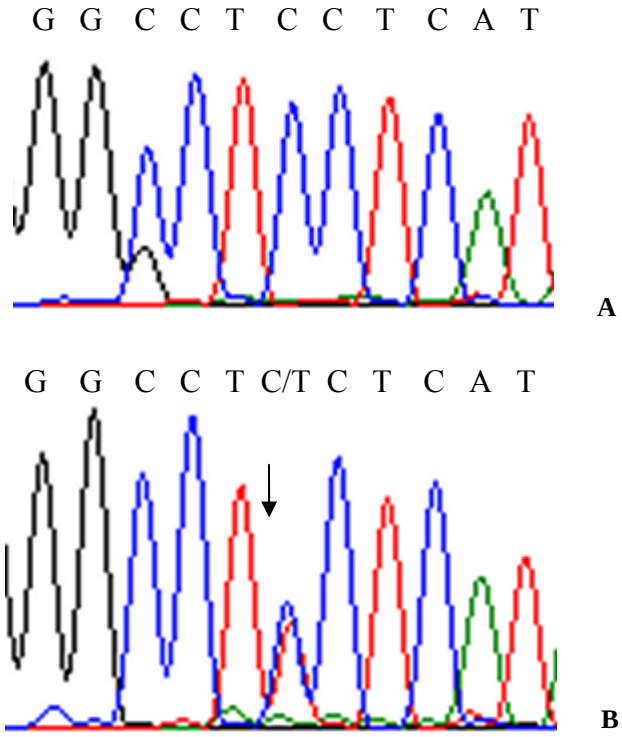


Figure 7.12 A-B. Forward sequence exon 2, wild type (A) and variant (B)
B The arrow indicates the C>T 116 variant

A

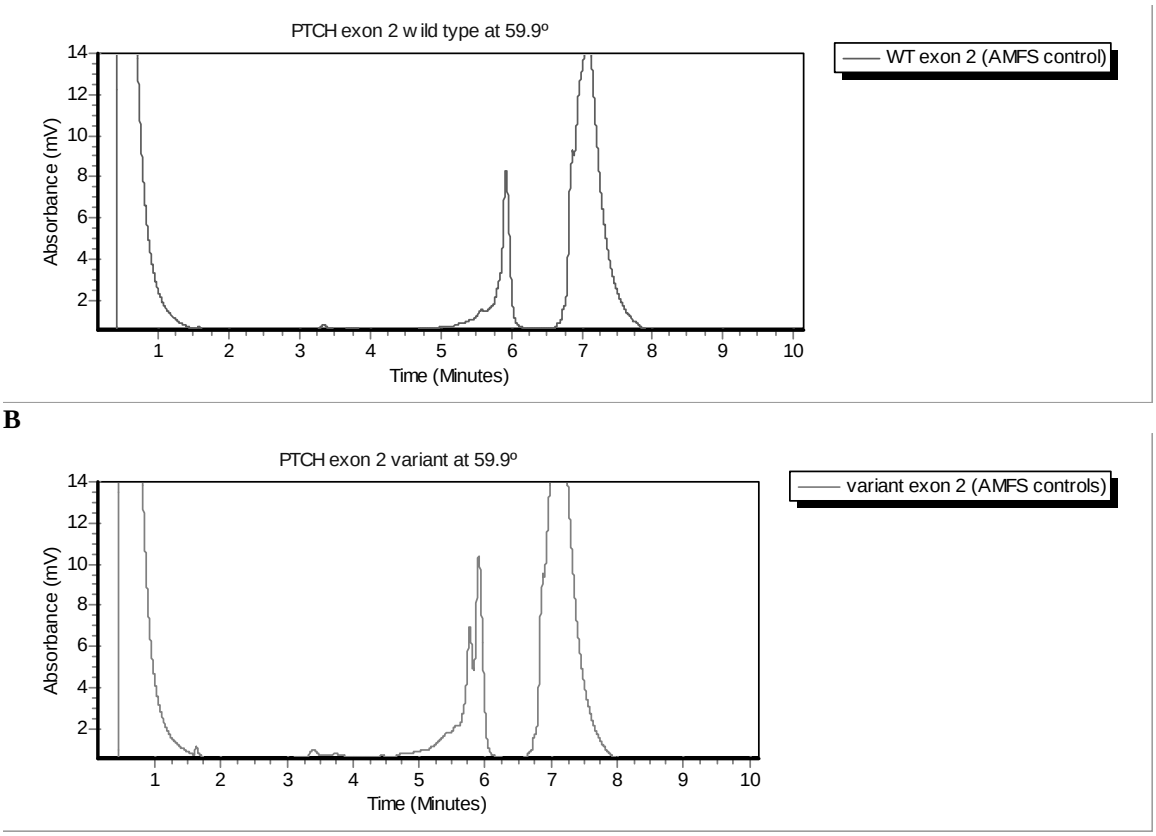


Figure 7.13 A-B dHPLC elution profiles for AMFS controls PTCH exon 2; wild type (A) and variant (B)
B Variant looks similar to variant profile of probands

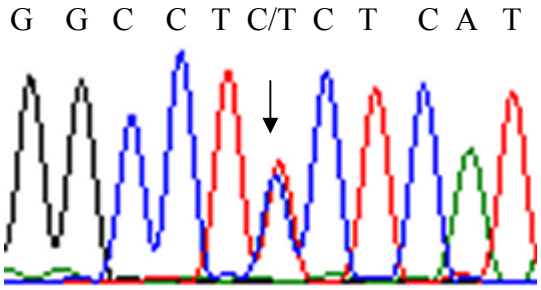


Figure 7.14 Forward sequence exon 2 AMFS control, variant
The arrow indicates the C>T 116 variant

Exon 5

A

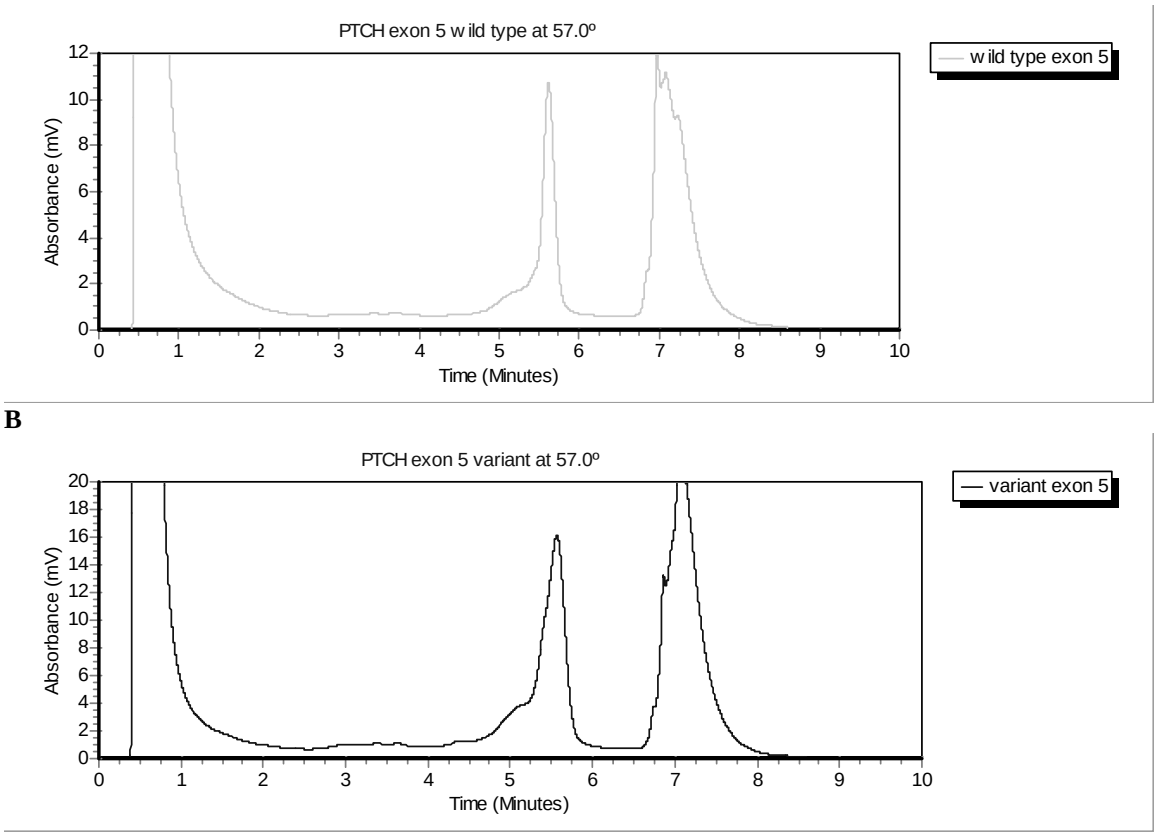
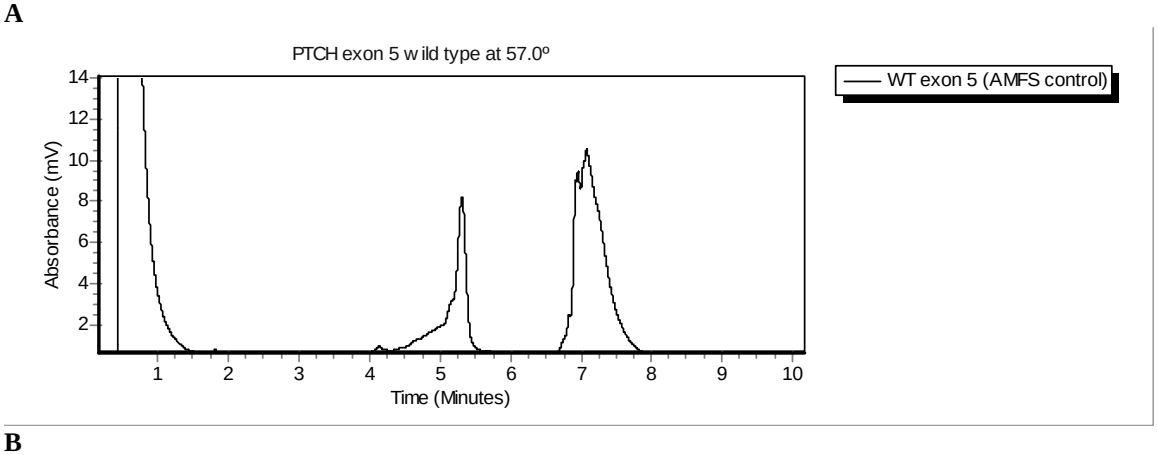


Figure 7.15 A-B dHPLC elution profiles for PTCH exon 5, wild type (A) and variant(B)
B Similar elution profiles as this were obtained for samples with ID numbers 8781, 8825, 9094, 9273, 9274, and 8779



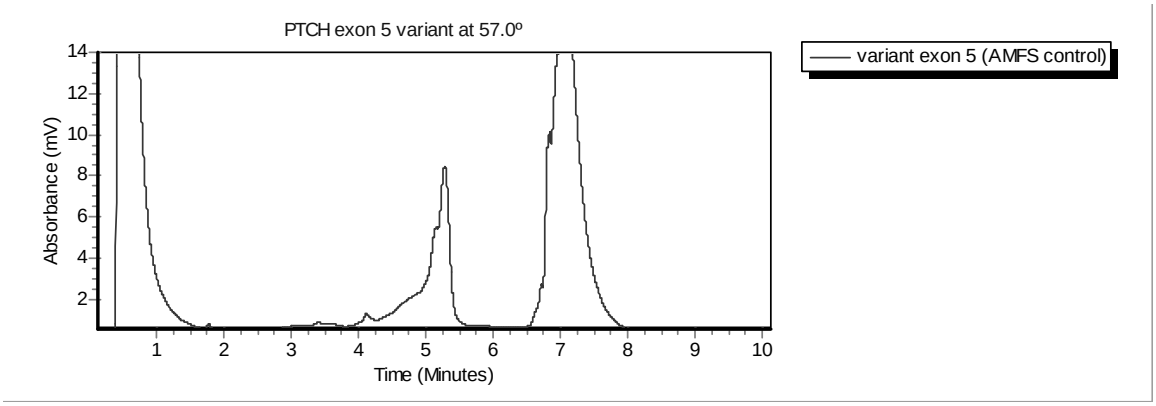


Figure 7.16 A-B dHPLC elution profiles for AMFS controls PTCH exon 5; wild type (A) and variant (B)
B Similar elution profiles to this were obtained for samples with ID numbers 11089, 21027, 7877, 6658, and 310

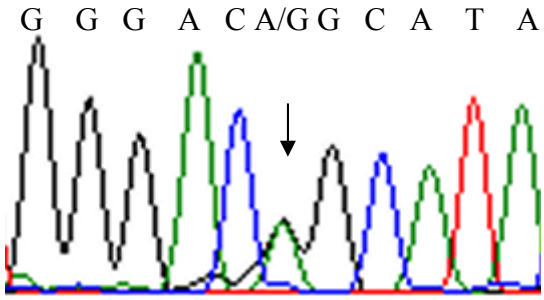
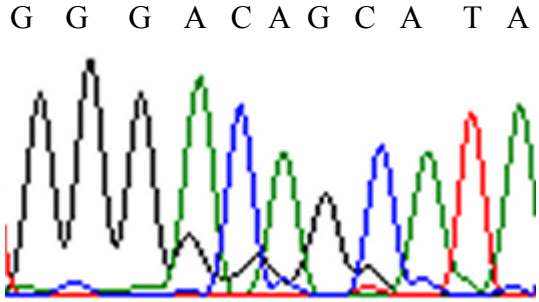
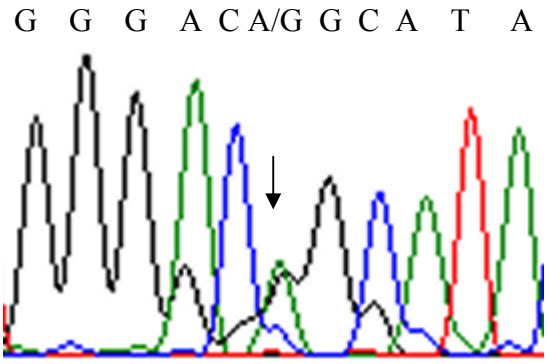


Figure 7.17 Forward sequence exon 5 AMFS control, variant
The arrow indicates the A>G 80 variant



A



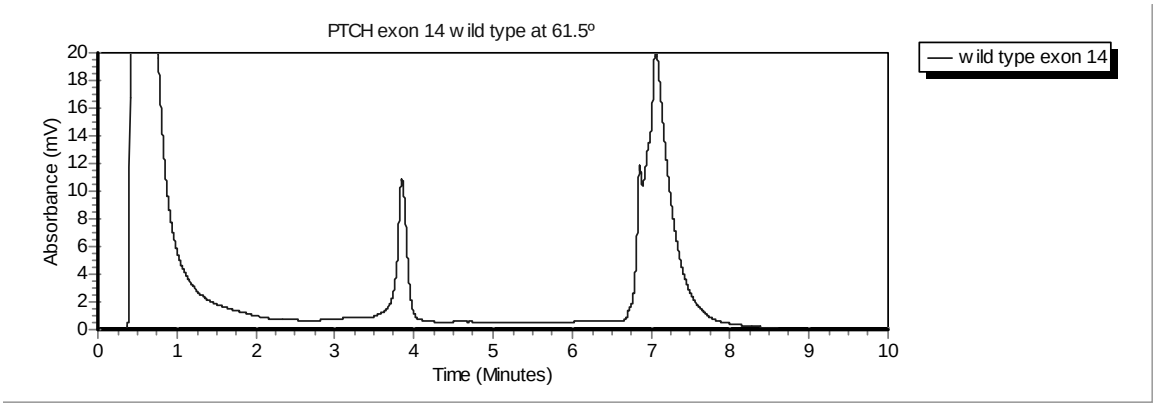
B

Figure 7.18 A-B Forward sequence exon 5, wild type (A) and variant (B)

B The arrow indicates the A>G 80 variant

Exon 14

A



B

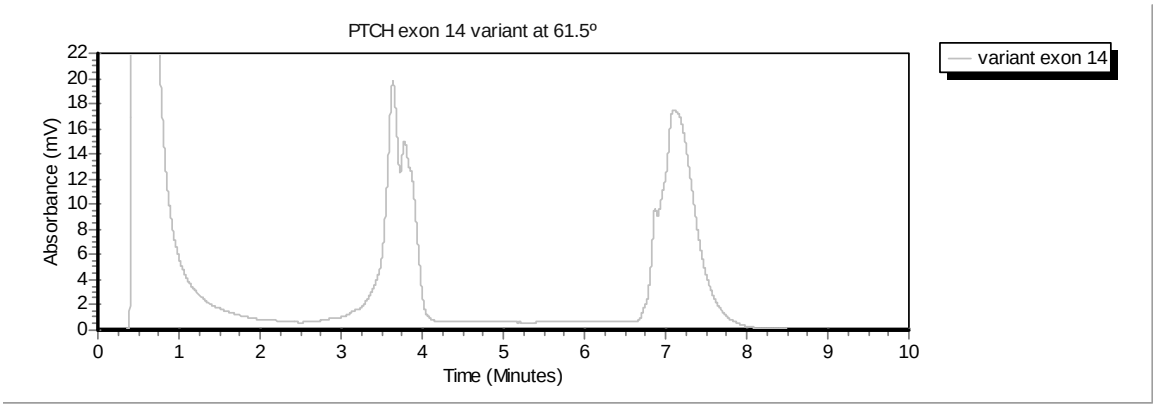
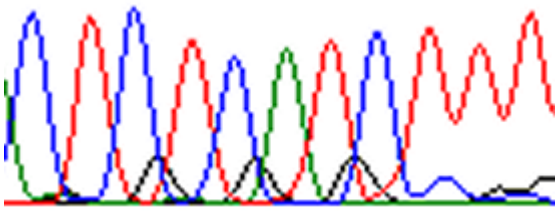
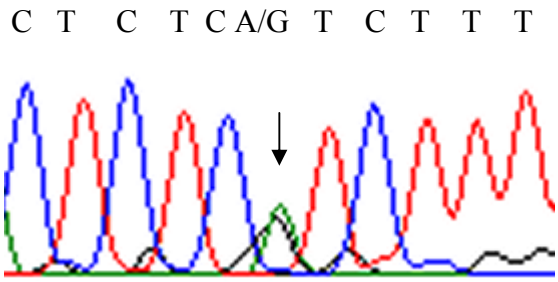


Figure 7.19 A-B dHPLC profile for PTCH exon 14, wild type (A) and variant (B)
B Elution profile for sample ID 9465

C T C T C A T C T T T



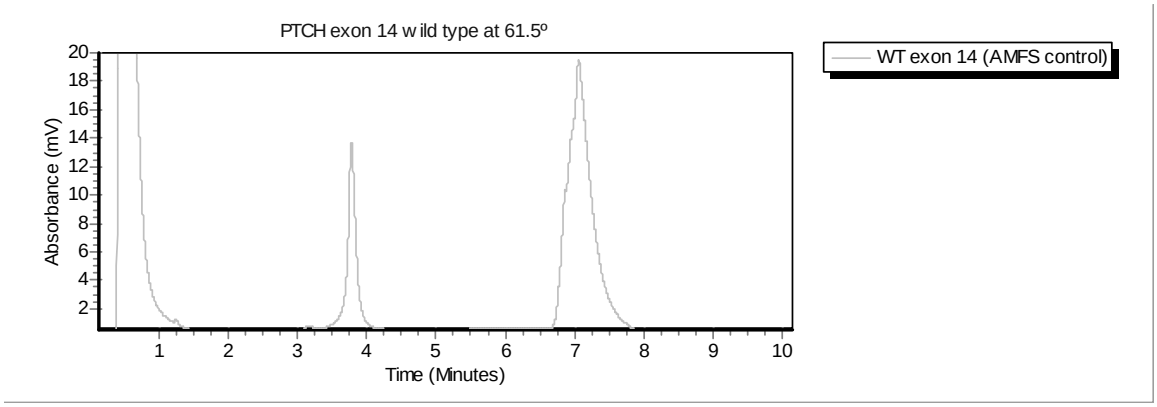
A



B

Figure 7.20 A-B Forward sequence exon 14, wild type (A) and variant (B)
B The arrow indicates the A>G 351 variant

A



B

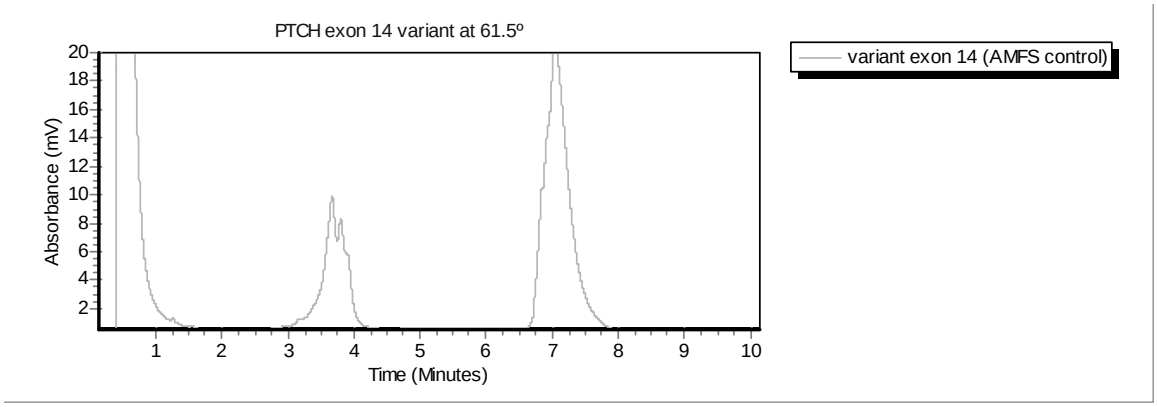


Figure 7.21 A-B dHPLC elution profiles for AMFS controls PTCH exon 14; wild type (A) and variant (B)
B Similar elution profiles to this were obtained for samples with ID numbers 12835 and 12836

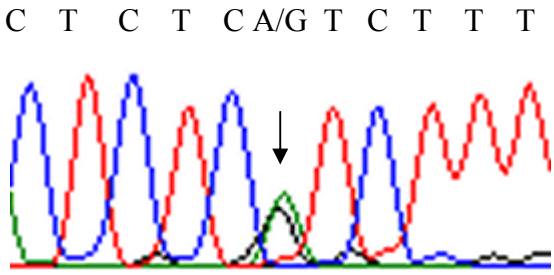
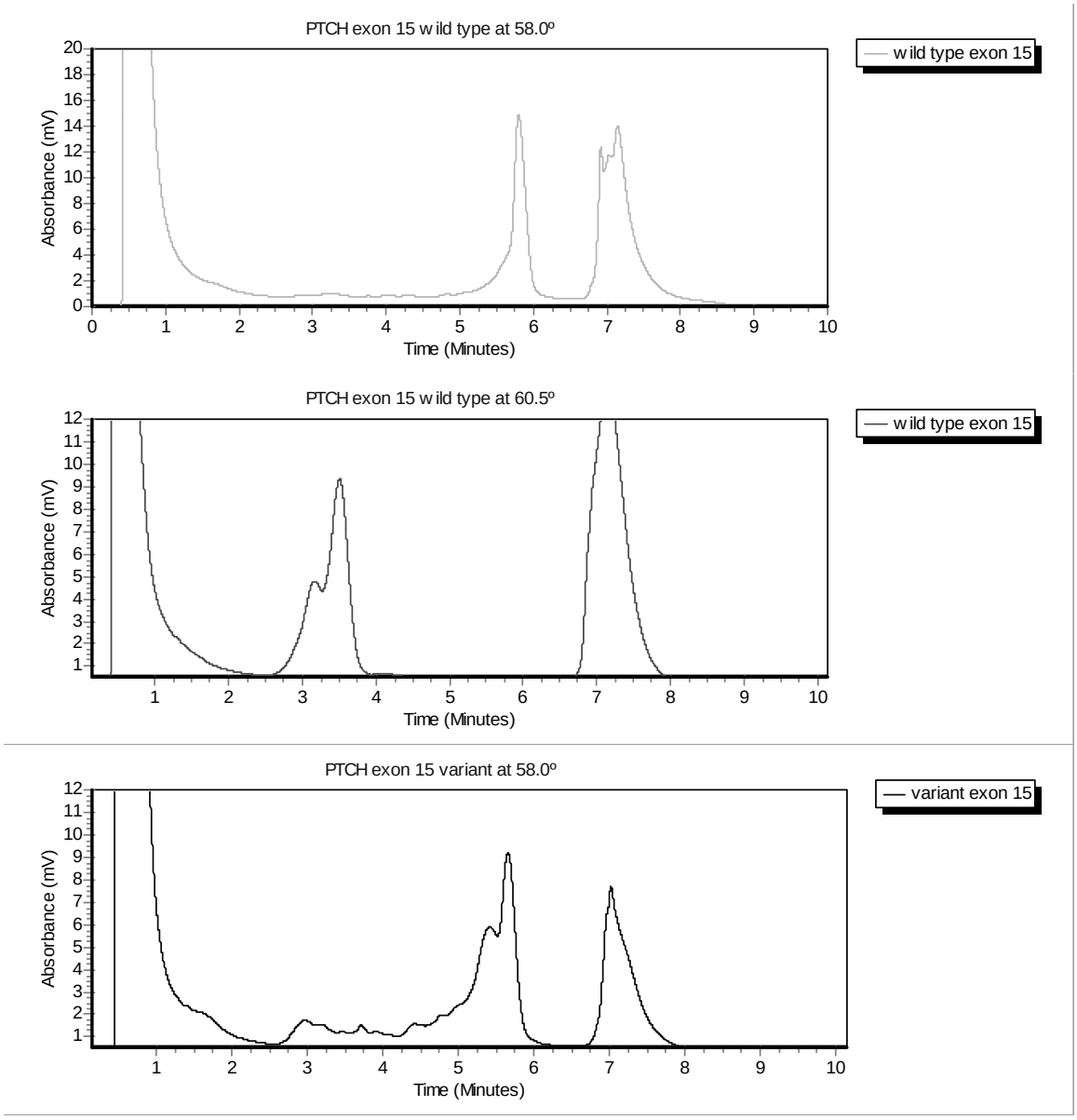


Figure 7.22 Forward sequence exon 14 AMFS control, variant
The arrow indicates the G>A 351 variant

Exon 15


PTCH exon 15 wild type at 58.0º
wild type exon 15
Absorbance (mV)
Time (Minutes)
PTCH exon 15 wild type at 60.5º
wild type exon 15
Absorbance (mV)
Time (Minutes)
PTCH exon 15 variant at 58.0º
variant exon 15
Absorbance (mV)
Time (Minutes)

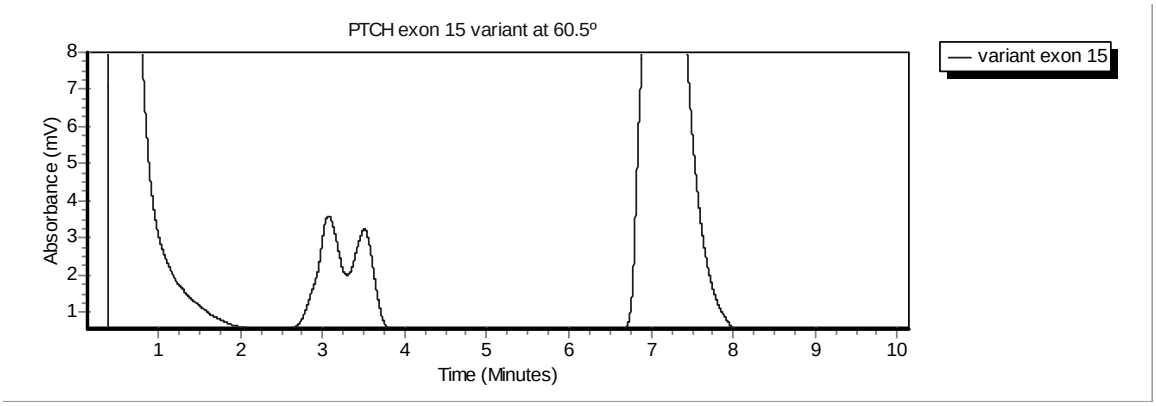


Figure 7.23 A-D dHPLC elution profile for PTCH exon 15, wild types (A, B) and variants (C, D)
C, D Same sample seen at two temperatures. Similar elution profiles seen at both temperatures for all 8 variants.

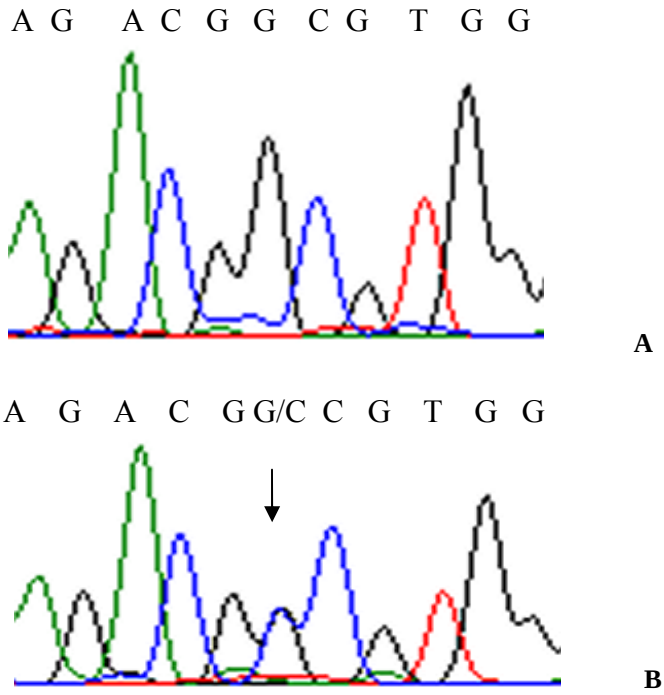


Figure 7.24 A-B. Forward sequence exon 15, wild type (A) and variant (B)
B The arrow indicates the IVS15+9 G>C variant

Chapter 8: References

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