Molecular Pathology as a Tool to Determine Features of Clinico-Pathological Progression and Malignancy in Melanocytic Neoplasia.

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Abstract

Understanding tumour progression in cutaneous malignant melanoma is important because the stage of the disease dramatically affects prognosis. This thesis broadly comprises two investigations of melanocytic tumour progression.

1. The signalling ligand Wnt5a has been shown to promote motility and invasiveness in melanoma *in vitro*. To expand upon this data, expression of Wnt5a was examined in matched and unmatched tumour samples comprising different stages of melanoma progression. In order to place the alterations of Wnt5a in context, expression of $p16^{INK4a}$ and mutations of B-raf exon 15 were determined. The effect of Wnt5a expression on outcome was assessed. Wnt5a demonstrated a trend of increasing expression with progression ($p = 0.013$) however, expression in naevi was comparatively strong. When this was compared with $p16^{ink4a}$ expression and BRAF mutation, the changes supported a multi-step process of carcinogenesis. Survival analysis showed that Wnt5a is a an independent marker of time to metastasis ($p=0.041$) and death ($p=0.047$)

2. B-raf and N-ras mutations are common in most melanocytic tumours, but are rare in Spitz naevi, where occasional H-ras mutations are found. The hypotheses were that Spitzoid tumours comprise a distinct subgroup of lesions that progress in the absence of B-raf and N-ras mutations, unlike most other melanocytic tumour types; but that progression from Spitz naevus to Spitzoid melanoma is unlikely because there is no subset of H-ras mutant Spitzoid melanomas. B-raf and N-ras mutations were found at lower frequency in Spitzoid melanomas ($p=0.009$) and in all Spitzoid tumour types ($p=0.001$) compared to non-Spitzoid melanomas, indicating that Spitz naevi and non-classical Spitzoid tumours are genetically distinct. H-ras mutations were not seen in malignant Spitzoid and non-Spitzoid melanomas indicating that H-ras mutant Spitzoid tumours do not progress to malignancy.

The findings of these investigations demonstrate the utility of molecular pathology in characterising neoplastic progression in archival melanocytic tumour tissue.

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Many thanks to my family for their immeasurable support over the years and I can only apologise for not becoming a proper doctor. Most importantly, thank you to Katharine for being so supportive and putting up with me during the difficult times.

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Abbreviations

ABC	streptavidin-biotin complex
AJ	Professor Sir Alec Jeffreys
AJCC	American Joint Committee on Cancer
ALM	acral lentigenous melanoma
ATCC	American Type Culture Collection
BAC	bacterial artificial chromosome
bp	base pairs
CAN	common acquired naevus
CDK	cyclin dependent kinase
CGH	comparative genomic hybridisation
CI	confidence interval
CISH	colorimetric *in situ* hybridisation
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
GDP	guanosine diphosphate
GSS	Dr Gerald S Saldanha
GTP	guanosine triphosphate
H+E	haematoxylin and eoisin
HCl	hydrochloric acid
HR	hazard ratio
IAA	isoamylalcohol
ICC	intra-class correlation coefficient
IMS	industrial methylated spirit
ISH	*in situ* hybridisation
JHP	Dr J Howard Pringle
kb	kilobase

LCM	laser capture microdissection
LMM	lentigo maligna melanoma
LOH	loss of heterozygosity
MAPK	mitogen activated protein kinase
MDE	mutation detection enhancement
MLPA	multiplex ligation-dependent probe amplification
mm	millimetres
mM	millimoles
NBT-BCIP	nitro blue tetrazolium chloride – 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt
NCBI	National Centre for Biotechnology Information
PCR	polymerase chain reaction
PDF	Dr Philip Da Forno
RGP	radial growth phase
RPM	revolutions per minute
SD	standard deviation
SSCP	single-stranded conformational polymorphism
SSMM	superficial spreading malignant melanoma
SPSS	statistical package for the social sciences
STUMP	Spitzoid tumour of uncertain malignant potential
TAE	tris acetate buffer
TBE	tris borate EDTA buffer
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
UPR	unfolded protein response
UV	ultraviolet
VGP	vertical growth phase
VAB	veronal acetate buffer

V	volts
v/v	volume by volume
w/v	weight by volume
W	watts
Yrs	years

1 General Introduction

1.1 Cutaneous malignant melanoma

The genetic basis of melanocytic tumours has interested scientists for a long time. In 1820 William Norris, a general practitioner from the British Midlands, described a pedigree where many members had large numbers of 'moles', many of whom subsequently died from tumours that originated within them[1]. Countless other observations concerning progression in melanocytic tumours have been added to the literature since this time and yet the collection of malignant melanocytic lesions that together are called cutaneous melanoma continue to provide challenges for scientists and clinicians alike (hereafter, all use of 'melanoma' implies cutaneous melanoma unless otherwise stated).

Disease progression is an important aspect of melanoma biology because prognosis varies considerably depending on the stage of progression in the primary lesion and the presence or absence of metastatic disease[2-6]. This thesis examines progression in melanocytic tumours by applying molecular pathological techniques to archival tissue samples from a range of melanocytic tumours. Firstly, there is an investigation of Wnt5a expression during melanoma progression, followed by correlation of the expression data with clinical outcome. Studies suggest that Wnt5a, a signalling ligand, may be important in mediating aggressive behaviour in melanoma[7-9]. Next, there is an investigation of Spitzoid tumours, a specific subset of melanocytic lesions in which malignancy can be difficult to predict. The hypothesis here is that Spitzoid tumours are a genetically distinct subset of tumours that progress to malignancy in an alternative manner to non-Spitzoid melanocytic tumours.

Epidemiological data suggests that the need to learn about progression in melanoma is becoming increasingly important. Epidemiological aspects of cutaneous melanoma in the United Kingdom are described in the following section.

1.1.1 Epidemiology of cutaneous melanoma in the United Kingdom

Melanoma is the least common of the main skin cancers but accounts for the majority of skin cancer deaths. With 8100 new cases per year, melanoma represents 3% of all cancers[10]. In the last 25 years the incidence of melanoma has increased more than that of any other cancer, with the rate during this time quadrupling in males and tripling in females (Figure 1-1). Whilst the incidence has increased in all ages, the rate of increase has been greatest in patients over the age of 65. This increased incidence has been mainly due to an increase in thin melanomas, which is partly the result of increased public awareness and surveillance of the disease. However, it is believed that some of the increase has arisen due to changes in exposure to the main environmental risk factor for cutaneous melanoma, the sun [10].

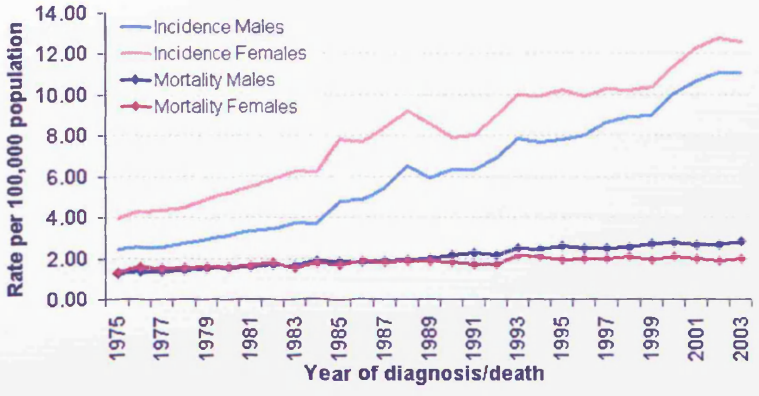


Figure 1-1 European age-standardised incidence and mortality rates, malignant melanoma, by sex, Great Britain, 1975-2004. Image taken from[10]

In 2005 there were 1817 deaths from melanoma with an age standardised mortality rate of 2.4 per 100,000 (95%CI 2.3-2.5)[10]. The mortality rate rises steadily with age but, as can be seen in Figure 1-2, deaths do occur in young people with 116 deaths in people under 40 years, and half of the deaths in patients under 70 years. The age standardised mortality rate has risen steadily in the past 30 years but not to the same extent as incidence, which reflects improvements in diagnosis and treatment.

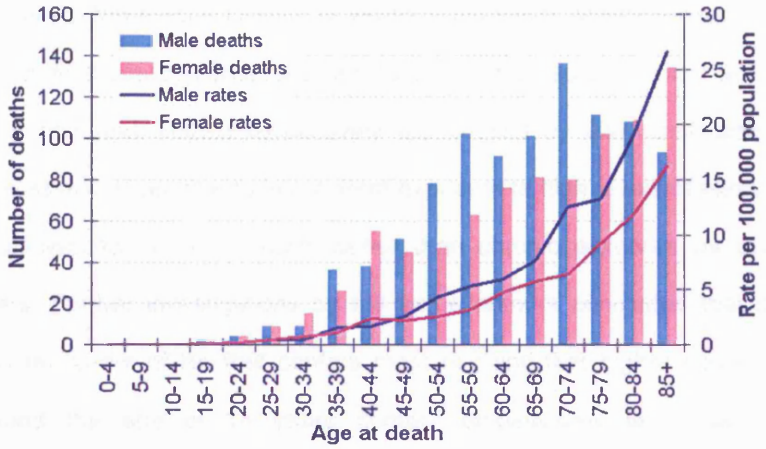


Figure 1-2 Number of deaths and age-specific mortality rates, malignant melanoma, by sex, UK, 2005. Image taken from[10]

The five-year relative survival for patients diagnosed with melanoma in 2000-01 was 78% for men and 91% for women. The trend of better survival in women than in men has been a consistent feature of epidemiological studies for the past 30 years[10]. It should be noted however, that survival is closely linked to tumour stage which, along with other important clinical spects of melanoma, will be discussed in the following section.

1.1.2 Risk factors, classification, and prognostication in cutaneous melanoma

1.1.2.1 Risk factors

The strongest risk factors for melanoma are a family history of melanoma, multiple benign or atypical naevi, and a previous melanoma[11]. In 25-40% of melanoma prone families, mutations of the CDKN2A gene are found, while a smaller percentage have germ line CDK4 mutations. The significance of these genes is discussed later, but the genetic characterisation of such kindreds has provided vital insights for the investigation of the molecular basis of melanoma. Other hereditary risk factors for melanoma include skin photo type, hair colour and eye colour[10]. Aside from inherited genetic predispositions, environmental risk factors for melanoma

have been identified, the most important of which is ultraviolet radiation exposure. Studies of the population in Australia by Armstrong and Kricker estimated that about 90% and 80% of cases in white men and women respectively are caused by ultraviolet radiation[12], and it appears that most risk arises due to intermittent exposure to high levels of ultraviolet radiation such as is sufficient to cause sunburn, rather than chronic exposure as experienced by outdoor workers. Further investigations by the same authors concluded that it is exposure during the first ten years of life that confers most risk and that higher doses of ultraviolet radiation beyond the age of 15 years confer comparatively less risk[13]. While the epidemiological evidence for the role of ultraviolet radiation exposure in melanoma is overwhelming, some key genetic mutations in melanoma do not conform to the common ultraviolet mutation signature[14].

1.1.2.2 Classification of cutaneous melanoma

Histologically, melanoma comprises a proliferation of atypical melanocytes that can either be confined to the epidermis and dermo-epidermal junction, or can, to varying degrees, invade the dermis, subcutis, and beyond[15]. Lesions can be classified clinicopathologically into one of four main categories namely; superficial spreading melanoma (SSMM), acral lentiginous melanoma (ALM), lentigo maligna melanoma (LMM) and nodular melanoma. SSMM is the most common variant, and is most frequently found on the back and legs[16]. ALM accounts for around 8% of all melanomas in Caucasians but is the predominant sub-type in Afro-Carribeans and Orientals. It is found on the digits, and plantar surfaces. LMM accounts for around 4% of cases, is typified by a prolonged *in situ* phase and typically develops on chronic sun damaged skin in the elderly. Nodular melanoma represents around 4% of tumours and clinically presents as a nodular or polypoid lesion that is often ulcerated. This morphological classification appears to have a genetic basis, with distinct differences seen between some tumour types[17, 18]. Whether such a classification is of prognostic significance remains to some degree a contentious issue. Multivariate analyses have shown that histological sub-type is not

of independent prognostic significance[19], yet some authors argue to the contrary[20] stating for example that prognosis in LMM is inherently better because they are more likely to be clinically obvious, have a prolonged *in situ* stage and are therefore curable by excision at an early stage compared to nodular melanoma, which tends to show quicker tumour progression.

Primary melanoma can also be classified clinicopathologically as being in the radial growth phase (RGP) or vertical growth phase (VGP). In the RGP, tumours are confined either entirely to the epidermis (in *in situ* melanoma) or lie predominantly in the epidermis with minimal invasion of the papillary dermis. By contrast, in VGP melanoma the tumour is centred on the dermis with an expansile dermal nodule containing mitotic activity that may supervene the pre-existing RGP. Nodular melanomas for example are considered to have little or no RGP and progress very early to the VGP[15]. Identifying the growth phase of a tumour is considered by some to be the most important determinant of prognosis[15, 21] and the transition from RGP to VGP is considered to be a crucial event in melanoma progression[6].

1.1.2.3 Prognostication

Determining the stage of melanoma is, as in most other malignancies, highly predictive of survival. Staging broadly comprises Stages I and II where the tumour is localised to the primary lesion with no evidence of regional or distant metastases, Stage III where there are regional lymph node metastases, and Stage IV where there are distant metastases. Fifteen-year survival curves of the different stages are shown in Figure 1-3.

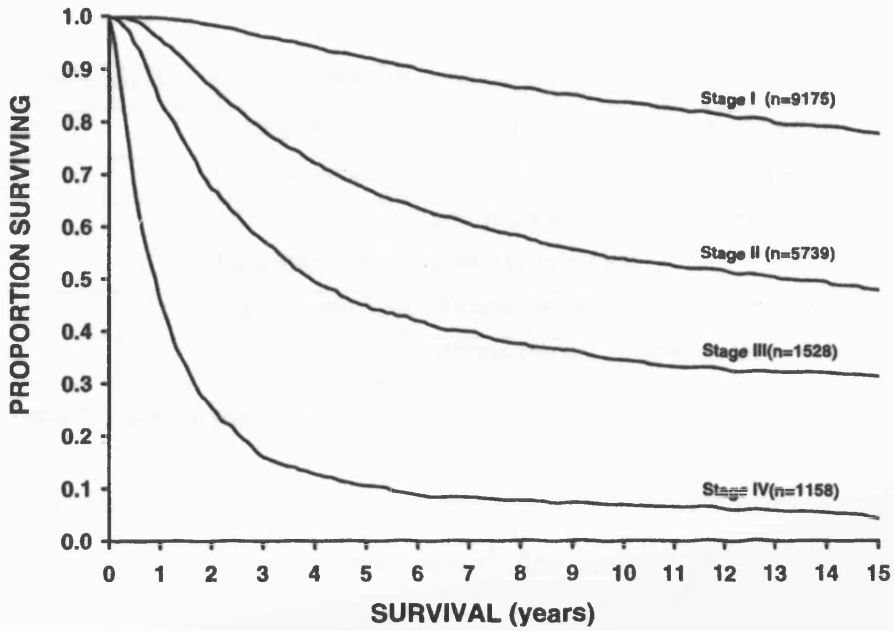


Figure 1-3 Fifteen year survival curves for the melanoma staging system. The numbers in parentheses are the numbers of patients in the AJCC melanoma staging database used to calculate the survival rates[3].

In 2003 the American Joint Committee on Cancer (AJCC) implemented a revised staging system for malignant melanoma[3]. The revision was validated by analysing prognostic factors in 17,600 melanoma patients from prospective databases. The analysis showed tumour thickness to be the most powerful predictor of biological behaviour. The significance of depth of invasion in relation to outcome in melanoma has been appreciated since the early 1950's[22], however in 1969 Clark *et al* described 5 anatomical levels of invasion that permitted accurate assignment of prognosis[5] (see Table 1-1). The determination of Clark's level of invasion remains a fundamental piece of prognostic information in the reporting of melanoma today, particularly in the context of melanoma less than 1mm in thickness, where it remains a powerful independent prognostic variable[20]. In 1970 Breslow first described the value of measuring the depth of tumour invasion in determining prognosis in melanoma[4]. In a study of 98 patients, tumour thickness measured in millimetres was found to correlate well with likelihood of recurrence, especially in the context of Clark's level II and III lesions. In a group of 45 tumours combined from levels II and III, all of which were less than 0.76mm, only one tumour was shown to be recurrent ($p<0.05$).

Clark's Level	Anatomical site
I	Malignant melanocytes are confined to the epidermis
II	Partial infiltration of the papillary dermis by single cells or small nests of cells
III	Tumour cells fill and expand the papillary dermis with extension of tumour to the papillary– reticular dermal interface
IV	Melanoma cells infiltrate the reticular dermis in a significant fashion.
V	Melanoma cells infiltrate the subcutaneous fat

Table 1-1 Clark's levels describing anatomical compartments of invasion.

Following tumour thickness the next most important prognostic factor is ulceration, followed by patient age, anatomical site and gender[2]. The presence or absence of ulceration impacts significantly on prognosis, for example the five year survival rate in stage I or II tumours (i.e. primary tumours with no evidence of lymph node metastases) drops from 80% to 55% in the presence of ulceration[20]. A possible explanation for this association is that genuine ulceration, i.e. non-traumatic ulceration, reflects rapidly proliferating tumours and tumour ischaemia. Other prognostic indicators include mitotic index, which was shown by Azzola *et al* to be second only to tumour thickness as a prognosticator of survival[23], and the nature of the host immune response characterised by tumour infiltrating lymphocytes. Survival has been shown to be better in patients with a brisk immune response, which is characterised by a diffuse infiltrate of lymphocytes throughout the vertical growth phase or the presence of infiltrating lymphocytes along 90% of the circumference of the lesional base[20, 21].

The presence and number of involved regional lymph nodes is of considerable prognostic significance in melanoma and is a reflection of the lack of effective therapeutic strategies for metastatic disease[3]. Clinically apparent lymph node metastases are of greater significance than microscopic metastases and the overall metastatic tumour burden in lymph nodes is also predictive of survival. The sentinel lymph node status is an important prognosticator, particularly in the setting of primary lesions greater than 4mm in thickness. Whether sentinel lymph node biopsy is a therapeutic as well as prognostic procedure, has been the subject of

some debate. The controversy in this area arises from a failure, as yet, to ascertain what regional lymph node metastases represent. The Incubator Hypothesis states that regional lymph nodes harbour metastatic melanoma cells which remain localized while they undergo further clonal expansion until a clone emerges with the ability to metastasise to distant sites in the body. In this setting, clearance of the regional lymph nodes, as indicated by a positive sentinel node, would be of therapeutic utility in the early stages of the disease and increase survival[24]. Conversely, the Marker Hypothesis states that a positive sentinel node is merely a marker of the metastatic phenotype and indicates the likelihood of occult metastases elsewhere in the patient. In such a scenario, clearance of the lymph node basin would be of palliative benefit only. Morbidity associated with lymph node dissection includes lymphoedema and, it has been suggested[25], an increase in in-transit metastases and therefore the procedure should only be performed if indicated. A number of prospective trials have been or are currently addressing this research question however conclusive evidence as to the role of sentinel node biopsy is yet to emerge[24, 26, 27].

Determination of whether a tumour is in the RGP or VGP is prognostically important because this strongly implies whether or not a tumour has metastatic potential[21]. The growth phase model which, as described below, is of conceptual and biological importance, has been criticised by some because assessment of this criterion can be can be subjective in individual tumours.

It is hoped that the molecular characterisation of melanoma will identify new prognostic markers that can be used as an adjunct to those described above [20]. In particular the identification of factors that are associated with aggressive behaviour will be of considerable prognostic importance and may enable the development of new therapeutic agents.

1.1.3 Melanocyte development and melanocytic neoplasia

Melanocytes are pigment-producing cells that normally reside in the basal layer of the epidermis, where they are interspersed with basal keratinocytes and anchored to the basement membrane. The dendritic processes of melanocytes project into the overlying layers of keratinocytes, and develop contacts with them, forming the 'epidermal melanin unit' that typically comprises 20-35 cells[28]. Upon exposure to ultraviolet light, the melanocyte produces melanin pigment, which is transported to the keratinocyte via the dendritic processes. Melanin absorbs and dissipates ultraviolet energy.

During vertebrate embryogenesis, melanocytes arise from cells of the neural crest, a transitory structure that forms at the dorsal borders of the neural plate[29]. The cells of the neural crest migrate along definitive pathways and stop at different locations to give rise to a variety of derivatives including melanocytes, glial cells, peripheral neurons, cartilage and adrenergic cells. Pluripotent cells of the neural crest give rise to lineage restricted oligopotent precursors, which may differentiate along neural or melanocytic lineages. The oligopotent precursors finally give rise to unipotent progenitors called melanoblasts that colonise the epidermis. During expansion of the skin, for example in childhood, there is a need for melanocyte proliferation to ensure the correct ratio with keratinocytes is maintained. In adulthood, melanocytes retain some replicative ability but, in general, are in a resting state.

Dysregulation of normal pathways of melanocyte development is likely to play a fundamental role in the pathogenesis of melanocytic tumours. Characterising the components of these pathways is likely to enhance understanding of how melanocytic tumours arise, how they progress from early lesions to advanced cancers and may identify potential therapeutic targets. This thesis comprises investigations into five genes that are considered to be important in the development of melanocytic tumours, namely N-ras, H-ras, B-raf, $p16^{ink4a}$ and Wnt5a. These genes are of interest to researchers of melanocytic tumours on account of the

regulatory effects they are considered to have on melanocyte proliferation, differentiation and motility, and the effect this has on melanoma progression[7, 30-32].

While the number and complexity of signal transduction pathways within human cells is vast and ever expanding, within melanocytic tumours a proportionately small number are considered to be of prime importance [33]. The importance of these pathways is not specific to melanocytic neoplasms, indicating the central role they play in the regulation of cellular functions that are deregulated during all neoplastic transformation. The pathways of interest here are the mitogen activated protein kinase (MAPK) pathway, the retinoblastoma (Rb) regulatory pathway and the Wnt signalling pathway. These are described in the following section.

1.1.3.1 The mitogen activated protein kinase pathway

The ras-raf-mek-erk mitogen activated protein kinase (MAPK) pathway is a highly conserved signalling cascade that transmits signals from the cell surface to the nucleus through a series of intermediate cytoplasmic proteins. Growth factors such as platelet-derived growth factor activate receptor tyrosine kinases such as c-kit at the cell surface that, via adaptor proteins and exchange factors, activate the membrane bound proteins N-ras, H-ras or K-ras. The ras proteins are inactive in their guanosine diphosphate (GDP)-bound state and active when bound to guanosine triphosphate (GTP). The ras-GTP complex can bind to several effector proteins including PI3K, thereby activating the PI3K-Akt3 pathway, and also the raf serine/threonine-specific protein kinases[14]. The raf proteins phosphorylate and activate mek which in turn activates erk. The erk proteins are serine/threonine specific protein kinases that phosphorylate both nuclear and cytoplasmic substrates. In the cytoplasm erk phosphorylates proteins that regulate cell shape and migration, while in the nucleus erk phosphorylates transcription factors such as Ets1, Ets2 and Elk(Figure 1-4).

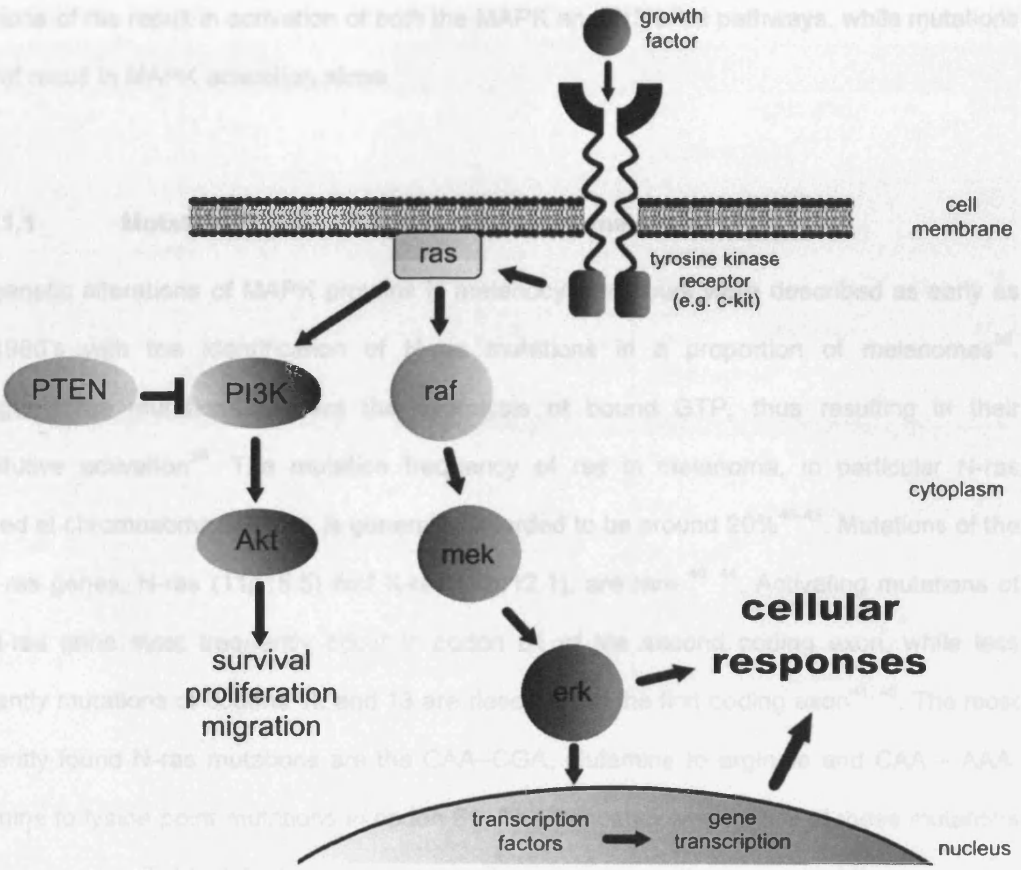


Figure 1-4 Schematic of the MAPK pathway and the ras-activated PI3K-Akt pathway.

Activation of nuclear substrates by erk can have effects on proliferation, differentiation, senescence and apoptosis and it appears that the degree of activation of the pathway influences the outcome. For example excessive pathway activation has been shown to induce premature senescence in different cell types including cells of melanocyte lineage [34-36] by up-regulation of cyclin dependent kinase inhibitors such as $p16^{ink4a}$. Low level activation of the pathway however, has been shown to stimulate proliferation in pc12 cells (from phaeochromocytoma), while sustained higher levels induce neuronal differentiation[37]. The outcome of MAPK signalling is not only influenced by the duration and intensity of signalling but also by cross signalling from other pathways[14]. It is important therefore, that MAPK signalling is carefully regulated in order to preserve cell homeostasis and allow the correct cell responses to signals from the external environment. As can be seen in Figure 1-4, activating

mutations of ras result in activation of both the MAPK and PI3K-Akt pathways, while mutations of B-raf result in MAPK activation alone.

1.1.3.1.1 Mutations of ras and raf in cutaneous melanoma

Key genetic alterations of MAPK proteins in melanocytic tumours were described as early as the 1980's with the identification of N-ras mutations in a proportion of melanomas[38]. Oncogenic ras mutations prevent the hydrolysis of bound GTP, thus resulting in their constitutive activation[39]. The mutation frequency of ras in melanoma, in particular N-ras (located at chromosome 1p13.1), is generally regarded to be around 20%[40-43]. Mutations of the other ras genes, H-ras (11p15.5) and K-ras (12p12.1), are rare [40, 44]. Activating mutations of the N-ras gene most frequently occur in codon 61 of the second coding exon, while less frequently mutations of codons 12 and 13 are described in the first coding exon[41, 45]. The most frequently found N-ras mutations are the CAA–CGA, glutamine to arginine and CAA – AAA, glutamine to lysine point mutations in codon 61. The frequency and nature of these mutations is summarised in Table 1-2. There is a non-coding exon preceding exon 1 of the ras genes, which has lead to some authors to refer to exons 1 and 2 as exons 2 and 3 respectively.

Gene	Exon	Codon	Mutation
N-ras (~20%)	1	12	GGT (glycine) – GAT (asparagine)[41]
		13	GGT (glycine) - GAT (asparagine)[46] GGT (glycine) – GTT (valine) [46]
		18	GCA (alanine) – ACA (threonine)[47]
	2	61	CAA (glutamine) – CGA (arginine)[41] CAA (glutamine) – AAA (lysine)[41] CAA (glutamine) – CAT (histidine)[43] CAA (glutamine) – TTA (leucine)[43]
B-raf (~60%)[30]	11	468	GGA (glycine) – AGC (serine)[45]
	15 (>90%)[45]	600	GTG (valine) – GAG (glutamate) (>90%)[30] GTG (valine) – GAA (Glutamate)[42] GTG (valine) – AAG (lysine)[42] GTG (valine) – AGG (arginine)[42]

Table 1-2 Mutations of N-ras and B-raf in melanoma. Where generally accepted, the mutation frequencies have been given.

Mutations of ras in common acquired naevi (CAN) are found at a similar frequency to those in melanoma [48, 49], however the frequency in congenital naevi appears to be higher than that of CAN or melanoma[50]. This suggests that congenital naevi are genetically distinct from naevi acquired later in life. ras mutations are described in early stages of other malignancies such as colonic polyps, which frequently harbour mutations of K-ras[51], and superficial tumours of the urinary bladder that have H-ras mutations[52]. These findings suggest that activated ras contributes to tumour maintenance as well as transformation[53].

raf proteins have three isoforms, A-raf, B-raf and C-raf. Common to all isoforms are three conserved regions CR1, CR2 and CR3[14]. The ras binding domain lies within CR1 and the kinase domain lies within CR3. The kinase domain contains an activation segment that in B-raf requires phosphorylation of codons 598 and 601 to induce kinase activity via a conformational change in the protein. In addition, all of the ras isoforms contain a negative charge regulatory region (N-region) in CR3 that in A-raf and C-raf requires phosphorylation, along with the activation segment, for kinase activity. By contrast, the N-region of B-raf is constitutively phosphorylated and so requires fewer steps to become activated. This makes B-raf susceptible to oncogenic mutations compared to A-raf and C-raf.

Davies *et al* reported mutations of B-raf in several tumour types, with a frequency of 66% in a series of melanomas that were primarily cell lines[30]. Several other authors have subsequently confirmed this, describing mutation frequencies typically between 50 and 70%[42, 44, 45, 49, 54, 55] although lower frequencies have been described by some authors[56, 57]. It has been suggested that the variation in mutation frequency may, in part, be attributable to differences in the methods of mutation detection[58]. Greater than 90% of the mutations of B-raf occur at codon 600[14] (this is referred to as codon 599 in earlier literature, however NCBI gene-bank re-named the mutation following the acquisition of new sequencing data[34]) in exon 15, with a point mutation, GTG-GAG, producing a valine to glutamate substitution (V600E). Codon 600 lies within the activation segment in the kinase domain of B-raf and mutations constitutively increase its activity by a factor of 500[59]. A number of other mutations of B-raf exon 15 have been described but individually account for less than 1% of mutations. Similarly, activating mutations in exon 11, which encodes the glycine rich loop, are described but are very rare[45].

Despite exposure to UV radiation being the only known environmental risk factor for melanoma, the majority of B-raf mutations that have been described, including V600E do not show the UV damage "signature" of C to T or CC to TT transversions at pyrimidine dimers.

Furthermore, B-raf mutations are not common in other skin cancer types whose incidence also correlates with UV radiation exposure such as basal cell carcinoma[60], but they are found in congenital naevi, which arise prior to UV exposure[57, 61], although these mutations were not found by one author[50]. The B-raf V600E mutation may also be found in tumours that receive no UV exposure at all such as ovarian, colorectal and thyroid cancer[30]. This evidence suggests that UV radiation does not directly damage the B-raf gene, but that the mutations occur secondarily; for example through the generation of reactive oxygen species during melanogenesis[14].

Mutations of N-ras and B-raf in melanoma tend to be mutually exclusive, a feature consistent with being effectors in the same pathway, however, double mutant cases have been reported by a number of authors [42, 44, 61-64]. Consequently, because of the high frequency of these mutations, the majority of melanomas appear to have mutations of one or the other gene. This indicates the fundamental nature of MAPK pathway activation in melanoma development, but there are notable exceptions to this rule. For example, melanomas that arise at sites protected from the sun such as the acral skin or mucosal surfaces, show low frequencies of these mutations[65-67]. Furthermore, desmoplastic melanoma, which is typified by chronic levels of high sun exposure, also rarely shows these mutations[68]. These are most important findings for they indicate that malignant melanoma is not a single disease but an entity composed of a number of subtypes that may have unique genetic pathways of tumour progression. Expanding on this data, Curtin *et al* examined 126 melanomas that were grouped according to the degree of UV exposure they received (chronic exposure, intermittent exposure, acral skin that receives very little exposure and mucosal that receives none). Through a combination of B-raf and N-ras mutation analysis, and analyses of DNA copy number, they were able to demonstrate significant differences between these groups, which enabled them to classify lesions into one of the four groups with 70% accuracy. This research provides further evidence of distinct tumour progression pathways within melanoma, some of which appear to progress without B-raf or N-ras activation. It must be noted however, that the majority of melanomas are

not of acral or desmoplastic type and therefore mutations of B-raf and N-ras are likely to comprise parts of a progression pathway of fundamental importance in most melanomas.

Aside from melanoma, mutations of B-raf and N-ras are also described in up to 80% of naevi [48, 50, 61, 63, 69]. Many authors have regarded this as evidence that mutations of B-raf and N-ras are early events in melanoma progression, because some melanomas are considered to arise within naevi[70, 71]. This conclusion may not be applicable to all melanomas, because less than a third of cases are seen in association with a pre-existing naevus[71, 72], and it is possible that some of these lesions are in fact melanomas that arise *de novo* and collide with a pre-existing naevus. Further support for B-raf and N-ras mutations arising early in progression are reports of a high frequency of these mutations in RGP tumour components that are sometimes found adjacent to B-raf or N-ras mutant VGP tumours (see section 1.1.4)[41, 45]. However, much lower mutation frequencies in purely RGP tumours have been reported by one author[56]. While the precise timing of B-raf and N-ras mutations in melanoma progression is unclear, there is a large amount of evidence indicating that these mutations are very common in a large number of melanocytic tumours, including melanoma; and in melanoma, B-raf and N-ras mutations are common in primary tumours.

The presence of B-raf and N-ras mutations in benign as well as malignant melanocytic tumours indicates that these alterations alone are not sufficient to cause malignancy, but may be important for the initiation of neoplasia[61]. Chudnovsky *et al*[73] have demonstrated that mutations of ras, in association with activated telomerase and disruption of either the Rb or p53 pathway, result in invasive tumours in human skin models grafted onto immunodeficient mice. They subsequently demonstrated that when mutated ras was replaced with mutant B-raf, there was no invasive phenotype, merely mild junctional nesting. The authors conclude that ras is more oncogenic than raf, on account of its ability to activate both the MAPK and PI3K pathways but conceded that the alterations used in the models were introduced simultaneously, not in sequence, as is likely to happed *in vivo.* This finding supports the notion

that B-raf is important for the initiation of neoplasia and maintenance of tumour growth, and hence it is a frequent feature of melanocytic tumours, but alone is not sufficient to produce malignancy.

Investigating ras and raf mutations has demonstrated the importance of activation of both the MAPK and PI3K-Akt pathways in many melanomas. Both pathways are either activated by simultaneous alterations of B-raf and PTEN (see Figure 1-4) or by mutations of N-ras alone. PTEN mutations are rarely seen in association with mutations of N-ras[74-76]. It is apparent therefore that increased MAPK signalling requires alterations of other signal transduction pathways in order for melanoma to progress.

It is clear that B-raf and N-ras are important oncogenes that are frequently activated in melanoma, which makes these molecules attractive therapeutic targets. Sorafenib is an oral small molecule multikinase inhibitor which targets tumours and tumour vasculature. The drug was discovered in a screen for inhibitors of C-raf but also inhibits wild type and mutant B-raf, as well as other receptor tyrosine kinases[77]. Sorafenib has been shown to block erk activation and cell proliferation in immortalised murine melanocytes transformed with either ras or raf, and retard the growth of melanoma cell line xenografts in mice. Unfortunately Phase II trials of the drug as monotherapy were disappointing with little or no anti-tumour activity demonstrated in patients with advanced melanoma[78]. There are a number of potential reasons why sorafenib has performed poorly in melanoma patients[77]; one explanation is that sorefanib acts as a cytostatic agent and therefore may need to be used in combination with cytotoxic drugs to produce an antitumour effect. Another possibility is that because sorafenib was developed as a C-raf inhibitor, it may not be a sufficiently potent inhibitor of mutant B-raf. In support of this is the efficacy of the drug against renal cell carcinoma, which can harbour mutations of C-raf. Finally, only a proportion (six of 17) of the patients in the Phase II clinical trial had V600E mutations of B-raf, while the rest of the patients were wild type at exons 15 and 11. While these initial studies of inhibitors of MAPK pathway kinases have been disappointing, it is vital

that research continues because it is likely that more potent and selective inhibitors of B-raf will be of significant benefit to patients with metastatic melanoma.

1.1.3.2 Wnt5a and Wnt signalling

Wnt proteins are a family of cystein rich ligands that show considerable signalling heterogeneity. The first member of the Wnt family to be discovered was Wnt1, which was shown to be located adjacent to the integrated mouse mammary tumour virus in a large proportion of murine mammary carcinomas[79]. The action of Wnt ligands has been characterised in *Drosophila and Xenopus,* where their importance in embryonic development was first discovered[80].

Wnt ligands bind to the *Frizzled* family of receptors, which are seven-pass, G-protein coupled transmembrane receptors that can utilise a variety of G-protein sub units. In addition, there are *Frizzled* co-receptors that are necessary for, and can modulate the effects of, Wnt signalling, as well as non-*Frizzled* receptors that transduce Wnt signalling such as the transmembrane tyrosine kinases Derailed and Ror2. The complexity of signalling that arises due to different combinations of Wnts, *Frizzled*s, co-receptors and non-*Frizzled* receptors is therefore substantial. As well as activators of Wnt signalling, additional modulation comes from Wnt pathway antagonists such as secreted frizzled related proteins, which bind and inhibit Wnts, and the Dickkopf family of proteins as well as related proteins such as Soggy. Wnt ligands signal through at least 3 different pathways, which are outlined below. The two pathways of most relevance to this work are illustrated in Figure 1-5.

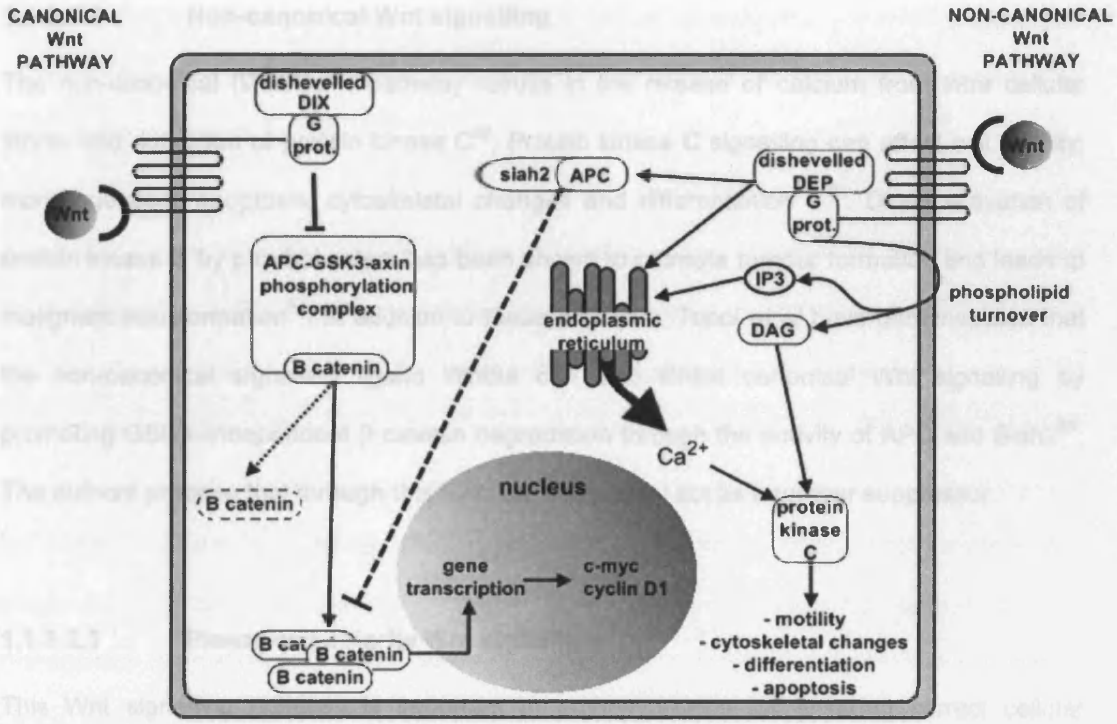


Figure 1-5 Simplified Wnt signalling pathways. The canonical pathway inhibits GSK3-β, which stabilises β-catenin resulting in its accumulation in the nucleus where it can activate transcription. The non-canonical pathway results in phospholipid turnover in the cell membrane resulting in activation of protein kinase C and the release of intra cellular calcium. In addition, the non-canonical pathway can inhibit the canonical pathway

1.1.3.2.1 Canonical Wnt signalling

Canonical (or classical) Wnt signalling inhibits the degradation of β catenin, thereby allowing it to accumulate in the cytoplasm and translocate to the nucleus where it initiates gene transcription[81]. Wnt-*Frizzled* binding results in activation of the cytoplasmic phosphoprotein Dishevelled, which inhibits GSK3β mediated β catenin phosphorylation. If GSK3β is not inhibited, β catenin is ubiquitinated and subsequently destroyed. GSK3β mediated phosphorylation occurs while β catenin sits in a complex with APC, Axin, protein phosphate 2A and β-TrCP. Therefore canonical Wnt signalling can regulate the expression of β catenin activated genes such as c-Myc and cyclin D1, that promote cell proliferation. Wnts that can activate the canonical pathway include Wnt1 and Wnt8.

1.1.3.2.2 Non-canonical Wnt signalling

The non-canonical (Wnt/Ca^{2+}) pathway results in the release of calcium from intra cellular stores and activation of protein kinase C[82]. Protein kinase C signalling can affect cell motility, morphogenesis, apoptosis, cytoskeletal changes and differentiation[81, 83]. Direct activation of protein kinase C by phorbol esters has been shown to promote tumour formation and leads to malignant transformation[84]. In addition to these functions, Topol *et al* have demonstrated that the non-canonical signalling ligand Wnt5a can also inhibit canonical Wnt signalling by promoting GSK3–independent β catenin degradation through the activity of APC and Siah2[85]. The authors propose that through this function Wnt5a may act as a tumour suppressor.

1.1.3.2.3 Planar cell polarity Wnt signalling

This Wnt signalling pathway is important in embryogenesis for ensuring correct cellular orientation, such as in receptor cells that contain sensory hairs in the ear of vertebrates and cells of the hair bulb[81]. The pathway is activated by receptor ligand binding between *Frizzled* and Wnts such as Wnt7a. No oncogenic role for this pathway has been described.

1.1.3.2.4 Wnt5a and cutaneous melanoma

Increased expression of Wnt5a has been described in a number of human malignancies including cancers of the lung, breast, prostate, and melanoma[86, 87]. There are a number of studies indicating Wnt5a may be important in the progression of melanoma with particular emphasis placed on its influence on cell morphology, epithelial to mesenchymal transition and invasiveness[7-9, 86, 88]. Protein kinase C signalling is an important down-stream effect of non-canonical Wnt signalling. In melanoma, protein kinase C affects cell growth and metastatic potential with different isoforms having various stimulatory and inhibitory effects on these processes[84]. Disseneyaka *et al*[8] demonstrated that many of the metastasis and motility promoting effects of Wnt5a are mediated via the actions of protein kinase C. In their study, by stimulating protein kinase C activity directly in melanoma cell lines using phorbol esters, they

demonstrated subsequent increased expression of the transcription suppressor snail with down regulation of E-cadherin, increased expression of vimentin and increased expression of MMP-2 with increased motility.

A key study concerning the role of Wnt5 in melanoma is that of Bittner *et al*[7] who analysed gene expression in 31 melanoma samples and, within these, identified a cluster of 19 with similar gene expression profiles. The gene with the most power to define this cluster of 19 samples was Wnt5a, which showed markedly decreased expression. *In vitro* studies showed reduced cell motility and invasiveness within this group compared with samples from outside the cluster, indicating that Wnt5a influences aggressive behaviour. The precise role of Wnt5a in tumour progression is unclear because altered expression does not appear to be a prerequisite for malignancy and decreased expression has been described in pancreatic cancer and neuroblastoma[82, 86]. In those tumours where Wnt5a is over expressed, the gene is not amplified or rearranged. [86].

Following on from the work of Bittner *et al*, Weeraratna *et al* described increased motility and invasion along with specific morphological changes *in vitro* in melanoma cells that constitutively express Wnt5a. Furthermore, in vivo, they describe high Wnt5a expression in cells with high-grade cytological features and, in one case, increased expression with tumour progression. In the same paper the authors state that Wnt5a expression correlated strongly with both survival and time to metastasis, but there was no statistical analysis to support this statement, with only 5 deaths recorded in total, and no correction for baseline variables such as Breslow depth. The authors acknowledge that this was a pilot study that precluded statistical analysis in some instances and elsewhere may have accounted for some observations achieving limited statistical significance. Despite this the results indicate that further studies of Wnt5a in melanoma progression and outcome are warranted.

Following on from this paper, Dissaneyaka *et al* have characterised some of the effects of Wnt5a expression and down regulation in melanoma cell lines[8]. Wnt5a expression was shown to down-regulate the expression of KISS-1 an inhibitor of melanoma metastasis and up-regulate CD44, an important mediator of cell invasion and metastasis homing. The authors also found that Wnt5a could influence 'epithelial to mesenchymal transition' via protein kinase C signalling. This promoted expression of the intermediate filament and mesenchymal marker vimentin, as well as snail, a transcriptional suppressor of E-cadherin. E cadherin expression is an important feature of the epithelial phenotype and is down regulated in melanoma progression[11, 33]. Histopathologists may note however, that the term 'epithelial to mesenchymal transition' is inappropriate in the setting of melanoma cells because they are not epithelial in origin.

1.1.3.3 p16^{ink4a} and the retinoblastoma pathway

The transition from G1 to S phase of the cell cycle commits the cell to DNA replication and subsequent division. The G1-S checkpoint is therefore an important gateway where a number of pathways that control cell division converge[11]. Central to control of the G1-S transition is pRb a tumour suppressor that sequesters E2F transcription factors thereby inhibiting transcriptional activation of genes vital for progression into S phase. The ability to inhibit E2F transcriptional activation is regulated by phosphorylation of pRb, which is controlled by a group of constitutively expressed enzymes called cyclin dependent kinases (CDK's). The kinase activity of CDK's is regulated by cyclins, a family of proteins that are expressed at specific points of the cell cycle[89]. The kinase activity of CDK's is activated upon complexing with cyclins, with different combinations of CDK's and cyclins regulating each of the important transitions of the cell cycle. G1 to S transition is under the regulation of cyclins D and E and CDK's 2, 4 and 6. Upon activation, the CDK's hyperphosphorylate pRb, which releases the E2F transcription factors; which are then able to transcribe genes necessary for S phase. The CDK inhibitor p16^{INK4a} introduces a further layer of control by inhibiting CDK4[90]. The role of pRb, CDK's, cyclins and p16^{INK4a} in the G1 to S phase transition is illustrated in Figure 1-6.

Alterations of the genes that regulate this phase of the cell cycle are found in many cancers including melanoma. Cyclin D1 lies downstream of the MAPK pathway and it has been reported to be over expressed in a variety of cancers including breast, prostate and lymphoma[53]. In melanoma, cyclin D1 amplification has been described most frequently in acral tumours[18, 91]. Furthermore, mutations that render CDK4 resistant to inhibition by $p16^{INK4a}$ have been described in melanoma cell lines and cases of familial melanoma[53]. The importance of these alterations in melanocytic neoplasia is not as great, however, as that of the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene.

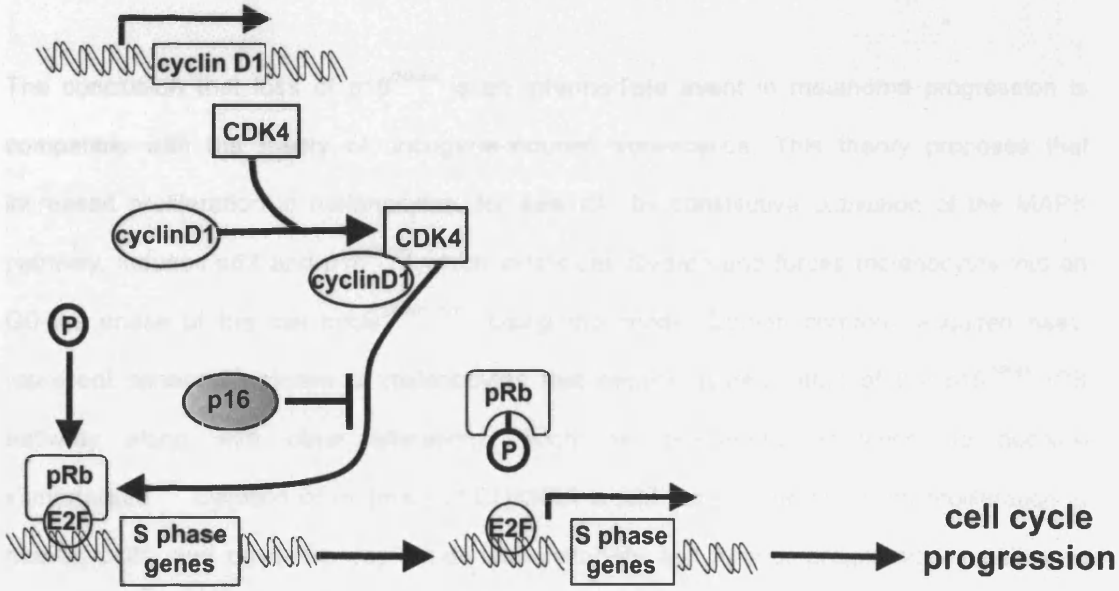


Figure 1-6 Control of the G1/S phase checkpoint of the cell cycle. Hyperphosphorylation of retinoblastoma protein by cyclin dependent kinase 4 is inhibited by $p16^{INK4a}$.

The CDKN2A locus on chromosome 9p21 not only encodes $p16^{INK4a}$, but also the tumour suppressor $p14^{ARF}$. Mutation, loss of heterozygosity and deletion of the CDKN2A gene is well described in both sporadic and familial melanomas [15, 31, 92-94]. While germ line mutations of CDKN2A are primarily associated with melanoma susceptibility, they may also be associated with pancreatic cancer [95]. Melanoma associated CDKN2A mutations and deletions can involve $p16^{INK4a}$, $p14^{ARF}$ or both transcripts.

The $p16^{INK4a}$ protein is expressed in virtually all melanocytic naevi but there is progressive loss of expression as tumours progress from RGP melanoma to VGP melanoma to metastatic melanoma[32, 96-98]. The presence of $p16^{INK4a}$ in a significant number of RGP melanomas and some VGP melanomas suggests that alteration is not necessary for tumour initiation, but may be an intermediate event necessary for further progression to invasion and metastasis [32, 99]. Loss of $p16^{INK4a}$ expression appears to be a frequent event in advanced primary and metastatic melanoma, but genetic alterations in the CDKN2A gene appear to be less frequent, indicating that there are other influences on CDKN2A expression that are yet to be determined[93, 98, 100, 101].

The conclusion that loss of $p16^{INK4a}$ is an intermediate event in melanoma progression is compatible with the theory of oncogene-induced senescence. This theory proposes that increased proliferation in melanocytes, for example by constitutive activation of the MAPK pathway, induces p53 and $p16^{INK4a}$ which inhibit cell division and forces melanocytes into an G0-like phase of the cell cycle[34-36, 102]. Using this model, benign common acquired naevi represent senescent clones of melanocytes that require dysregulation of the $p16^{INK4a}$ /RB pathway along with other alterations, such as telomerase activation, to become immortalised.[95]. Deletion or mutation of CDKN2A would remove the break on proliferation in naevus cells, and pave the way for genetic instability and tumour progression, resulting in malignant behaviour.

$p14^{ARF}$ derives its name from the use of an alternate reading frame (hence ARF) of the exons it shares with $p16^{INK4a}$. Its function is to control via sequestration the levels of the protein MDM2, which ubiquitinates and therefore promotes the degradation of the key tumour suppressor gene p53.

1.1.4 Concepts of tumour progression in cutaneous malignant melanoma

Clark *et al* proposed that primary melanoma should be separated into tumours in the RGP, in which there is minimal metastatic potential; and tumours in the VGP, where there is a significant risk of metastasis[21]. This observation has lead to a theoretical model of melanoma progression, called Clark's model. Clark's model is illustrated in Figure 1-7

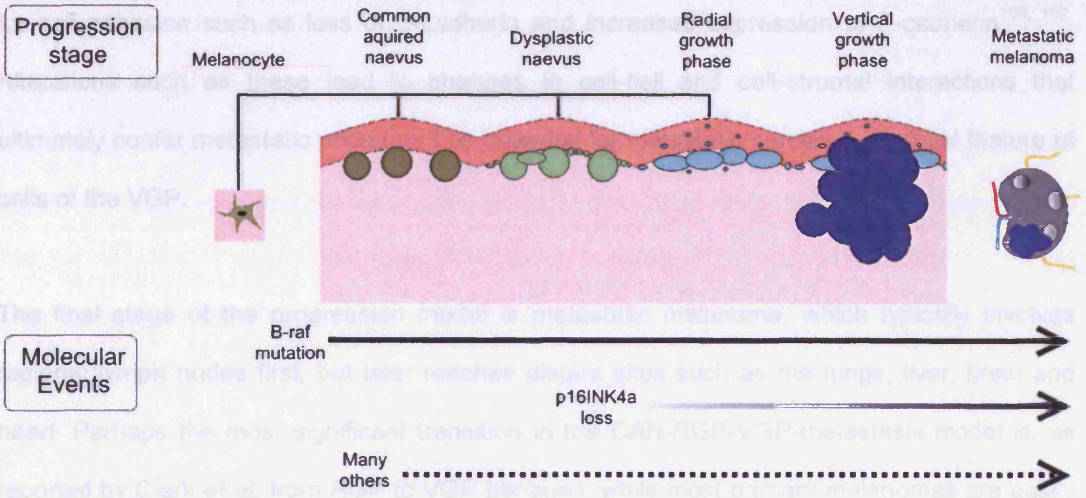


Figure 1-7 Clark's progression model

The common acquired naevus (CAN) is often included as the first step in Clark's model, however only a proportion of melanomas are believed to arise within these lesions [70, 71]. CAN are benign tumours of melanocytes called naevus cells. The next step is the dysplastic naevus, a premalignant lesion that shows cytological and architectural atypical features. Dysplastic naevi can arise *de novo* or within CAN and can regress as well as progress[11, 70]. Multiple dysplastic naevi arise in patients with the dysplastic naevus syndrome of which 25-40% have germ line mutations of $p16^{INK4a103}$. The RGP is the first malignant stage of the model and here the tumour cells acquire the ability to proliferate intraepidermally and may invade into the superficial dermis. Crucially, RGP melanomas are believed to lack the ability to metastasise[21]. Loss of $p16^{INK4a}$ expression has been demonstrated in RGP melanoma relative

to CAN and dysplastic naevi[32, 96, 104] and for this reason loss of $p16^{INK4a}$ is considered an intermediate event in progression by some authors. Reduced expression of $p16^{INK4a}$ can also be demonstrated in later stages of melanoma progression[105].

Following the RGP, tumours progress to the VGP where there can be widespread dermal invasion and the development of an expansile, proliferating, dermal tumour nodule. In VGP tumours a variety of molecular alterations have been demonstrated, notably in molecules vital for cell adhesion such as loss of E-cadherin and increased expression of P-cadherin[106, 107]. Alterations such as these lead to changes in cell-cell and cell-stromal interactions that ultimately confer metastatic potential. The potential for metastatic spread is a critical feature of cells of the VGP.

The final stage of the progression model is metastatic melanoma, which typically involves regional lymph nodes first, but later reaches distant sites such as the lungs, liver, brain and heart. Perhaps the most significant transition in the CAN-RGP-VGP-metastasis model is, as reported by Clark *et al,* from RGP to VGP because, while most primary melanomas are easily cured by excision, metastatic melanoma is frequently resistant to current therapy. Characterising molecular alterations that underlie the clinicopathologically recognisable stages of Clark's model, such as those that enable progression from the RGP to VGP, may present new targets for prognostication and therapy.

At the periphery of VGP melanoma dermatopathologists frequently observe atypical melanocytes in either junctional nests or the superficial papillary dermis, which are reminiscent of the pre-existing RGP in which the VGP tumour apparently arose. This has lead some researchers to use RGP-like tumour components adjacent to the VGP, as a research tool for identifying molecular differences between these two growth phases in the same tumour. It is of note however, that while RGP like components of VGP tumours bear histological similarity with

purely RGP melanomas, at a molecular level some similarities with the VGP have been demonstrated[41, 43].

1.2 The Spitz naevus and Spitzoid tumours

The Spitz naevus is a benign, usually acquired, melanocytic tumour that is characteristically composed of spindle and / or epithelioid melanocytes, which may appear similar to cells seen in some cases of melanoma. The incidence of these lesions in the population is difficult to ascertain, being variously described as 'fairly common'[16] and 'relatively uncommon'[108], and this uncertainty is likely to arise from the fact that many of the lesions that do come to clinical attention are not removed for histological assessment. Spitz naevi are most prevalent in the first two decades of life and decrease in incidence thereafter[109]. In one series 39% (78 of 200) of cases were in patients younger than 15 years, but importantly 29% were in patients over thirty (57 of 200)[109]. There appears to be no sex prediliction[108].

Clinically Spitz naevi are usually asymptomatic, dome shape with a smooth surface and typically pink or tan, but may also be brown or black. They are usually less than 1cm in diameter and often appear to be vascular lesions and hence can be mistaken for pyogenic granulomas. They may occur anywhere on the body, but the face and extremities are most frequently involved.

Lesions showing features of a Spitz naevus comprise a small proportion of the workload of most dermatopathologists, but assume a disproportionate degree of importance because of their histological similarity to malignant melanoma. This diagnostic difficulty is discussed in greater detail below.

1.2.1 Difficulties of diagnosis and taxonomy in Spitzoid tumours

Histological criteria to discriminate Spitz naevi from the major differential diagnosis of cutaneous malignant melanoma were first published in 1947 by Sophie Spitz[110]. In this seminal study it was reported that these lesions were a juvenile variant of melanoma in which the prognosis was frequently excellent. This opinion was revised in a subsequent study, which concluded that 'juvenile melanoma' was a benign tumour[22]. In 1960 Kernan and Ackerman published a series of 27 cases and concluded that the title 'spindle/ epithelioid cell naevus' was more befitting of a lesion which they regarded to be a variant of the benign naevus[111]. While the concept that such tumours were benign naevi became central dogma, the eponym of Spitz naevus has been preferred by most. Sixty years have passed since criteria to help differentiate Spitz naevi from melanoma were first published and, while these have been considerably refined, there is no consensus of opinion on specifically which criteria define the entity of Spitz naevus. Disagreement between expert dermatopathologists concerning the biological potential of Spitzoid tumours (lesions with a Spitz-like histological morphology) is well described [112-114] and melanoma misdiagnosed as Spitz naevi constitute a large proportion of dermatopathology malpractice claims [115, 116].

Numerous histological features have been described that may be present or absent in classic Spitz naevi (see section 2.2.1), but these are often subjective and non-specific, and, as a consequence, diagnosis requires the interpretation of a constellation of criteria[40, 112, 117-119]. The relative importance placed upon these features also varies considerably between pathologists. The lack of diagnostic clarity is greater still in tumours that deviate from the classical appearance and show features more commonly associated with the main differential diagnosis, malignant melanoma. Atypical Spitz naevus, atypical Spitz tumour, ambiguous Spitzoid tumour, Spitzoid tumour of uncertain malignant potential (STUMP) and Spitzoid melanoma are entrenched terms for the classification of lesions that are neither classic Spitz naevus nor 'common' melanoma (i.e. non-Spitzoid cutaneous melanoma)[120-122], however their use is staunchly resisted by some [123, 124]. For the purpose of this introduction, all such lesions

will be referred to as non-classical Spitzoid tumours, when not referring to a specific series of published cases. The application of these terms lacks reproducibility because there is little guidance on what features delineate 'classical' Spitz naevi from non-classic Spitzoid lesions and non-classic Spitzoid lesions from 'common' melanoma[112, 117, 119, 123, 125, 126].

Poor histological reproducibility adversely affects research into Spitzoid tumours. It is difficult to compile well-defined, standardised tumour series and therefore experimental differences between types of Spitzoid tumour can go unnoticed. Furthermore, when comparing data from different studies, direct comparisons between supposedly identical types of lesions may be invalid due to variations in the criteria applied to compile them[113, 114]. Categorising lesions as unequivocally benign, of uncertain malignant potential or unequivocally malignant may be the most reproducible way of comparing different data sets, however this risks oversimplifying this diverse group of lesions. Figure 1-8 indicates how the various diagnostic terms used within the literature may relate to the spectrum of histological features and the risk of malignancy within Spitzoid tumours, but this is merely the author's interpretation.

Histological Features	Classical Spitz Naevus				Malignant Melanoma
Diagnostic Term Applied*	Spitz Naevus	Atypical Spitz Naevus	Atypical Spitz Tumour/STUMP	Spitzoid Melanoma	'Common' Melanoma
Implied Likely Behaviour	Benign	Probably Benign	Uncertain	Malignant	

Figure 1-8 The spectrum of histological features, diagnostic terminology and clinical behaviour encountered in Spitz naevi, malignant melanoma and intermediate lesions. *Of these entities only Spitz naevus and 'common' melanoma (i.e. non-Spitzoid melanoma) have widely accepted histological criteria.

1.2.2 The molecular pathology of Spitz naevi and Spitzoid tumours

Examination of cell functions that are commonly altered during carcinogenesis supports the notion that Spitz naevi are benign, while similar investigations in non-classical Spitzoid tumours suggest they may represent a stage of tumour progression. Many of these observations come from characterising hallmark features of neoplasia[127] in Spitzoid tumours and comparing the findings with those of better-characterised lesions such as common acquired naevi (CAN) and 'common' melanoma. Investigations of proliferation[128], apoptotic rate[129, 130] p53 expression[131-135] and inhibition of replicative senescence via telomerase activity[136-138] have shown differences between Spitz naevi and melanoma and similarities between Spitz naevi and CAN. In some instances, non-classical Spitzoid tumours have shown features intermediate to Spitz naevi and melanoma, which suggests they may be transitional lesions. This work is summarised in more detail by Da Forno *et al*[139] (see Chapter 8 Appendix 3: Publications arising from this work).

In addition to replicative senescence, which results from cell ageing, cells may also undergo premature senescence. A premature senescent mechanism, oncogene induced senescence, has been characterised in common naevus cells and is described above (see section 1.1.3.1.1). In Spitz naevi, two oncogene induced premature senescent mechanisms have been proposed. Bastian *et al* have correlated H-ras activation (which is found in around a fifth of Spitz naevi) with cytological features of malignancy such as marked nuclear pleomorphism and prominent nucleoli[140]. They postulate that this is a manifestation of partial transformation towards malignancy, but crucially malignancy does not develop because the H-ras induced hyper-proliferation drives these lesions into senescence via increased expression of cell cycle inhibitory proteins. Alternatively, Denoyelle *et al* suggest that in the context of H-ras activation, senescence results from a stress induced response of the endoplasmic reticulum (ER), termed the unfolded protein response (UPR)[141]. The UPR produces structural alterations within the ER that may account for the morphological changes observed in H-ras activated Spitz naevi by Bastian *et al*. Further work is needed in this area because the senescent mechanisms in non-

H-ras activated Spitz naevi are yet to be described and it remains to be shown whether dysregulation of the above mechanisms occur in non-classical Spitzoid tumours or Spitzoid melanoma.

The Investigations described above concern hallmark features of neoplasia and common end points of carcinogenesis that may be seen in many types of benign and malignant tumours. By contrast, investigations of MAPK pathway genes and DNA gains and losses, have shown alterations that appear specific to Spitzoid tumours. Distinct sets of genetic alterations have been shown in other melanocytic tumour types[18], indicating that they comprise discreet genetic subtypes, which provides a rational basis for postulating that Spitzoid tumours could also be a distinct subset of melanocytic lesions.

1.2.2.1 Are Spitzoid tumours genetically distinct from other melanocytic tumours?

1.2.2.1.1 Spitz naevi

As described in section 1.1.3.1.1, a high frequency of B-raf and N-ras mutations in benign and malignant melanocytic skin tumours has been demonstrated since the initial investigation by Davies *et al.*[30, 61]. Subsequent reports of a complete absence of B-raf mutations and a very low frequency of N-ras mutations in Spitz naevi were therefore surprising for a lesion considered by many to arise via mechanisms similar to melanoma and CAN (Table 1-3)[57, 63, 142]. A large number of authors have subsequently confirmed this observation[17, 30, 44, 45, 54-56, 61, 69, 122, 143-149] and so distinctive is the absence of these mutations within the context of melanocytic tumours, that one might consider the presence of a B-raf or N-ras mutation incompatible with a true Spitz naevus. It should be noted however, that two studies have described B-raf mutant Spitz naevi (Table 1-3). Fullen *et al* reported B-raf mutations in 10 of

48 Spitz naevi and in support of this is the finding of B-raf mutations in a small series of Spitz naevi by La Porta *et al*[150]. These are most surprising results when one considers that no mutations of B-raf had been reported in seven previous studies that have analysed Spitz naevi, on a total of 204 cases, using different methods of mutation detection[54, 57, 63, 122, 142-144]. It is possible that differences of diagnostic criteria, as described earlier, may account for these outlying cases, but, in general, it appears that B-raf or N-ras mutations in Classic Spitz naevi are extremely uncommon.

Study	Common acquired naevi	Spitz naevi	Atypical Spitz naevi	Suspected for melanoma/ STUMP	Primary Spitzoid melanoma	Spitzoid melanoma metastasis	'Common' Melanoma
Palmedo *et al*[143]	-	-	-	-	2/6	-	-
Gill *et al*[120]	-	0/10	-	-	0/9	-	-
Lee *et al*[121]*	8/11	-	-	-	1/33*	0/2	8/12
van Dijk *et al*[122]	-	0/14	0/16	8/23	30/36	6/7	-
Fullen *et al*[151]	-	5/23	5/25	0/7	2/13	-	-
Takata et al[152]		0/12	2/16**				15/24
Other studies[17, 30, 44, 45, 54-57, 61, 63, 69, 122, 142-150]	249/319	9/212 +	-	-	-	-	281/534
Total	257/330 (78%)	14/271 (5%)	5/31 (16%)	8/30 (27%)	35/97 (36%)	6/9 (67%)	304/570 (53%)

Table 1-3 Comparison of B-raf and/or N-ras mutation frequency in published studies of melanocytic tumours. The studies summarised in detail have analysed atypical Spitzoid tumours or Spitzoid melanomas, while studies examining common acquired naevi, Spitz naevi or common melanomas are grouped together. Note that the mutation frequencies for atypical Spitzoid tumours and Spitzoid melanoma are based on a comparatively small number of samples and show considerable variation between studies. STUMP, Spitzoid tumour of uncertain malignant potential. *Authors concede some cases might be diagnosed as suspected for melanoma/ STUMP by other dermatopathologists.** Cases not specifically categorised and excluded from column totals. +8 of these mutant cases were identified in one study[150].

Characterisation of DNA gains and losses in Spitz naevi has also shown clear differences to melanoma and CAN. One of the first techniques employed to examine for genomic imbalances in melanocytic tumours was analysis of ploidy via flow cytometry. This was shown to have some discriminatory power between Spitz naevi and melanoma[153, 154] but failed to show significant specificity for routine diagnostic use. Subsequently, studies of chromosome 9p via *in situ* hybridisation (ISH), and loss of heterozygosity (LOH) also demonstrated an ability to differentiate between classic Spitz naevi and 'common' melanoma[155, 156]. A study using multiplex ligation-dependent probe amplification (MLPA), also demonstrated multiple copy number aberrations in melanoma but minimal alterations in Spitz Naevi[157]. Comparative genomic hybridisation (CGH) shows that Spitz naevi, along with other benign naevi, have virtually no alterations of DNA copy number, while in melanoma these changes can be numerous[158-160]. Bastian *et al* showed that around 20% of Spitz naevi have a characteristic increase of chromosome 11p copy number, the site of the H-ras gene, which in 67% of amplified tumours is also concurrently mutated[140, 159]. No such amplifications were seen in other naevus types and H-ras mutations are extremely uncommon in melanoma[161]. Common mutations of H-ras exons 1 and 2 are shown in Table 1-4.

H-ras exon	Codon	Mutation
1	12	GGT (glycine) – arginine*[140]
2	61	CAG (glutamine) – CTG (leucine)[140] CAG (glutamine) – arginine*[140] CAG (glutamine) – lysine*[122]

Table 1-4 Mutations of codons 1 and 2 of the H-ras gene that have been described in Spitzoid tumours. * specific mutation sequence not stated.

The evidence from B-raf and N-ras mutations and H-ras amplification has made for a compelling argument that most Spitz naevi arise via different mechanisms to CAN and melanoma. The identification of these important differences has lead to speculation that non-classical Spitzoid lesions may also differ from common naevi and "common" melanoma[123, 125]. Genetic changes in Spitzoid melanomas will be considered in the next section.

1.2.2.1.2 Spitzoid melanoma

Gill *et al*, examined nine Spitzoid melanomas, alongside ten age matched 'typical' Spitz naevi, for mutations of B-raf, N-ras and H-ras (Table 1-3)[120]. All cases were from children aged ten years or younger. Upon initial histological assessment none of the cases were reported as unequivocally malignant, the diagnosis was only made retrospectively upon spread to local lymph nodes. None of the cases exhibited visceral involvement (follow-up 2-78 months). No activating mutations of B-raf, N-ras or H-ras were found in the Spitzoid melanomas or classical Spitz naevi and the authors concluded that all Spitzoid tumours might be biologically different from non-Spitzoid melanomas and CAN. Subsequently, Lee *et al* expanded on this investigation by examining 33 Spitzoid melanomas from patients with an age range of 6 years to 71 years (mean 35 years)[121]. Five of the cases were metastatic tumours, with two of the patients dying from their disease. Cases of melanoma and CAN were included, but no classic Spitz naevi were examined. They found one Spitzoid melanoma with a B-raf mutation, but no N-ras mutations. The mutation frequencies in the CAN and melanomas were in keeping with those reported previously in the literature. They concluded that their study, along with that of Gill *et al,* indicates that Spitzoid lesions are biologically separate entities from CAN and 'common' melanoma. These data provide a convincing argument that classic Spitz naevi along with Spitzoid melanoma comprise a distinct subtype of melanocytic tumour in which mutations of B-raf or N-ras are very rare (Table 1-3). In stark contrast to these conclusions however, other studies indicate that there is some genetic heterogeneity within Spitzoid tumours.

Palmedo *et al* described two B-raf mutant Spitzoid melanomas in a series of six, but no mutations in 21 'classical' Spitz naevi [143]. They concluded that while Spitz naevi appear genetically different, Spitzoid melanomas have a mode of tumour progression similar to other melanomas. A larger investigation by van Djik *et al* concurs with this finding; they examined 36 primary Spitzoid melanomas and 7 Spitzoid metastases and found a B-raf or N-ras mutation frequency of 83% but no mutations of H-ras. By contrast, they found no N-ras or B-raf mutations in 14 Spitz naevi or 16 atypical Spitz naevi, but within these lesions mutations of

H-ras were found. They concluded that Spitz naevi and atypical Spitz naevi are genetically distinct from Spitzoid melanomas. In keeping with this, Fullen *et al* reported B-raf mutations in two of 13 Spitzoid melanomas[151]. In the light of the conflicting conclusions arising from these investigations and those of Gill *et al* and Lee *et al*, it is apparent that further investigations of B-raf and N-ras mutations in Spitzoid melanomas are warranted.

Data concerning DNA gains and losses in Spitz naevi have not been expanded to include Spitzoid melanomas. In spite of this one might speculate that, because genomic instability is a common feature of malignancy, such lesions would show frequent changes of DNA copy number like any other type of melanoma.

1.2.2.1.3 Non-classical Spitzoid tumours

There is limited data concerning the frequency of MAPK pathway gene mutations in non-classical Spitzoid tumours, and that which is available is contradictory. van Djik *et al* and Fullen *et al* have examined a number of intermediate lesions that appear to fit into this category. Both authors analysed atypical Spitz naevi, while van Djik *et al* looked at 23 cases of 'suspected Spitzoid melanoma' and Fullen *et al* at seven 'atypical Spitz tumours of uncertain biologic potential'. The latter two groups comprised lesions showing atypia beyond than that of an atypical Spitz naevus but not sufficient for a diagnosis of Spitzoid melanoma and therefore might be considered STUMPs (see Figure 1-8). The results conflict considerably, with van Djik *et al* finding mutations in roughly a third of their 'suspected Spitzoid melanoma' group but no mutations in atypical Spitz naevi, while Fullen *et al* found mutations in a fifth of their atypical Spitz naevi but none in 'atypical Spitz tumours of uncertain biologic potential' (see Table 1-3). Takata *et al* examined 16 cases of 'ambiguous Spitzoid lesions' for mutations of B-raf, N-ras and H-ras alongside classic Spitz naevi and 'common' melanoma[152]. They employed a panel of two dermatopathologists to independently diagnose the tumours and disagreements between the two were common, with a variety of diagnostic terms used. Two mutant lesions were found. The first was a mutation of B-raf in a lesion regarded as 'melanoma' by one reviewer and 'atypical Spitz tumour (favours melanoma)' by

the other. The second case involved an N-ras mutation in a lesion regarded as malignant by both reviewers. The authors conclude that the majority of atypical Spitz tumours are probably not different from conventional Spitz naevi. Clearly, further work is needed to characterise the nature of this heterogeneic group of tumours.

Investigations into DNA gains and losses in non-classical Spitzoid tumours are also limited but some interesting reports have emerged. De Wit *et al* found that the frequency of aberrations of chromosome 1, as detected via ISH, were significantly different between Spitz naevi and nodular melanoma but, when applied to metastatic lesions originally diagnosed as Spitz naevi (and hence possibly STUMPs), the frequency of aberrations varied considerably [162]. Similarly, examination of LOH shows features intermediate between Spitz naevi and melanoma in the context of non-classical Spitzoid tumours [163]. Using CGH, Bauer and Bastian examined two lesions that might be classified as STUMP in so much as histological assessment could not assign them as being unequivocally benign or malignant. They found CGH to be diagnostically useful in this setting, with one lesion demonstrating no genomic aberrations, as in classic Spitz naevi, and consequently being considered benign, while the other lesion had many genetic aberrations, similar to 'common' melanoma, and was consequently considered malignant[164]. Neither case showed increased chromosome 11p copy number, as previously described in a minority of Spitz naevi, and crucially no follow-up data on the patients was given. Mihic-Probst *et al* describe a lesion initially diagnosed as a Spitz Naevus that was re-diagnosed as melanoma upon metastasising. Using CGH they demonstrated a number of genetic aberrations including loss of chromosome 9p (which contains the $p16^{INK4a}$ tumour suppressor gene locus), and concluded that if CGH had been used for the initial diagnosis, the malignant nature of the lesion would have been revealed[144]. Takata *et al,* in addition to the mutation analysis described above, analysed the same series of 'ambiguous' Spitzoid lesions for DNA copy number aberrations[152]. They found that a tumour with a B-raf mutation also showed multiple copy number alterations, which suggested that this lesion was in fact 'common' melanoma. No aberrations were found, however, in an N-ras mutant tumour with clinical evidence of distant metastases. Loss of the CDKN2A gene was found in three 'ambiguous' Spitzoid tumour cases and was not seen in 12 Spitz naevi.

The authors conclude that the loss of this tumour suppressor gene may account for the clinical and histological deviations of these tumours from typical Spitz naevi. With so few cases of non-classical Spitzoid tumours in the literature, no significant patterns of DNA copy number change can be identified to show similarity or differences to Spitz naevi, but from a diagnostic standpoint these case reports are encouraging.

In summary therefore, the bulk of evidence suggests that Classic Spitz naevi are indeed a distinct type of melanocytic tumour, arising via B-raf or N-ras independent mechanisms, with a subset of these having amplified H-ras, which is in stark contrast to 'common' melanoma and CAN. This conclusion cannot however be extended to other Spitzoid lesions because the molecular findings are contradictory, possibly due to inherent difficulties in establishing criteria for case selection.

1.2.2.2 Does the histological spectrum of Spitzoid tumours represent a progression from Spitz naevus to Spitzoid malignant melanoma?

Clark's progression model[21] (see Figure 1-7), is accepted by many as a model of step-wise carcinogenesis in melanocytic tumours. This has lead to speculation that a similar or indeed identical process occurs in Spitzoid tumours, whereby the Spitz naevus becomes a non-classical Spitzoid tumour and finally melanoma that is morphologically different, but at the molecular level similar, to 'common' non-Spitzoid melanoma[165, 166] (see Figure 1-9 a). In support of this, non-classical Spitzoid tumours show a spectrum of histological features intermediate between classic Spitz naevi and melanoma, and while most non-classical Spitzoid tumours do not behave like melanoma, there is unquestionably a small percentage that do and are referred to retrospectively, by some, as Spitzoid melanoma[120-123]. Alternatively, the broad histological spectrum of Spitzoid tumours might simply comprise a dichotomy of Spitz naevi and 'common' melanoma that look Spitzoid. If this hypothesis were correct, non-classical Spitzoid tumours would merely represent a mix of Spitz naevi and melanomas that cannot be separated by current pathological techniques (see Figure 1-9 b). In this setting, Spitz naevi would never progress to malignancy and lesions that had a Spitzoid

appearance and showed aggressive behaviour would actually be 'common' melanoma mimicking the Spitz naevus phenotype.

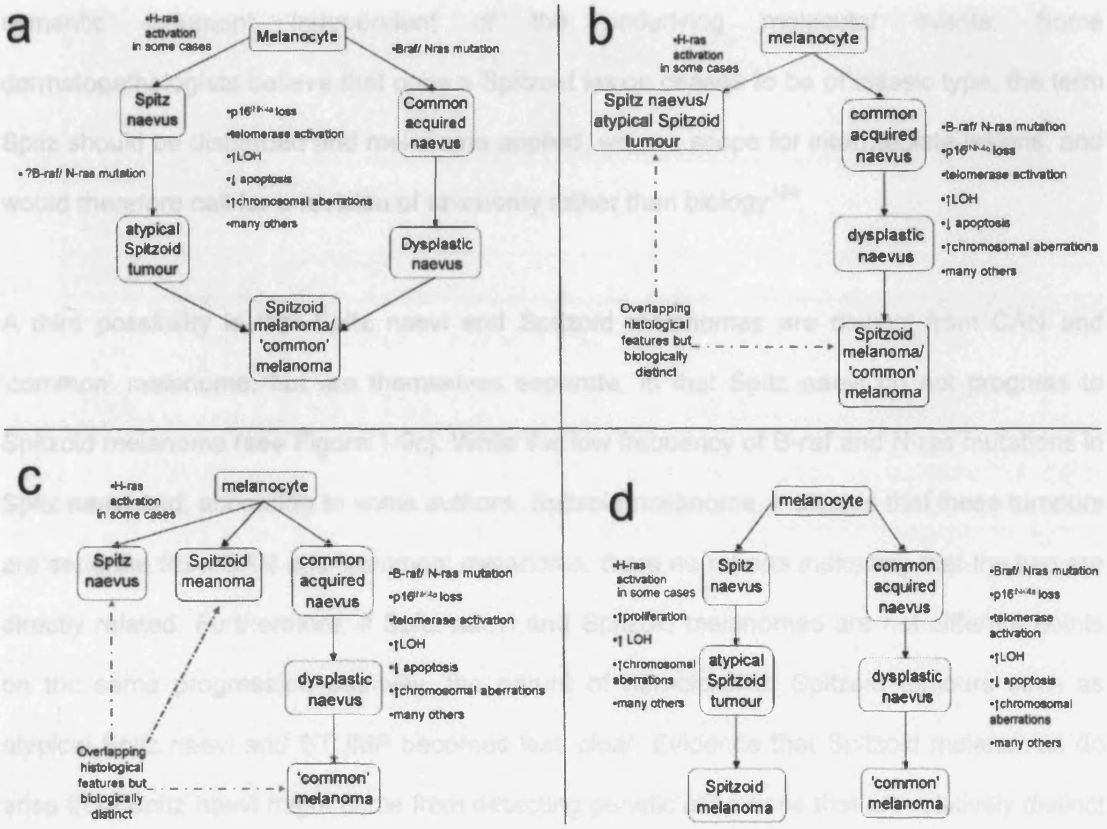


Figure 1-9 Hypothetical mechanisms of tumour progression in Spitzoid tumours. a) Tumour progression that is very similar to that of the progression of common acquired naevi to 'common' malignant melanoma. b) Spitzoid lesions comprise a dichotomy of benign Spitz naevi that do not undergo malignant progression and 'common' melanomas with a Spitzoid appearance. c) Spitz naevi and Spitzoid melanomas are distinct from CAN and 'common' melanoma, but are themselves separate entities d) A separate Spitzoid progression pathway, distinct from the 'common' melanoma pathway of Clark's progression model.

It will not be possible to distinguish between these two scenarios by analysis of B-raf or N-ras mutation status alone, because a mutation could be interpreted as both evidence that a tumour is progressing towards malignancy or that the lesion is actually malignant *ab initio*. If Spitz naevi and non-classical Spitzoid tumours do progress to malignancy by acquiring B-raf or N-ras mutations, in a manner similar to Clark's progression model, one might expect the frequency of these mutations to increase with progression, as was found by van Dijk *et al*[122](Table 1-3). In further support of this model, a study by the same authors found an increasing frequency of LOH from atypical Spitz naevi (33%) through lesions suspicious of

melanoma (65%), to unequivocal Spitzoid melanomas (88%)[163]. The alternative situation in which there is a dichotomy of Spitz naevi and melanoma with no borderline lesions has been fiercely championed by some authors[109, 124, 167]. Whether this dichotomy exists is in part a semantic argument independent of the underlying molecular events. Some dermatopathologists believe that once a Spitzoid lesion ceases to be of classic type, the term Spitz should be discarded and melanoma applied, with no scope for intermediate lesions, and would therefore call for a revision of taxonomy rather than biology[124].

A third possibility is that Spitz naevi and Spitzoid melanomas are distinct from CAN and 'common' melanoma, but are themselves separate, in that Spitz naevi do not progress to Spitzoid melanoma (see Figure 1-9c). While the low frequency of B-raf and N-ras mutations in Spitz naevi and, according to some authors, Spitzoid melanoma, indicates that these tumours are separate from CAN and 'common' melanoma, there no reports indicating that the two are directly related. Furthermore, if Spitz naevi and Spitzoid melanomas are not different points on the same progression pathway, the nature of non-classical Spitzoid tumours such as atypical Spitz naevi and STUMP becomes less clear. Evidence that Spitzoid melanomas do arise from Spitz naevi might come from detecting genetic alterations that are relatively distinct in Spitz naevi, such as H-ras mutations, in non-classical Spitzoid tumours and, ultimately, Spitzoid melanoma. This is yet to be described, but H-ras mutations have been reported in atypical Spitz naevi and a single Spitzoid tumour suspected of melanoma (see Table 1-5) [122].

A final possibility is that there is a separate Spitzoid progression pathway, distinct from the 'common' melanoma pathway of Clark's progression model (see Figure 1-9d). In this case the whole family of Spitzoid tumours would be a distinct subset of melanocytic tumours.

Study	Spitz naevi	Atypical Spitz naevi	Suspected for melanoma/ STUMP	Primary Spitzoid melanoma
Bastian et al[140]	9 of 33* (27%)	-	-	-
Takata et al[152]	0/11 (0%)	0/12** (0%)		
Gill *et al*[120]	0/10 (0%)	-	-	-
van Dijk *et al*[122]	4/14 (29%)	2/16 (13%)	1/23 (4%)	0/34(0%)
Total	**13/68* (19%)**	**2/16 (13%)**	**1/23 (4%)**	**0/34(0%)**

Table 1-5 H-ras mutation frequency in published studies of melanocytic tumours.*12 cases selected because they showed H-ras gene amplification.** Cases not specifically categorised and therefore excluded from column totals. STUMP, Spitzoid tumour of uncertain malignant potential

The possibility of a distinct Spitzoid pathway is made more likely by the increasing body of evidence supportive of alternative melanoma progression pathways in the various melanoma sub-types[17, 18]. The fact that some reports find frequent B-raf and N-ras mutations in Spitzoid melanoma [121, 122, 143, 151] would argue against Spitzoid tumours being a distinct subset.

Reports of improved prognosis in Spitzoid melanomas in young people is regarded by some as supportive of a separate Spitzoid progression pathway [126, 168, 169]. The oxymoronic concept of the 'malignant/metastasising' Spitz naevus, a lesion histologically in keeping with a Spitz naevus, that shows local lymph node metastasis but no spread beyond this, is a concept that polarises opinion. It appears reasonable to regard metastasis as a highly specific but poorly sensitive marker of malignancy, yet with the advent of the 'malignant/metastasising' Spitz naevus, even the specificity of metastasis for malignancy is questioned. It should be noted that patients with regional nodal metastases (stage 3 disease) from 'common' melanoma show greater than 30% fifteen-year survival (Figure 1-3), but these tumours would not be regarded as benign[2]. Perhaps the most robust criteria for malignancy in a young person would be widespread metastasis, but fortunately such instances are rare. Due to the limited number of published cases, often with minimal follow-up, the 'malignant/metastasising' Spitz

naevus cannot currently be considered robust evidence in support of Spitzoid tumours being a distinct group. The most forceful opponents of this entity tend to advocate the dichotomy of benign Spitz naevi and malignant melanoma and do not acknowledge the possibility of intermediate lesions[167].

1.3 Aims and objectives

Malignant melanoma is a common cancer that can be cured easily by excision when localised, but is frequently fatal once metastatic[3]. In addition, melanocytic lesions, particularly Spitzoid tumours, are a frequent area of diagnostic and therapeutic difficulty for dermatopathologists and dermatologists respectively. It is important therefore to improve our understanding of these tumours, in particular the molecular events underlying their progression because this may provide opportunities to improve diagnosis, prognostication and treatment. Improved understanding of the molecular mechanisms that control melanocytic tumour progression might also inform our understanding of the molecular basis of human cancer as a whole.

Current understanding of mechanisms of progression in malignant melanoma have been described, together with some of the difficulties of identifying features of progression using standard histological techniques. With this information in mind, the aims and objectives of the current investigations are outlined below.

1.3.1 Characterisation of Wnt5a expression during cutaneous melanoma progression: aims and objectives

The principle hypothesis for this study was:

- If expression of Wnt5a is important for motility and invasiveness in cutaneous melanoma, expression should increase with tumour progression and high expression will predict poor survival.

The expression of Wnt5a during Clark's progression model was characterised in a series of melanocytic tumours. The tumour series included matched cases of primary and

metastatic melanoma from the same patient. The effect of Wnt5a protein expression on metastasis-free and overall survival was assessed. In the same tumour series, expression of $p16^{INK4a}$ and mutations of B-raf exon 15 were characterised to provide benchmarks against which Wnt5a expression could be compared throughout the stages of Clark's model and assess whether a process of multi-step carcinogenesis in melanoma was valid. Furthermore, because alterations of $p16^{INK4a}$ and B-raf are well characterised during certain stages of melanoma progression, replication of these changes, alongside those of Wnt5a, would validate the experimental methods, particularly in terms of case selection.

1.3.2 Characterisation of B-raf, N-ras and H-ras mutations in Spitzoid tumours: aims and objectives

The principle hypotheses for this study were:

- Spitzoid lesions comprise a distinct genetic subgroup of melanocytic tumours that progress in the absence of B-raf and N-ras mutations, unlike most other melanocytic tumour types.
- Progression from Spitz naevus to Spitzoid melanoma is unlikely because there are no H-ras mutant Spitzoid melanomas

A series of diagnostically difficult Spitzoid tumours was compiled along with classic Spitz naevi and non-Spitzoid melanoma. A panel of dermatopathologists reviewed the tumour series and assigned them to diagnostic categories, having first agreed criteria for atypical histological features. The tumours were screened for mutations of B-raf exon 15, N-ras exons 1 and 2 and H-ras exons 1 and 2. The mutation data were used to examine whether Spitzoid tumours represent a distinct group of melanocytic neoplasms and whether Spitz naevi can progress to Spitzoid melanoma.

2 Materials and methods

2.1 Characterisation of Wnt5a expression during cutaneous melanoma progression

2.1.1 Tissue samples

The study used a collection of melanoma **samples** within which there were three subsets of matched **cases** (see Figure 2-1). A case comprised a series of samples, all derived from the same patient, that characterised all, or parts of, Clark's model.

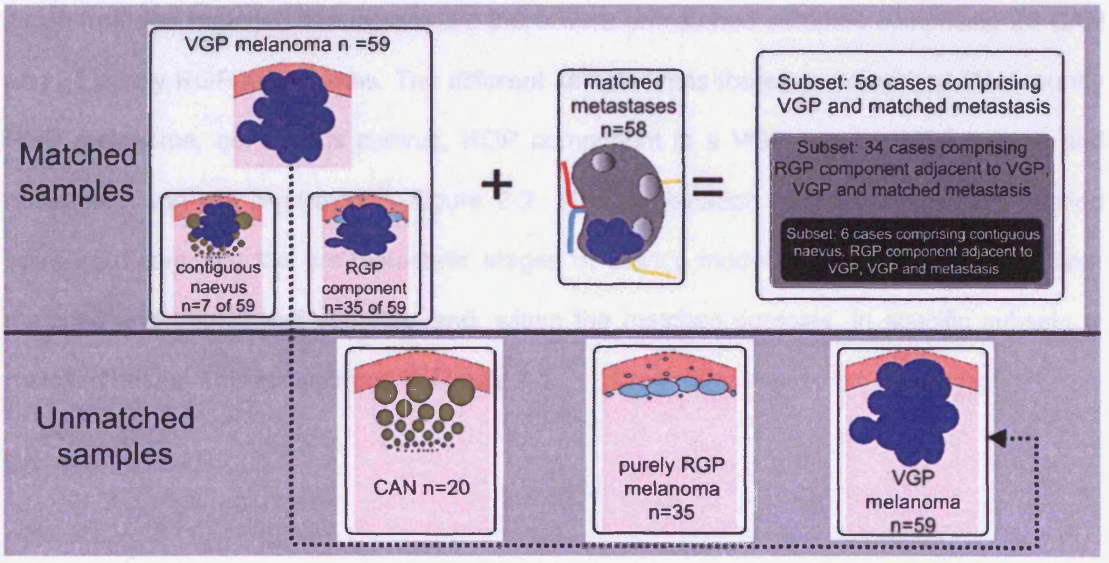


Figure 2-1 Diagram to illustrate the composition of the tumour series. There were VGP melanoma samples with matched metastases, and unmatched samples of purely RGP melanoma and CAN. Some of the matched VGP tumours had RGP components adjacent to the VGP and/or an area of contiguous naevus. This meant that within the matched samples there were three subsets of 58, 34 and 6 cases that characterised all, or parts of, Clark's model. This also meant that the non-metastatic phases of Clark's model were also characterised by unmatched samples.

There were 59 metastatic VGP melanoma samples with tissue samples from matched metastases. One of the metastatic tumours was subsequently found to be almost entirely necrotic and therefore unsuitable for assessment, giving a subset of 58 cases of matched primary VGP and metastatic melanoma. In 35 of the VGP tumour samples there were adjacent lateral areas of junctional or intra-epidermal melanoma reminiscent of a pre-existing

RGP component (hereafter such areas will be referred to as a 'radial growth phase (RGP) component'). Therefore, from the initial subset of 58 cases, this gave a secondary subset of 34 cases comprising RGP component adjacent to the VGP, VGP and matched metastasis. There were not 35 cases because, as mentioned above, one case was lost due to the matched metastasis being necrotic. In addition, 7 of the VGP melanoma samples appeared to arise within naevi (the benign naevus part of these tumours will be referred to as a 'contiguous naevus', see Figure 2-2). This meant that within the secondary subset of 34 cases with an RGP component, there was a tertiary subset of 6 cases comprising contiguous naevus, RGP component adjacent to the VGP, VGP and matched metastasis. There were not 7 cases because one case was lost due to there being no identifiable RGP component.

Aside from the matched tissue samples there were unmatched samples comprising 20 CAN and 35 purely RGP melanomas. The different sample types therefore comprised CAN, purely RGP melanoma, contiguous naevus, RGP component in a VGP tumour, VGP tumour and metastasis and are illustrated in Figure 2-2. This combination of matched and unmatched samples meant that the pre-metastatic stages of Clark's model could be examined in both matched and unmatched samples, and, within the matched samples, in specific subsets of matched cases. This is illustrated in Figure 2-1.

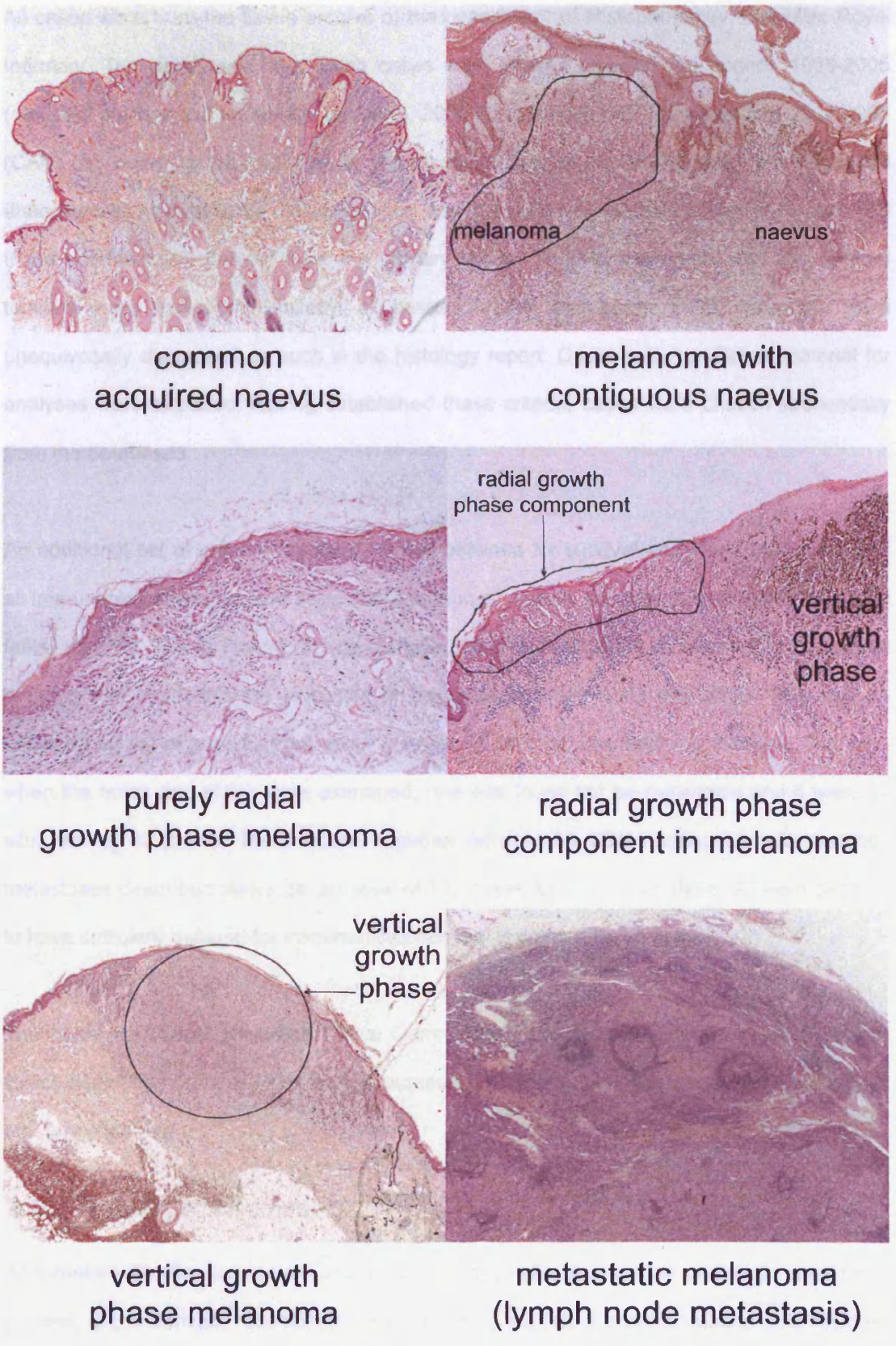


Figure 2-2 Photographs illustrating the six different sample types analysed for the study.

All cases were from the tissue archive of the department of Histopathology, Leicester Royal Infirmary. The databases from which cases were chosen covered the periods 1995-2005 (matched primary and metastatic tumours), 2002-2004 (purely RGP tumours) and 2004-2005 (CAN). In order to be included in the study, cases of melanoma had be diagnosed unequivocally as cutaneous melanoma and, where a case was metastatic, the metastasis had to be unequivocally derived from the primary lesion. Therefore, patients with two primary tumours were excluded. Similarly, all cases of CAN and purely RGP melanoma were unequivocally diagnosed as such in the histology report. Cases with insufficient material for analyses were excluded. Having established these criteria, cases were chosen sequentially from the databases.

An additional set of primary melanomas was obtained for survival analysis. For these cases all immunohistochemistry and semi-quantification of protein expression were performed by a fellow student, Qiang Huang. These samples were retrieved from a database of cases that presented to the University Hospitals of Leicester between 1983 and 2000. This was an unbiased cohort of patients from which a set of 50 patients was randomly selected. Of these, when the notes and slides were examined, one was found not be melanoma and 6 were in-situ, leaving 43 cases. These cases, together with the 59 VGP melanomas with matched metastases described above gave a total of 102 cases for survival analysis. All were deemed to have sufficient material for immunohistochemical staining.

The study had Local Research Ethics Committee approval(Leicestershire Local Research Ethics committee number 6926, project number UHL 8485). All tissue was paraffin embedded and formalin fixed.

2.1.2 Primary antibodies

Anti-mouse Wnt5a: anti-mouse polyclonal antibody, IgG, produced in goats immunized with purified, E-coli-derived, recombinant mouse Wnt5a peptide containing amino acid residues Gln254-Cys 334 of mouse Wnt5a (AF645, R&D Systems). It has been shown that intracellular components of the Wnt signalling pathway in various species have been highly conserved

during evolution, and that human and mouse Wnt5a protein exhibit 90% homology at the amino acid level [9, 106].

Mouse anti-human p16^{INK4a}: Mouse monoclonal antibody, clone G175-405, IgG1k (550834, BD Pharmingen)[90, 94, 170].

2.1.3 Secondary antibodies

Rabbit anti-goat immunoglobulins: biotinylated secondary antibody (E0466, Dako)

Rabbit anti-mouse immunoglobulins: biotinylated secondary antibody (E0413, Dako)

ABC kit: Streptavidin ABC complex (K0391, Dako)

2.1.4 Other materials

Other materials used for immunohistochemistry, along with the manufacturers, are listed in Table 2-1

Material	**Manufacturer**
Aquatex aqueous mounting agent	Merck
Xylene	Genta Environmental
IMS	Genta Medical
Naphthol AS BI Phosphate	Sigma
Dimethylformamide	Fischer Scientific
Fast Red TR	Sigma
Filter paper	Whatman
Levamisole	Sigma
Mayer's haematoxylin	BDH
NBT-BCIP tablets	Roche
Normal rabbit serum	Dako

Table 2-1 Other materials and their manufacturers used for immunohistochemistry.

2.1.5 Buffers

TBS (tris-buffered saline): 50 mM tris, 150 mM Sodium chloride, the pH was adjusted to 7.65 with concentrated HCl.

10mM Citrate buffer: 10mM citric acid, pH adjusted to 6.0 with 5M sodium hydroxide.

VAB (veronal acetate buffer): 30 mM sodium acetate trihydrate, 30 mM sodium barbitone, 0.1M sodium chloride, pH adjusted to 9.2 with concentrated HCl

2.1.6 Rehydration of tissue sections

Sections were heated at 65°C for 10 minutes and then dewaxed in xylene and rehydrated through graded alcohols as follows: xylene, 2x5 minutes; 99% IMS, 2x3minutes; 95% IMS, 3 minutes. Sections were washed in running tap water for 5 minutes and immersed in TBS for 2x5 minutes.

2.1.7 Antigen retrieval

Antigen retrieval of $p16^{INK4a}$ was achieved by micro waving sections immersed in 10mM citrate buffer for 20 minutes at 750W. This was followed by cooling for 1 hour. No antigen retrieval was used for the Wnt5a antibody.

2.1.8 Immunostaining method

Sections were placed in a humidified slide tray, non-specific staining was blocked by normal rabbit serum diluted 1:5 in TBS, 100 µl was applied to each section and they were incubated for 10 minutes. Normal serum was drained from the sections and 100µl of the primary antibody was applied to each section. Wnt5a primary antibody was applied at a concentration of 1:40 and $p16^{INK4a}$ primary antibody at a concentration of 1:30. Sections were incubated overnight in a cold room at 4°C.

Primary antibodies were drained from the sections, which were then rinsed in TBS for 2x5 minutes. For sections incubated with Wnt5a primary antibody, the secondary antibody was biotinylated rabbit anti-goat diluted 1:600 in TBS, with100µl applied to each section. For sections incubated with $p16^{INK4a}$ primary antibody, the secondary antibody was biotinylated

rabbit anti-mouse diluted 1:400 in TBS, with100µl applied to each section. The secondary antibodies were incubated at room temperature for 30 minutes.

The ABC solution was prepared immediately after applying the secondary antibody:

TBS, 1000 µl

Streptavidin, 9 µl

Biotinylated horseradish peroxidase, 9 µl

After rinsing in TBS for 2x5 minutes, 100 µl of the ABC solution was applied to each section and incubated for 30 minutes. Following this, a further rinse 2x5 minutes in TBS was done.

Wnt5a expression was visualised using fast red developer solution composed of:

25 mg Naphthol AS BI phosphate

250 µl dimethylformamide

25 mg fast red TR

12 mg levamisole

50 ml VAB

The solution was poured through filter paper into a hellendahl jar in which the sections were incubated for 1hour. Sections were rinsed in running tap water for 5 minutes, counterstained with Mayer's Haematoxylin for 2 minutes followed by rinsing in running tap water for 5 minutes to remove excess dye. Sections were mounted with Aquatex aqueous mounting agent.

$p16^{INK4a}$ expression was visualised using NBT-BCIP. One NBT-BCIP tablet was dissolved in 10 ml of ultra pure water and the resulting solution was run through filter paper. 100 µl of the filtered NBT-BCIP solution was applied and incubated in a humidified slide tray for 10 minutes. Sections were rinsed in running tap water for 5 minutes, counterstained with Mayer's Haematoxylin for 15 seconds, followed by rinsing in running tap water for 3 minutes to remove excess dye. Sections were mounted with Aquatex aqueous mounting agent.

2.1.9 Controls

Negative controls: Each section had a corresponding negative control serial section, which was incubated with normal rabbit serum, overnight at 4°C, instead of the primary antibody.

Positive controls: For Wnt5a expression, sections of normal breast tissue were used as a positive control[87, 171], while for $p16^{INK4a}$ sections of CAN previously determined to express $p16^{INK4a}$ were used[104]. Positive controls were included with each batch of staining.

2.1.10 Quantification of immunohistochemical staining

Cytoplasmic Wnt5a: Cytoplasmic Wnt5a was scored via a semi quantitative method based on the H-SCORE[172, 173]. An eyepiece graticule with a grid was employed to compartmentalise the tumour or tumour growth phase in question (contiguous naevus, RGP component adjacent to VGP or VGP), and the following formula was applied based on the percentage area of tumour staining and the intensity of the staining reaction[172, 173].

Score = 1(percentage staining weakly) + 2(percentage staining moderately) + 3 (percentage staining intensely)

This provided a score between 0 (no staining) and 300 (all areas intense) for each tumour growth phase examined. Two pathologists (GS, PDF) agreed on reference fields of tumour that were graded as weak, moderate and intense staining and an intra-class correlation coefficient (ICC) was calculated . An ICC was also calculated prior to scoring of the cases used for survival analysis by fellow student, Qiang Huang, to ensure agreement was good. The reference fields are illustrated in Figure 2-3

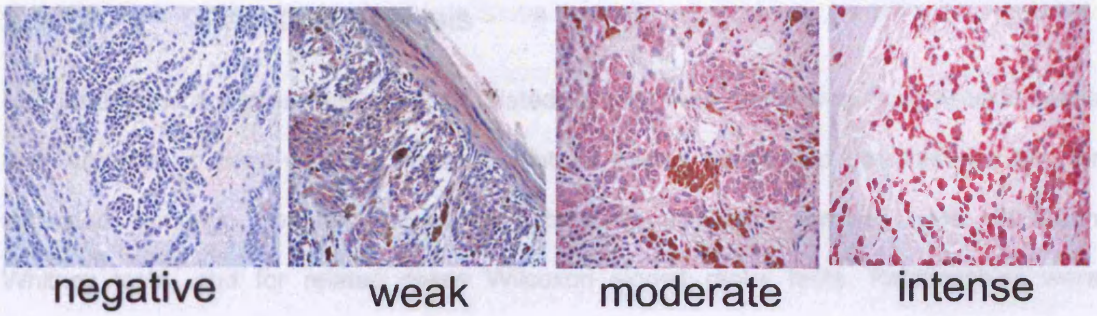


Figure 2-3 Wnt5a immunostaining: negative, mild, moderate and intense areas.

p16^{INK4a}: Five hundred cells from random areas of the tumour or tumour growth phase in question were scored as having either positive or negative nuclear staining. Positive and negative nuclear staining are shown in Figure 2-4

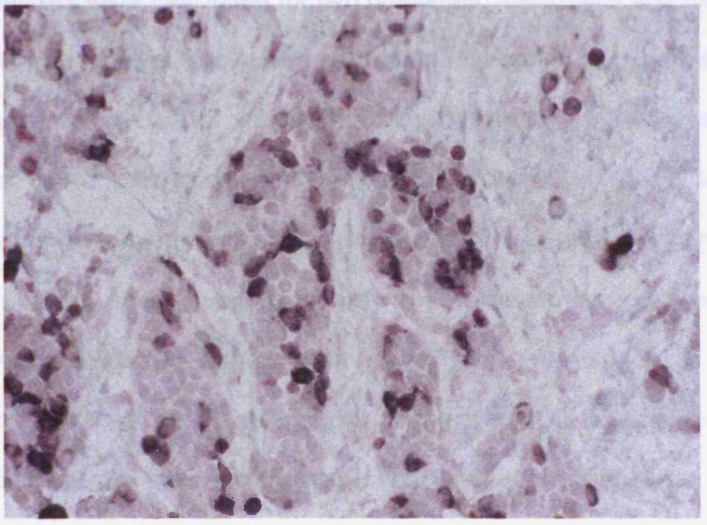


Figure 2-4 Positive and negative nuclear p16^{INK4a} immunostaining

From this, a percentage of p16^{INK4a} positive cells for the area examined was derived. The criteria for positive nuclear staining were agreed by 3 observers (PDF, GS, JHP) prior to scoring. All sections were immunoscored by a histopathologist (PDF). Cytoplasmic staining was not assessed.

2.1.11 DNA mutation detection

See section 2.2.3

2.1.12 Statistical analysis

Non-parametric analyses were used. Unrelated samples were compared using Kruskal-Wallis test. Analysis of trend was via Page's L test for related cases and Jonckheere's test for unrelated samples. Post-hoc comparisons between unrelated samples were via Mann Whitney tests, and for related cases Wilcoxon signed ranks tests. Relationships were analysed using Spearman's rank correlation. $p<0.05$ was considered significant except where a Bonferroni correction was applied to post-hoc analyses. Analysis of categorical data was via Fisher's exact and Cohen's κ tests. Analyses were performed using SPSS for Windows release 12.0 except Page's L test which was calculated manually[174].

Time to event analyses were calculated for two outcomes: metastasis and death from any cause. Metastasis-free survival times were calculated in months from the date of primary melanoma diagnosis to the date of metastasis, with those patients without metastasis or who died from a cause unrelated to melanoma being censored. Time to death was calculated in months from the date of primary diagnosis to date of death from any cause, with patients remaining alive being censored. The samples analysed were the 102 primary melanomas described above. Four cases with metastases at the time of diagnosis and one that died in the same month of diagnosis (i.e. time in months to event was 0) were excluded from time to metastasis and time to death analyses respectively. The survival rates were obtained based on Kaplan-Meier estimates. Survival curves were generated according to the Kaplan-Meier product-limit method and were compared using the log-rank test. Multivariate analyses of prognostic factors were based on the Cox proportional hazards model. The prognostic factors included in the multivariate analysis of primary melanoma were: age at diagnosis (coded as 0 ≤ 60 years, and 1 greater than 60 years); sex (coded as 0, female; 1, male), primary melanoma site (coded as 0, extremities; 1, trunk, head and neck or acral sites), Breslow thickness (coded as 0 ≤ 2.00 mm; 1 > 2 mm); nuclear Wnt5a (0 ≤ median score of 84; 1, > median score) and cytoplasmic Wnt5a (0 ≤ median score of 96, 1 > median score). The coding for clinical covariates was based on commonly used definitions[175]. All variables were entered into the model without any variable selection procedures. A model was also constructed without

the variable 'site', to allow inclusion of 8 cases where this data was missing. The main effect of cytoplasmic Wnt5a obeyed the assumption of proportionality and there was no multicollinearity. All analyses were performed using SPSS 12.0 for windows.

2.2 B-raf, N-ras and H-ras mutations in Spitzoid tumours

2.2.1 Tissue samples

Diagnostically difficult Spitzoid skin tumours are infrequently encountered during routine dermatopathological practice. In order to obtain sufficient cases for the study it was necessary to collaborate with a number of histopathology departments, as well as identify suitable cases from our own tissue archives. A list of collaborators and the departments in which they worked is shown in Table 2-2. Many of the collaborators were contacted via the Children's Cancer and Leukaemia Group (formerly the United Kingdom Children's Cancer Study Group).

Collaborator	Department
Dr A Evans	Dept of Histopathology, Ninewells Hospital and Medical School, Dundee
Mr R B Berry	Dept. Plastic Surgery, University Hospital of North Durham
Dr C T C Kennedy	Dept. Paediatric Dermatology, Bristol Royal Hospital for Children
Dr K Hollowood	Dept. Cellular Pathology, Oxford Radcliffe Hospital NHS Trust
Dr P Barrett	Dept. Histopathology, North Durham Acute Hospitals NHS Trust
Dr B McCann	Dept. Histopathology, Norfolk & Norwich University Hospital NHS Trust
Dr M Gerrard	Dept. Paediatric Oncology, Sheffield Children's NHS Trust
Mr J M Gollock	Surgical Directorate, Borders General Hospital
Dr B Gostelow	Dept. Histopathology, Kettering General Hospital
Dr A Krajewski	Dept. Pathology, The Lothian University Hospitals NHS Trust
Pathologists	Dept Histopathology, Swansea NHS Trust
Dr C Mitchell	Dept Paediatric Oncology, Oxford Radcliffe Hospital NHS Trust
Dr L Su	University of Michigan, Ann Arbor, MI, USA
Dr M Cohen	Dept Histopathology, Sheffield Children's NHS Trust
Dr Pramila Ramani	Dept. Histopathology, Bristol Royal Infirmary

Table 2-2 Collaborators and their departments

Multicentre Research Ethics Committee approval was obtained to facilitate these collaborations (reference number: MREC/03/11/085). There is considerable variation in practice in the reporting of Spitzoid tumours between histopathologists, and therefore broad diagnostic criteria were distributed to potential collaborating departments in order to help them identify lesions that might be suitable for inclusion in the study. Having acquired the cases and noted the initial diagnosis provided by the locally reporting histopathologist, there followed a second stage of diagnosis that was performed specifically for the study. The second diagnoses were given by two dermatopathologists (Dr Alan Fletcher and Dr Mark Bamford, Consultant Histopathologists, Leicester Royal Infirmary) who classified the lesions with reference to predetermined criteria drawn from published accounts of Spitzoid tumours. The purpose of the second stage of diagnosis was to standardise the terminology applied to the Spitzoid lesions, in order to remove inconsistencies of reporting practice that may have existed between the numerous collaborating histopathologists. A flow diagram summarising how cases were categorised for the first and second stages of histological assessment is shown in Figure 2-5.

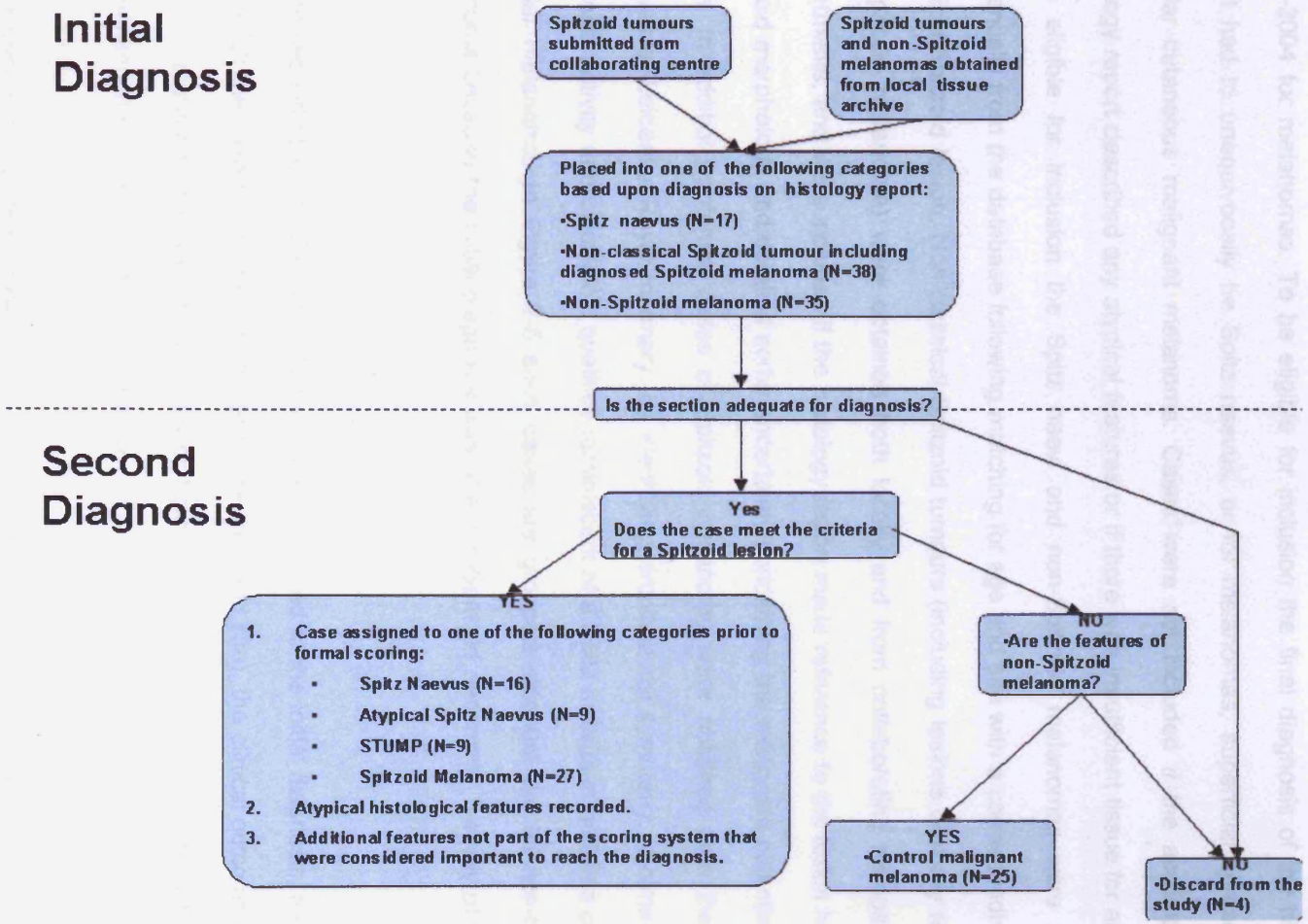


Figure 2-5. Flow diagram summarising how cases were categorised initially and following histological review and scoring.

Initially, 90 lesions, broadly classified as Spitz naevi (n=17), non-classical Spitzoid tumours (n=38) (including Spitzoid melanoma) and non-Spitzoid melanoma (n=35), were obtained. Classical Spitz naevi and non-Spitzoid melanomas were obtained from the tissue archives of Leicester Royal Infirmary from a database spanning the years 1987-2005 for Spitz naevi and 1987-2004 for melanomas. To be eligible for inclusion the final diagnosis of the histology report had to unequivocally be Spitz naevus, or for melanomas, superficial spreading or nodular cutaneous malignant melanoma. Cases were not included if the accompanying histology report described any atypical features or if there was insufficient tissue for analysis. Once eligible for inclusion the Spitz naevi and non-Spitzoid melanomas were chosen sequentially from the database following matching for age and site with a corresponding non-classical Spitzoid lesion. Non-classical Spitzoid tumours (including lesions clinically reported as Spitzoid melanoma) were obtained both locally and from collaborating histopathology departments, and were included if the histology report made reference to the lesion having a Spitzoid morphology and detailed some uncertainty concerning the malignant potential of the lesion. In addition, potential cases of Spitzoid melanoma were obtained from the tissue archives of Leicester Royal Infirmary, by identifying architectural symmetry, minimal lateral junctional activity and cytological qualities reminiscent of a Spitz naevus in archived cases of nodular melanoma. In Figure 2-5 such cases are grouped amongst the 35 non-Spitzoid melanoma because the initial diagnosis was of a non-Spitzoid melanoma, usually of nodular type.

For the second stage of diagnosis, reviewers were blinded to the initial diagnosis, the centre from which the case came, the clinical data (age, sex, and site), the clinical outcome and the results of mutation analysis. The process of how the second diagnoses were achieved is summarised in Figure 2-5 and detailed below.

LeBoit comments that "Key to determining whether a Spitz nevus is a "typical" one or not is an adequate biopsy specimen. This means a biopsy specimen that displays the edges of the lesion (so one can tell if it is well circumscribed or not) and enough of the dermal component so that one can determine if maturation is present and whether there are deep mitotic

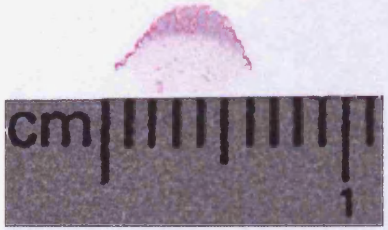
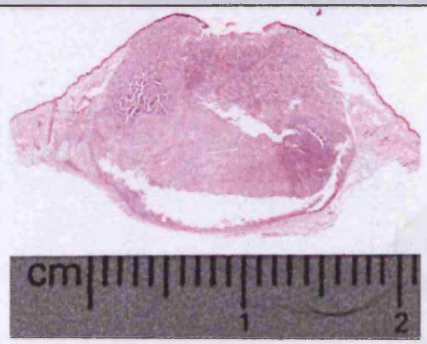
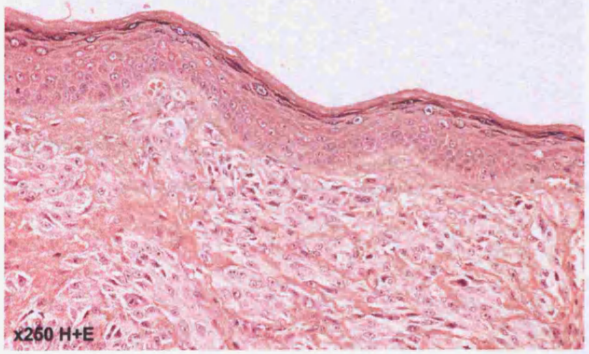
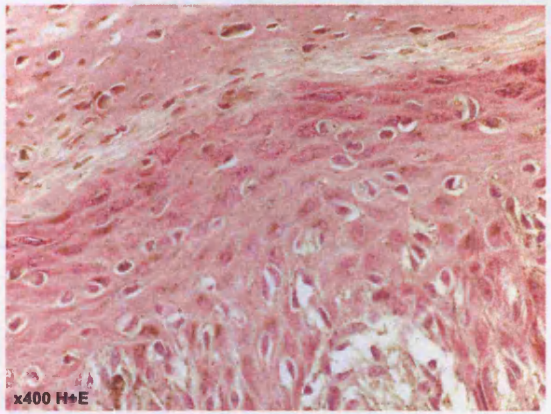
figures."[118] Unfortunately some of the cases included in the study did not meet these criteria and in such circumstances the best possible assessment on the material available was made, however if the amount of tissue available for assessment was judged to be so inadequate as to prevent reasonable histological assessment, it was discarded.

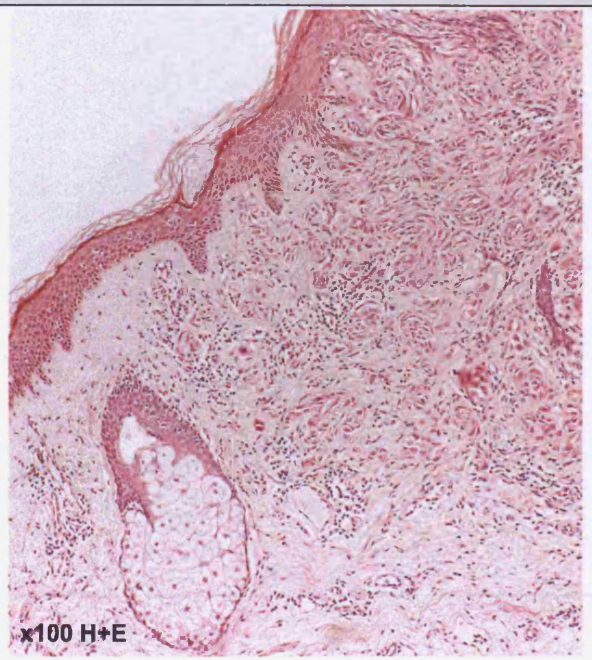
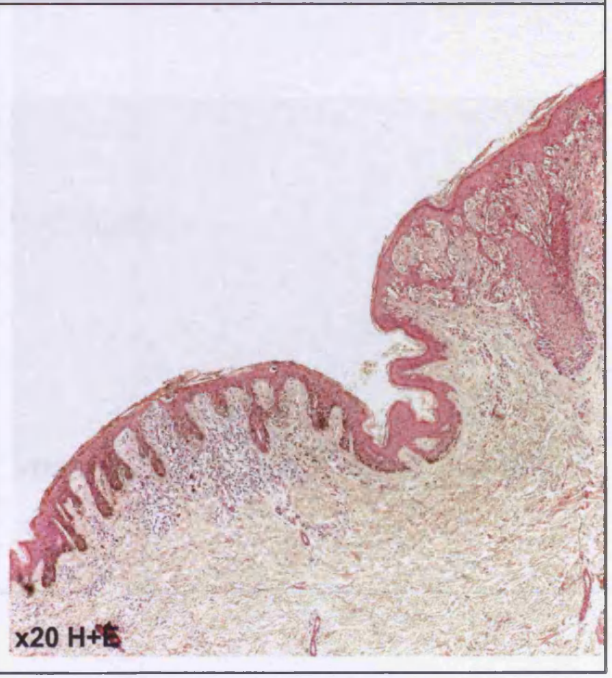
Having assessed that the submitted sections were of sufficient quality for diagnosis, the first step involved determining if a case had a Spitzoid morphology. There is no universally accepted definition of a Spitzoid morphology but the following criteria were applied based upon frequently described features of Spitzoid lesions:

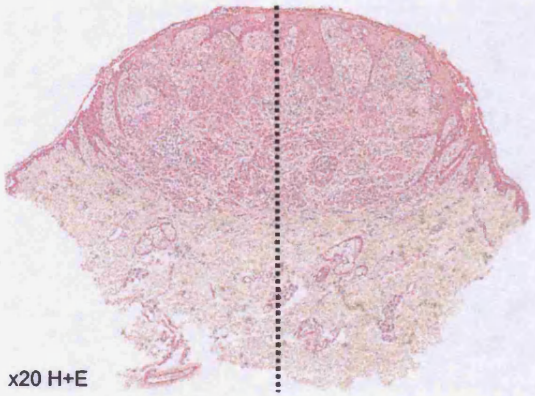
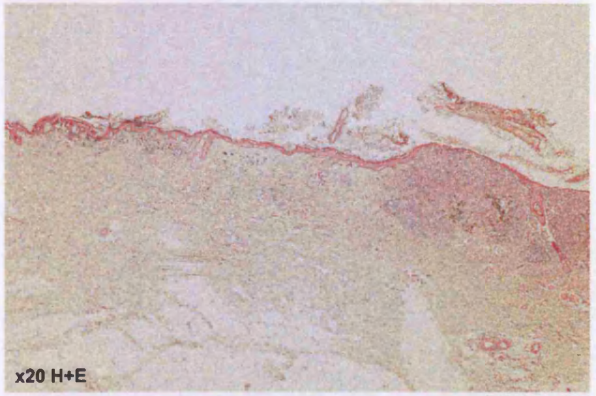
1. The overwhelming majority of cells have a spindle and/or epithelioid morphology[110, 111].
2. Where there is a dermal component, the lesion is reasonably circumscribed and reasonably symmetrical[108, 109, 111, 118, 123, 167, 176, 177].
3. The junctional features do not favour a diagnosis of SSMM, ALM or LM (M).

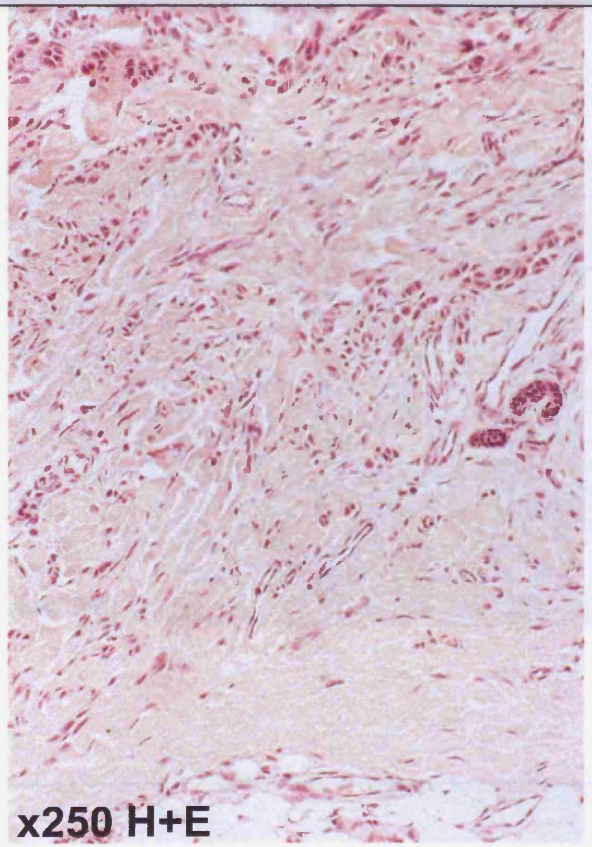
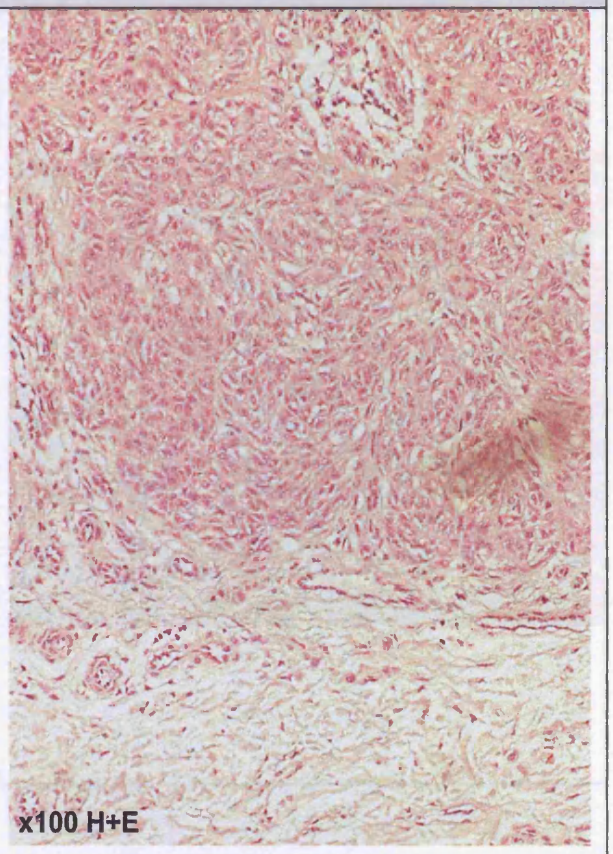
Cases that were not considered to meet the criteria for 'Spitzoid' were either diagnosed as non-Spitzoid melanomas or, if this diagnosis was not appropriate, discarded from the study. The cases that did have a Spitzoid morphology were then examined further by the two histopathologists, who assigned them to one of the following categories: Spitz naevus, atypical Spitz naevus, Spitzoid tumour of uncertain malignant potential and Spitzoid melanoma.

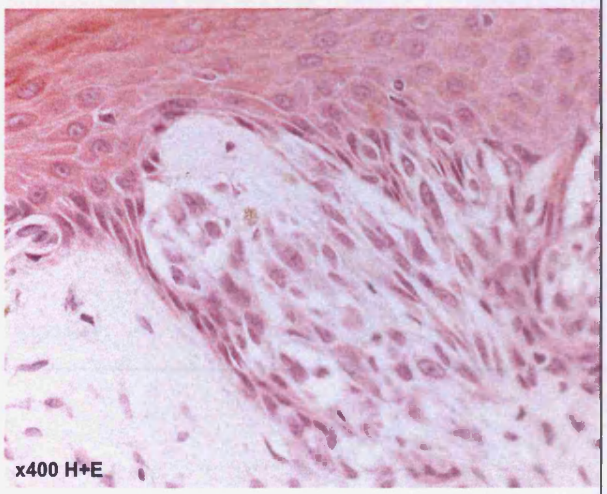
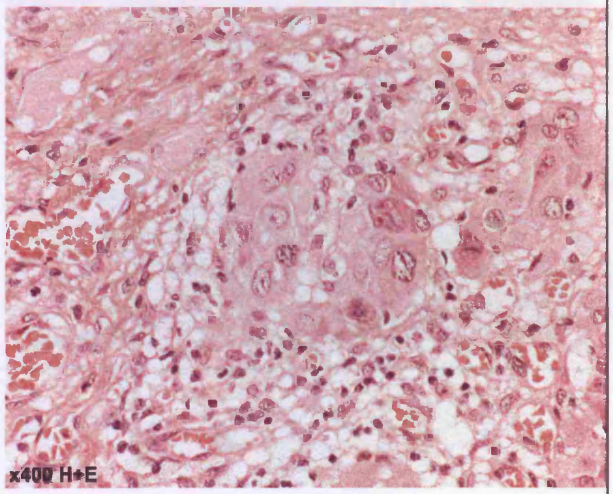
Next, a scoring system based upon that employed by van Dijk *et al*, was applied to the lesions[122]. This was modified to include criteria drawn from published methods of risk assessment in Spitzoid tumours and histological features considered to be clinically useful by the two reviewers[119, 126]. Where possible, definitions of the histological features were taken from the literature. The definitions were agreed by both reviewers and are shown below in Table 2-3.

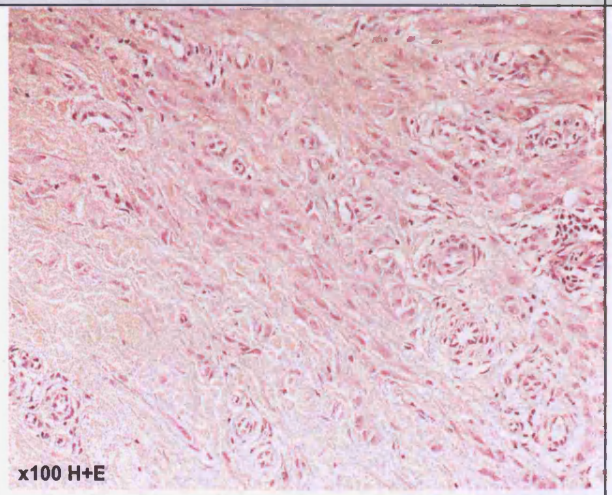
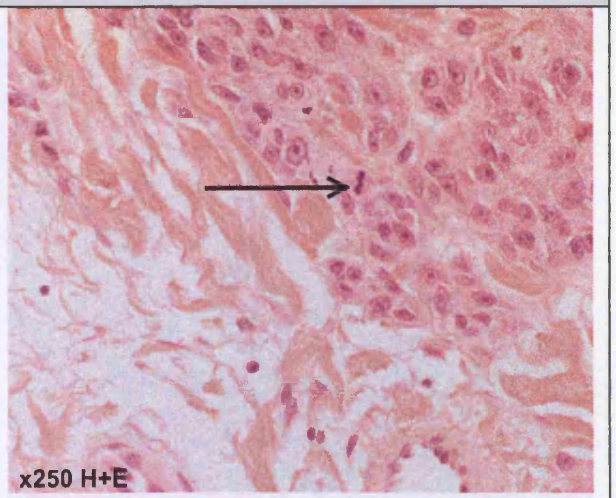
Histological Feature	Definition	Classical appearance	Atypical appearance
Size greater than 10mm[119]	In either the horizontal or vertical plane		
Pronounced Pagetoid Spread	Defined by Paniago-Peirera *et al* as randomly scattered cells throughout the epidermis, often the upper part, as is commonly seen in SSMM [109]. Mooi adds that transepidermal elimination of nests and focal single cells that are small in number relative to the junctional component are acceptable and not considered abnormal [177].		

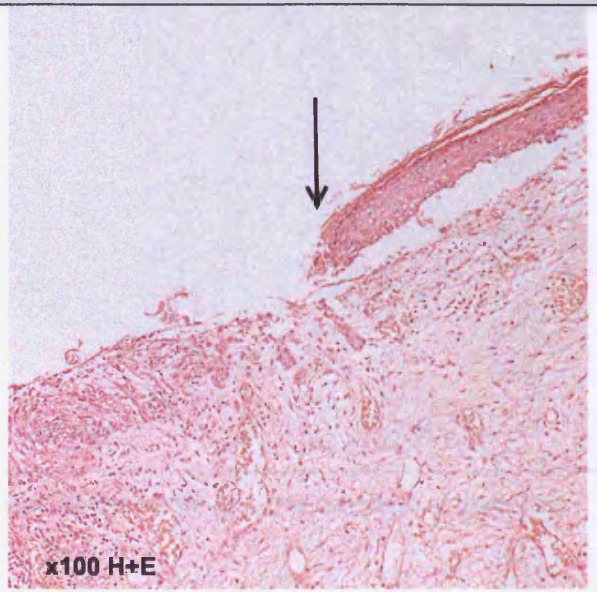
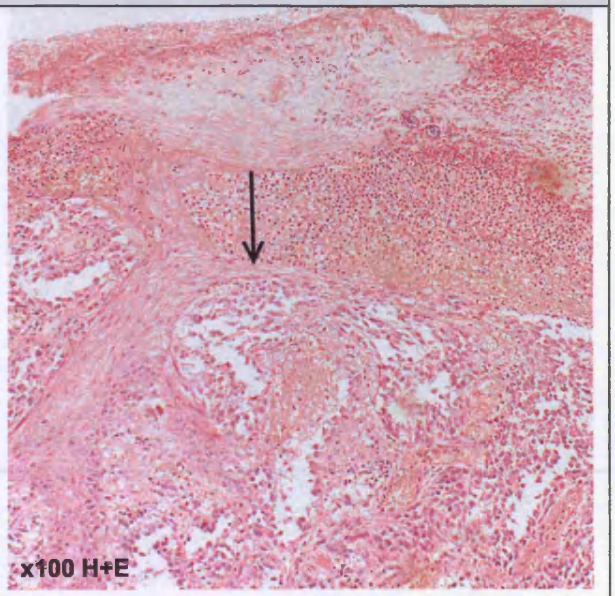
Histological Feature	Definition	Classical appearance	Atypical appearance
Poor Circumscription	Defined by Paniago-Peirera et al as being where the junctional component is not sharply demarcated laterally from the contiguous uninvolved epidermis, similar to SSMM where individual melanocytes are scattered along and above the dermoepidermal junction[109].		

Histological Feature	Definition	Classical appearance	Atypical appearance
Marked side to side asymmetry[126, 176, 178]	No explicit definitions of this have been published, however this was considered to be substantial deviation from a mirror image silhouette pattern, where the line of symmetry is perpendicular to the epidermis. The silhouette incorporates areas of involved epidermis, any inflammatory response associated with the lesion and any stromal response.	 x20 H+E	 x20 H+E

Histological Feature	Definition	Classical appearance	Atypical appearance
Lack of Maturation	Defined by Mooi as failure of individual cells to show gradual diminution with increasing depth in the lesion[177]. Maturation can lead to an increased nuclear to cytoplasmic ratio. Other authors add that where there is lack of maturation the nests of cells do not decrease in size with increasing depth and that there may be nodules or sheets of cells at the base of the lesion[108, 125, 176, 178].		

Histological Feature	Definition	Classical appearance	Atypical appearance
Cytological Atypia	Cytological features beyond those considered acceptable in a Classic Spitz naevus. It is best appreciated by assessing side to side homogeneity, and may be referred to as 'zonation'[126]. It may comprise the following: - Marked variation in the size and shape of nuclei[179] . - Absence of a delicate or dispersed chromatin pattern[125]. - Large numbers of cells with a high nuclear to cytoplasmic ratio in the superficial aspect of the lesion.		

Histological Feature	Definition	Classical appearance	Atypical appearance
Atypical Mitoses and/or deep mitoses[176, 180]	Atypical mitoses at any level of the lesion were considered significant. Typical mitoses in the lower third of the dermal component are rare in Spitz naevi but common in melanoma according to Elder and Murphy[176].	 x100 H+E	 x250 H+E

Histological Feature	Definition	Artefactual ulceration	True ulceration
Ulceration[119]	This was considered to be genuine when there was gradual thinning and loss of the epidermis and an inflammatory exudate or polymorph infiltrate. Artefactual ulceration and true ulceration are shown here.		

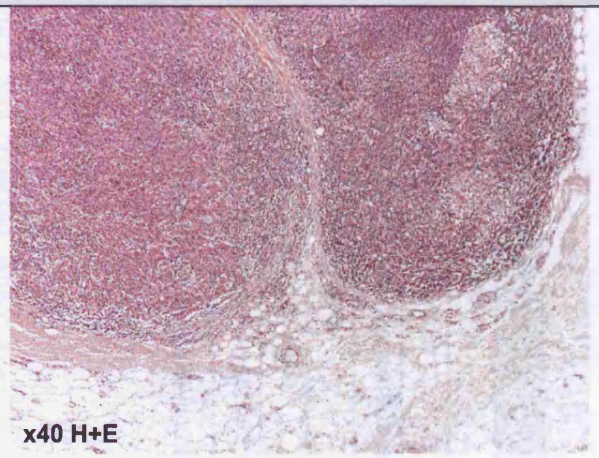
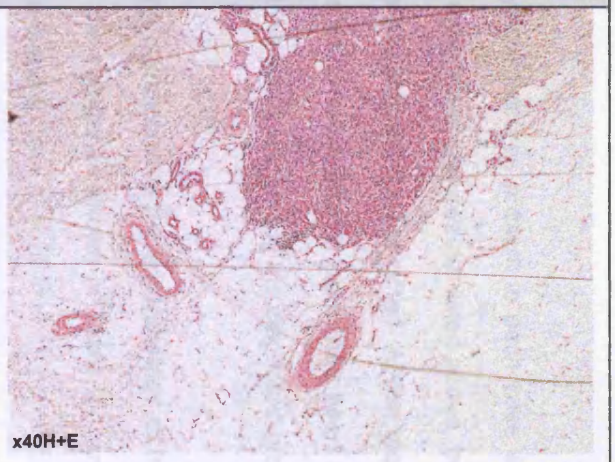
Histological Feature	Definition	Classical appearance	Atypical appearance
Involvement of the subcutis[119]	A case that invades close to, but not into, the subcutis is shown, along with a case showing genuine involvement of the subcutis	 x40 H+E	 x40H+E

Table 2-3 Definitions of atypical histological features used for the histological review stage of diagnosis.

The atypical features present in each case were recorded and a total score calculated. In addition any other features not listed above, but which the reviewers considered important to achieve the diagnosis, were noted. At the request of the reviewers, the atypical histological features were noted after a lesion had been assigned to a diagnostic category, in order to prevent the score influencing the diagnostic process.

Following completion of the second stage of histological review, all lesions were assigned to one of the categories shown in Table 2-4 and, if Spitzoid, had an atypical features score between 0 and 9.

Diagnostic category
Classic Spitz naevus
Atypical Spitz naevus
Spitzoid tumour of uncertain malignant potential (STUMP)
Spitzoid melanoma
Non-Spitzoid melanoma
Non of the above –discarded from the study

Table 2-4 Diagnostic categories into which the cases were placed following the second stage of histological review

2.2.2 Clinical data

Clinical data was collected, where available, for each case. This was either retrieved from patient notes and the histopathology database of University Hospitals of Leicester NHS Trust or submitted by collaborating histopathologists. The data set items collected are shown in Table 2-5.

Data Set Item
Patient age at diagnosis
Sex
Date of Diagnosis
Histological diagnosis
Tumour site
Breslow depth (if applicable)

Data Set Item
Presence or absence of local recurrence
Interval between recurrence and initial diagnosis (if applicable)
Histological diagnosis of recurrence (if applicable)
Presence or absence of metastatic disease
Site of metastasis/ metastases (if applicable
Size of metastasis (if applicable)
Histological diagnosis of metastasis (if applicable)
Duration of disease free survival
Date of death (if applicable)
Cause of death (if applicable)

Table 2-5 Clinical data items collected for each case

2.2.3 DNA mutation detection

2.2.3.1 Materials

The materials for mutation detection, along with the manufacturers, are listed in Table 2-6.

Material	Manufacturer
SeaKem LE agarose	Cambrex Bio Science
Ammonium Persulfate	Sigma
BigDye Mix	PNACL, University of Leicester
Boric Acid	Fluka
Bromophenol blue	Sigma
Performa DTR spin columns	Edge BioSystems
dNTP's	Invitrogen
EDTA	Sigma
Formamide	Sigma
Glisseal	Borer Chemie
Glycerol	Sigma
100 bp ladder	Gibco
MDE gel solution	Cambrex Bio Science
Magnesium Chloride	Sigma
β-mercaptoethanol	BDH
N,N,N',N'-tetramethylethylenediamine (TEMED)	Sigma
Proteinase K	Boehringer Mannheim
QIAquick PCR Purification Kit	Qiagen

Material	Manufacturer
Silver Nitrate	Sigma
Sodium Hydroxide	Sigma
GoTaq Taq DNA polymerase	Promega
Tris	Roche
Phenol/ Chloroform/ IAA 25:24:1	Sigma
Chloroform/ IAA 24:1	Sigma
Glycogen	Sigma
Sodium Chloride	Sigma
Absolute Ethanol	Sigma
Xylene Cyanol	Sigma

Table 2-6 Materials and manufacturers for mutation detection

2.2.3.2 *Buffers and solutions*

Rapid Extraction Buffer

0.05%(v/v) 1M KCL, 0.01%(v/v) 1M Tris (pH8.3), 0.0025% (v/v) $MgCl_2$, 0.0045%(v/v) Tween 20, 0.0045%(v/v) NP40

AJ PCR Buffer (Working Concentration)

45mM Tris-HCl (pH 8.8), 11mM $(NH_4)_2SO_4$, 4.5mM $MgCl_2$, 110µg/ml BSA, 16.7mM β-mercaptoethanol, 4.4mM EDTA (pH 8.0), 200mM dNTP's in water

Ethidium Bromide Solution

10 mg/ml Ethidium Bromide in Water

1x TAE Buffer

In 1 litre of ultra pure water: 4.84g/L Tris, 100ml 0.01M EDTA, 1142µl glacial acetic acid

10x TBE Buffer

In 1 litre of ultra pure water: 108g/L Tris, 55g/L boric acid, 5mM pH8.0 EDTA

Silver Staining

Fixer	10% IMS, 0.5% Glacial Acetic Acid
Stain	0.1% Silver Nitrate
Developer	1.5%NaOH, 0.16% Formaldehyde
Neutraliser	0.075% Na_2CO_3

SSCP Loading Buffer

95% Formamide, 0.25% Bromophenol Blue, 0.25% xylene cyanol, 10mM NaOH

1M Sodium Chloride

1x TE

1mM EDTA, 10mM Tris in ultra-pure water, adjusted to pH 8.0 with concentrated HCl.

2.2.3.3 DNA extraction

In the majority of cases tumour cells were separated from the surrounding tissue by naked-eye manual microdissection with a 20µl pipette tip, following identification of tumour-rich areas by examining corresponding H+E sections. Single stranded conformational polymorphism (SSCP) using this protocol has been shown to be able to detect mutations where tumour cells comprise as little as 10% of the total sample without false negative results[63]. An H+E section of a manually dissected case is illustrated in Figure 2-6.

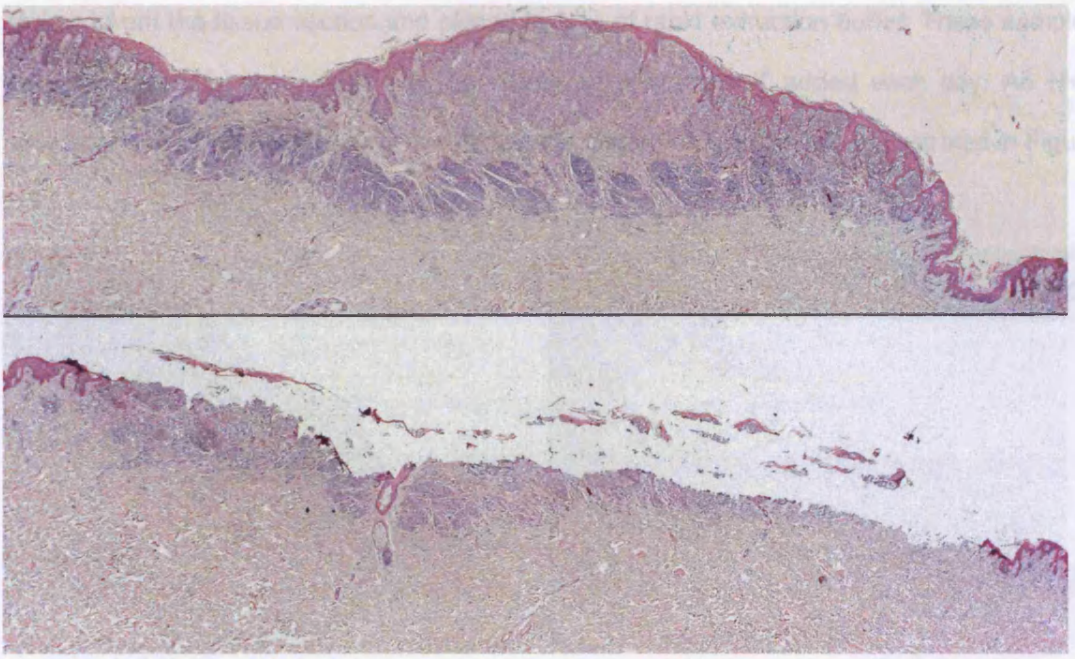


Figure 2-6 A manually microdissected tumour. This case was dissected by naked eye using a 20µl pipette tip.

Up to ten 4µm tissue sections, depending on the amount of tumour present, were de-waxed in xylene twice for five minutes and rehydrated by immersion in 99% industrial methylated spirit (IMS) twice for three minutes, 95% IMS for three minutes and left to air dry. Rapid extraction buffer containing 5µl 10mgl/ml proteinase K was applied to tumour rich areas of the tissue section, which were subsequently manually dissected with a pipette tip. The dissected tissue was aspirated from the section and placed in rapid extraction buffer to achieve a final volume of 100µl. Samples were incubated at 37˚C for up to seven days until all the tissue appeared to have been digested. A further 5µl 10mgl/ml proteinase K was added each day. Following digestion, cases were heated to 98˚C for 15 minutes to denature the enzyme, and stored at 4˚C

For the investigation of Wnt5a expression during melanoma progression, cases of RGP melanoma that were too thin to allow adequate extraction of tumour DNA by manual microdissection, and the RGP components of VGP tumours were micro dissected using a dissecting microscope and a 26G needle mounted on a 10ml syringe according to a method modified from Zhuang *et al*[181]. Once the relevant tumour areas were identified they were

scraped from the tissue section and placed in 30µl of rapid extraction buffer. These samples were incubated for three days with 5µl 10mgl/ml proteinase K added each day. An H+E section of a case dissected with a needle using a dissecting microscope is illustrated in Figure 2-7

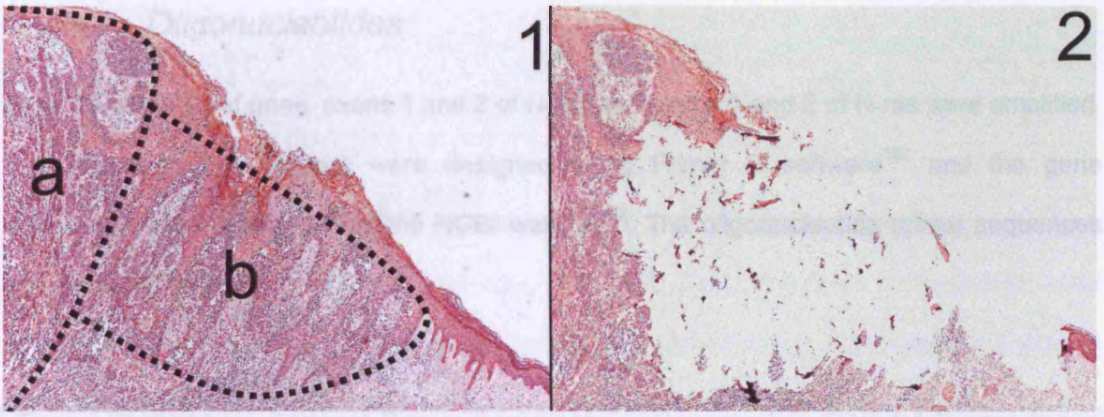


Figure 2-7 A manually micro dissected tumour. This case was dissected by using a dissecting microscope and a 26G needle mounted on a 10ml syringe. In image 1, area a shows the VGP part of the tumour, while area b shows the RGP component adjacent to the VGP. In image 2 the RGP component adjacent to the VGP has been discreetly micro dissected.

For the investigation of Spitzoid tumours, before amplification of N-ras exons 1 and 2, and H-ras exons 1 and 2, the DNA was cleaned using phenol-chloroform. PCR amplification of B-raf was found to be less efficient following phenol-chloroform treatment and so B-raf amplification was performed prior to this step.

To clean the DNA, the 100µl DNA extract was mixed with 100µl phenol/ chloroform/ IAA and micro centrifuged at 13,000 rpm for 2 minutes. The aqueous layer was removed, mixed with 100µl chloroform/ IAA, mixed and micro centrifuged at 13,000rpm for 2 minutes. Again, the aqueous layer was removed and mixed with 10µl 1M Nacl, 200µl cold absolute ethanol and 1µl glycogen and rested for 1 hour at -20°C. The mixture was micro centrifuged for 15 minutes at 13,000rpm and the supernatant removed. The remaining pellet was re-suspended in 200µl 70% ethanol and microfuged at 13,000rpm for 10 minutes. The supernatant was removed and the remaining pellet air dried and suspended in 30µl 1xTE buffer.

2.2.3.4 *Thermal cyclers*

Thermal Cycling was performed with the GeneAmp PCR system 9700 (Perkin Elmer, Oak Brook, IL).

2.2.3.5 *Oligonucleotides*

Exon 15 of the B-raf gene, exons 1 and 2 of N-ras and exons 1 and 2 of H-ras were amplified. The oligonucleotide primers were designed using Primer 3 software[182] and the gene sequences were obtained from the NCBI website[183]. The oligonucleotide primer sequences are shown in Table 2-7

Gene	Exon	Forward Primer	Forward Primer Sequence	Reverse Primer	Reverse Primer Sequence
B-raf	15	Fsh1	TTTCCTTTACTTACTACACCTC	Rsh	TCAGGGCCAAAAATTTAATCA
N-ras	1	F2	CTCGCCAATTAACCCTGATT	R2	CCGACAAGTGAGAGACAGGA
	2	Fsh1	CACCCCCAGGATTCTTACAG	Rsh1	TCGCCTGTCCTCATGTATTG
H-ras	1	shF	AGGAGACCCTGTAGGAGGA	shR	CGCTAGGCTCACCTCTATAGTG
	2	F2	AGAGGCTGGCTGTGTGAACT	R	TCACGGGGTTCACCTGTACT

Table 2-7 Oligonucleotide primer used for PCR amplification of the exons of interest

2.2.3.6 *PCR amplification of the exons of interest*

PCR was carried out in 50µl reactions containing 0.2pm/µl of each primer, 1 unit of Taq polymerase and 1-5µl target DNA (depending on the amount and quality of DNA yielded by extraction, which could vary considerably – see section 6.3.1 in Appendix 1: Optimisation of techniques) in AJ PCR buffer. The amplification protocol consisted of an initial denaturation step of 98°C for 3 minutes followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds and a final incubation at 72°C for 7 minutes. PCR products were stored at 4°C

2.2.3.7 *Agarose gel electrophoresis*

PCR products were run on a 3% SeaKem LE agarose gel consisting of 1x TAE and 0.05% ethidium bromide. Gels were run at 100V for approximately 1 hour in a tank containing 1x TAE and 0.05% ethidium bromide. The gels were visualised and photographed using a UV transilluminator.

2.2.3.8 *SSCP gel preparation*

20x20cms gel plates were cleaned alternately with water and IMS 3 times. 1mm spacers were placed between the plates and given a watertight seal with Glisseal. The plates were fixed in a gel-pouring rack and the gel solution prepared. MDE gel solution was used at 0.5x final concentration in 0.6x TBE buffer. 10% ammonium persulfate solution was added to a final concentration of 5µl/ml and N<N<N',N'-tetramethyethylethylenediamine (TEMED) was added to a final concentration of 0.5µ/ml. The gel was poured between plates using a syringe and a 1mm comb added. The gel was allowed to set at room temperature for 2 hours. Prior to loading the samples the gel was pre-run at 350V for approximately 15 minutes.

2.2.3.9 *Sample preparation*

The volume of PCR product included in each sample varied according to the concentration of product obtained from the PCR reaction, as determined by band intensity on agarose gel electrophoresis. Three to 9µl of PCR product was added to SSCP denaturing loading buffer to give a final volume of 12µl. Samples were heated to 95°C for 5 minutes, chilled on ice and loaded onto the gel. Gels were run in 0.6% w/v TBE buffer at 4°C. The gels were run at 350V overnight except for N-ras exon 2 analysis, where gels were run at 270V overnight; and H-ras exon 2 where gels were run at 350V for 20 hours.

2.2.3.10 Silver staining of SSCP gels

SSCP gels were removed from the gel plates and washed twice in fixer for 3 minutes with fresh fixer used for each wash. The gel was shielded from light, placed in silver nitrate solution for 15 minutes and then rinsed twice in water. The gel was then shielded from the light and placed in developer until sufficiently developed for up to 20 minutes. The gel was then placed in neutralising solution for 1 hour and digitally scanned.

Aberrantly migrating bands were picked from the gel with a 25G needle and amplified by PCR before sequencing.

2.2.3.11 Sequencing

The PCR reactions derived from the aberrantly migrating bands were purified using the QIAquick PCR purification kit according to the manufacturers instructions, and quantified using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Delaware, USA). 20µl BigDye sequencing reactions were set up containing 20ng of PCR product, 8µl BigDye mix and 3.5pM of oligonucleotide primer. The primers used for the different exons are shown in Table 2-8. Samples were then amplified by PCR for 35 cycles with 96°C denaturation, 50°C annealing for 10 seconds and extension at 60°C for 4 minutes. Unincorporated DyeDeoxy terminators were removed using Performa DTR columns, and the sequence analysed by the Protein and Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester. Sequences were examined using Chromas v1.45 software and the Basic Local Alignment Search Tool[184] from the National Centre for Biotechnology Information website[127].

Gene	Exon	Primer
B-raf	15	Rsh
N-ras	1	R2
	2	Fsh1
H-ras	1	shR
	2	R

Table 2-8 Primers used for sequencing DNA amplified from aberrantly migrating bands

2.2.3.12 Controls

Negative controls: Sections of normal skin were included with each round of extractions and run alongside the corresponding cases throughout the protocol.

Positive controls: Exon-specific positive controls were run with each SSCP gel. These are detailed in Table 2-9

Exon	Control name	Control ATCC number
B-raf 15	Skmel 28 cell line	HTB-72
N-ras 1	Molt4 cell line	CRL-1582
N-ras 2	HT1080 cell line	CCL-121
H-ras 1	T24 cell line	HTB-4
H-ras 2	Positive case H59/06	n/a

Table 2-9 Positive control DNA used for SSCP analyses

2.2.4 Statistical analysis

Analysis of categorical data was via chi-square and Fisher's exact tests. For 2x2 tables, Yates' corrected chi-square was calculated except where assumptions were not met and Fisher's exact tests were used. Non-parametric analyses were used for continuous data. Analysis of trend was via Jonckheere's test. Samples were compared using Mann Whitney U and Kruskal-Wallis tests. Probabilities in binomial data were determined via Bernoulli trial. $p<0.05$ was considered significant. Analyses were performed using SPSS for Windows release 12.0.

3 Results of characterisation of Wnt5a expression during cutaneous melanoma progression

The primary aims of this study were to provide detailed information on Wnt5a expression during melanoma progression according to Clark's model and determine the effect of Wnt5a expression on outcome. Changes in Wnt5a expression were compared with two comparatively well-characterised alterations, namely $p16^{INK4a}$ expression and B-raf mutation.

3.1 Optimisation of techniques

See Appendix 1: Optimisation of techniques

3.2 Clinicopathological data

The CAN samples were predominantly intradermal (n=15; 75%) with the rest compound (n=5; 25%), and the patients had a mean age of 36.9 years. The purely RGP tumour samples were predominantly of superficial spreading type (n=32; 91.4%) with the remainder comprising acral lentigenous tumours (n=3; 8.6%), and were excised from the trunk in 14 cases (40%), lower extremity in 8 cases (22.9), upper extremity in six cases (17.1%) and head and neck in six cases (17.1%). This data was missing in one case (2.9%). The mean age of these patients was 54.9 years. The VGP tumour samples comprised 40 nodular melanomas (67.8%), 18 superficial spreading melanomas (30.5%) and one case of lentigo maligna melanoma (1.7%). VGP tumours were excised from the lower extremities in 21 cases (35.6%), the trunk in 14 cases (23.7%), the upper extremities in 8 cases (13.6%) and head and neck in 8 cases (13.6%). The majority of the matched metastases were from lymph nodes (n=44; 74.6%) and the rest from skin (n=14; 23.7%) and liver (n=1; 1.7%). The mean age of patients with VGP melanoma and matched metastases was 56.5 years. The clinicopathological data is summarised in Table 3-1.

Tumour Type	Mean age (years)	Sex	Site of tumour	Histological Sub-type	Mean Breslow Depth (mm)
CAN n=20	36.9	M - 6 (30%) F - 14 (70%)		Compound - 5 (25%) Intradermal - 15 (75%)	n/a
Purely RGP n=35	54.9	M - 14 (40%) F - 21 (60%)	Head & neck - 6 (17.1%) Upper extremity - 6 (17.1%) Trunk - 14 (40%) Lower Extremity - 8 (22.9%) Unknown – 1 (3%)	SSMM - 32 (91.4%) ALM - 3 (8.6%)	0.39 (SD = 0.31)
VGP n=59	56.5	M - 26 (44.1%) F - 33 (55.9%)	Head & neck - 8 (13.6%) Upper extremity - 8 (13.6%) Trunk - 14 (23.7%) Lower Extremity - 21 (35.6%) Unknown – 8 (13.6%)	Nodular - 40 (67.8%) SSMM - 18 (30.5%) LMM - 1 (1.7%)	4.34 (SD = 3.14)

Table 3-1 Clinicopathological data for the three primary tumour types. SSMM = superficial spreading malignant melanoma, ALM = acral lentigenous melanoma, LMM = lentigo maligna melanoma.

There were 43 additional cases of primary melanoma that were used only for the survival analysis. Immunostaining and semi-quantification was performed by a colleague (Qiang Huang). These cases had a mean Breslow thickness of 1.68 mm (SD = 1.49 mm). Of these, 3 were from the head and neck, 7 from the lower limb, 8 from the upper limb, 18 from the trunk and in 7 the site was not documented.

3.3 Results of Wnt5a and $p16^{INK4a}$ immunohistochemistry

3.3.1 Morphological aspects of Wnt5a and $p16^{INK4a}$ immunohistochemistry

Immunohistochemical staining of Wnt5a was often limited to the cytoplasm, however occasional areas of nuclear staining were encountered. In some instances this appeared more prominent at the edge of tissue sections and was considered artefactual. Only cytoplasmic staining was scored for analysis of Wnt5a in progression.

There was some heterogeneity of staining within samples, and this appeared more prominent in the primary tumours than the metastases. An example of tumour heterogeneity is shown in Figure 3-1. Within primary tumours there was no stratification of staining in relation to the depth of the lesion, and neither was there any appreciable difference of the staining in cells with a particular morphology, such as spindle cells, as has been suggested by one author[9]. In addition to staining melanocytes, there was often Wnt5a staining within the epidermis that was most marked in the stratum spinosum, as well as a degree of expression in sebaceous epithelial cells.

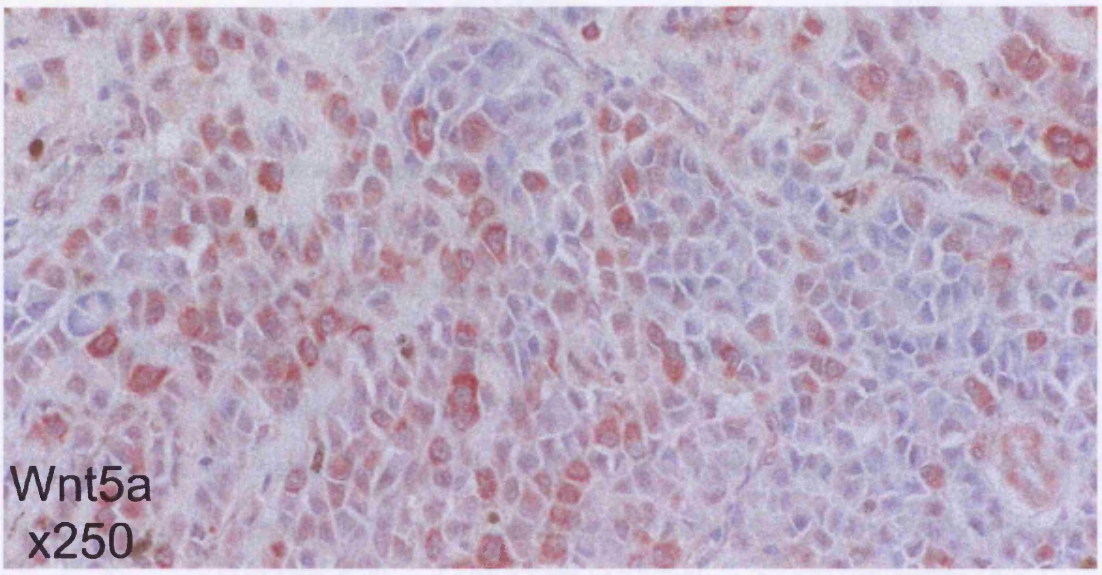


Figure 3-1 Heterogeneic Wnt5a staining

By comparison, $p16^{INK4a}$ had a greater propensity for heterogeneous staining, which again was most apparent in primary tumours. In some instances, basal non-neoplastic melanocytes within the epidermis would show positive staining but this was an infrequent finding (Figure 3-2A). A striking finding which has been previously described[34, 102] was that, even in comparatively high expressing tumours, expression of $p16^{INK4a}$ would be present in some cells but absent in those immediately adjacent, producing a mosaic-like pattern (Figure 3-2B). Diffuse nuclear staining was not found in any tumour. Some tumours would show cytoplasmic staining (Figure 3-2C), however this was disregarded because the nature of this is unclear and may represent normal protein, but may also represent mutant $p16^{INK4a}$ protein or non-specific binding of the antibody[32, 97, 185, 186]. Some of these features are shown in Figure 3-2.

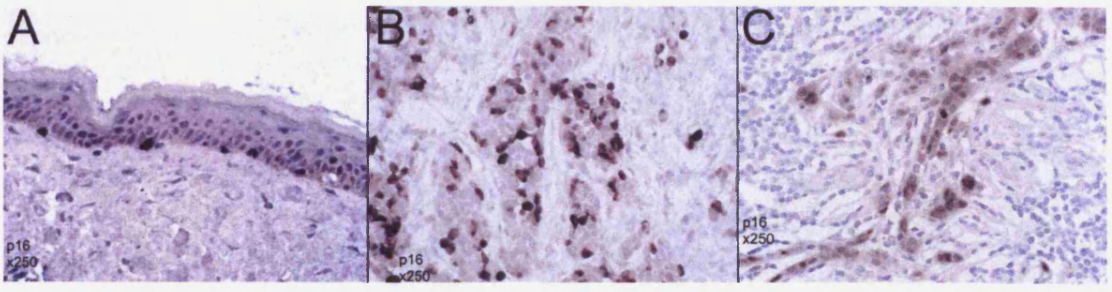


Figure 3-2 Immunohistochemistry for $p16^{INK4a}$. A) Positive staining in non-neoplastic basal melanocytes, B) mosaic-like staining, a commonly seen feature, C) cytoplasmic staining.

3.3.2 Expression of Wnt5a and $p16^{INK4a}$ in matched samples

In the 59 VGP melanoma samples, areas of contiguous naevus and RGP components adjacent to the VGP were identified alongside the collection of 58 matched metastases (one metastasis was discarded because it was entirely necrotic). The median Wnt5a expression scores for all matched samples were 200.0 (n=7) in contiguous naevi, 100.0 (n=35) for RGP components in VGP melanomas, 123.4 (n=59) for VGP melanoma and 148.8 (n=58) in metastases. In the same samples, for $p16^{INK4a}$, the median expression was 6.6% in contiguous naevi, 1.26% in RGP components in VGP melanomas, 0.2 in VGP melanomas and 0.0 in metastases. This data is illustrated in Figure 3-4 and immunohistochemistry from representative cases is shown in Figure 3-3.

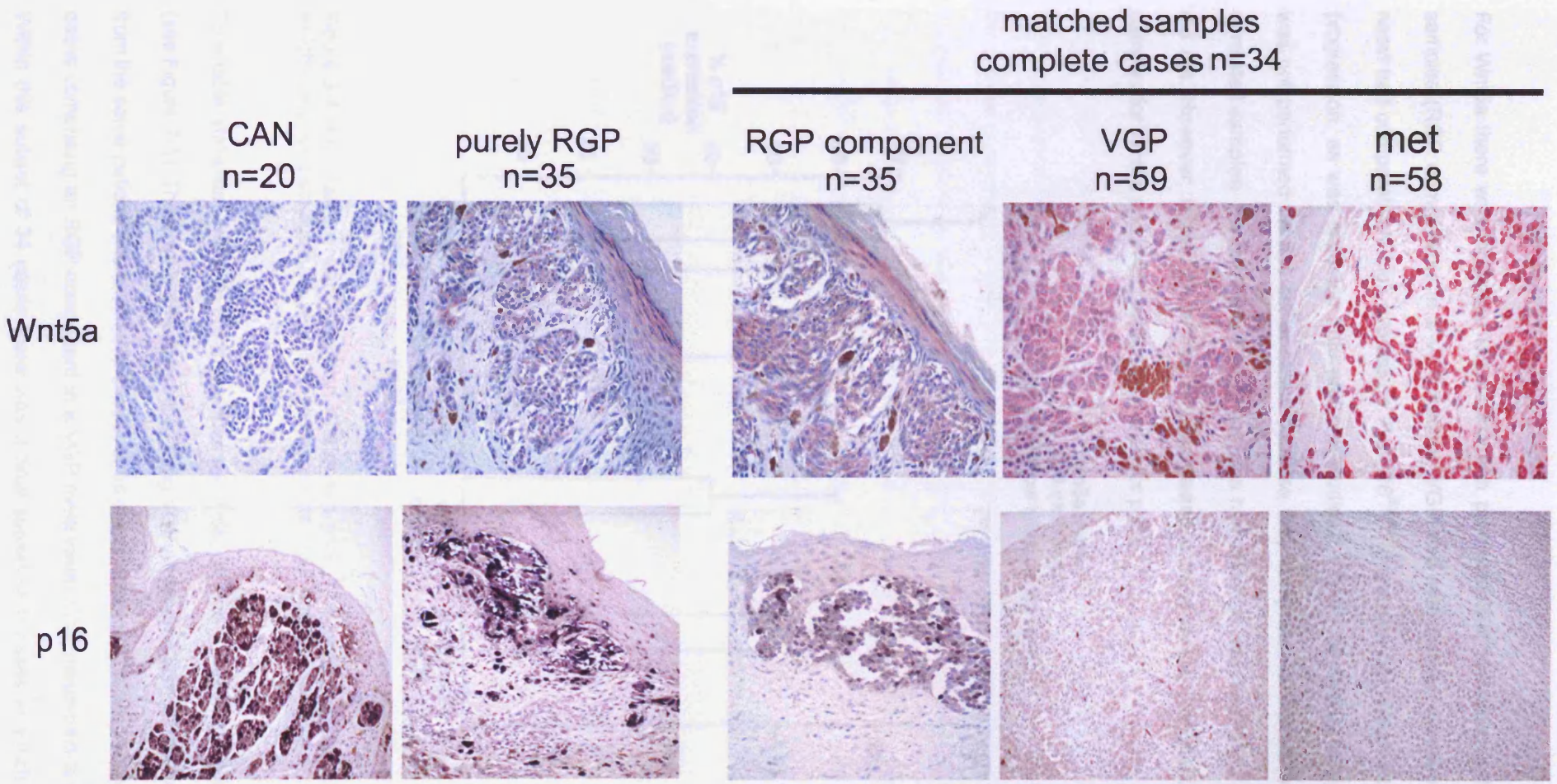


Figure 3-3 Wnt5a and $p16^{INK4a}$ immunohistochemistry

For Wnt5a there was increased expression with progression in the malignant parts of the samples (RGP components in a VGP tumour, VGP and metastases), however, contiguous naevi had comparatively high expression. For $p16^{INK4a}$, there was decreased expression with progression, as was expected from previous studies[32, 104, 105]. Statistical analysis for trend was not performed on this set of cases because they comprised a mixture of related and unrelated samples. For example, 7 VGP samples had matched naevi while the remaining 52 did not. However, inspection of the data suggested a trend in the malignant parts of the samples for Wnt5a and in all parts of the lesion for $p16^{INK4a}$.

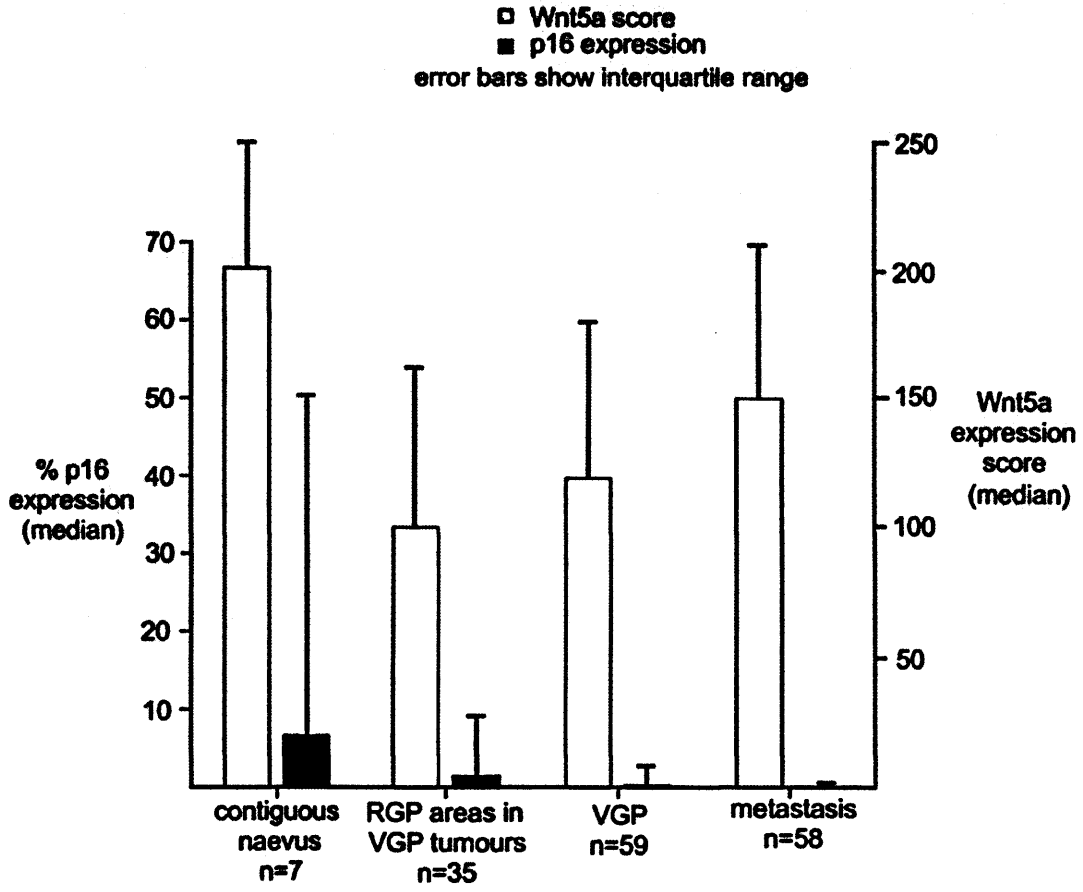


Figure 3-4 Bar chart comparing median Wnt5a and $p16^{INK4a}$ scores in all matched samples, which comprised contiguous naevi, RGP components, VGP and metastases.

To enable statistical analysis, the three subsets of 58, 34 and 6 matched cases were used (see Figure 2-1). There were 58 cases comprising VGP melanoma with a matched metastasis from the same patient, and within this subset of 58 cases, there was a secondary subset of 34 cases comprising an RGP component in a VGP melanoma, VGP melanoma and metastasis. Within this subset of 34 cases there was a final subset of 6 cases in which all stages of

Clark's model were represented. This enabled a within subject analysis of trend to be performed to test whether the trend observed in all the matched samples was statistically significant.

In these perfectly matched subsets, the key finding was that the 34 cases containing an RGP component, a VGP and a metastasis showed a significant trend of increasing Wnt5a expression with progression (p=0.013 Page's L test). This confirmed the impression gained from analysing all the matched samples (see Figure 3-4), where statistical analysis could not be performed. There was no significant trend of Wnt5a expression in the 6 matched cases with a contiguous naevus (p= 0.42 Page's L test) because of the relatively high expression in the naevus components. When only the malignant parts of these cases were analysed (RGP component, VGP and metastasis), there was a nearly significant trend of increasing expression with progression (n=6, p=0.075, Page's L test). In the 58 cases comprising VGP and matched metastasis, expression tended to be greater in the metastases, but this difference was not significant (n=58, p=0.13 Wilcoxon signed-rank test).

For $p16^{INK4a}$ there was a significant trend of decreasing $p16^{INK4a}$ expression with progression in matched cases comprising an RGP component in a VGP tumour, VGP and metastasis (n=34, p=0.006 Page's L test) and cases of VGP and matched metastasis (n=58, p=0.015 Wilcoxon signed-rank test). A nearly significant trend of decreasing $p16^{INK4a}$ expression was seen in matched cases with a contiguous naevus (n=6, p=0.052 Page's L test).

The distribution of Wnt5a and $p16^{INK4a}$ expression within these subsets of matched cases and the significance of the trends are illustrated in Figure 3-5.

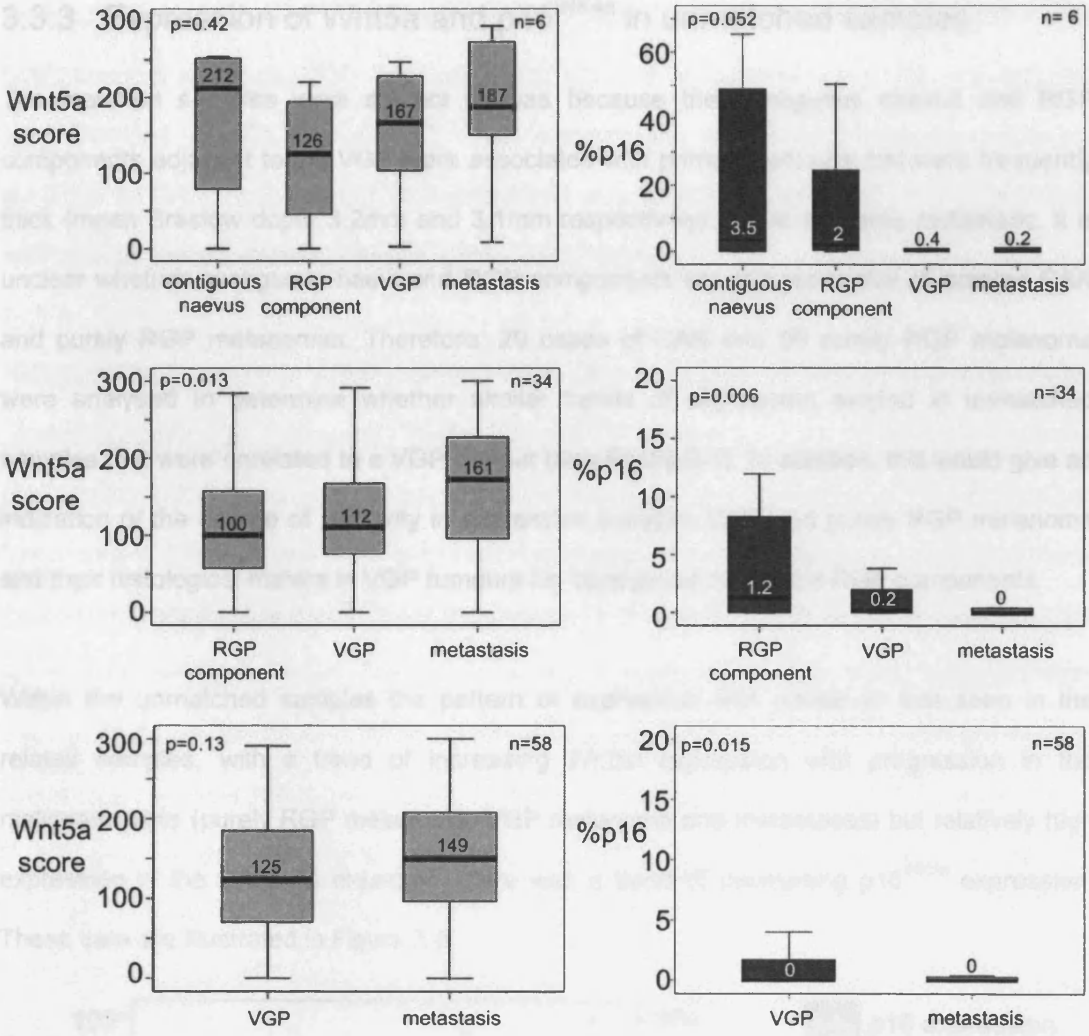


Figure 3-5 Box plots showing Cytoplasmic Wnt5a and nuclear p16^{ink4a} immunostaining in the three subsets of matched cases. Note that the 6 cases with a contiguous naevus component are a subset of the 34 cases with a radial growth phase component, which in turn are a subset of the total of 58 cases of matched VGP melanomas and metastases. The median values are given above the median lines. The significance of the associated trends are also shown.

Within the subset of 34 cases, where all the samples were malignant (RGP components, VGP and metastases), post hoc analyses showed significantly different Wnt5a expression between RGP components and metastatic tumours (p=0.015 Wilcoxon signed-rank test, Bonferroni correction). For p16^{INK4a} there were significant differences of expression between both RGP components and VGP tumours, and their matched metastases (p=0.021 and p=0.045 respectively (as above)).

3.3.3 Expression of Wnt5a and p16^{INK4a} in unmatched samples

The matched samples were subject to bias because the contiguous naevus and RGP components adjacent to the VGP were associated with primary tumours that were frequently thick (mean Breslow depth 3.2mm and 3.1mm respectively) and in all cases metastatic. It is unclear whether contiguous naevi and RGP components are representative of isolated CAN and purely RGP melanomas. Therefore, 20 cases of CAN and 35 purely RGP melanoma were analysed to determine whether similar trends of expression existed in unmatched samples that were unrelated to a VGP tumour (see Figure 2-1). In addition, this would give an indication of the degree of similarity in expression between CAN and purely RGP melanoma and their histological mimics in VGP tumours i.e. contiguous naevi and RGP components.

Within the unmatched samples the pattern of expression was similar to that seen in the related samples, with a trend of increasing Wnt5a expression with progression in the malignant parts (purely RGP melanoma, VGP melanoma and metastases) but relatively high expression in the CAN. As expected, there was a trend of decreasing p16^{INK4a} expression. These data are illustrated in Figure 3-6.

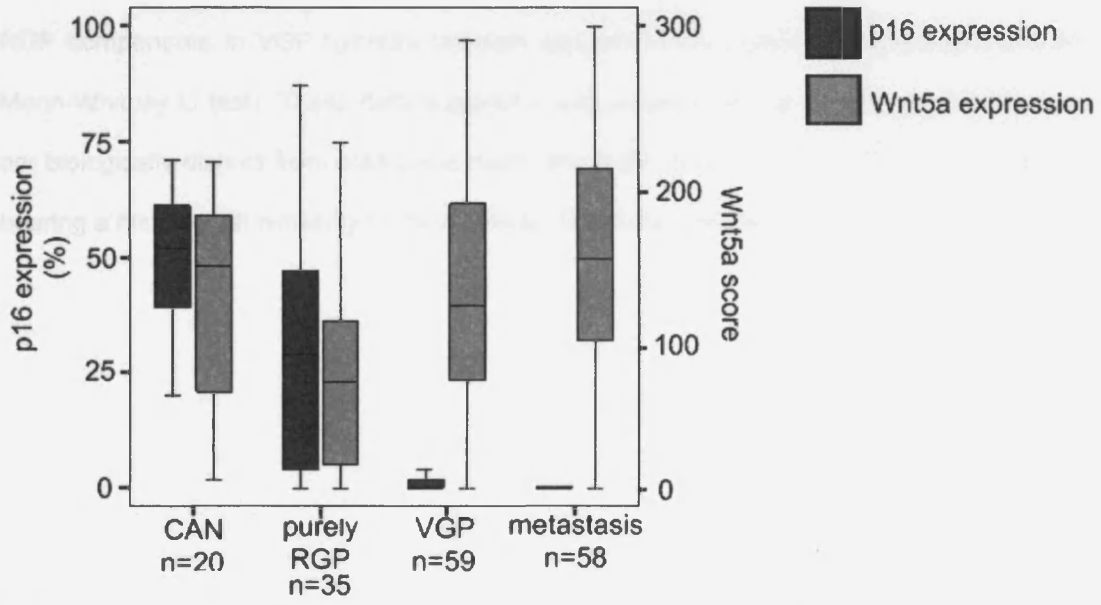


Figure 3-6 Box plots showing expression of Wnt5a and p16^{INK4a} in unmatched samples of CAN, purely RGP melanoma, VGP melanoma and metastases.

Statistical analysis of trend could not be performed because the cases were a mix of related and unrelated samples. However, for Wnt5a, significant differences of expression were seen

between CAN and purely RGP tumours (p=0.04 Mann-Whitney U test), and between purely RGP tumours and both VGP and metastatic tumours respectively (p=0.002 and p<0.001 Mann-Whitney U test). For $p16^{INK4a}$ these comparisons were also statistically significant (p=0.008 for CAN versus purely RGP melanoma, and between purely RGP tumours and both VGP and metastatic tumour, both p<0.001, Mann-Whitney U test).

Histologically, contiguous naevi resemble CAN, and RGP components in VGP melanomas resemble purely RGP melanoma. Wnt5a and $p16^{INK4a}$ expression were characterised in these four sample types to determine whether they were similar at the level of protein expression. The cases of CAN showed lower expression of Wnt5a than that observed in contiguous naevi (median expression 144.3 and 200.0 respectively) however this difference was not significant (p=0.18 Mann-Whitney U test). Wnt5a expression in purely RGP tumours was also lower than that of RGP components in VGP tumours, but again this was not significant (median expression 69.2 and 100.0, p=0.9 Mann-Whitney U test). Expression of $p16^{INK4a}$ was analysed in a similar manner, and the expression in CAN was significantly higher than that seen in contiguous naevi (median expression 52.1 and 6.6 respectively, p=0.017 Mann-Whitney U test) and a similar difference of expression was identified between purely RGP tumours and RGP components in VGP tumours (median expression 29.1 and 1.4 respectively, p<0.001 Mann-Whitney U test). These data suggested that isolated CAN and purely RGP melanoma are biologically distinct from contiguous naevi and RGP components of VGP tumours, despite bearing a histological similarity to them. These differences are illustrated in Figure 3-7.

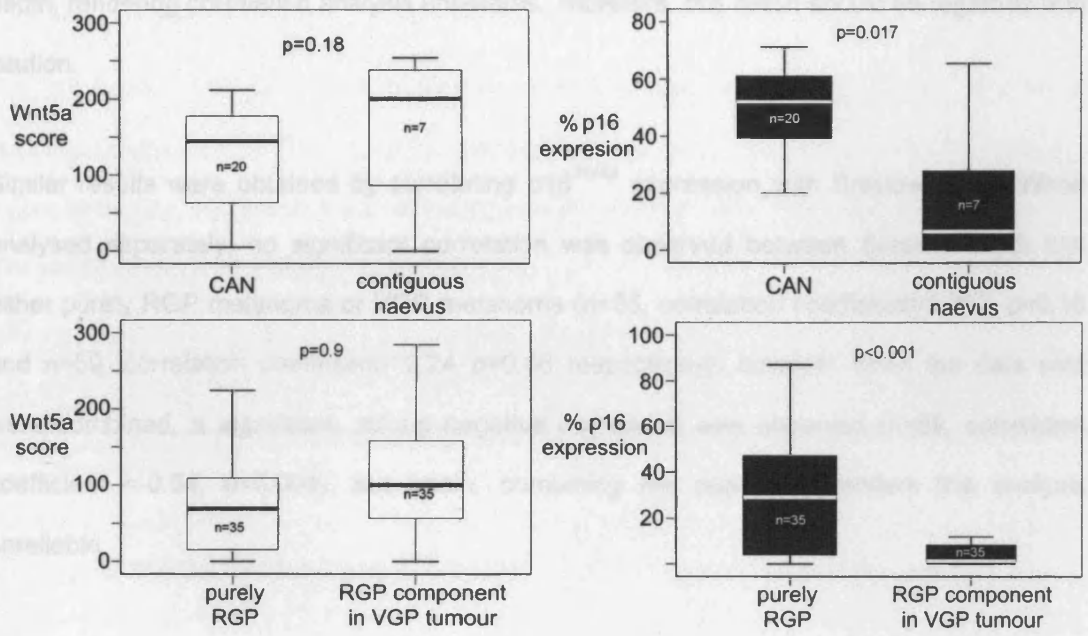


Figure 3-7 Box plots showing differences in expression of Wnt5a and $p16^{INK4a}$ between CAN and purely RGP melanoma and their equivalent components in VGP tumours, namely contiguous naevi and RGP components in VGP tumours. The significance of these differences is shown.

Nevertheless, the key finding in this part of the study was that in both contiguous naevi and in isolated CAN, there was relatively high Wnt5a expression, which was not in keeping with the trend seen in the malignant components of Clark's progression model.

3.4 Correlation of Wnt5a and $p16^{INK4a}$ expression with clinical parameters

3.4.1 Breslow depth

Correlation of Wnt5a expression in purely RGP melanomas and VGP melanoma samples with Breslow depth was not significant (n=35, correlation coefficient=0.26, p=0.126 and n=59, correlation coefficient=-0.11 p=0.43 respectively, Spearman's Rank Order Correlation). When the data were combined, a moderately positive correlation was observed and significance was achieved (n=94, correlation coefficient =0.3, p=0.015) [187] however, this figure is derived from the combination of two separate groups of tumours that vary in distribution of Breslow

depth, rendering correlation analysis unreliable. Therefore, this result should be regarded with caution.

Similar results were obtained by correlating $p16^{INK4a}$ expression with Breslow depth. When analysed separately, no significant correlation was observed between Breslow depth and either purely RGP melanoma or VGP melanoma (n=35, correlation coefficient=0.262, p=0.16 and n=59, correlation coefficient=-0.24 p=0.06 respectively), however when the data sets were combined, a significant, strong negative correlation was observed (n=59, correlation coefficient =-0.54, p<0.001), but again, combining the data sets renders this analysis unreliable.

When the purely RGP and VGP samples were grouped by the current T-category thresholds of the American Joint Committee on Cancer TNM Staging system [2, 3] a significant trend of increasing Wnt5a expression with stage of tumour invasion was observed (p=0.041, Jonckheere's test). For $p16^{INK4a}$, there was a significant trend of decreasing expression with stage of tumour invasion (p<0.001). The distribution of Wnt5a and $p16^{INK4a}$ expression for the different tumour stages is illustrated in Figure 3-8.

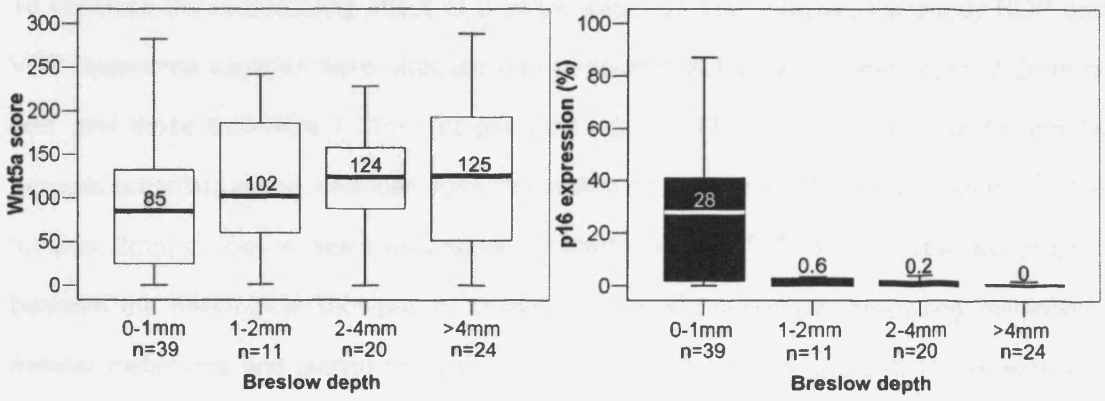


Figure 3-8 Box plots showing expression of Wnt5a and $p16^{INK4a}$ at different stages of tumour depth. Median expression values are shown above the median bars.

3.4.2 Tumour site

For the purely RGP and VGP melanoma samples there was no significant relationship between Wnt5a or $p16^{INK4a}$ expression and tumour site when categorised as head and neck, upper extremity, trunk and lower extremity (n=85, p=0.081 and n=85, p=0.96 respectively). These data are summarised in table Table 3-2.

Site	Wnt5a score (±SD)	mean $p16^{INK4a}$ expression (%)(±SD)
head & neck n= 14	110.2 (81.0)	13.8 (24.2)
upper extremity n=14	97.8 (82.3)	11.4 (16.4)
trunk n=28	89.0 (75.4)	13.9 (25.2)
lower extremity n=29	142.0 (83.9)	12.9 (23.2)

Table 3-2 Mean expression of Wnt5a and $p16^{INK4a}$ in purely RGP and VGP melanoma samples from different anatomical sites

3.4.3 Melanoma histological subtype

To minimise the confounding effect of Breslow depth on this analysis, the purely RGP and VGP melanoma samples were stratified into tumours that had a Breslow depth of 2mm or less, and those that were 2.01mm or greater in depth (T1 and T2 tumours or T3 and T4 tumours according to the American Joint Committee on Cancer TNM Staging system [2, 3]). For tumours 2mm or less in depth expression of Wnt5a and $p16^{INK4a}$ did not differ significantly between the histological subtypes of melanoma; namely superficial spreading melanoma, nodular melanoma and lentigo maligna melanoma (n=50, p=0.21 and p=0.11 respectively). Similarly, for tumours 2.01mm or greater in depth there was no significant difference in Wnt5a or $p16^{INK4a}$ expression between the histological sub-types (n=44, p=1.0 and p=0.55 respectively).

3.5 Results of B-raf exon 15 mutation analysis

Following immunohistochemistry, there were 43 cases comprising VGP melanoma with matched metastases, in which there was sufficient tissue available for mutation analysis of B-raf Exon 15 via SSCP. The overall mutation frequency in the VGP primary tumours was 51.2%(n=22 of 43) of which 20 were common GTG→GAG point mutations at codon 600 resulting in valine being replaced by glutamate (V600E). The remaining 2 cases were both double mutations at codon 600 with GTG→AAG resulting in valine being replaced by lysine (V600K). The mutation frequency in metastatic tumours was 53.5% (n=23 of 43). There was 93% (40 of 43) concordance for mutation status between VGP and matched metastasis. Importantly, in both cases of the less common V600K mutation, the primary and metastatic tumours were concordant, as illustrated in Figure 3-9.

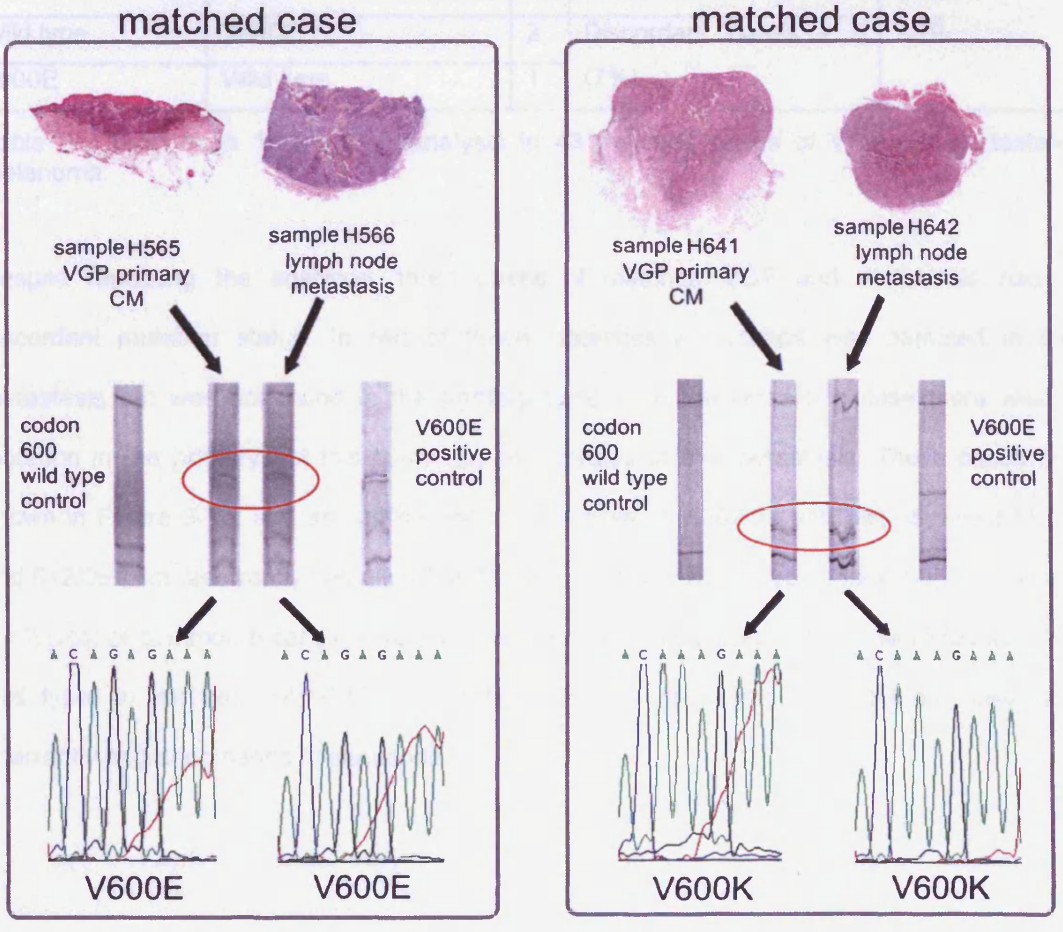


Figure 3-9 Examples of B-raf mutation analyses in matched cases of VGP and metastatic melanoma. H+E sections from the tumour samples, SSCP gels and sequencing electropherograms are shown. The aberrantly migrating bands on the SSCP gel are encircled in red. In the case on the right, note the unusual position of the aberrant bands indicating the uncommon V600K mutation.

This indicated that the primary and metastatic tumours were clonally related, because to find two cases with concordant rare mutations by chance would be most unlikely. The Cohen's Kappa score of 0.86 indicated almost perfect agreement between cases of primary and metastatic melanoma [188] and showed that when B-raf mutation occurred, the overwhelming majority of mutations arose prior to the development of metastases. The results of this analysis are shown in Table 3-3.

VGP mutation status	**Metastasis mutation status**	**No. cases**		
Wild type	Wild type	19	Concordant cases = 40 (93%)	Kappa score = 0.86
V600E	V600E	19		
V600K	V600K	2		
Wild type	V600E	2	Discordant cases = 3 (7%)	
V600E	Wild type	1		

Table 3-3 B-raf exon 15 mutation analysis in 43 matched cases of VGP and metastatic melanoma.

Despite repeating the analyses, three cases of matched VGP and metastasis had a discordant mutation status. In two of these instances a mutation was detected in the metastasis but was not found in the primary tumour. In the remaining case there was a mutation in the primary but this could not be detected in the metastasis. These cases are shown in Figure 3-10, and as can be seen in the case of H587/05 and 588/05, and 511/05 and 512/05 faint, aberrantly migrating bands were seen in the wild type cases, but these were not typical of common B-raf exon 15 mutations and upon sequencing these were found to be wild type. In the case of H549/05 and H550/05, the primary tumour did not have any aberrantly migrating bands to sequence.

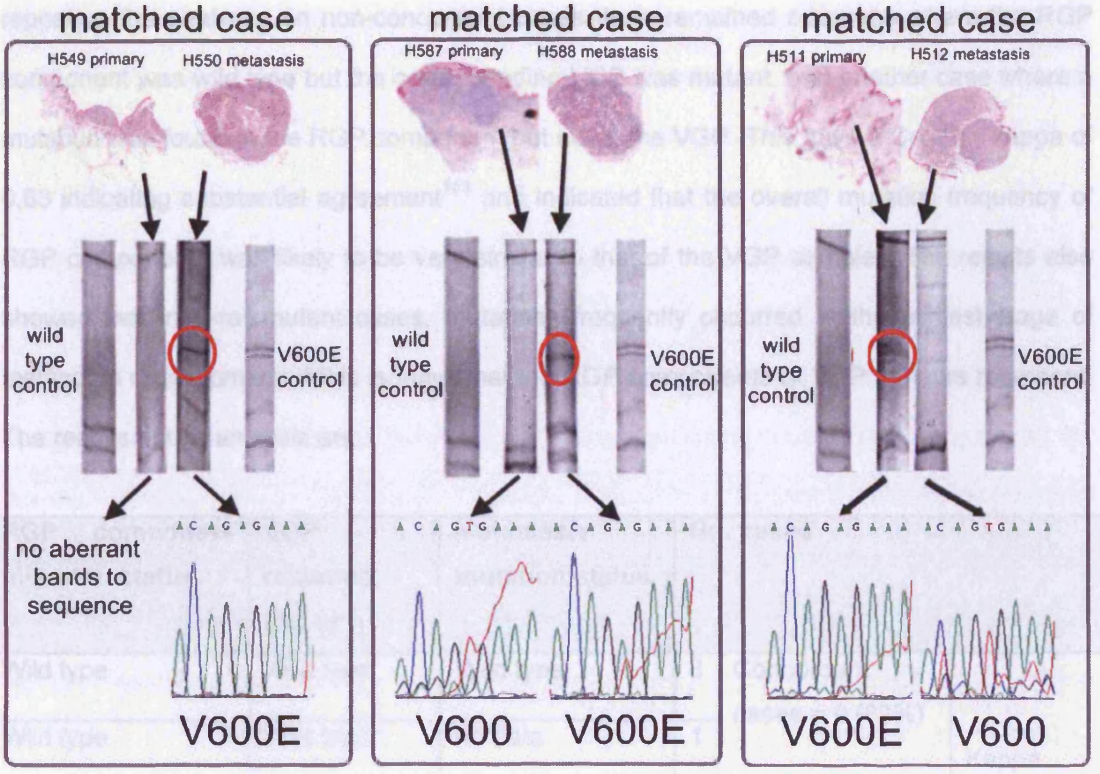


Figure 3-10 Examples of B-raf mutation analyses in discordant cases of VGP and metastatic melanoma. H+E sections from the tumour samples, SSCP gels and sequencing electropherograms, where sequencing was performed, are shown. The aberrantly migrating bands on the SSCP gel, where present are encircled in red.

Eleven cases comprising an RGP component adjacent to the VGP, a VGP and metastasis were analysed for B-raf exon 15 mutations. A mixture of cases with known mutant and wild type VGPs were selected to establish that the mutation status of RGP components adjacent to the VGP are usually concordant with the mutation status of the VGP, as has been suggested by other authors[41, 45]. The two cases with uncommon V600K mutations, which showed concordant mutations of the VGP and metastatic samples, could not be included in this subset because in both cases the primary samples lacked an RGP component. This was unfortunate because, as shown in the analysis of matched cases of VGP and metastatic melanoma, these uncommon mutations are a useful tool for demonstrating within cases the clonality common to different stages of progression.

Nine of the 11 cases (82%) with an RGP component showed a concordant mutation status with the corresponding VGP and two cases were discordant. Six of 11 (55%) RGP components were B-raf mutant (all V600E mutations) and five (45%) were wild type. Despite

repeating the analyses on non-concordant cases there remained one case where the RGP component was wild type but the corresponding VGP was mutant, and another case where a mutation was found in the RGP component but not in the VGP. This gave a Cohen's kappa of 0.63 indicating substantial agreement[188] and indicated that the overall mutation frequency of RGP components was likely to be very similar to that of the VGP samples. The results also showed that in B-raf mutant cases, mutations frequently occurred at the earliest stage of melanoma development, if this is truly what the RGP components of VGP tumours represent. The results of this analysis are shown in Table 3-4.

RGP component mutation status	VGP mutation status	Metastasis mutation status	No. cases		
Wild type	Wild type	Wild type	3	Concordant cases = 9 (82%)	Kappa score = 0.63
Wild type	Wild type	no data	1		
V600E	V600E	V600E	5		
Wild type	V600E	V600E	1	Discordant cases = 2 (18%)	
V600E	Wild type	Wild type	1		

Table 3-4 B-raf exon 15 mutation analysis in 11 cases of VGP melanoma with an RGP component. In one of these cases the corresponding metastasis was not available for analysis.

There was a single case comprising contiguous naevus, RGP component, VGP and metastasis that was analysed for B-raf exon 15 mutation. In this case, all components were found to be wild type.

Data from the current study, and others[41, 45], indicated that the overall mutation frequency in RGP components of VGP tumours was likely to be very similar to that observed in the VGP melanoma samples due to the high concordance seen between the two. Next, the aim was to determine whether a similar mutation frequency was present in purely RGP tumours. Mutation analysis of B-raf Exon 15 was successful in 22 of the 35 samples of purely RGP melanoma and of these, 4 (18.2%) were mutant and 18 (81.8%) wild type. The mutation

frequency of the purely RGP melanoma samples was significantly different to that seen in the VGP samples (p=0.021 Fisher's exact test) and was therefore predicted to be considerably lower than that seen in RGP components adjacent to VGP tumours.

Melanomas arising in chronically sun damaged skin, such as lentigo maligna melanoma, have been shown to have a low frequency of B-raf mutations[17, 18], although, mutation frequencies in excess of 50% have been reported [189]. Three (13.6%) of the purely RGP samples were lentigo maligna melanomas and rest were superficial spreading melanomas. By contrast, for the VGP samples, all but one case (1.7%) were of superficial spreading or nodular type, with one case of acral lentigenous melanoma. It was possible therefore, that the significantly lower frequency of mutations seen in purely RGP melanoma samples compared to the VGP samples resulted from a bias of case selection. However, when the cases of lentigo maligna melanoma, all of which were wild type, were removed from the analysis, the difference between the mutation frequencies of purely RGP melanomas and VGP melanomas. (p=0.049 chi^2) remained significant. The different B-raf exon 15 mutation frequencies between purely RGP melanomas, VGP tumours and metastases are illustrated in Figure 3-11.

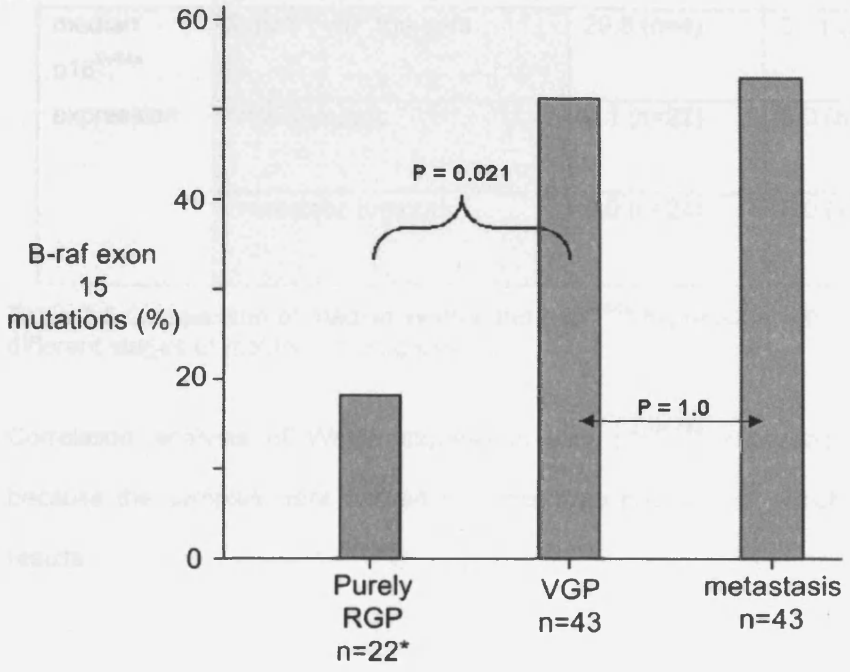


Figure 3-11 B-raf exon 15 mutation frequency in purely RGP tumours, VGP tumours and metastatic melanoma. The statistical significance of differences in mutation frequency between these sample types is shown. The mutation frequency in RGP components is predicted to be similar to that of the VGP samples, due to their high level of concordance. * three cases were of lentigo maligna sub-type, the rest superficial spreading melanoma.

3.6 Relationships between Wnt5a expression and $p16^{INK4a}$ expression, and B-raf mutation

There was no significant difference in Wnt5a expression between B-raf mutant and wild type purely RGP melanomas (n=35, p=0.87 Mann–Whitney U test), VGP melanomas (n=59, p=0.91) or metastatic melanomas (n=58, p=0.99). Similarly, no association between $p16^{INK4a}$ expression and B-raf mutation status could be shown in these groups of samples (n=35, p=0.79; n=59, p=1.0 and p=0.41 respectively). The median Wnt5a and $p16^{INK4a}$ expression scores in B-raf mutant and wild type tumours for these different growth phases are shown in Table 3-5.

		B-raf mutant	**B-raf wild type**
median Wnt5a expression	Purely RGP tumours	27.6 (n=4)	33.1 (n=18)
	VGP tumours	125.2 (n=22)	102.0 (n=21)
	metastatic tumours	146.0 (n=24)	124.5 (n=19)
median $p16^{INK4a}$ expression	Purely RGP tumours	29.8 (n=4)	31.1 (n=18)
	VGP tumours	0.1 (n=22)	0.0 (n=21)
	metastatic tumours	0.0 (n=24)	0.0 (19)

Table 3-5 Comparison of median Wnt5a and $p16^{INK4a}$ expression with B-raf mutation status in different stages of melanoma progression.

Correlation analysis of Wnt5a expression with $p16^{INK4a}$ expression was not performed because the samples were derived from separate populations, which may have biased the results.

3.7 Comparison of Wnt5a expression with $p16^{INK4a}$ expression and B-raf mutation frequency

Loss of $p16^{INK4a}$ and activating mutations of B-raf are considered important events in melanoma progression, while data on the role of Wnt5a is limited. Several studies have shown that expression of $p16^{INK4a}$ diminishes with progression[31, 32, 93, 96-98], while B-raf mutation is generally regarded as a putative early event in melanoma progression[30, 45, 56, 57, 61]. This analysis replicated, and expanded upon the findings for $p16^{INK4a}$ and B-raf, while also characterising Wnt5a expression, in the same tumour series. For the first time in a large number of samples therefore, it was possible to compare changes of Wnt5a alongside alterations of $p16^{INK4a}$ and B-raf at different stages of Clark's model. The expression of Wnt5a along with $p16^{INK4a}$ expression and B-raf mutation frequency in the matched samples is shown in Figure 3-12A. Here, the naevus and radial stages of Clark's model are represented by contiguous naevi and RGP components of VGP melanoma samples, both of which lie adjacent to the VGP tumour. It was from these matched samples that the matched cases were derived (see Figure 2-1). Wnt5a expression along with $p16^{INK4a}$ expression and B-raf mutation frequency in the unmatched samples is shown in Figure 3-12B. Here the naevus and radial stages of Clark's model are represented by CAN and purely RGP melanoma samples.

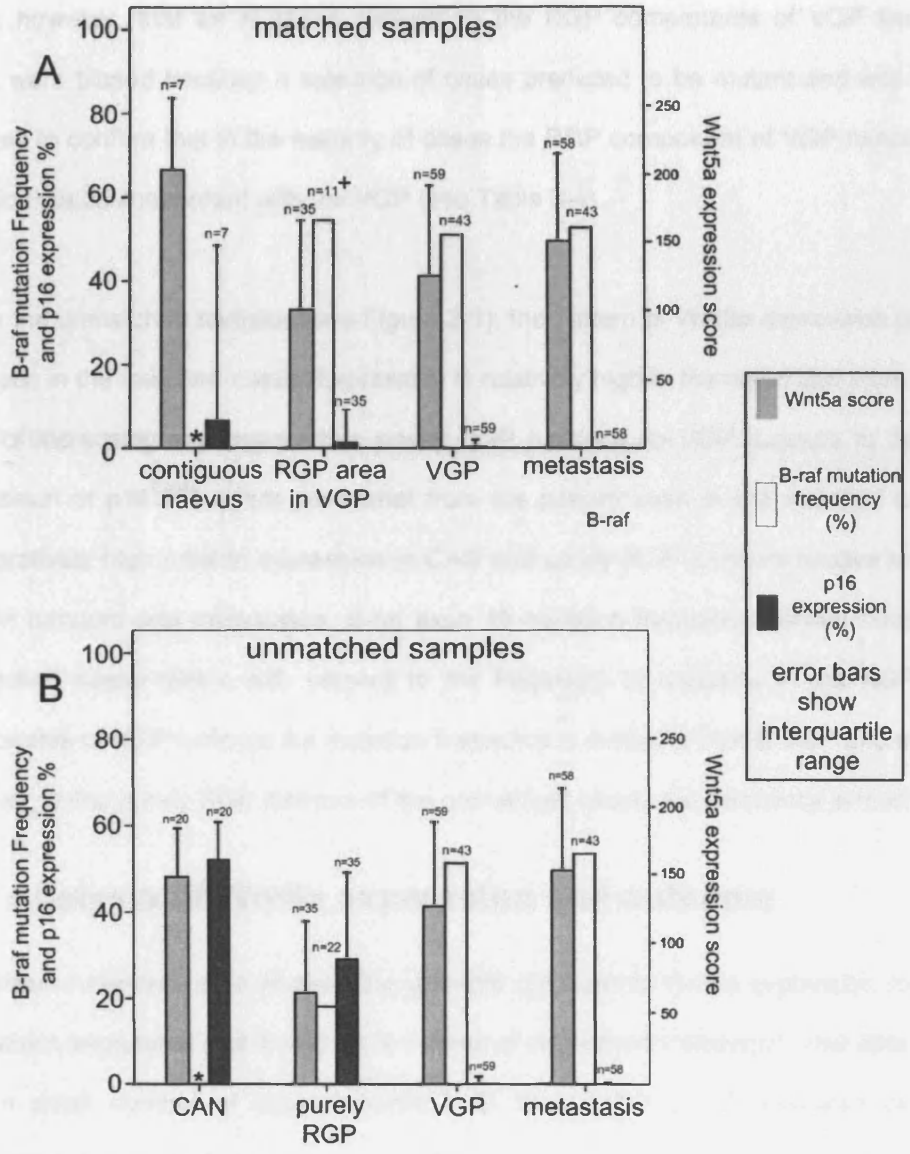


Figure 3-12 Median expression of Wnt5a and $p16^{INK4a}$, and frequency of B-raf mutation, in different phases of Clark's melanoma progression model in matched and unmatched samples. + biased sample of selected cases, but the frequency is predicted to be similar to that of the VGP because a kappa statistic showed substantial agreement between RGP components and the adjacent VGP. * indicates that B-raf mutation analysis was not performed.

Within the malignant matched samples (RGP component adjacent to VGP, VGP and metastasis) there is a linear trend of increasing Wnt5a expression with progression, but overall expression is greatest in naevi. There is a gradual reduction of $p16^{INK4a}$ expression in the matched samples, but even in the contiguous naevi this expression is low when compared to the unmatched samples of independent naevi. The B-raf exon 15 mutation frequencies of the RGP components in VGP tumours, VGP tumours and metastases are similar. It should be

noted, however, that for mutation analysis in the RGP components of VGP tumours, the cases were biased because a selection of cases predicted to be mutant and wild type were selected to confirm that in the majority of cases the RGP component of VGP tumours have a mutation status concordant with the VGP (see Table 3-4).

Within the unmatched samples (see Figure 2-1), the pattern of Wnt5a expression is similar to that seen in the matched cases. Expression is relatively high in the naevi and there is a linear trend of increasing expression from purely RGP tumours, to VGP tumours to metastases. Expression of $p16^{INK4a}$ differs somewhat from the pattern seen in the matched cases, with comparatively high median expression in CAN and purely RGP tumours relative to that seen in VGP tumours and metastases. B-raf exon 15 mutation frequency between matched and unmatched cases differs with respect to the frequency of mutation in the RGP. In RGP components of VGP tumours the mutation frequency is similar to that of VGP and metastases however, in the purely RGP tumours of the unmatched cases, the frequency is much lower.

3.8 Analysis of Wnt5a expression and outcome

Metastatic melanoma cells showed the greatest cytoplasmic Wnt5a expression (see section 3.3), which suggested that it may be a marker of aggressive behaviour. This data, and data from a small number of cases examined by Weeraratna *et al*[9], indicated that primary melanomas having high cytoplasmic Wnt5a expression may also be more aggressive and therefore have a poorer prognosis. To test this, the 59 primary melanomas with matched metastases were combined with a further 43 melanomas from a database of consecutive cases with known outcome, yielding a total of 102 cases. While this dataset was biased towards thick melanomas, making it difficult to extrapolate results to melanomas in general, the specific question of whether Wnt5a expression might affect metastasis-free and overall survival could be addressed. In addition to cytoplasmic Wnt5a, nuclear Wnt5a was also assessed, as this has been previously analysed by Bachmann *et al* [106]. Other important clinico-pathological variables were analysed (age, gender, site, and Breslow depth). Univariate and multivariate models were constructed and the results are summarised in Table 3-6 and Table 3-7.

Outcome: metastasis						
			Univariate model		Multivariate model †	
	No. of patients*	Metastases	Mean survival (months) (95% CI)	p value (log rank test)	HR (95% CI)	Adjusted p value (Cox proportional hazard model)
Age (years)						
> 60	41	23	102 (77, 126)	0.002	2.33 (1.16, 4.71)	0.018
<= 60	57	15	180 (156, 206)			
Sex						
Male	46	21	133 (102, 165)	0.114	1.71 (0.85, 3.47)	0.134
Female	52	17	155 (130, 181)			
Site†						
Axial	48	19	147 (117, 176)	0.989	0.83 (0.40, 1.72)	0.621
Extremity	42	17	147 (115, 179)			
Breslow thickness (mm)						
> 2	52	28	114 (85, 144)	0.0001	2.38 (1.01, 5.31)	0.034
<= 2	46	10	184 (159, 209)			
Cytoplasmic Wnt5a						
> median score	50	24	124 (94, 155)	0.020	1.91 (0.96, 3.83)	0.066§
<= median score	48	14	169 (142, 196)			
Nuclear Wnt5a						
> Median score	49	21	129 (97, 161)	0.286	1.33 (0.66, 2.70)	0.425
<= median score	49	17	161 (131, 190)			

Table 3-6 Results of univariate and multivariate analyses of Wnt5a expression and other important clinico-pathological variables in melanoma. The outcome event is metastasis. *4 patients with metastasis at time of melanoma diagnosis and one patient who died within a month of diagnosis were excluded from respective survival analyses. † For 8 patients, the site was unknown. §Becomes significant when site is not included in multivariate model (p=0.041).

Outcome: death						
			Univariate model		Multivariate model †	
	No. of patients*	Deaths	Mean survival (months) (95% CI)	p value (log rank test)	HR (95% CI)	Adjusted p value (Cox proportional Hazard model)
Age (years)						
> 60	43	24	106 (80, 132)	0.002	2.4 (1.21, 4.83)	0.012
<= 60	58	15	181 (156, 206)			
Sex						
Male	46	21	133 (102, 165)	0.117	1.66 (0.82, 3.33)	0.158
Female	55	18	155 (131, 180)			
Site†						
Axial	49	20	144 (114, 173)	0.753	0.97 (0.47, 2.00)	0.932
Extremity	44	17	152 (120, 183)			
Breslow thickness (mm)						
> 2	55	29	117 (89, 146)	0.0001	2.29 (1.04, 5.06)	0.041
<= 2	46	10	184 (159, 209)			
Cytoplasmic Wnt5a						
> median score	51	24	124 (95, 155)	0.028	1.83 (0.93, 3.62)	0.080§
<= median score	50	15	168 (142, 195)			
Nuclear Wnt5a						
> Median score	50	21	130 (98, 162)	0.345	1.23 (0.61, 2.46)	0.614
<= median score	51	18	160 (131, 188)			

Table 3-7 Results of univariate and multivariate analyses of Wnt5a expression and other important clinico-pathological variables in melanoma. The outcome event is death. *4 patients with metastasis at time of melanoma diagnosis and one patient who died within a month of diagnosis were excluded from respective survival analyses. † For 8 patients, the site was unknown. §Becomes significant when site is not included in multivariate model (p=0.047).

High cytoplasmic Wnt5a expression (defined by scores greater than the median from the 102 cases) was associated with reduced metastasis-free survival (p = 0.020) and reduced overall survival (p = 0.028) while nuclear Wnt5a was not a significant predictor of either outcome. In the mutivariate model, age at diagnosis and Breslow thickness were significant predictors of both time-to metastasis and overall survival. No other variables were significant. However, cytoplasmic Wnt5a staining was very close to significant for metastasis-free and overall survival (p = 0.066 and 0.080 respectively). The multivariate model required exclusion of 8 cases where the site was unknown, leading to a loss of power. When site was removed from the multivariate model, thus increasing the power of the analysis, cytoplasmic Wnt5a staining joined age and Breslow thickness as significant predictors of both metastasis-free survival, p = 0.041, HR 2.01 (95% CI 1.03 to 3.92), and overall survival, p = 0.047 (HR 1.95, 95% CI 1.01 to 3.76), while nuclear Wnt5a and other variables were still not significant factors. Survival curves for cytoplasmic and nuclear Wnt5a are shown in Figure 3-13.

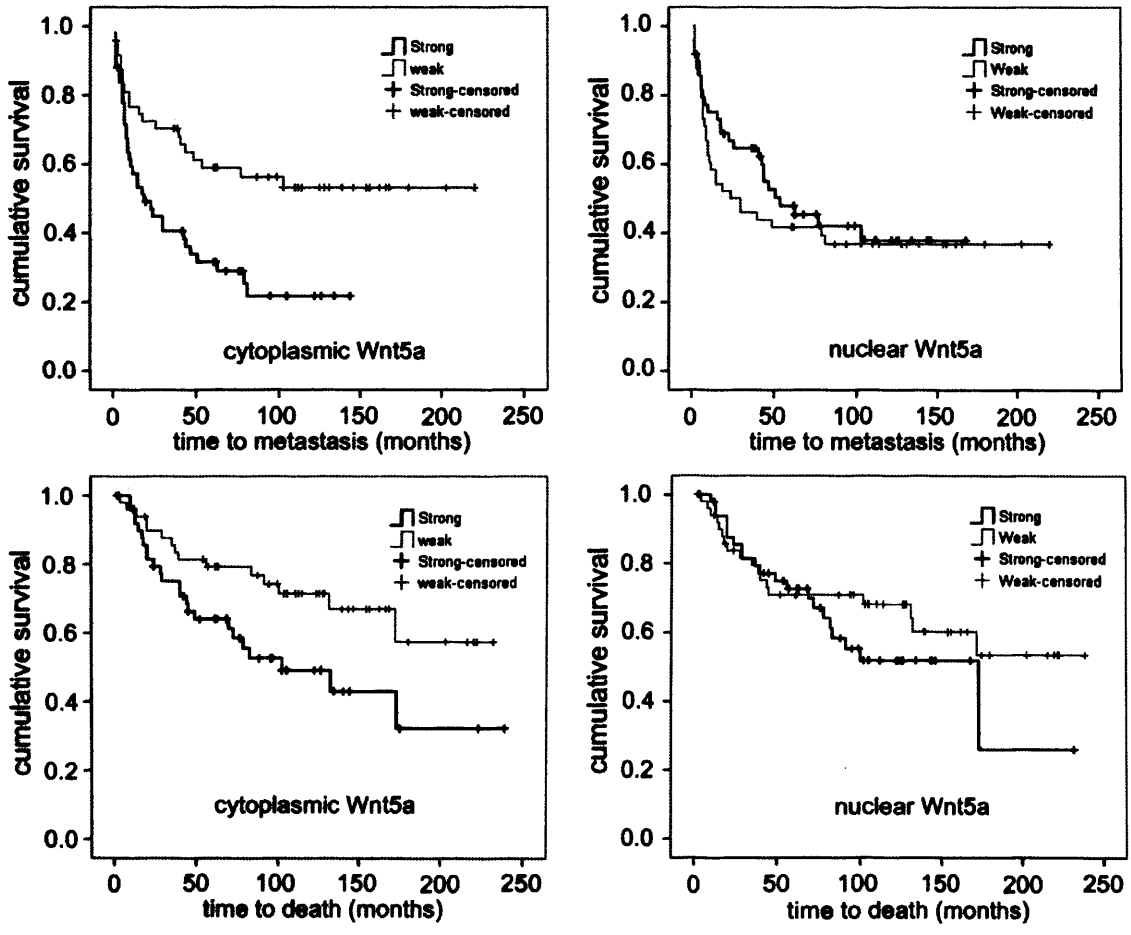


Figure 3-13 Time to metastasis and time to death survival curves for cytoplasmic and nuclear Wnt5a expression.

4 Results of characterisation of B-raf, N-ras and H-ras mutations in Spitzoid tumours

4.1 Introduction

Spitzoid tumours are a heterogeneous group that show a variety of histological features common to both classical Spitz naevi, which are benign, and malignant melanoma. Distinct genetic differences have been demonstrated between Spitz naevi and non-Spitzoid melanoma[57, 63, 158, 159], however, whether all Spitzoid tumours represent a genetically distinct group, and whether within this group there is progression from Spitz naevus to Spitzoid melanoma is unclear[112, 118, 165]. For this study there were 2 main research questions:

1. Are Spitzoid tumours a genetically distinct group?
2. Do Spitz naevi progress to Spitzoid melanoma

Based on current evidence[120-122, 190, 191], the hypotheses were that Spitzoid tumours are a distinct group of tumours, and that Spitz naevi do not progress to Spitzoid melanoma. The first research question was tested by examining the frequency of B-raf and N-ras mutations, in a series of Spitzoid tumours and non-Spitzoid melanomas. The second research question was addressed by looking for a putative early event in the development of Spitz naevi, namely H-ras mutation, in a group of Spitzoid melanomas. In addition, relationships between mutation status, tumour type and clinicopathological data were explored. Mutations in the exons of interest were identified via SSCP analysis of PCR products, followed by dideoxy terminator sequencing.

4.2 Optimisation of techniques

See Appendix 1: Optimisation of techniques

4.3 Histopathological review of cases

Histological review of the tumour series was performed to address the problem of poor reproducibility in the classification of Spitzoid tumours. The histological definition of a Spitzoid

lesion and the definitions of atypical features used during the case review are described in section 2.2.1.

Following the formal histological review, there were four different ways of categorising the tumour series.

1. Clinical outcome

The cases were categorised retrospectively according to whether or not the patient developed metastatic disease. This is a gold standard against which other methods of diagnosis can be compared and it would be expected that metastatic tumours would show a number of atypical histological features. Importantly however, while metastasis is very specific for malignancy it lacks sensitivity in the case of cutaneous melanocytic tumours because some malignant lesions may be cured prior to the development of metastases by the diagnostic excision biopsy and any subsequent wide local excision of the scar.

2. Initial diagnosis by reporting dermatopathologist

This was the diagnosis provided on the histology report when the case was first reported. A variety of diagnostic terms were used for the lesions and in some cases a specific diagnosis was not reached. Furthermore, in some cases, the diagnosis was altered upon seeking a specialist referral.

3. Histological review diagnosis

Two dermatopathologists from the department of Histopathology, Leicester Royal Infirmary, were blinded to the clinical details of the cases, and placed them into one of the following categories:

- Spitz naevus
- atypical Spitz naevus
- STUMP
- Spitzoid melanoma
- Non-Spitzoid melanoma
- none of the above/ insufficient material for diagnosis

This ensured that there was diagnostic consistency within the tumour series. The reviewers approached the cases as if they were part of their routine reporting and the diagnoses were made from an overall impression of the lesion, rather than using limited criteria.

4. Histological review score

The reviewers assessed the presence or absence of nine features used for assessing atypia in Spitzoid tumours (as described in section 2.2.1), to provide a numerical score of the degree of atypicality in a case. Similar methods have been employed by other authors to categorise Spitzoid tumours for both clinical[119, 123] and research purposes[122] but such methods inevitably impose artificial thresholds on what is likely to be a continuum of lesions.

The distribution of the cases within these four methods of categorisation is shown in Table 4-1. Detailed information concerning the classification of individual cases is shown in Appendix 2: Complete clinicopathological and mutation analysis data. Four cases were excluded from the histological review because the original H+E sections were not available and repeat sections were not considered to be representative of the lesion. This left 86 cases where a review diagnosis was given.

Method of categorisation	Number of cases within diagnostic category	Number of metastatic cases (median follow up 6.8 years, interquartile range 8.1 years)
Clinical outcome n=90	disease free = 78 metastatic disease = 12	metastatic disease = 12
Initial diagnosis n= 90	Spitz naevus = 17 (18.9%) Non-classical Spitzoid Tumours = 56 (62.2%) comprising: • atypical Spitz naevus = 7 (7.8%) • atypical Spitz tumour = 4 (4.4%) • Probably Spitz naevus but treat as malignant melanoma = 1 (1.1%) • Features of Spitz naevus but probably malignant melanoma = 2 (2.2%) • Metastasising Spitz naevus = 1 (1.1%) • Severely atypical melanocytic lesion with a Spitzoid component = 1 (1.1%) • Spitz naevus (revised diagnosis following referral to a specialist) = 3 (3.3%) • Spitz naevus of uncertain behaviour = 1 (1.1%) • Spitzoid melanocytic lesion = 1 (1.1%) • Spitzoid lesion = 1 (1.1%) • Spitzoid melanoma = 9 (10%) • Spitzoid melanoma (revised diagnosis following referral to a Specialist) = 2 (2.2%) • Spitzoid tumour of uncertain malignant potential = 1 (1.1%) • Spitzoid lesion cannot exclude malignant melanoma = 2 (2.2%) • Malignant melanoma with Spitzoid features = 19 (21.1%) Malignant melanoma = 17 (18.9%)	atypical Spitz naevus = 1 atypical Spitz tumour = 3 Metastasising Spitz naevus = 1 Malignant melanoma = 7

Method of categorisation	Number of cases within diagnostic category	Number of metastatic cases (median follow up 6.8 years, interquartile range 8.1 years)
Review diagnosis n = 90	Spitz naevus = 16 (17.8%) Atypical Spitz naevus = 9 (10%) STUMP = 9 (10%) Spitzoid melanoma = 27 (30%) Non-Spitzoid melanoma = 25 (27.7%) Insufficient material to formulate a diagnosis = 4 (4.4%)	Atypical Spitz naevus = 1 STUMP = 2 Spitzoid melanoma = 6 Non-Spitzoid melanoma = 3
Review score n = 90	Score of 0 = 15 (16.6%) Score of 1 = 6 (6.7%) Score of 2 = 9 (10%) Score of 3 = 14 (15.6%) Score of 4 = 8 (8.9%) Score of 5 = 8 (8.9%) Score of 6 = 1 (1.1%) Non-Spitzoid melanoma = 25 (27.8%) Insufficient material to formulate a diagnosis = 4 (4.4%)	Score of 2 = 2 Score of 3 = 3 Score of 4 = 1 Score of 5 = 2 Score of 6 = 1 Non-Spitzoid melanoma = 3

Table 4-1The distribution of cases from the tumour series using four different methods of diagnostic categorisation, the number of metastatic cases within each category is also shown.

The reviewers found in some cases that the sum score of atypical features did not accurately reflect their impression of the likelihood of malignancy. The distribution of atypical histological features compared with the reviewers' diagnoses for all Spitzoid tumours is shown in Figure 4-1.

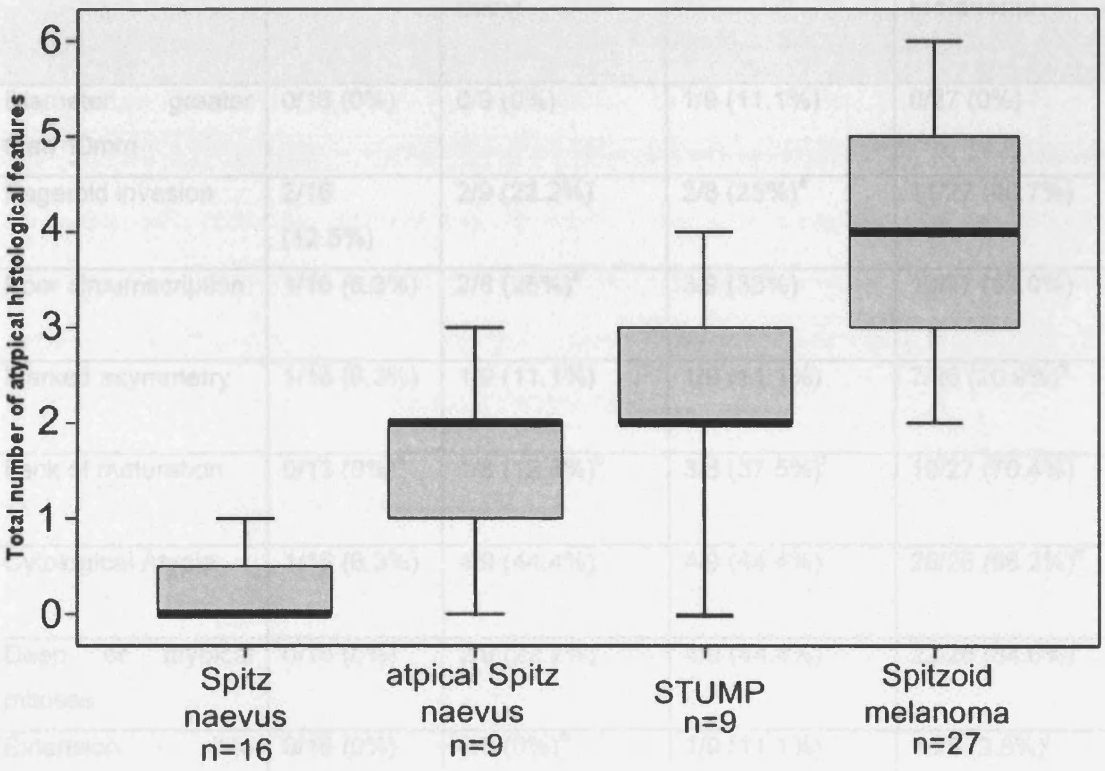


Figure 4-1 Box plots showing the distribution of total atypical histological features between categories of Spitzoid tumour.

There was a significant trend of increasing total score across the four categories from Spitz naevi to Spitzoid melanomas ($p<0.001$ Jonckheere's test). The mean total number of atypical histological features identified within the Spitzoid lesions differed significantly between the four tumour categories ($p<0.001$ Kruskal-Wallis test), and post-hoc comparisons showed significant differences between Spitz naevi and atypical Spitz naevi ($p=0.005$, Mann-Whitney U test), and STUMP and Spitzoid melanomas ($p=0.002$). Crucially, there was no significant difference between atypical Spitz naevi and STUMP, and the ranges of the total scores between the tumour categories showed considerable overlap. These results indicated that there were either histological features other than those listed which the reviewers used to achieve a diagnosis, or that there were subtle degrees of severity within the atypical

histological features that informed the reviewing diagnosis. The individual frequencies of the atypical features identified during histological review and mean total score for each review diagnosis category are shown in Table 4-2.

	Spitz naevi	Atypical Spitz naevi	STUMP	Spitzoid melanoma
Diameter greater than 10mm	0/16 (0%)	0/9 (0%)	1/9 (11.1%)	0/27 (0%)
Pagetoid invasion	2/16 (12.5%)	2/9 (22.2%)	2/8 (25%)[e]	11/27 (40.7%)
Poor circumscription	1/16 (6.3%)	2/8 (25%)[b]	3/9 (33%)	10/27 (37.0%)
Marked asymmetry	1/16 (6.3%)	1/9 (11.1%)	1/9 (11.1%)	7/26 (26.9%)[g]
Lack of maturation	0/13 (0%)[a]	1/8 (12.5%)[c]	3/8 (37.5%)[f]	19/27 (70.4%)
Cytological Atypia	1/16 (6.3%)	4/9 (44.4%)	4/9 (44.4%)	25/26 (96.2%)[h]
Deep or atypical mitoses	0/16 (0%)	2/9 (22.2%)	4/9 (44.4%)	22/26 (84.6%)[i]
Extension into subcutaneous fat	0/16 (0%)	0/8 (0%)[d]	1/9 (11.1%)	1/26 (3.8%)[j]
Ulceration	0/16 (0%)	3/9 (33.3%)	2/9 (22.2%)	8/27 (29.6%)
Mean total score (±SD) Range	0.25 (0.45) 0-1	1.7 (1.13) 0-3	2.33 (1.12) 0-4	3.89 (1.05) 2-6

Table 4-2 Frequencies and mean total scores of atypical features identified by the histological review.

[a] Unable to assess maturation in three cases

[b]Unable to assess circumscription in one case

[c]Unable to assess maturation in one case

[d]Unable to assess extension into subcutaneous fat in one case

[e]Unable to assess pagetoid invasion in one case

[f]Unable to assess maturation in one case

[g]Unable to assess symmetry in one case

[h]Unable to assess cytological atypia in one case
[i]Unable to assess mitoses in one case
[j] Unable to assess extension into subcutaneous fat

The main purpose of the review was to apply consistency of diagnosis to the categorisation of the tumour series. It became apparent that to apply a numerical scoring system to categorise the tumours, while more objective and reproducible for future studies, would risk tumours being incorrectly categorised with respect to the likelihood of malignancy. This could confound correlation with molecular features and, because of this, it was decided that the tumours would be classified primarily according the diagnosis given by the reviewing pathologists. The tumour series therefore comprised 16 Spitz naevi, 9 atypical Spitz naevi, 9 STUMP, 27 Spitzoid melanomas and 25 non-Spitzoid melanomas.

4.4 Clinicopathological data

Clinicopathological data for the tumour series is shown in table Table 4-3.

Clinical and pathological parameters	Spitz naevi n=16	Atypical Spitz naevus n=9	STUMP n=9	Spitzoid melanoma n=27	non-Spitzoid melanoma n=25
Sex					
- Male	8 (50%)	5 (55.6%)	2 (22.2%)	13 (48.1%)	11 (44%)
- Female	8 (50%)	4 (44.4%)	6 (66.7%)	14 (51.9%)	14 (56%)
- Missing data	-	-	1 (11.1%)		
Age at diagnosis (years) mean ± SD	30.3 ± 13.3	35.4±15.6	31.7 ± 11.2	31.3 ± 22.8	37.3 ±17.1
Tumour site					
- Head & neck	2 (12.5%)	2 (22.2%)	-	6 (22.2%)	5 (20%)
- Trunk	1 (6.3%)	-	2 (22.2%)	5 (18.5%)	3 (12%)
- Upper extremity	1 (6.3%)	2 (22.2%)	2 (22.2%)	5 (18.5%)	4 (16%)
- Lower extremity	10 (62.5%)	4 (44.4%)	4 (44.4%)	9 (37.5%)	12 (48%)
- Missing data	2 (12.5%)	1 (11.1%)	1 (1.1%)	2 (11.1%)	1 (4%)
Histological Subtype (from initial histology report)					
- Junctional	3 (18.8%)	-	-	-	
- Compound	8 (50%)	4 (44.4%)	5 (55.6)	9 (33.3%)	
- Dermal	3 (18.8%)	1 (11.1%)	-	-	
- Desmoplastic	-	-	-	-	
- SSMM	-	-	-	2 (7.4%)	15 (60%)
- Nodular MM	-	2 (22.2%)	1 (11.1%)	6 (22.2%)	10 (40%)
- Spitzoid MM	-	-	1 (11.1%)	6 (22.2%)	
- None	2 (12.5%)	2 (22.2%)	2 (22.2%)	4 (14.8%)	
Tumour thickness (mm) mean (± SD)	1.8 (± 1.2)	2.0(± 1.7)	4.2 (± 3.4)	3.2 (± 2.9)	2.5 (± 2.6)
Outcome					
- Disease Free	16 (100%)	8 (88.9%)	7 (77.8%)	21 (77.8%)	22 (88%)
- Recurrence or metastasis	-	1 (11.1%)	2 (22.2%)	6 (22.2%)	3 (12%)
Follow up period (yrs) mean ± SD	9.5 ± 6.2	8.2 ± 5.3	5.7 ± 3.2	9.8 ± 6.9	7.1 ± 4.2

Table 4-3 Clinicopathological data for the tumour series

The mean age was 32.2 years in patients with Spitzoid tumours and 37.3 in patients with non-Spitzoid melanomas. The mean age of patients with non-Spitzoid melanoma was low compared with national melanoma statistics, where the incidence is greatest in people over 65 years[10]. This difference arose because when acquiring the tumours, the non-Spitzoid melanoma cases were age-matched with the Spitzoid tumours to minimise age bias. There was no significant difference between the ages of the patients in the different tumour groups ($p=0.9$, Kruskal-Wallis test) and no significant difference specifically between the Spitzoid and non-Spitzoid melanomas ($p=0.5$, Mann-Whitney U test).

The site of a malignant melanoma and hence the degree of sun exposure it receives has been shown to have a significant impact on B-raf and N-ras mutation frequency[18, 49, 161]. For this reason the Spitzoid tumours and Non-Spitzoid melanomas were site-matched where possible. There were no significant differences in the anatomical site between the different tumour categories ($p=0.8$, Fisher's exact test) and when the tumours were classified as Spitzoid or non-Spitzoid, similarly there was no significant difference shown ($p=1.0$, Fisher's exact test) nor was there a significant difference between the Spitzoid and non-Spitzoid melanomas ($p=0.9$, Fisher's exact test).

The thickness of the lesions did not differ significantly between the groups ($p=0.09$, Kruskal-Wallis test). Clinical data and the results of mutation analyses for all cases are listed in Appendix 2: Complete clinicopathological and mutation analysis data.

4.5 Mutations of B-raf and N-ras

Mutations of B-raf and N-ras are frequently found in cutaneous melanoma[30, 41, 45, 189] and are very rare in Spitz naevi[17, 30, 44, 45, 54-56, 61, 69, 122, 143-149], but little is known about the frequency of these mutations in non-classical Spitzoid tumours and Spitzoid melanoma. The presence of these mutations would suggest that these tumours arise via similar mechanisms to common acquired naevi and 'common' melanoma while their absence would suggest that the Spitzoid tumours arise via a novel progression pathway. The presence of hotspot mutations of B-raf and N-ras was characterised in the tumour series which, following the histological review, comprised 86 cases in total. Example SSCP analysis and sequencing electropherograms from wild type and mutant cases are shown in Figure 4-2.

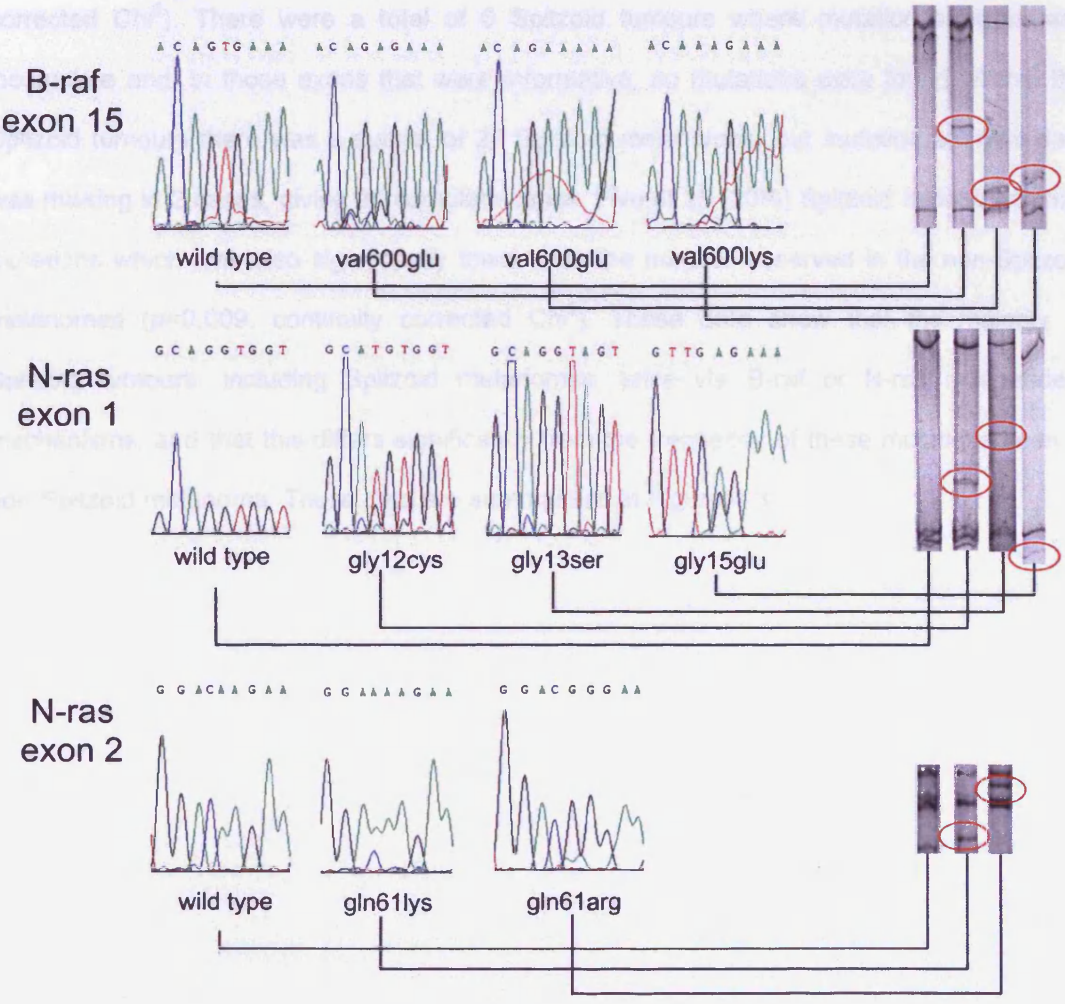


Figure 4-2 SSCP analysis with accompanying sequencing electropherograms from cases with wild type and mutant B-raf exon 15, N-ras exon 1 and N-ras exon 2. Aberrantly migrating bands are encircled in red.

The results of mutation analysis in individual cases are shown in Appendix 2: Complete clinicopathological and mutation analysis data. From the 25 cases of non-Spitzoid melanoma, there were 2 cases where PCR amplification did not work in one or more of the exons of interest, and B-raf or N-ras mutations were not found where PCR was successful. In these cases the possibility that a mutation may have been present in the uncharacterised exon could not be excluded and so they were removed from the analysis. A total of 14 of the 23 remaining cases (60.9%) had mutations of either B-raf exon 15 or N-ras exons 1 and 2. This high frequency of mutations is in keeping with previous studies[30, 45, 122]. By contrast, in the 55 Spitzoid tumours that had complete mutation analysis data, 10 (18.2%) were B-raf or N-ras mutant, which was significantly lower than in the non-Spitzoid melanomas (p=0.001, continuity corrected Chi^2). There were a total of 6 Spitzoid tumours where mutation analysis was incomplete and, in those exons that were informative, no mutations were found. Within the Spitzoid tumours there was a subset of 27 Spitzoid melanomas, but mutation analysis data was missing in 2 cases, giving 25 complete cases. Five of 25 (20%) Spitzoid melanomas had mutations which was also significantly lower than the number observed in the non-Spitzoid melanomas (p=0.009, continuity corrected Chi^2). These data show that the majority of Spitzoid tumours, including Spitzoid melanomas, arise via B-raf or N-ras independent mechanisms, and that this differs significantly from the frequency of these mutations seen in non-Spitzoid melanoma. These data are summarised in Figure 4-3.

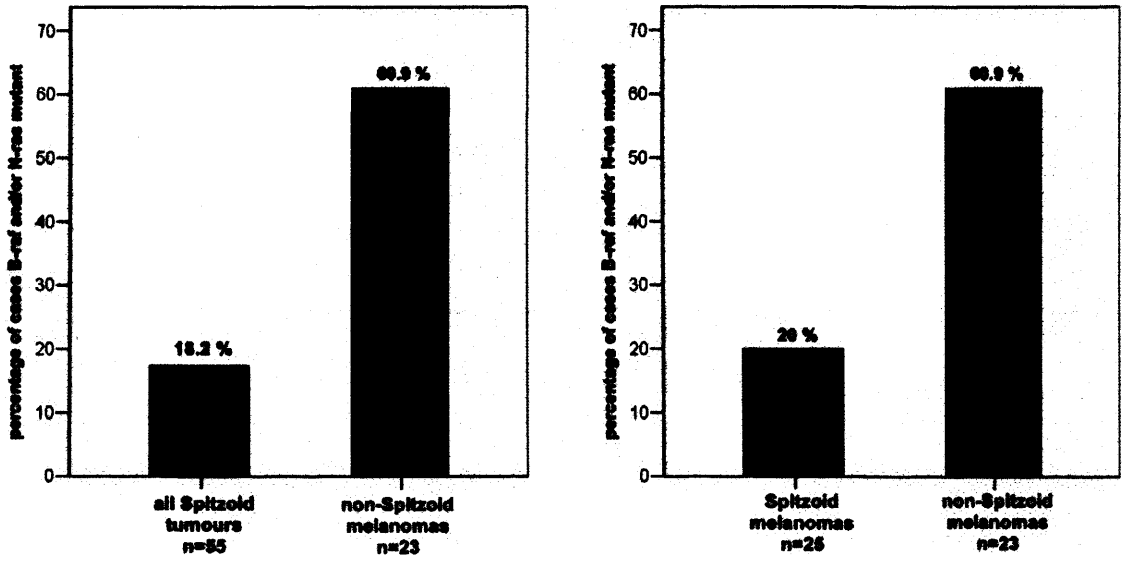


Figure 4-3 Bar charts comparing the frequency of B-raf exon 15 and/or N-ras exons 1 and 2 mutations in all Spitzoid tumours and non-Spitzoid melanomas, and Spitzoid melanomas and non-Spitzoid melanomas.

When the Spitzoid tumours were grouped by the specific review diagnosis, there was a single Spitz naevus (1 of 14, 7.1%) and a single atypical Spitz naevus (1 of 8, 12.5%) that harboured co-existent B-raf and N-ras 1 mutations. The low frequency of mutations in Spitz naevi is well documented, indeed to find a mutation at all was unusual but this result was repeated to confirm the finding. B-raf or N-ras mutant Spitz naevi have been reported by other authors[150, 151]. The B-raf and N-ras mutant Spitz naevus is shown in Figure 4-4; this lesion came from the thigh of a 32 year old male and has shown no clinical evidence of aggressive behaviour after 11.7 years follow up.

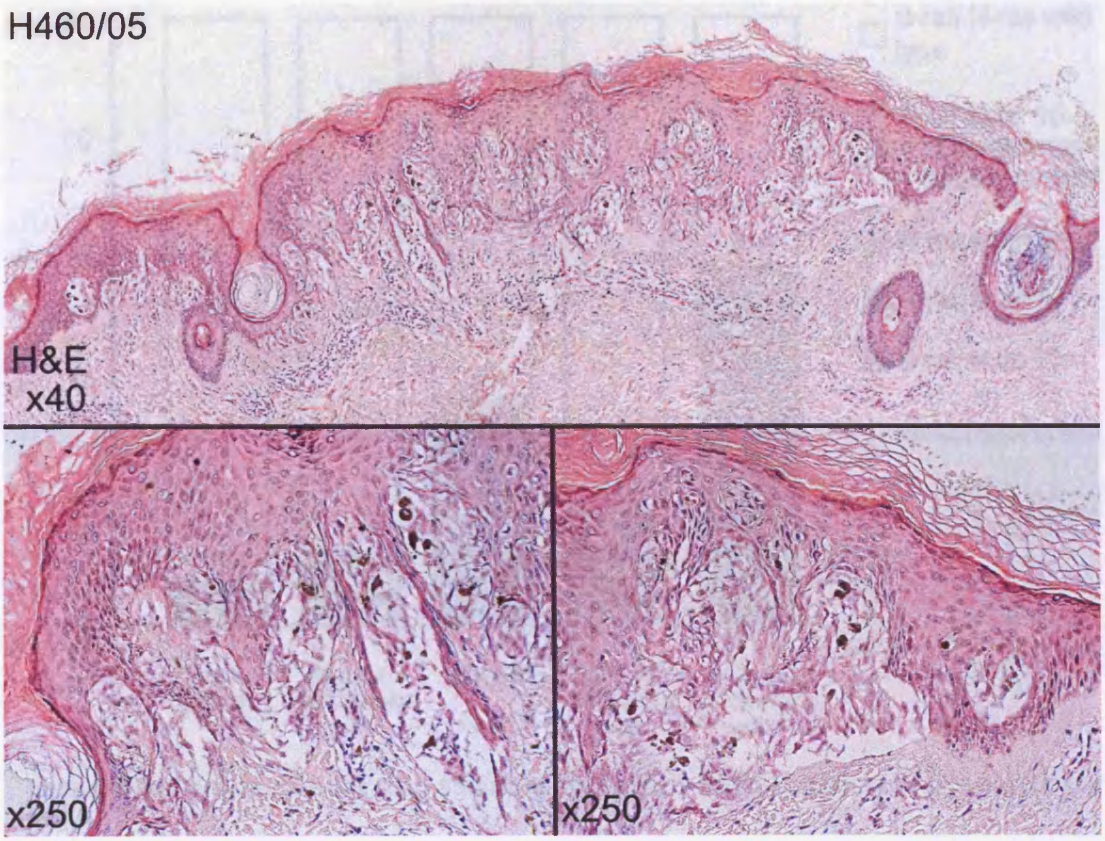


Figure 4-4 Case H460/05. This case was diagnosed as a junctional Spitz naevus but harboured mutations of B-raf and N-ras.

Three of 8(37.5%) STUMP's harboured mutations, one B-raf exon 15, one N-ras exon 2 and one with co-existent B-raf and N-ras 1 mutations. In lesions where co-existent mutations were found, the analysis was repeated with re-extraction of DNA to confirm the result however, while uncommon, double mutants are described in malignant melanoma and common acquired naevi [42, 44, 61-64]. None of the double mutant cases had clinical evidence of aggressive behaviour (median follow up = 6.9 years). Of the 25 Spitzoid melanomas, 5 (20%) were mutant with four cases having mutations of B-raf exon 15 and one an N-ras exon1 mutation. The mutations in the non-Spitzoid melanomas comprised eleven in B-raf exon 15, two in N-ras exon 2 and one in N-ras exon 1. The numbers of B-raf and N-ras mutations encountered in the tumour series are illustrated in Figure 4-5.

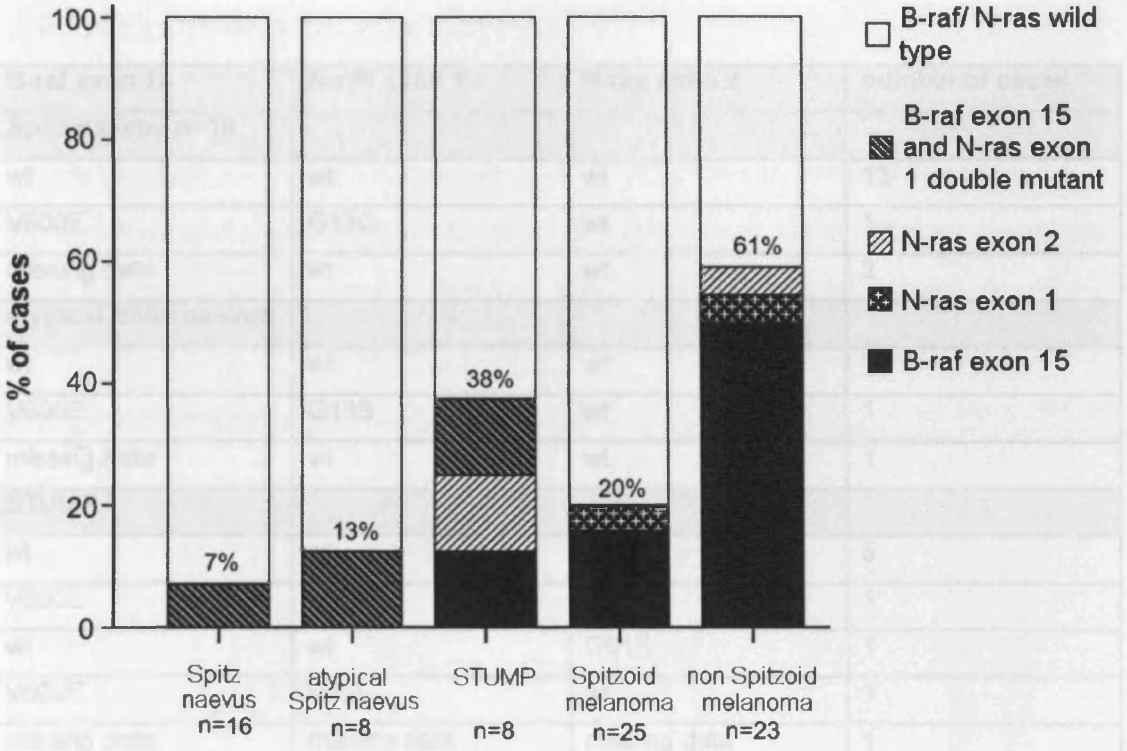


Figure 4-5 Percentage of B-raf exon 15 and N-ras exons 1 and 2 mutations in different tumour types.

There was no significant difference in B-raf and/or N-ras mutation frequency between the different categories of Spitzoid tumour ($p=0.35$, Chi^2 test). The combined mutation frequency of STUMP and Spitzoid melanomas (i.e. tumours showing higher amounts of atypia) was greater than that of Spitz naevi and atypical Spitz naevi (i.e. those with no or lower atypia) however this difference was also not significant ($p=0.28$, Chi^2 test). These data indicate that B-raf or N-ras mutations are uncommon in Spitzoid tumours and, where they do occur, the frequency does not significantly differ between the different Spitzoid tumour types. The frequency of the different types of mutations within the different tumours is shown in Table 4-4.

B-raf exon 15	N-ras exon 1	N-ras exon 2	number of cases
Spitz naevus n=16			
wt	wt	wt	13
V600E	G12C	wt	1
missing data	wt	wt	2
atypical Spitz naevus			
wt	wt	wt	7
V600E	G13S	wt	1
missing data	wt	wt	1
STUMP			
wt	wt	wt	5
V600E	wt	wt	1
wt	wt	G61K	1
V600E	G12C	wt	1
missing data	missing data	missing data	1
Spitzoid melanoma n=27			
wt	wt	wt	20
V600E	wt	wt	3
V600K	wt	wt	1
wt	G13S	wt	1
missing data	wt	wt	2
non-Spitzoid melanoma n=25			
wt	wt	wt	9
V600E	wt	wt	11
wt	G15E	wt	1
wt	wt	G61R	1
wt	missing data	G61R	1
missing data	wt	wt	1
wt	missing data	wt	1

Table 4-4Mutations of B-raf exon15, N-ras exon 1 and N-ras exon 2 in the tumour series.

4.6 H-ras mutation frequency

Mutations of the H-ras gene have been described in a minority of classic Spitz naevi[36, 122, 140] but are not found in non-Spitzoid melanoma. H-ras mutations can therefore be used a tool to identify neoplastic progression in non-classical Spitzoid tumours. Gene mutations are unlikely to be lost during progression and therefore, if progression to malignancy were to occur in Spitzoid tumours, H-ras mutations should be detectable in some cases. Example SSCP analysis and sequencing electropherograms from wild type and mutant cases are shown in Figure 4-6.

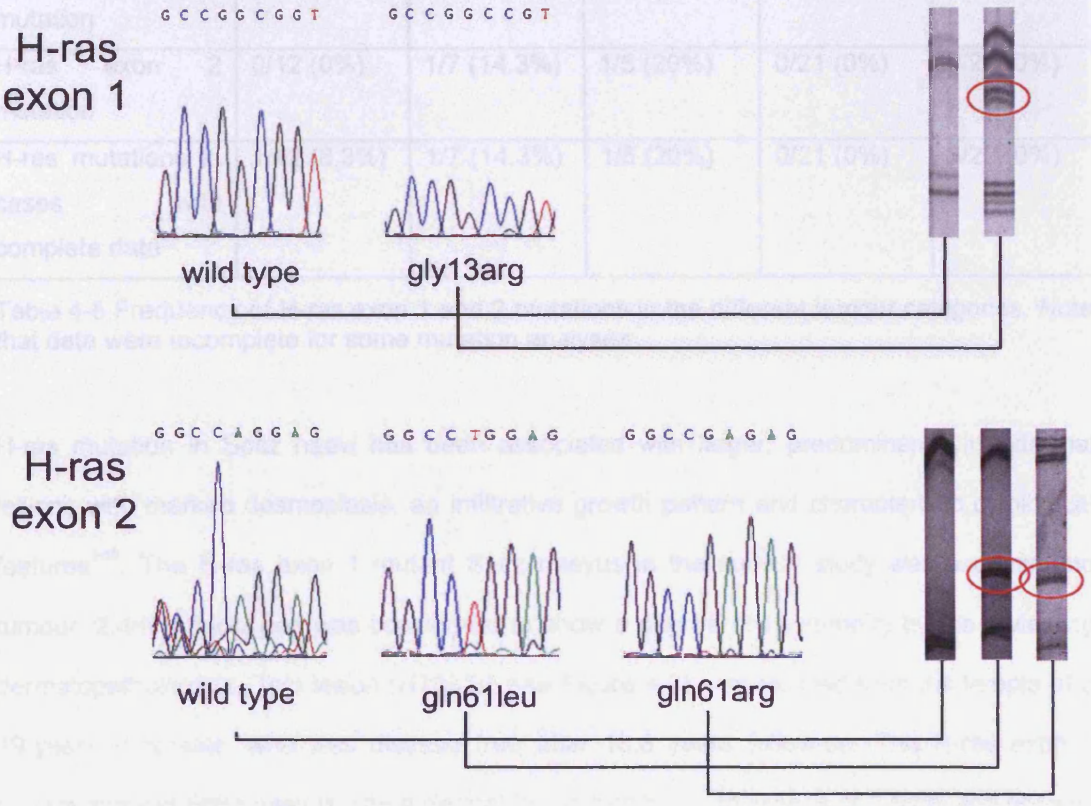


Figure 4-6 SSCP analysis with accompanying sequencing electropherograms from cases with wild type and mutant H-ras exons 1 and 2. Aberrantly migrating band are encircled in red.

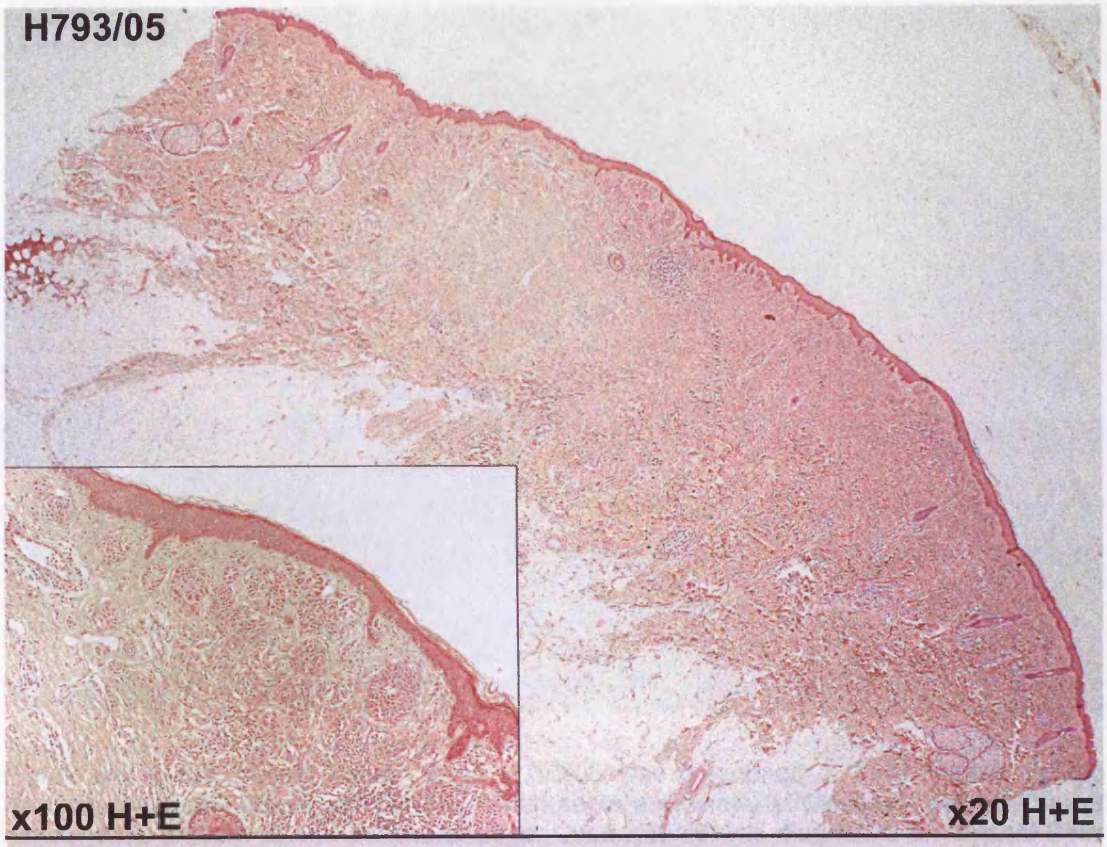
The H-ras exon 1 and 2 mutation status could not be determined in all cases, with the most data missing from analyses of exon 2. From a total of 90 cases analysed there were 67 cases where complete mutation data for H-ras exons 1 and 2 was available. A total of three mutations of H-ras were found, with one G13R exon 1 mutation in a classic Spitz naevus , one Q61R exon 2 mutation in an atypical Spitz naevus and one G61L exon 2 mutation in a

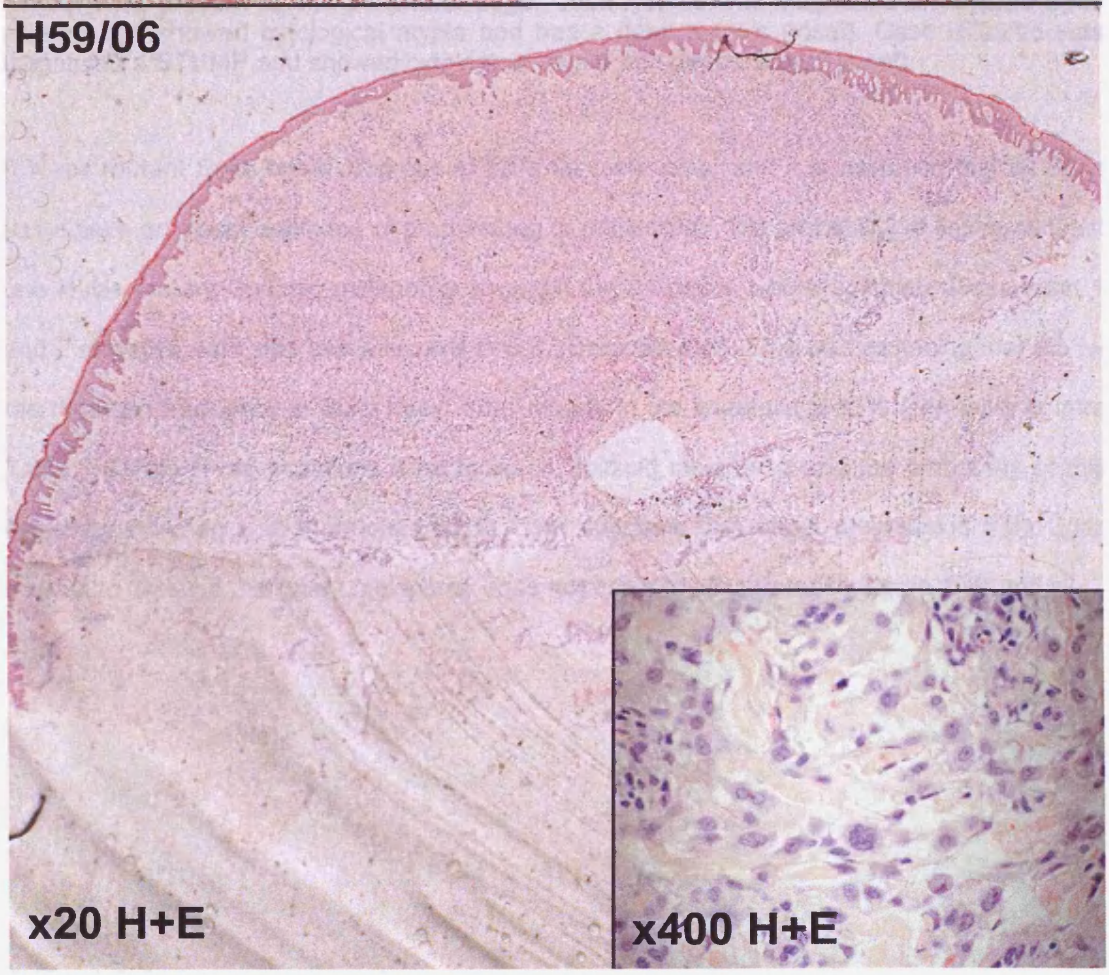
STUMP. No H-ras mutations were found in Spitzoid or non-Spitzoid melanomas and none of the H-ras mutant cases exhibited gold-standard evidence of malignancy i.e. metastases (median follow-up 8.5 years, see Appendix 2: Complete clinicopathological and mutation analysis data). The frequency of H-ras mutations is shown in Table 4-5.

	Spitz naevus n=16	**atypical Spitz naevus n=9**	**STUMP n=9**	**Spitzoid melanoma n=27**	**non-Spitzoid melanoma n=25**
H-ras exon 1 mutation	1/15 (6.3%)	0/9 (0%)	0/8 (0%)	0/27 (0%)	0/25 (0%)
H-ras exon 2 mutation	0/12 (0%)	1/7 (14.3%)	1/5 (20%)	0/21 (0%)	0/22 (0%)
H-ras mutations in cases with complete data	1/12 (8.3%)	1/7 (14.3%)	1/5 (20%)	0/21 (0%)	0/22 (0%)

Table 4-5 Frequency of H-ras exon 1 and 2 mutations in the different tumour categories. Note that data were incomplete for some mutation analyses.

H-ras mutation in Spitz naevi has been associated with larger, predominantly intradermal lesions with marked desmoplasia, an infiltrative growth pattern and characteristic cytological features[140]. The H-ras exon 1 mutant Spitz naevus in the current study was a compound tumour, 2.4mm thick, and was considered to show a degree of asymmetry by the reviewing dermatopathologists. This lesion (H793/05 see Figure 4-7) was excised from the temple of a 19-year-old female, who was disease free after 13.8 years follow-up. The H-ras exon 2 mutant atypical Spitz naevus was a dermal lesion that had a thickness of 1.5mm and showed cytological atypia with a deep mitosis. This lesion (H59/06 see Figure 4-7) was excised from an unknown site in a 36-year-old female, who was disease free after 8.5 years follow up. The H-ras exon 2 mutant STUMP was a compound lesion, 3 mm thick and also showed cytological atypia with a deep mitosis. This lesion (H795/05 see Figure 4-7) was excised from the shoulder of a 19 year old female, who was disease free after 7.3 years follow up.


H793/05
x100 H+E
x20 H+E


H59/06
x20 H+E
x400 H+E

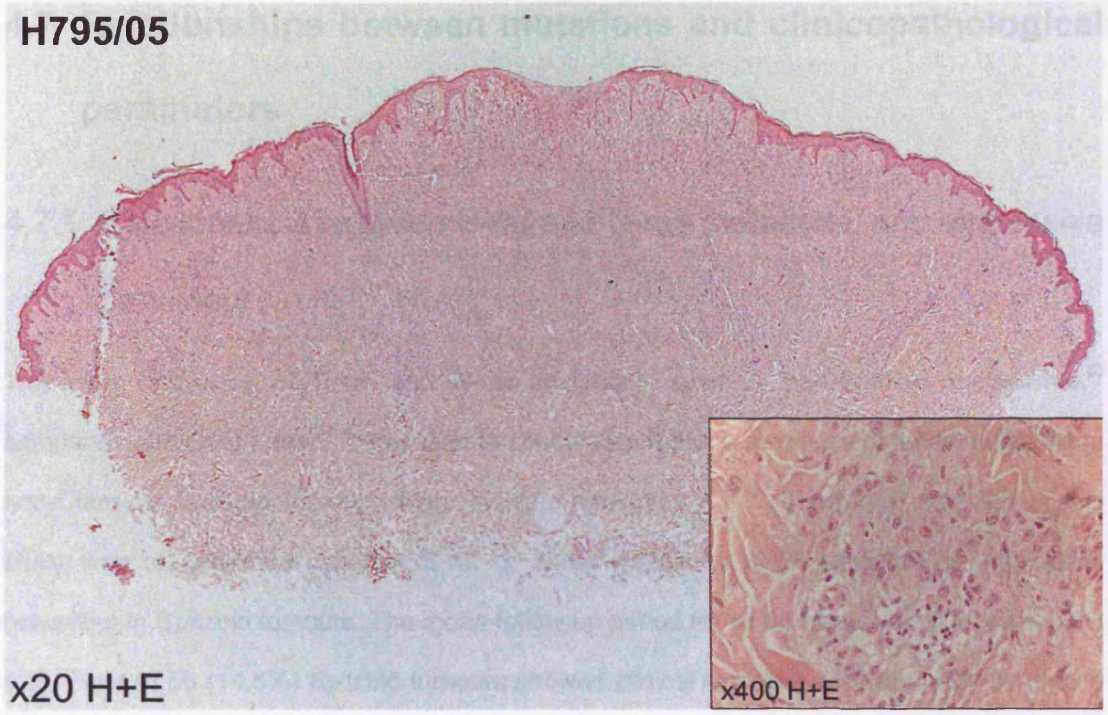


Figure 4-7 H-ras mutant cases. Case H793/05 was diagnosed a Spitz naevus by the reviewing dermatopathologists but they noted some asymmetry of the lesion with nodules of tumour cells present at one peripheral edge. Case H59/06 was diagnosed an atypical Spitz naevus and showed cytological atypia and had a deep mitosis (inset). Case H795/05 was diagnosed a STUMP and showed cytological atypia with deep mitoses (inset).

If H-ras mutant Spitz naevi progress to Spitzoid melanoma, and it is assumed that all Spitz naevi have an equal likelihood of progressing to malignancy, the probability of finding at least one H-ras mutant Spitzoid melanoma amongst the 21 cases where complete H-ras exon 1 and 2 mutation data was available was p=0.97(Bernoulli trial). This was assuming that the H-ras mutation frequency in Spitz naevi from reports in the literature is 15% (see table in intro Table 1-5). No H-ras mutations were found in Spitzoid melanoma and the probability of this occurring by chance is therefore p=0.03. This suggests that direct progression from Spitz naevus to Spitzoid malignant melanoma does not occur (as illustrated in Figure 1-9b and c).

4.7 Relationships between mutations and clinicopathological parameters

4.7.1 Relationships between B-raf and N-ras mutations, and aggressive behaviour

The high frequency of B-raf and N-ras mutations seen in non-Spitzoid melanomas[30] compared with Spitz naevi[57, 63] has lead to speculation that the presence of these mutations in non-Classical Spitzoid tumours might indicate malignancy[57, 63]. Therefore, one aim of this study was to determine whether B-raf or N-ras mutations were predictive of aggressive behaviour in Spitzoid tumours. The mean follow-up period for all 86 cases was 8.1 years (±SD 5.3). Eight of 55 (14.5%) Spitzoid tumours showed clinical evidence of malignancy by having histologically confirmed metastases to either regional lymph nodes or a distant site. Clinicopathological and mutation analysis data from these cases is shown in Table 4-6, the same data for the 47 Spitzoid tumours that did not display malignant behaviour during follow up is given in Appendix 2: Complete clinicopathological and mutation analysis data.

Case	Sex	Age	Site	Depth	Review diagnosis	*Number of atypical features	Clinical outcome	Duration of follow up (median 8.5, IQR 14.9)	Time to disease progression	B-raf 15	N-ras 1	N-ras 2	H-ras 1	H-ras 2
H140/06	M	10	Scalp	3.2	Atypical Spitz naevus	2	Sentinel node positive	5.2	0	Wt	Wt	Wt	Wt	Wt
H51/06	F	36	Back	4	STUMP	2	axillary node metastases	3.8	1.7	Wt	Wt	M	Wt	Wt
H138/06	F	40	Cheek	3	Spitzoid melanoma	3	Sentinel node positive	3.2	0	Wt	Wt	Wt	Wt	Wt
H12/06	M	14	Neck	14.2	Spitzoid melanoma	5	Cervical node and lung metastases	26.2	10.9	Wt	Wt	Wt	Wt	Wt
H142/06	M	12	Ankle	4.7	Spitzoid melanoma	4	Sentinel node positive	6.2	0	Wt	Wt	Wt	Wt	Wt
H239/06	M	29	Back	3.6	Spitzoid melanoma	6	axillary node metastases	17.2	4.0	M	Wt	Wt	Wt	Wt
H242/06	F	76	Ankle	3.6	Spitzoid melanoma	5	Skin metastasis	19.7	2.8	M	Wt	Wt	Wt	Wt
H258/06	F	3	Face	2.6	Spitzoid melanoma	3	Cervical node metastasis	10.8	1.8	Wt	Wt	Wt	Wt	ND

Table 4-6 Clinicopathological data and results of mutation analyses in metastatic Spitzoid tumours. Wt, wild type; m, mutant; ND, no data available.

In three cases (37.5%) the metastases were detected on sentinel lymph node biopsy with no distant recurrences reported after a median follow up period of 5.2 years. Of the remaining five cases, two patients developed axillary lymph node metastases, one developed cervical lymph node and lung metastases, one developed cervical lymph node metastases only and one developed a skin metastasis. Three of the eight metastatic Spitzoid tumours (37.5%) harboured a B-raf or N-ras mutation, while 7 of the 47 (14.9%) non-metastatic Spitzoid tumours were mutant. While the frequency of mutations was greater in the metastatic Spitzoid tumours compared with the non-metastatic group, this difference was not significant (p=0.15, Fisher's exact test).

The number of metastatic cases within the Spitzoid melanomas was greater than in the non-Spitzoid melanomas because the Spitzoid melanoma group was enriched for cases that had metastasised and defied prediction of behaviour. Three cases of non-Spitzoid melanoma had clinical evidence of metastases and they all had V600E mutations of B-raf exon 15, while 2 of 6 (33%) metastatic Spitzoid melanomas harboured B-raf or N-ras mutations (p=0.17, Fisher's exact test).

4.7.2 Relationships between B-raf and N-ras mutations, and age

Some authors have suggested that age may influence the development of B-raf and N-ras mutations in Spitzoid melanoma[122]. One study of metastatic Spitzoid melanoma in children found no mutations in nine cases[120], which suggests that children may develop melanoma via B-raf and N-ras independent mechanisms. One aim of this study therefore, was to investigate possible relationships between B-raf or N-ras mutations and patient age in Spitzoid tumours.

In the current study, there was no significant difference between the mean age of patients with B-raf and/or N-ras mutant Spitzoid melanomas (29.0 years ± 27.7, n=5), and those with wild type tumours (32.7 years ± 22.0, n=20) (p=0.68, Mann-Whitney U test). Furthermore, when the analysis included all Spitzoid tumours, the mean age of B-raf and/or N-ras mutant patients (32.3 years ± 19.4, n=10) did not differ significantly from those with wild type tumours (30.7 years ± 17.7, n=45) (p=0.77, Mann Whitney U test).

Next, patients were grouped according to whether they were greater or less than ten years of age, to examine whether there were differences between tumours in pre and post pubertal patients. The cut-off of ten years of age has been utilised by other authors to define these two groups[167]. The biology of melanoma in children is poorly understood and it has been suggested by some authors that melanoma in children may behave differently from that of adults[126, 192, 193]. There were four patients aged under 10 years of age with Spitzoid melanomas, of which one (25%) harboured a B-raf mutation. This mutation was in a tumour removed from the buttock of a five-year-old female who was disease free after 5.3 years follow-up. The tumour is shown in Figure 4-8. There were 21 Spitzoid melanomas in patients over ten years of age, 4 (19%) of which harboured B-raf or N-ras mutations. There was no significant difference in the proportion of mutant cases between the two groups (p=1 Fisher's exact test) although the small number of cases in the group under ten years of age precludes definitive comment. Similarly the proportion of mutant cases varied little between the two groups when the analysis was expanded to include all Spitzoid lesions.

The number of cases in patients aged less than ten years was small and so, to increase the number of cases in the group of young patients, the age cut-off was increased to 20 years of age. There were ten Spitzoid melanomas in patients under 20 years of age and fifteen in patients greater than twenty years of age. In both of these age groups the proportion of B-raf or N-ras mutant cases was the same, with 2 of ten mutant cases in patients under twenty and three of fifteen cases in patients over twenty years old. When all Spitzoid lesions were examined in this way, 2 of 16 (12.5%) tumours in patients less than twenty years of age were mutant, while 8 of 39 (20.5) cases were mutant in those greater than twenty years of age. While mutations were seen at a greater frequency in the patients over twenty years of age, this difference was not significant (p=0.71 Fisher's exact test).

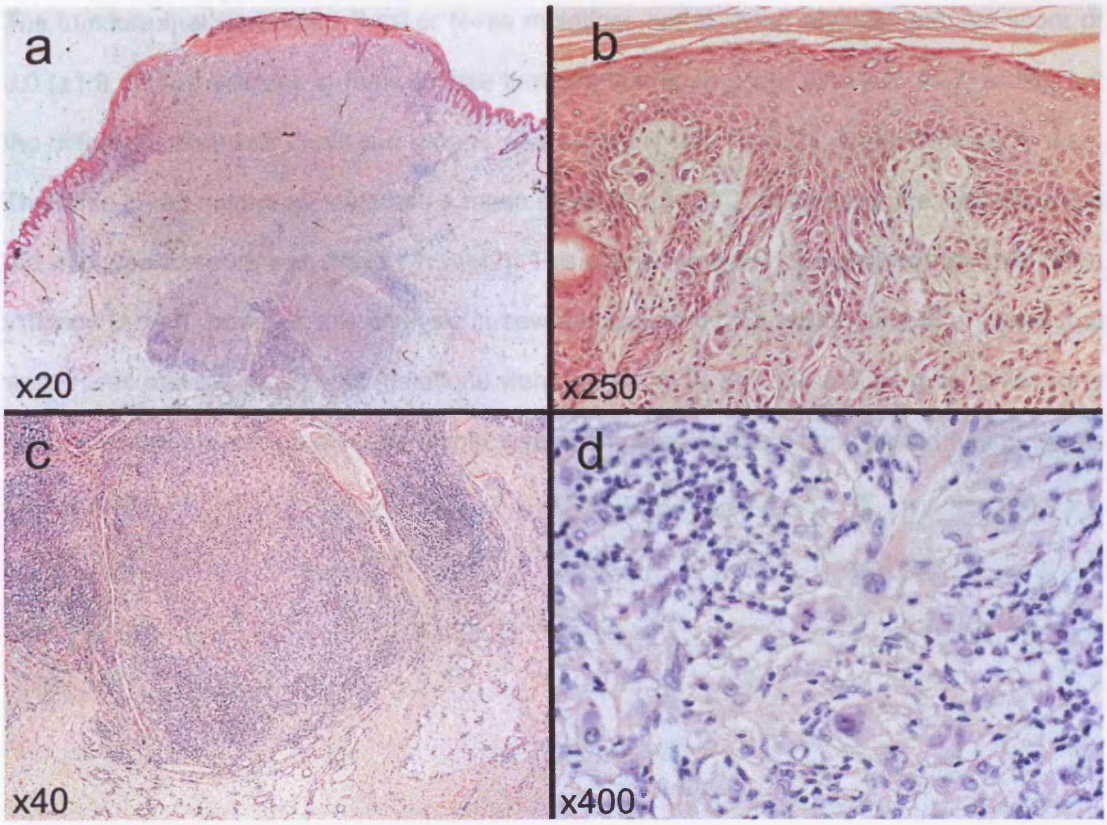


Figure 4-8 B-raf V600E mutant Spitzoid melanoma in a child aged five years. This tumour was described as a "severely atypical lesion with a Spitzoid component" on the initial histology report and was diagnosed as a Spitzoid melanoma on histological review. The patient is disease free after 5.3 years of follow-up. a) low power view, b) Spitzoid junctional component c) atypical, expansile deep component d) cytological atypia and mitotic figure in the deep component.

These data indicate that, within Spitzoid lesions, mutations of B-raf or N-ras are not more likely to be encountered in older patients when compared with tumours from younger patients.

4.7.3 Relationships between B-raf and N-ras mutations, and atypical histological features

Bastian *et al* reported increased atypia in Spitz naevi with amplification and concurrent mutations of H-ras[140]. One aim of the study was to determine whether mutations of H-ras, or B-raf and N-ras, were more likely to be encountered in Spitzoid tumours that showed histologically atypical features.

The tumours that harboured B-raf or N-ras mutations had a mean atypical features score of 3.0 (±1.9, n=45) whereas in the wild type tumours the mean score was 2.2 (±1.7, n=10), but the difference between these two groups was not significant (p=0.28, Mann Whitney U test). The three H-ras mutant tumours had a mean score of 1.3 (±1.2, n=3), while the mean score in the wild type tumours was 2.5 (±1.7, n=42). This difference was not significant (p=0.22 Mann Whitney U test), however this analysis is severely limited by the small number of cases that were H-ras mutant, plus H-ras mutations were not found in Spitzoid melanomas, which as a group showed greater amounts of atypia when compared to the other Spitzoid tumour types (see Figure 4-1).

5 Discussion

5.1 Characterisation of Wnt5a expression during cutaneous melanoma progression

The results of this investigation show that expression of Wnt5a increases with tumour progression in cutaneous melanoma. This is the first study of Wnt5a expression during melanoma progression to describe this finding in a large series of matched and unmatched samples. This is a key finding, which indicates that Wnt5a may be an important mediator of tumour progression in cutaneous malignant melanoma. In support of this, analysis of the effect of Wnt5a expression on outcome shows that this protein affects metastasis free and overall survival. Alongside these findings, the current study shows that expression of $p16^{INK4a}$ decreases and where present, mutations of B-raf frequently occur in the RGP and VGP phases. These trends indicate that alterations of these genes are non-random events that tend to occur during specific phases of Clark's model, and as such, may be part of a multi-step model of melanoma progression.

5.1.1 Wnt5a expression during melanoma progression:

Comparison of the Wnt5a data with that of previous pilot studies has lead to some similar but also some conflicting conclusions. Bittner *et al*[7] concluded that melanoma cell lines expressing higher levels of Wnt5a were more invasive and motile. In support of this, the current study has shown significantly higher levels of Wnt5a expression in VGP and metastatic melanomas, which are more aggressive phenotypically in comparison with RGP melanoma. In addition, a significant trend of increasing expression with Breslow depth was shown, which also suggests a role for Wnt5a in tumour invasion. Weeraratna *et al*[9] describe one case where increased expression of Wnt5a correlated with intra-tumoural progression. This study has expanded on this preliminary data by demonstrating in 34 cases of matched primary and metastatic melanoma, that expression of Wnt5a increases significantly during melanoma progression. It should be noted that Weeraratna *et al* detected membranous

Wnt5a expression via immunoflourescence, but their photomicrographs clearly show cytoplasmic staining.

Bachmann *et al*[106] examined Wnt5a expression in 202 cases of nodular melanoma, 20 cases of superficial spreading melanoma, and 32 cases of benign naevi via immunohistochemistry. Unlike the current study, they used antigen retrieval in their immunohistochemistry method and different conditions for incubating the primary antibody. Expression of Frizzled, E-cadherin, P-cadherin, N-cadherin and beta catenin were also characterised. The authors found nuclear Wnt5a staining to be more prevalent than membranous or cytoplasmic. The possible significance of this is discussed in section 5.1.2. Where cytoplasmic staining was recorded, expression in melanomas was found to be significantly greater than that in naevi. Similarly, this study found the cytoplasmic expression of Wnt5a in VGP melanomas, the majority of which in this study were nodular tumours, to be greater than in CAN, but Wnt5a expression in purely RGP tumours, which comprised predominantly SSMM, was lower than that in CAN. Bachmann *et al* found the difference of expression in naevi compared with melanomas to be highly significant, while the association in the current study was not significant. This difference may have arisen due to the greater power of the study by Bachmann *et al,* who analysed a total of 222 cases of CM (via tissue micro array), compared with 94 in this study.

In contrast to their findings concerning cytoplasmic Wnt5a, Bachmann *et al* found that nuclear expression of Wnt5a reduced significantly from naevi to melanoma and suggest this indicates that where present, nuclear Wnt5a exerts a tumour suppressor function. The current study also suggests that during specific parts of Clark's progression model, particularly in naevi, Wnt5a may act as a tumour suppressor, in this case, when expressed in the cytoplasm.

Expression of Wnt5a in naevi was greater than that observed in purely RGP tumours and in RGP components adjacent to VGP melanoma. In support of this finding, is a report of high levels of Wnt5a mRNA expression in naevi in a pilot study by Pham *et al* [88]. It is curious that while Wnt5a expression in melanoma shows a trend of increasing expression, naevi do not

appear to follow the trend and show greater levels of expression than that observed in the early phases of malignant progression. The data indicate that within melanoma progression, Wnt5a may be something of a paradox, acting as a tumour suppressor in benign melanocytic lesions, yet promoting progression in advanced melanoma. The role of Wnt5a in Wnt signalling is complex and it appears that the ligand signals through different non-canonical pathways as well as the canonical Wnt pathway [83, 85] to mediate both pro and anti-proliferative signals via the stabilisation or degradation of β-catenin. This may also account for the differences of expression observed between different cancer types[86]. What may occur in melanoma is a situation analogous to that of the transcription factor MITF, as described by Garraway et al[194]. In the normal state MITF, inhibits proliferation and promotes differentiation, but in the setting of malignancy, where ordered alterations of genes such as B-raf and CDKN2A ($p16^{INK4a}$) occur, there is gene amplification and subsequent oncogenic activity. Expression data from the current study suggests that similar context-specific changes may occur with Wnt5a.

The work of Hoek *et al* [195] suggests that Wnt5a may actually play a role in the regulation of MITF by upstream inhibition of β-catenin, and thereby promote a metastatic phenotype. In a series of 86 cultures of melanoma they performed DNA micro array analyses and identified three different sample cohorts that represented melanomas with differing metastatic potential. Between these cohorts there was a switch from a highly proliferative, weakly metastatic state to a weakly proliferative, highly metastatic state, which was considered to arise from relative differences in the activity of signalling by the Wnt/β-catenin pathway and TGF-β. They concluded that the highly proliferative yet weakly metastatic phenotype arose from canonical Wnt signalling promoting nuclear accumulation of β-catenin, which subsequently increased MITF transcription leading to increased proliferation. Conversely in the weakly proliferative yet highly metastatic cohort they concluded that up-regulation of inhibitors of canonical Wnt signalling such as Wnt5a via TGF-β-like signalling, reduced MITF transcription and promoted metastasis. This provides an alternative mechanism whereby Wnt5a can promote tumour progression in the later stages of melanoma i.e. by inhibiting classical Wnt signalling and

MITF transcription, as well as promoting motility and invasion via non-canonical Wnt signalling.

The above model however, does not provide an explanation for the role of Wnt5a in naevi, where high levels of expression were also observed. A possible explanation for this could be a tumour suppressor function for Wnt5a, acting as an inhibitor of β-catenin mediated proliferation via GSK3β independent mechanisms[85, 196]. One can speculate that in naevi, Wnt5a may act as a tumour suppressor, with the pro-metastatic effects observed by Hoek *et al* silenced via other tumour suppressor mechanisms. In the early stages of melanoma, in those naevi that progress to malignancy, this tumour suppressor function may be lost as a result of decreased Wnt5a expression, as was seen in this study. Finally, in the latter stages of melanoma progression, increased Wnt5a expression results predominantly in non-canonical Wnt signalling, producing increased motility, invasion and hence tumour progression.

Dissanayake *et al* [8], through the use of recombinant Wnt5a, protein kinase C activation with phorbol esters and siRNA to the WNT5A gene, have demonstrated that Wnt5a may promote cell motility and invasion via a number of mechanisms. Many of its pro-metastatic effects are mediated via protein kinase C, for example, activation of Snail, a transcriptional suppressor of the E-cadherin; increased expression of vimentin; and down regulation of E-cadherin. The use of siRNA against the WNT5A gene to effectively block the actions of Wnt5a protein, suggests it may be a useful therapeutic target[8].

The mechanisms underlying alterations of Wnt5a expression during melanoma progression cannot be determined from this study because alterations of the WNT5A gene or up-stream components of Wnt5a signalling were not examined. Iozzo *et al* have shown in a number of human cancers with increased Wnt5a expression, that gene rearrangements or amplifications of the WNT5A gene do not occur [86]. This suggests that the alterations may arise in genes upstream of Wnt5a that regulate its transcription.

Wnt5a expression was not formally correlated with cytological features in the tumour samples. Interpreting grade and behaviour in melanoma via cytological features is likely to be unreliable because there is a paucity of robust evidence concerning the independent prognostic significance of such criteria *in vivo,* when compared to other markers of prognosis such as Breslow depth or level of invasion[21, 197-199]. However, while reviewing the sections for semi-quantification of protein expression, there were no apparent trends. In contrast, Weeraratna *et al*[9] observed increased Wnt5a expression in melanoma cells with 'high grade' cytological features and in cells at the invasive edge of tumours. Why a similar pattern was not apparent in this study, despite using the same antibody for immunohistochemistry, is unclear. A possible explanation is that the features observed by Weeraratna et al in a small number of cases were not apparent in this more robust analysis on a larger sample. In contrast to the observations of Weeraratna *et al*, Pham *et al*[88] found increased Wnt5a RNA expression, as defined by in situ hybridisation, in melanoma characterised by small, uniform cells and comparatively low expression in large pleomorphic cells. The reason for the differences between these two studies is unclear, however, it is possible that protein expression does not correlate well with expression of mRNA due to modifications during mRNA translation and protein synthesis.

5.1.2 Wnt5a expression and outcome

Cytoplasmic Wnt5a expression was found to predict outcome for both time to metastasis and overall survival, but was not significant in a multivariate model of survival that included other predictors known to be important. However, cytoplasmic Wnt5a was very close to significant and became so when the covariate "site of origin" was removed from the analysis, allowing 8 cases with missing data to be included. Taken together with the assessment of expression during progression, the data suggest that cytoplasmic Wnt5a leads to aggressive behaviour in cutaneous melanoma.

The samples in this study were biased towards thick lesions because 59 of the samples were from relatively thick primary VGP tumours that were known to have metastasised and were used in the preceding analysis of matched primary and metastatic melanoma cases. Nevertheless, the analysis was helpful in providing a preliminary assessment of the

contribution of cytoplasmic Wnt5a expression to outcome. To determine whether this conclusion can be extended to cutaneous melanoma in general would require a study of unbiased melanoma samples whose baseline features match those of melanomas in clinical practice. Such an investigation would require a much larger number of cases to obtain sufficient outcomes (and hence power) for multivariate analysis. Despite this possible shortcoming of the survival data, poor outcome in primary melanomas with strong cytoplasmic Wnt5a expression does appear logical given that the most aggressive cells in a melanoma, those from metastases, show high expression and that enforced Wnt5a expression promotes melanoma cell migration *in vitro* [9].

Previous studies of Wnt5a expression and survival have been performed. Weeraratna *et al* performed a small study of tumour tissue including 8 naevi, 10 primary melanomas and 9 metastases[9], studying membranous Wnt5a (although, as stated above, their photomicrographs clearly show cytoplasmic staining). These authors stated that Wnt5a expression correlated strongly with both survival and time to metastasis. However, there was no statistical analysis to support this statement, with only 5 deaths recorded in total, and no correction for baseline variables such as Breslow depth. The data of the current study therefore extend that of Weeraratna *et al*, and provide more robust evidence that high cytoplasmic expression of Wnt5a predicts a worse prognosis compared to cases with weak staining. Bachmann *et al* performed a study that included both cytoplasmic and nuclear Wnt5a staining. Nuclear Wnt5a was tested as a predictor of 5-year and 10-year survival rate and was not significant. The impact of cytoplasmic Wnt5a on outcome is not explicitly stated, although P-cadherin was reported as a significant predictor of survival and this correlated positively with cytoplasmic Wnt5a.

Given that Wnt5a is a secreted ligand produced in both naevus and melanoma cells [88], its presence in the cytoplasm of these cells would be expected and be a reasonable marker of expression. However, the significance of its presence in the nucleus is unclear. In particular, it is difficult to be sure that this does not represent an artefact (e.g. due to non-specific staining or cross-reactivity with another protein) rather than genuine presence of Wnt5a. There are no other reports of Wnt5a expression in the nucleus, although it is known that secreted ligands

can be found in the nucleus, as with fibroblast growth factor 2 [200]. In the current study, nuclear Wnt5a was scored in all the primary melanomas and included in the outcome analysis because it served as useful comparison with cytoplasmic Wnt5a and also as a comparison with the findings of Bachmann *et al.* Whatever the role of nuclear Wnt5a, if indeed any, our results suggest that it is not important in melanoma survival. Further work will be needed to assess whether nuclear Wnt5a is genuinely found in the nucleus and, if so, what its function is.

5.1.3 B-raf mutation during melanoma progression

Despite the large amount of published data concerning B-raf mutation in melanoma, these alterations were determined in the current tumour series in order to provide a context in which to examine the expression of Wnt5a. The results indicate that the point at which B-raf mutation occurs in melanoma progression may not be as well characterised as the literature suggests. The prevailing attitude is that B-raf mutations occur early in melanoma progression due to the high frequency of mutations observed in naevi and the RGP components of B-raf mutant VGP melanomas[45, 61]. By contrast, the current study found the frequency of B-raf exon 15 mutations in purely RGP melanoma to be significantly lower than that observed in VGP tumours. Furthermore, expression in purely RGP melanomas was likely to be significantly lower than in RGP components adjacent to the VGP, where mutations tended to be concordant with those in the adjacent VGP and are therefore predicted to be comparatively high. In support of this prediction is a study by Omholt *et al* who described a relatively high frequency of B-raf mutations in RGP components that tended to be concordant with the adjacent VGP[45]. As a result of this finding, Omholt *et al* concluded that B-raf was an early event in melanoma pathogenesis. Conversely by examining purely RGP melanomas, Dong *et al* have shown, as in the current study, that there is a significantly lower frequency of B-raf mutations in purely RGP melanomas compared to VGP and metastatic tumours.

A possible explanation for these conflicting results is that in the minority of cases where melanoma arises within a naevus[71], mutation of B-raf is a very early event[61]. However, in the majority of melanomas which arise *de novo,* mutations of B-raf occur either as an

intermediate event (i.e. in the VGP) or else B-raf mutant RGP lesions are positively selected for progression and thus are enriched in the VGP and beyond. An alternative explanation, as acknowledged by Demunter *et al* who performed a similar study in N-ras mutations, is that cells from the VGP may repopulate the RGP components and so may not truly be derived from the pre-existing RGP[41]. In the current study, RGP components in VGP tumours were only micro dissected from the tumour periphery, and not from the central areas overlying the dermal VGP nodule. This was done because it is possible that VGP melanoma invades upwards as well as down into the dermis, and in this situation is more likely to become interspersed with junctional tumour nests than at the edge of the tumour away from the VGP nodule.

Cases in which a mutation was present in the metastatic phase of progression but not in the corresponding primary lesion have been previously described [41, 45]. Three such cases were found in this study. Omholt *et al* concluded that such events can be explained by a failure to detect mutations in a small clone of cells in the primary that rapidly acquires metastatic potential after becoming mutant. In the converse situation, it is possible that some tumours progress via subclones, which develop alternative means of MAPK dysregulation. In this scenario a B-raf wild-type subclone (perhaps with N-ras mutation) gives rise to B-raf wild type metastases, while the dominant sub-clone in the primary lesion is B-raf mutant. This situation could only occur if B-raf mutation is an intermediate, rather than very early event.

5.1.4 $p16^{INK4a}$ expression during melanoma progression

$p16^{INK4a}$ expression in malignant melanoma has been reported in several studies. In the current study, as for B-raf, $p16^{INK4a}$ was assessed to provide a context in which to analyse expression of Wnt5a. Expression of $p16^{INK4a}$ decreased with tumour progression, a finding supported by previous studies[32, 97-99, 105]. The greatest decrease in expression in this study was observed during the RGP to VGP transition, and hence loss of $p16^{INK4a}$ appears to be an intermediate event. A significant decrease in $p16^{INK4a}$ expression between early primary melanoma (i.e. RGP melanoma) and more advanced primary tumours (i.e. VGP melanoma)

has also been observed by Reed *et al*[32]. Alonso *et al* more recently concluded that $p16^{INK4a}$ expression was most significantly decreased between the VGP and metastatic phase[105]. This conflicting conclusion may have arisen due to use of different antibodies, different detection methods or a smaller series of tumours being investigated in their study. Regardless of the precise timing, the evidence for loss of $p16^{INK4a}$ expression as melanoma progresses appears strong. Importantly however, immunohistochemical evaluation of protein expression fails to elucidate the mechanisms that underlie this reduction. In a large proportion of cases it is thought to arise due to deletion of the CDKN2A gene, but other evidence suggests that it is not purely due to changes at this locus[18, 93, 95, 98, 100, 101].

The mosaic pattern of staining found in this study, where only a proportion of the tumour cells expressed $p16^{INK4a}$, has been previously described [34, 102]. Importantly in spite of this patchy expression of $p16^{INK4a}$, Michaloglou *et al* have shown that all the cells in a naevus are growth arrested[34], which lead them to conclude that $p16^{INK4a}$ collaborates with other yet-to-be-identified factors to establish long-term growth arrest.

Despite subtle differences between the $p16^{INK4a}$ and B-raf data described in this study, and that of previous studies, in general the results appear comparable with the findings of others. This allows the Wnt5a expression data to be examined in the context of these well-described alterations.

5.1.5 Multi-step carcinogenesis and potential molecular interactions

This analysis of Wnt5a alongside $p16^{INK4a}$ expression and B-raf mutation enables some speculation on the sequence of these changes during melanoma progression. There is significantly increased expression of Wnt5a in the VGP and metastatic phases of melanoma relative to unmatched purely RGP melanoma samples, and in matched cases of metastatic melanoma relative to RGP components adjacent to a VGP melanoma. This indicates that the oncogenic effects of Wnt5a are likely to be most important in the later stages of progression. The significant decrease in $p16^{INK4a}$ expression coupled with the significant increase in B-raf mutation frequency between the RGP and VGP suggests that in some, if not most

melanomas, these are intermediate changes. There is evidence, however, that B-raf mutation is an early event in a subset of melanoma as shown by mutation in a minority of purely RGP melanomas.

The data also allow some speculation as to possible interplay between the three genes. Alterations in B-raf[30, 45, 57] and $p16^{INK4a}$ [31, 32, 93, 96] have been the subject of considerable investigation individually and are likely to play a central role in melanoma progression. In normal melanocytes, B-raf V600E mutation induces cell cycle arrest with corresponding induction of $p16^{INK4a}$, a process termed oncogene-induced senescence [34, 102]. In the current study there was no significant difference in the levels of $p16^{INK4a}$ expression between B-raf mutant and wild type melanomas. One explanation for this could be that a number of the B-raf wild type tumours may have alternative activation of the MAP kinase pathway due to mutations of N-ras or other components of the cascade. Another possibility, particularly in tumours at a later stage of progression, is that although the tumour is B-raf mutant, $p16^{INK4a}$ is lost due to subsequent deletion or mutation of the CDKN2A gene.

Loss of $p16^{INK4a}$ function removes melanocytes from their senescent state and allows uncontrolled proliferation which is driven by MAPK pathway activation. This is supported by the ability of the V600E mutation to transform cells that do not express $p16^{INK4a201}$. The acquisition of further genetic aberrations is then likely to be necessary for subsequent tumour progression. Chudnovsky *et al* have shown, that raf mutations and dysregulation of Rb alone are not sufficient to induce an invasive phenotype and other alterations, such as activation of hTERT or the PI3K pathway must also be acquired[73]. The current study suggests that Wnt5a expression may be another mechanism that promotes progression of the disease.

There is no evidence, as yet, of direct interactions between Wnt5a, and the MAP kinase or Rb pathways in cutaneous melanoma. Activating mutations of H-ras have been shown to down regulate Wnt5a in human mammary epithelial cells[202] but there was no evidence that B-raf mutations have a similar effect in the current study. Context specific changes in the function of Wnt5a during progression may arise indirectly from B-raf activation and $p16^{INK4a}$ loss, because

of the uncontrolled cell proliferation that ensues, and genetic damage that subsequently arises. Iozzo *et al* suggest that alterations to the WNT5A gene do not occur in malignancy, but there may be changes to other non-canonical Wnt signalling genes or regulatory genes upstream from them[86].

Although there is no evidence of close interactions between non-canonical Wnt signalling, B-raf activation and the retinoblastoma pathway, there is a common link in that they are associated with the normal and abnormal function of MITF, the so-called melanocyte "master regulator"[203]. MITF is believed to play a significant role in melanocytic differentiation and melanocytic tumourigenesis. MITF encodes a number of different isoforms including MITF-M, which is considered to be important in melanocytic differentiation [204]. In humans, germ line mutations of MITF are associated with Waardenburg syndrome, which is characterised by congenital deafness and disorders of pigmentation. MITF-M is believed to be vital in promoting the differentiation of neural crest cells along melanocytic lines and this function appears to be tightly regulated by several transcriptional regulators, including classical Wnt signalling ligands[80] and the transcription factor SOX10, which resides down stream of endothelin receptor-B. Endothelin receptor-B can also activate the mitogen activated protein kinase (MAPK) pathway[205]. As described above, Hoek *et al* have suggested that different Wnt signalling pathways can modulate the proliferative and metastatic potential of melanoma cultures by altering the expression levels of MITF[195]. Amplifications of MITF, when associated with loss of the tumour suppressor $p16^{ink4a}$ and activating mutations of B-raf, have recently been shown to produce malignant transformation in melanocytes *in vitro*[194].

5.1.6 Purely RGP tumours and RGP components in VGP tumours: morphologically similar yet biologically different

There are limited data comparing purely RGP melanoma to RGP components adjacent to VGP tumours. This study highlights an uncertainty concerning the nature of RGP components adjacent to VGP tumours and how they might relate to their putative antecedents, purely RGP melanoma. While purely RGP melanoma and the RGP components adjacent to VGP tumours appear histologically very similar, the expression data and mutation analysis indicates that

RGP components adjacent to VGP tumours differ biologically from purely RGP tumours. $p16^{INK4a}$ and Wnt5a both showed differential expression between purely RGP tumours and RGP components adjacent to VGP tumours. Indeed, a highly statistically significant reduction of $p16^{INK4a}$ expression was seen between purely RGP melanoma and RGP components adjacent to VGP tumours, with the level of $p16^{INK4a}$ expression observed in the RGP components more in keeping with that of the VGP. Similarly, the mutation frequency in purely RGP tumours was significantly lower than that predicted in micro dissected RGP components adjacent to VGP tumours and was likely to be comparable with that of matched VGP tumours and metastases. This is a potentially important finding because some researchers have used RGP components as a surrogate for purely RGP melanoma, to enable comparison of both progression phases in the same tumour [41, 43, 45]. In such studies, as described here, mutations of N-ras and B-raf, when present, are frequently found in both the RGP component and VGP, and are regarded as evidence that the mutation occurred during or prior to the radial phase of progression.

Similarly, Haqq *et al* correlated the gene expression profile of metastatic melanoma to either an RGP or VGP expression signature[206]. The reference RGP expression signature was derived from cells micro-dissected from the RGP component of a case adjacent to VGP melanoma. The data from this study suggest that the assumptions in these papers should be viewed cautiously and that the RPG component of a VGP tumour may represent an entity biologically, but not histologically, distinct from purely RGP melanoma. Furthermore, this finding is of biological significance because it suggests that the RGP components of VGP tumours are not dormant entities, but continue to evolve even though the VGP has established itself.

5.1.7 Summary

Principally this study demonstrates, in a large series of matched and unmatched samples, that expression of Wnt5a increases with melanoma progression and is likely to be of most importance late in progression. This confirms the conclusions of preliminary studies. In

parallel, trends in the expression of $p16^{Ink4a}$ and timing of B-raf mutations were shown, which provided context for the observed changes of Wnt5a. These trends suggest that such alterations occur in a somewhat predictable sequence during tumour progression and provide evidence of a multi-step pathway of progression to malignancy in melanocytic neoplasia. Further work is required to elucidate the additional alterations necessary for the development of melanoma within this pathway and the other progression pathways that are likely to exist within melanoma[18]. The findings also suggest that purely RGP melanoma and RGP components adjacent to VGP tumours are different, and data based on analysis of RGP components adjacent to VGP tumours should be viewed with caution. Finally, this study identifies Wnt5a as an independent predictor of time to metastasis and death.

5.2 Characterisation of B-raf, N-ras and H-ras mutations in Spitzoid tumours

5.2.1 Mutations of B-raf exon 15 and N-ras exons 1 and 2 in Spitzoid lesions

This study shows that the frequency of B-raf and N-ras mutations in Spitzoid tumours, including Spitzoid melanoma, is significantly lower than that of non-Spitzoid 'common' melanoma and that reported in published investigations of common acquired naevi[49, 55, 57, 61, 63], although these were not analysed in the current study. These results indicate that Spitzoid tumours are a distinct group of lesions not only histologically, but also at a genetic level. The results also show that metastatic disease in Spitzoid melanomas commonly occurs in the absence of these mutations, unlike non-Spitzoid melanoma. This precludes the use of B-raf and N-ras mutation analysis alone as a clinical tool for distinguishing between benign and malignant Spitzoid lesions, which has been proposed by some authors[63, 122].

As the current study and others have demonstrated, mutations of B-raf or N-ras are a very frequent occurrence in most malignant melanomas[30], with the exception of tumours that arise from non-sun exposed acral and mucosal sites[18, 65, 66], and in the setting of chronic sun

exposure[68]. B-raf mutations are also seen frequently in common acquired naevi[49, 55, 57, 61, 63], from which a minority of malignant melanomas arise[70, 71]. These mutations are consequently considered to be of fundamental importance for the initiation of most melanocytic lesions[41, 61], yet the data from the current study suggest that Spitzoid tumours arise via an alternative mechanism. The recognition that melanocytic tumours can progress via a number of divergent progression pathways is important for identifying new methods of prognostication and targeted therapy in melanocytic tumours. During the completion of this research, other studies have been published that have also examined the frequency of MAP kinase gene mutations in Spitzoid lesions and the results vary considerably between them [120-122, 151, 152].

In support of the current study, Lee *et al* and Gill *et al*[120, 121] also found that Spitzoid melanoma appears to share genetic similarities with Spitz naevi when compared to non-Spitzoid melanocytic tumours on account of their low incidence of B-raf and N-ras mutations. The current study has demonstrated that this conclusion can be extended to all lesions with a Spitzoid morphology, such as atypical Spitz naevi and STUMPs, which are histologically intermediate between classic Spitz naevi and Spitzoid melanoma. The results of the current study differ from those of Gill *et al* in that occasional mutant Spitzoid lesions were found, whereas they found no mutations in a series of 9 Spitzoid melanomas. In support of this, Lee *at al* found one mutant Spitzoid melanoma in their series of 33 (3%) and Takata *et al*[152] found two in a series of 16 'ambiguous Spitzoid lesions'. Despite such differences, these investigations generally confirm the findings of the current study that B-raf and N-ras mutations are uncommon in Spitzoid tumours, which indicates they are a distinct group. There are, however, other studies with contradictory results.

The results of van Djik *et al*[122] are considerably different from those of the current study. They examined 96 lesions that were classified as 'typical' Spitz naevus, atypical Spitz naevus, suspected Spitzoid melanoma (a category for tumours of borderline malignant appearance that might be considered analogous to STUMP), primary Spitzoid melanoma and Spitzoid metastasis. The authors report mutations of B-raf or N-ras in 30 of 36 Spitzoid melanomas (83%, although they state 86% in their paper), and 8 of 23 (35%) lesions suspected for

Spitzoid melanoma. In addition, Palmedo *et al*[143] found two B-raf mutant Spitzoid melanomas in a series of six cases (33%). In both cases the authors conclude that Spitz naevi and atypical Spitz naevi are unrelated to Spitzoid melanoma. Fullen *et al*[151] found mutations in 12 of 68 (18%) Spitzoid lesions, a frequency comparable to the current investigation; with two mutations in thirteen Spitzoid melanomas and none in seven atypical Spitzoid tumours. Their findings differ considerably with the current study, and most others however, in that they found the highest frequency of mutations in classic Spitz naevi or Spitzoid lesions showing minimal atypia (10 of 48, 21%). The authors conclude that Spitz naevi are not distinct from Spitzoid melanomas and non-Spitzoid melanocytic lesions. It can be seen therefore that there is considerable variation between investigations of MAP kinase gene mutations in Spitzoid tumours, with some supporting the findings of the current study, but others conflicting considerably. Possible explanations for this are considered in section 5.2.3, but first, the data from the current study will be discussed further.

This study enables some speculation concerning the nature of non-classical Spitzoid tumours. Some authors suggest that this group actually comprises a dichotomy of Spitz naevi and non-Spitzoid melanoma, and that Spitzoid melanomas and other atypical Spitzoid lesions do not exist as biologically distinct entities but merely serve as a diagnostic 'soft option' for vacillating dermatopathologists [124, 167]. The point of view that Spitzoid tumours comprise such a dichotomy has the consensus of opinion, with most investigations of Spitzoid tumours essentially aimed at differentiating the benign Spitz naevi from the malignant melanomas. Takata *et al* suggest that the majority of non-classical Spitzoid lesions are actually no different from classic Spitz naevi because they found very low frequencies of B-raf and N-ras mutations in 16 cases of 'ambiguous' Spitzoid tumours[152]. The findings of the current study appear to agree with this but the data also indicate that Spitzoid melanoma is a genuine entity in so far as there appears to be a subset of histologically malignant lesions with a Spitzoid morphology, that have a much lower incidence of B-raf and N-ras mutations compared with their non-Spitzoid counterparts. One could argue that histological diagnosis in the context of these tumours is not a very robust predictor of malignancy and that the Spitzoid melanoma group may have included lesions that had some features of malignancy but were actually

benign. In order to more robustly define a group of Spitzoid melanomas therefore, it is necessary to look at only those lesions that have metastasised i.e. the gold standard of malignant behaviour. Four of six (67%) metastatic Spitzoid lesions were wild type for B-raf and N-ras while there were mutations present in all metastatic non-Spitzoid melanomas (3 of 3, 100% see Appendix 2: Complete clinicopathological and mutation analysis data). The number of metastatic cases in both tumour groups was small, which may account for why the difference between the two did not achieve significance, but the results are supported by other studies that have shown a very low frequency of mutations in metastatic Spitzoid melanoma [120, 121] and a high frequency of B-raf and N-ras mutations in metastatic non-Spitzoid melanoma[45, 56, 64].

In the current study there was no significant difference in the frequency of B-raf and N-ras mutations between different types of Spitzoid tumour. This contrasts with the study by van Dijk *et al* who found an increasing mutation frequency from Spitz naevus to atypical Spitz naevus through to 'suspected Spitzoid melanoma' and Spitzoid melanoma. An explanation for the trend they observed could be that Spitzoid tumours require these mutations to progress and hence more mutations are found in the more atypical tumours. This implies that Spitzoid tumours progress in a manner similar to common naevi and dysplastic naevi (see Figure 1-9a), but the low frequency of mutations found in STUMP and Spitzoid melanoma in the current study argues against this.

5.2.2 Mutations of H-ras exons 1 and 2 in Spitzoid tumours - Do Spitz naevi progress to Spitzoid melanoma?

B-raf and N-ras mutation analysis indicated that Spitzoid tumours represent a distinct group of melanocytic tumours. The aim of H-ras analysis was to determine whether Spitzoid tumours show evidence of neoplastic progression, where Spitz naevi are the benign precursors to Spitzoid melanoma. Mutations of H-ras occur in a subset of Spitz naevi[140] and in one study were found in non-classical Spitzoid lesions [122], yet these mutations are distinct within melanocytic tumours because they are extremely rare in common acquired naevi and non-Spitzoid melanoma [161]. For this part of the study the hypothesis was that if Spitz naevi

progress to Spitzoid melanoma, it should be possible to identify H-ras mutations in some Spitzoid melanomas. Determining whether progression does occur in Spitzoid tumours is important, because it would indicate there are multiple pathways of melanocytic tumour progression, which may have implications for diagnosis, prognostication and therapy in malignant melanoma. A population of H-ras mutant Spitzoid melanomas would also provide further evidence that Spitzoid tumours are a distinct group of melanocytic lesions.

This study found no evidence that Spitz naevi progress to Spitzoid melanoma. A low frequency of H-ras mutations was found in the Spitzoid tumours, with a single mutation found in each of Spitz naevi, atypical Spitz naevi and STUMP, but no mutations were found in the Spitzoid and non-Spitzoid melanomas, and there were no metastases within the H-ras mutant tumours (median follow up 8.5 years). These results are in keeping with other studies (see Table 1-5)[120, 122, 140, 152]. van Dijk *et al* found H-ras mutations in atypical Spitz naevi and a single Spitzoid tumour suspected of melanoma. They conclude that the presence of such mutations indicates a benign lesion[122] and that this may be of diagnostic utility. The data from the current study appear to support this.

One explanation for why no H-ras mutant Spitzoid melanomas were found could be that not enough cases were examined. Combined analysis of H-ras exons 1 and 2 was not possible in all cases of Spitzoid melanoma, which meant that only 21 of the 27 cases had complete mutation data for both exons. Despite failing to derive a full data set from the cases it is still likely that the study was sufficiently powered. If the H-ras mutation frequency in Spitz naevi is assumed to be around 15% (see Table 1-5, note that 12 cases in the study by Bastian *et al* were enriched for likely H-ras mutants, hence a more conservative estimate of the overall frequency is used) the probability of finding a mutant in 21 cases of Spitzoid melanoma was p=0.97, which indicates that the study was sufficiently powered. Conversely, the probability of finding all Spitzoid melanomas wild type for H-ras was p=0.03.

If, as shown, the study was sufficiently powered, there are therefore two competing explanations for why no H-ras mutations were found in the Spitzoid melanomas. The first is,

as hypothesised, that Spitz naevi are unable to progress to Spitzoid melanoma, which is supported to some extent by the paucity of clinical reports of melanomas arising within lesions originally considered to be Spitz naevi. A second explanation is that H-ras mutation prevents tumour progression in Spitz naevi, but that H-ras wild type Spitz naevi are able to progress. Were this second scenario the case, a potential mechanism for this negative selection could be that that H-ras mutations induce expression of $p16^{INK4a}$ resulting in oncogene-induced senescence, as shown by Maldonado *et al*[36]. It is unlikely, however, that a $p16^{INK4a}$ dependent mechanism alone would place Spitz naevi in a state of irreversible senescence because this is not an insurmountable barrier to malignant transformation, as shown by the frequent loss of expression described in non-Spitzoid melanomas in the current study (see chapter 3), and by other authors [32, 96-98]. A more permanent mechanism of cycle arrest might be additionally induced by the unfolded protein response described by Denoyelle *et al*[141]. They found that the hyperproliferation that ensues from H-ras mutation induced a stress response, not seen with mutations of B-raf or N-ras. This resulted in structural changes of the endoplasmic reticulum and associated cell cycle arrest. It is possible therefore that these two mechanisms combine to render H-ras mutant Spitz naevi incapable of progressing to malignancy. If this is the case, the Spitz naevi that do progress to Spitzoid melanoma will be derived from the H-ras wild-type majority.

It is possible that the study may have been underpowered because within the group of Spitzoid melanomas there were some non-Spitzoid 'common' melanomas that were included erroneously due to diagnostic errors during case selection. This would mean that less than 21 genuine Spitzoid melanomas were analysed and the probability of finding an H-ras mutant lesion would therefore be less than expected. The problems of diagnostic reproducibility, and other potential explanations for the conflict between data from this study and other investigations of Spitzoid tumours, are discussed below.

5.2.3 Factors that may account for the variation between investigations of mitogen activated protein kinase pathway gene mutations in Spitzoid lesions

A most likely explanation for much of the inconsistency between investigations of Spitzoid tumours is variation in the criteria employed for case selection. This arises from a lack of universally accepted diagnostic criteria for these tumours, which creates a high degree of inter-observer variability[112-114]. When compiling Spitzoid tumours for research projects there are inevitably non-classical cases with borderline Spitzoid features, and whether they merit inclusion can be extremely subjective. Consequently, some non-Spitzoid melanocytic tumours (i.e. lesions likely to be B-raf or N-ras mutant) may be erroneously included in some tumour series. Were such instances to occur to a greater extent in some studies rather than others, the results of mutation analysis might vary considerably. Furthermore, because diagnostically challenging Spitzoid tumours are infrequently encountered in routine pathology practice, there is perhaps a pressure to be as inclusive as possible to ensure that the study has good statistical power.

In the current study a lesion was considered Spitzoid when it was composed of spindle and/or epithelioid cells, was reasonably circumscribed and symmetrical, and the junctional features did not better suit a diagnosis of superficial spreading melanoma, acral lentigenous melanoma or lentigo maligna melanoma. By contrast, van Dijk *et al* regard any lesion that is composed entirely of spindle and/ or epithelioid cells as Spitzoid, even if lateral radial growth is present (verbal communication). This approach was not adopted for the current study because by using these criteria some cases of superficial spreading melanoma or nodular melanoma (i.e. 'common' melanoma) might easily be considered Spitzoid lesions. This might explain the much higher frequency of B-raf and N-ras mutations encountered by van Dijk *et al* in comparison to the current study. Furthermore, during the reviewing process for this study, a mixture of potentially Spitzoid and non-Spitzoid lesions were given to the reviewing dermatopathologists, without any accompanying information. This meant that the reviewers

were blind as to whether an individual case was intended for inclusion within the Spitzoid or non-Spitzoid tumour groups, and hence avoided influencing their diagnosis.

Gill *et al* and Lee *et al* found a very low frequency of B-raf and N-ras mutations in Spitzoid melanoma compared with van Dijk *et al* and, to a lesser degree, the current study. This may have arisen from having highly specific criteria for case selection and consequently assembling a discrete series of 'true' Spitzoid melanomas that were all B-raf or N-ras wild type. Unfortunately the criteria they used for case selection are not explicitly detailed. In contrast, Van Dijk *et al* and possibly Palmedo *et al,* by applying more inclusive criteria, may have included lesions that were Spitzoid in certain aspects of their appearance, but genetically similar to non-Spitzoid naevi and melanomas. It is also notable that Lee *et al* [109, 112] and van Djik *et al* [176, 178] cite their criteria for diagnosis from different sources, which makes it possible that like has not been compared with like. Equally, in the current study, diagnostic uncertainty may account for some or all instances of B-raf or N-ras mutant Spitzoid tumours. Unfortunately, in the absence of universally accepted criteria, opinion about whether an individual case is or is not Spitzoid will always vary and it is unlikely that a consensus opinion will be achieved in the near future. One way of overcoming these ambiguities may be to illustrate all the lesions used in published studies as digital images in on-line addenda.

In the current study there was one case of a mutant Spitz naevus, which is unusual, although mutant cases have been previously described[150, 151](see Table 1-3). Diagnostic uncertainty may also account for this seemingly aberrant result because, as can be seen in Figure 4-4 this was a junctional lesion. Such lesions tend to be small and can cause diagnostic difficulty because of the lack of a dermal component, which deprives the pathologist of many criteria used for assessing Spitzoid lesions such as maturation, mitotic activity and lesional symmetry. Consequently there is considerable subjectivity when arriving at a diagnosis and, in the case of this tumour, junctional naevus or dysplastic junctional naevus would enter the differential diagnosis.

An interesting example of inter-observer variability in the diagnosis of Spitzoid lesions has been published recently. Takata *et al* examined 12 Spitz naevi and found no B-raf or N-ras mutations[152] yet Fullen *et al* found a surprisingly high number in their series of 48[151]. In the discussion of their paper, Takata *et al* suggest that the difference between these studies may have arisen due to case selection and propose that the lesions illustrated by Fullen *et al* as B-raf mutant Spitz naevi are actually dysplastic naevi in which B-raf mutations are commonly found [152]. Fullen and one of his co-authors, Lyndon Su are both dermatopathologists at the University of Michigan and are widely published in the field of dermatopathology. Dr Su kindly provided tissue from three metastatic Spitzoid tumours for the current study, all of which were considered to be Spitzoid by the blinded reviewing pathologists, and all of which were wild type.

The diversity of Spitzoid lesions may also explain some of the differing mutation frequencies observed between studies. Spitz naevi may be junctional, compound or intradermal in location[118] and a number of subtypes have been described including desmoplastic Spitz naevi, pigmented Spitz naevi, Reed naevi and pagetoid Spitz naevi[108, 117], all of which may have atypical variants. If there is variation in the frequency of B-raf and N-ras mutations within these subtypes, for example in a manner similar to superficial spreading melanoma and desmoplastic melanoma, this could confound the results of mutation analyses if not properly controlled for. However, the diagnosis of these subtypes is frequently subjective and therefore it is likely to be very difficult to control for such case variation.

Aside from inequalities of case selection, Van Dijk *et al*[122] suggest that patient age might explain the high frequency of mutations they found in Spitzoid melanomas compared with other studies. Their cases were predominantly derived from adults and they compare this with the study by Gill *et al* in which the tumours were solely from children[122] (see Table 1-3). It is logical to suggest that because classic Spitz naevi are more frequently encountered in young people this may extend to their malignant counterparts, while tumours with a similar histological appearance but found in older patients may be genetically different from such 'true' Spitzoid melanomas (i.e. B-raf or N-ras wild type Spitzoid melanoma). Data from the

current study suggests this is not the case. When the mutation frequency of Spitzoid tumours in patients under twenty years of age was compared with that of patients over twenty years, no significant difference could be demonstrated. Furthermore, in Spitzoid melanomas and all Spitzoid lesions, there was no significant difference between the mean age of patients with and without mutant tumours. In support of this conclusion, Lee *et al* included adult tumours in their series and found a very low mutation frequency in Spitzoid melanomas. Anecdotally, in the current study, a B-raf mutation was found in a Spitzoid lesion from a five-year-old child that exhibited a number of histologically atypical features but nevertheless retained a Spitzoid morphology (see Figure 4-8). This indicates that mutant lesions do occur in young children.

It is possible that the anatomical site and consequent degree of sunlight a Spitzoid lesion receives could influence the likelihood of acquiring a B-raf or N-ras mutation. Anatomical site has been shown to influence mutation frequency in non-Spitzoid melanomas [18, 65, 66, 68], although no such correlation has been found in naevi[207]. Disparities of anatomical site between tumour series in published investigations of Spitzoid tumours may explain some of the considerable variation in reported mutation frequency. In the current study, the combined mutation frequency of the non-Spitzoid melanomas was 61%, which is entirely in accordance with previous studies [30, 44, 45, 56, 208]. This not only validates the experimental method, but also indicates that the melanoma samples are unlikely to have been biased in terms of anatomical distribution. Therefore, because there was no significant difference between the anatomical distribution of the non-Spitzoid melanomas and the Spitzoid tumours (see Table 4-3), the anatomical site of the Spitzoid tumours is likely to be unbiased and will not have affected the results of mutation analysis.

Finally, another factor that may account for differences between the current study and other investigations is variation in experimental methods. Miller *et al*[58] have shown that this may account for discrepancies in the literature concerning the frequency of B-raf mutations in melanoma. In particular they observed that direct fluorescent sequencing was a relatively insensitive method of mutation detection. The majority of investigations into Spitzoid tumours have used direct sequencing for mutation detection[120-122, 151, 152] while the current study used

SSCP gel electrophoresis, from which aberrantly migrating bands were picked and sequenced. This method differs from direct sequencing in that it allows cases to be screened prior to sequencing. It is a sensitive method of mutation detection that can detect mutant DNA even when present as a small percentage of the total DNA extract [63, 189]. Aside from the means of mutation detection, studies are also likely to vary in other aspects of their method such as DNA extraction techniques and reagents, which may account for some variability in mutation frequency.

The sensitivity of SSCP analysis may account for the high frequency of B-raf and N-ras double mutant cases seen in the current study. No double mutant Spitzoid lesions have been reported in previous investigations although Takata *et al* screened their tumours first for mutations of B-raf and if one was found N-ras was not analysed, a strategy that may have been used by other authors. Double-mutant malignant melanomas and common acquired naevi are rare occurrences but have been described by a number of authors [42, 44, 61-64]. Sensi *et al* describe how conventional direct fluorescence sequencing, as used in most other investigations, was unable to detect co-existent mutations, but double mutant cases were found when the more sensitive method of mutant-allele-specific-amplification-PCR was used. In addition, they showed that at a single cell level, mutations of B-raf and N-ras are mutually exclusive and double mutants arise via separate clones, which suggest that concurrent mutations are incompatible with cell survival or induce irreversible growth arrest[62].

5.2.4 What promotes proliferation in Spitzoid tumours?

This investigation, and others, have shown that the frequency of B-raf and N-ras mutations in classic Spitz naevi and other Spitzoid tumour types is very low, yet activating mutations of B-raf and N-ras appear to be important for initiating and sustaining proliferation in a large proportion of melanocytic lesions[11, 30, 33, 59, 61]. This raises the question of what alternative mechanisms might drive proliferation in Spitzoid tumours. Identifying these mechanisms may further characterise this group of lesions as a distinct subtype. It has been shown that the MAP kinase pathway is activated in Spitz naevi through the measurement of ERK1/2 expression[36, 63], the activated downstream target of ras and raf, and the measurement of

cyclin D1 expression[36], the transcription of which is promoted by MAP kinase signalling. This finding underlines the central importance of the MAP kinase pathway in melanocytic neoplasia and also indicates there must be other mechanisms by which the MAP kinase cascade can be activated aside from mutations of B-raf exon 15 or N-ras exons 1 and 2.

In this study tumours were not screened for activating mutations of B-raf exon 11 because it was considered unlikely that these mutations would be found in the tumour series. B-raf exon 11 mutations are a rare event in melanoma and common acquired naevi, with 92% of mutations comprising V600E mutations of exon 15[14]. Mutations at other exons each account for less than 1% of all mutations[59]. Furthermore, investigations that have looked for B-raf exon 11 mutations in Spitz naevi and Spitzoid tumours have failed to find a single mutation[63, 120, 122]. For this investigation therefore, analysis of exon 11 was deemed to be an inefficient use of laboratory time, but it is possible that mutations in this exon could lead to constitutive MAP kinase activation in a very small proportion of cases. Similarly, activating mutations of H-ras are found in a minority of Spitzoid tumours, as shown by the current study, but this does not account for what drives proliferation in most cases. In the current study, only 7% of Spitzoid tumours had mutations of H-ras. These mutations were sought specifically because of their specificity to Spitz naevi and potential utility as a marker of malignant progression in Spitzoid tumours.

c-kit is a transmembrane tyrosine kinase receptor that lies upstream of ras and raf. Mutations and amplifications of c-kit are rare in malignant melanomas that arise on intermittently sun exposed skin, but they have been shown to be frequently present in melanomas on mucosal membranes, acral skin and chronically sun exposed skin[209]. In the majority of such cases there is increased c-kit expression. In a separate study it was found that melanomas with mutated c-kit did not have mutations of B-raf and the authors concluded that a proportion of melanomas can progress via somatic mutations of the c-kit gene independently of other MAP kinase gene mutations[210]. Furthermore, the level of c-kit expression in Spitz naevi has been shown to be similar to that of primary malignant melanoma[211]. It is possible therefore that proliferation in some Spitzoid tumours, including Spitzoid melanoma may be driven by mutant

c-kit in the absence of other MAP kinase gene mutations. If this were the case, effective therapy for metastatic disease may already be available in the form of the c-kit inhibitor imatinib [212].

Amplifications of chromosome 7, the site of the B-raf gene, have been shown to occur in up to 41% of malignant melanomas[213]. Willmore-Payne *et al*[214] found that 9 of 43 (21%) cases of B-raf mutant malignant melanoma had an increased copy number of the B-raf gene due to either polysomy or amplification of the gene itself. The authors also found that c-kit, was amplified in one of three c-kit mutant tumours. It is possible therefore that in Spitzoid tumours the MAP kinase pathway could become activated via amplification of one of its components in the absence of activating mutations, however such alterations are yet to be described and it appears that the majority of amplifications are found in association with mutations.

Curtin *et al* found that B-raf and N-ras wild type melanomas with reduced expression of activated ERK, frequently showed amplifications of the downstream genes CCND1 (cyclin D1) and CDK4. From this they concluded that increased cyclin D1 and CDK4 expression were important for melanoma progression, and that this could be acquired either by MAP kinase activation or independently via amplification of the CCND1 and CDK4 genes themselves. Cyclin D1 and CDK4 therefore could drive proliferation in the absence of MAP kinase signalling in some Spitzoid tumours via amplification, mutation or translocation. While amplifications of genes involved in MAP kinase signalling could, in theory, explain how this pathway is activated in B-raf and N-ras wild type Spitzoid tumours, it must be stated that such alterations have yet to be described in studies examining DNA gains and losses in Spitz naevi[158, 160].

While the question of what drives cell proliferation in most Spitzoid tumours remains unclear, there is evidence to indicate that in some cases an important inhibitor of cell proliferation, the CDK2NA gene, may be lost. Indsto *et al* found there to be a high frequency of loss of heterozygosity at chromosome 9p (the site of the CDKN2A gene) in Spitz naevi[215], while Takata *et al* found CDKN2A copy number loss in three of 14 ambiguous Spitzoid lesions, but

no losses in 8 Spitz naevi[152]. None of the three tumours with reduced CDKN2A copy number examined by Takata *et al* had B-raf or N-ras mutations. One product of the CDKN2A gene is $p16^{ink4a}$, which has been shown to be up regulated in naevi, including Spitz naevi, in the presence of a constitutively activate MAP kinase pathway. This is thought to be an important senescent mechanism in benign melanocytic tumours [34-36, 102]. It is likely therefore that Spitz naevi with loss of $p16^{ink4a}$ would be able to proliferate rapidly and may be a group at particular risk of progressing towards malignancy.

5.2.5 Summary

This study showed that mutations of B-raf exon 15 and N-ras exons 1 and 2 occur at a significantly lower frequency in Spitzoid tumours, including Spitzoid melanoma, when compared with non-Spitzoid melanoma, and at a lower frequency than reports of these mutations in common acquired naevi[49, 55, 57, 61, 63]. This indicates that Spitzoid lesions are a distinct group of melanocytic tumours that frequently arise in the absence of the genetic mutations that are fundamental for driving cell proliferation in most other melanocytic tumour types. Furthermore, the frequency of these mutations in metastatic Spitzoid lesions is lower than that observed in their metastatic non-Spitzoid counterparts, suggesting that non classical Spitzoid tumours are distinct entities rather than a dichotomy of Spitz naevi and 'common' non-Spitzoid melanoma. The study also found that mutations of H-ras are not seen in malignant Spitzoid tumours, which indicates that this may be a marker of benign behaviour in non-classical Spitzoid tumours, and that malignant progression does not occur in this subset.

5.3 Conclusion and further work

The experimental methods in this thesis utilised molecular pathological techniques to identify features associated with tumour progression and malignancy. These studies illustrate that by applying molecular techniques to formalin-fixed, paraffin embedded tissue there is a potentially vast amount of information available to researchers within histopathology archives. Using molecular pathology to unlock this great reserve of information is likely to lead to greater characterisation of the molecular alterations that occur during pre-malignant and malignant tumour progression. Ultimately this knowledge may translate into benefits for patients.

The investigation of Wnt5a expression was prompted by data produced from a cDNA expression array analysis, which indicated that Wnt5a might be important for promoting invasion and motility in melanoma[7]. The current study concluded that Wnt5a expression increases as cutaneous malignant melanoma progresses, and consequently may be an important mediator of progression and a marker of aggressive behaviour. This conclusion was supported by survival analyses. The timing of alterations of Wnt5a, $p16^{INK4a}$ and B-raf in Clark's model suggested that a progression model in malignant melanoma, akin to that described in colorectal cancer,[51] may be valid. Further characterisation of this model is particularly desirable because understanding factors that control the transition from an RGP primary lesion to a VGP tumour and then finally to a metastasis, may identify new therapeutic targets for advanced malignant melanoma, for which treatment is currently ineffective. The study of Spitzoid tumours also had the characterisation of progression in melanocytic tumours central to its aims. This study showed that most benign and malignant tumours with a Spitzoid morphology arise independently of B-raf and N-ras mutations, which are found in a great majority of non-Spitzoid melanomas and common acquired naevi. Furthermore the data suggests that an alternative method of MAP kinase pathway activation, H-ras mutation, might be incompatible with malignant behaviour in Spitzoid tumours. This implies that melanocytic tumours can arise via a number of molecular progression pathways rather than via a common sequence of genetic alterations. This adds to the already enlarging weight of evidence to

suggest that cutaneous malignant melanoma, at a molecular level, is not a single entity but comprises a collection of malignant diseases that arise within melanocytes.

Finally, it is likely that certain aspects of melanocytic dermatopathology will always remain problematic if investigation is limited to standard H+E assessment. By using molecular pathology as a tool to improve our understanding of mediators of progression in melanoma and to increase our appreciation of the molecular diversity of melanocytic tumour types, as this thesis has endeavoured to do, it is hope that some of the barriers to improved diagnosis in this field will be removed. While understanding the processes of neoplasia is in itself important, translating this knowledge to the bedside to assist clinical colleagues, and ultimately patients, must remain an important goal. Consequently, an understanding of the molecular basis of disease is likely to be of increasing importance for histopathologists and clinicians as a whole in the future.

5.4 Further work

For the study of Wnt5a expression, the series of VGP tumours was biased because all the cases were known to be metastatic. It would now be of interest to determine expression of Wnt5a in an 'incident series' of tumours where the outcome is unknown, and correlate this with survival prospectively. Furthermore, it would be of interest to correlate Wnt5a expression with other putative late changes in melanoma progression such as RhoC[216] and MITF[194] expression, to determine whether there are interactions present. Increased expression of RhoC has been shown to promote metastasis in a variety of human cancers including cutaneous melanoma, and its expression has been correlated with poor prognosis [217]. As described in section 5.1.5, the role of MITF in malignant melanoma is complex and appears to be context dependent, however there is evidence to suggest it may be important in the later stages of melanoma where it promotes cell survival[194]. There is speculation that high levels of MITF expression, and subsequent increased Bcl-2 expression, may explain the frequent resistance of metastatic melanoma to conventional chemotherapy [203].

Identifying areas of the genome with increased DNA copy number in Spitzoid melanomas and Spitz naevi may reveal the location of as yet unknown oncogenes that drive proliferation in these tumours. Furthermore, DNA copy number analysis could be used to identify features of genomic instability, which is frequently seen in malignant melanoma [158, 218] and may be used to identify aggressive Spitzoid tumours[144, 164, 219]. DNA copy number analysis, in combination with mutation analysis for H-ras, may have sufficient predictive value to be used in clinical decision-making in the setting of non-classical Spitzoid tumours. For the current study, attempts to develop *in situ* hybridisation probes to detect DNA copy number alterations of B-raf, N-ras and H-ras were unsuccessful (see Appendix 1: Optimisation of techniques), however work is on-going in this area. Due to the difficulties of developing *in situ* probes, the possibility of utilising real-time TaqMan PCR to measure copy number changes is being investigated and it is hoped this method can be applied to the Spitzoid tumour series and correlated with the mutation data reported here.

6 Appendix 1: Optimisation of techniques

6.1 Wnt5a immunohistochemistry

For optimisation, a range of dilutions of the primary antibody were used as recommended in the manufacturer's data sheets to give the best staining intensity as well as the least intensity of non-specific background staining. Concentrations of primary antibody at 1:100 and 1:200 were found to be sub-optimal with weak staining on positive control sections of normal breast. A primary concentration of 1:40 was found to give good staining intensity of breast myoepithelial cells and occasional luminal cells with minimal staining of background collagen. The results of these experiments are shown in figure Figure 6-1.

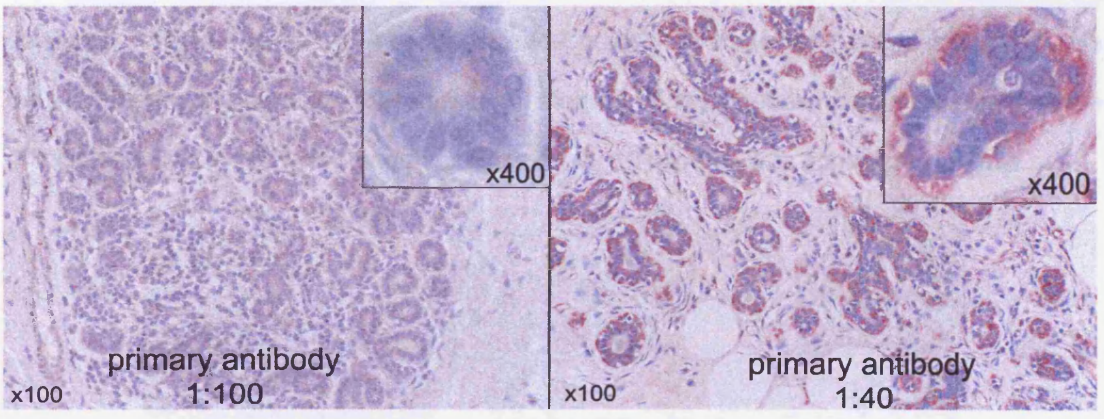


Figure 6-1 Immunohistochemistry for Wnt5a using different concentrations of primary antibody to optimise staining (tissue: normal breast).

A similar approach was used for determining the optimal concentration of secondary antibody. Dilutions of 1:200, 1:400 and 1:600 were used and a concentration of 1:600 was found to give the best result.

To determine if the semi-quantification of Wnt5a expression was reproducible, both pathologists (PDF and GSS) and the student (Qiang Huang) separately scored 12 randomly selected cases and an intra-class correlation coefficient (ICC) was calculated. This indicated a high degree of agreement (for PDF and GSS: ICC = 0.94 with 95%CI 0.82 – 0.98; for PDF and Qiang Huang: ICC=0.94 with 95%CI 0.89 – 0.97)[220, 221]. PDF scored the remaining cases except for the additional cases obtained for survival analysis, which were scored by Qiang

Huang. For the investigation of Wnt5a expression with melanoma progression, only cytoplasmic expression of Wnt5a was assessed. For the survival analysis, nuclear Wnt5a expression was also assessed by Qiang Huang.

6.2 $p16^{INK4a}$ Immunohistochemistry

For optimisation of $p16^{INK4a}$, similar methods were used to those described for Wnt5a. An antigen retrieval stage was required and microwave heating of sections was tried for durations of 10 and 20 minutes, with 20 minutes found to give the best result. A range of primary antibody concentrations was used (1:20, 1:30, 1:50 and 1:100) on positive control sections of common acquired naevus. A primary antibody concentration of 1:30 was found to be optimal as shown in figure Figure 6-2. A secondary antibody concentration of 1:400 was found to give the best staining. Microscopic observation of staining during incubation optimised the duration of incubation in the NBT chromagen. Incubation for 10 minutes was considered optimal.

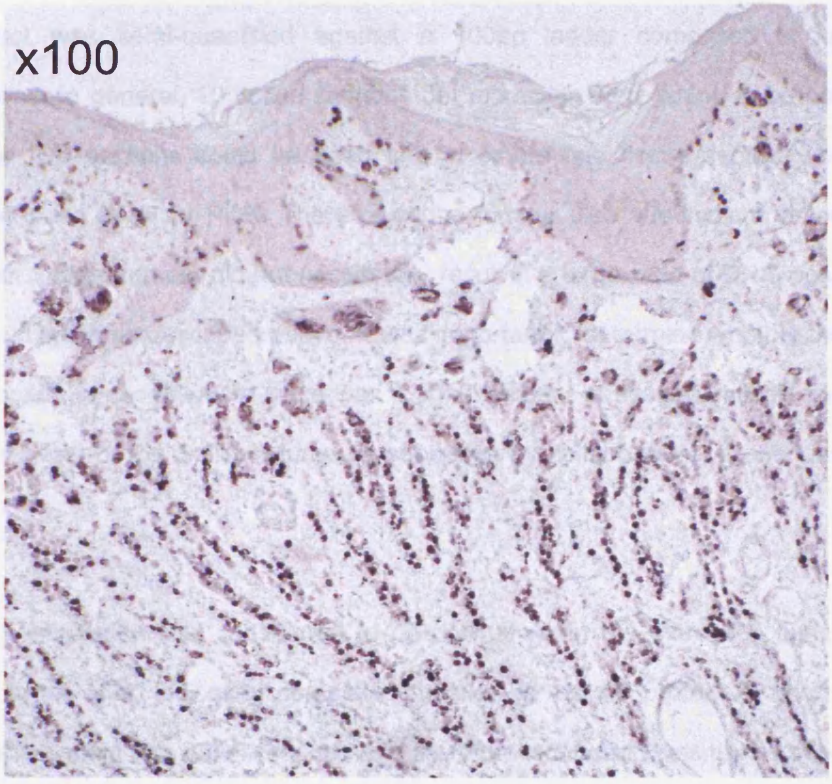


Figure 6-2 Optimised Immunohistochemistry for $p16^{ink4a}$ (tissue: common acquired naevus)

6.3 Mutation analysis

6.3.1 DNA extraction

The results from DNA extraction varied according to the amount of tissue used and duration of incubation of each extraction. Furthermore, there was considerable variation in the quality of the template DNA extracted despite optimisation of these two factors. This variation was considered to arise due to differences in the duration of formalin fixation, which can affect the quality of PCR [222], however the duration of fixation of was not documented in the histopathology records.

The amount of tissue needed for adequate DNA extraction was optimised, prior to the laboratory obtaining a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Delaware, USA), by assessing the product yield from PCR reactions run with 1µl of template DNA from extractions consisting of 1, 5 and 10 tumour sections. The yield of PCR product was semi-quantified against a 100bp ladder composed of known DNA concentrations. In general, 10 tissue sections per extraction were found to be optimal but in some cases five sections could be used and in others one tissue section would provide sufficient template DNA for PCR. The size of the tumour also affected the amount of DNA extracted but a large tumour did not necessarily result in a large yield of DNA, possibly due to variations in fixation as described above. It was important to determine whether the technique could be applied to cases where there was a small amount of tissue available for extraction, because for some of the Spitzoid tumours submitted by collaborators, tissue was extremely limited.

DNA extract incubation was considered to be optimal when the extraction buffer was clear when held to the light, indicating complete digestion of cellular protein by proteinase K. In some cases this point was never reached and therefore incubation was halted after 7 days.

The effect of the number of tissue sections used for DNA extraction on PCR product yield are illustrated in Figure 6-3 and the case-to-case variability in PCR product yield, despite similar

amounts of tissue being used for DNA extraction, is illustrated in Figure 6-4. In Figure 6-4 the amount of DNA within each extraction has subsequently been determined by spectrophotometry and is shown. It can be seen that in some instances the amount of DNA as determined by spectrophotometry does not correlate well with the PCR product yield. This was thought to have arisen due to variations in the quality of DNA, which may have been rendered sub optimal for PCR amplification due to formalin fixation [222].

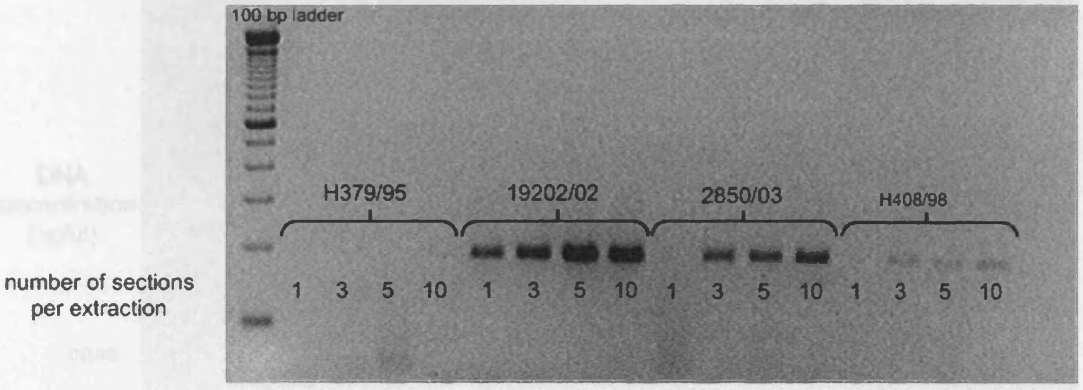


Figure 6-3 The effect of the number of tissue sections used for DNA extraction on PCR product yield. Four cases of formalin fixed paraffin embedded melanomas are shown with extractions consisting of 1,3,5 and 10 sections. B-raf Fsh1+Rsh primers.

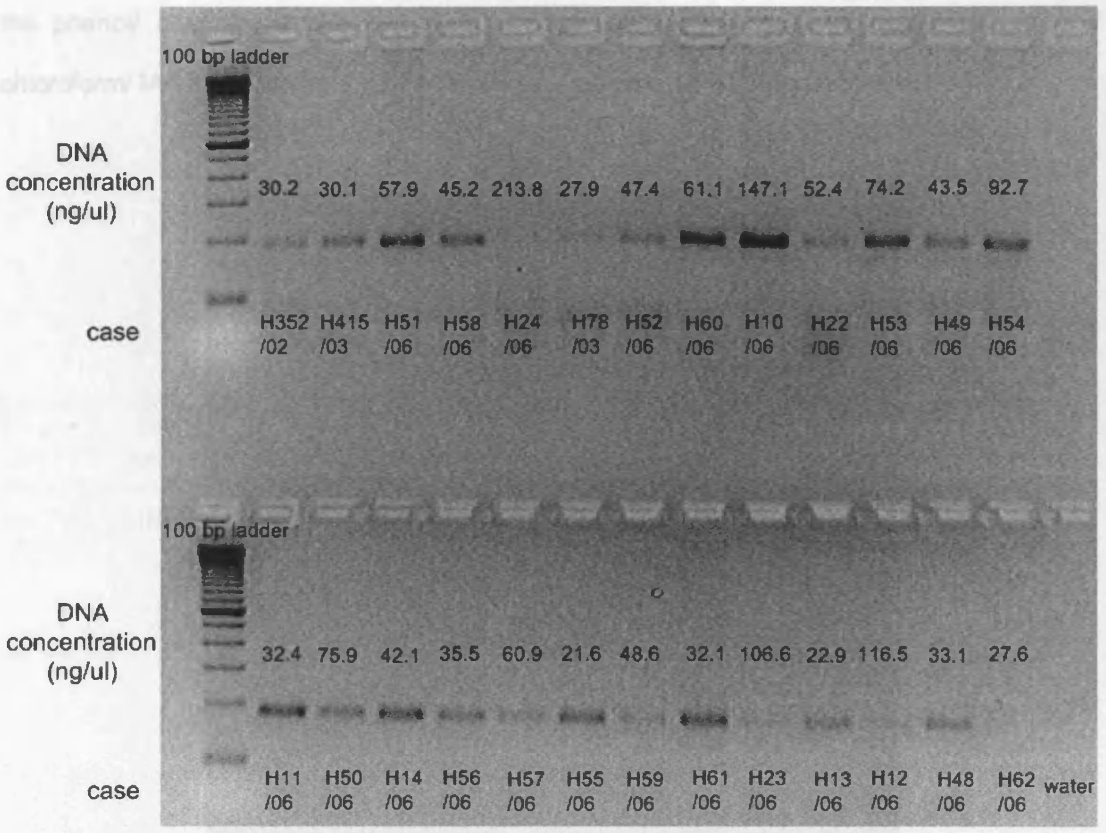


Figure 6-4 Variability in PCR product yield between cases despite similar amounts of tissue being used for extraction. It can also be seen that the quantity of DNA extracted as determined by spectrophotometry does not always correlate well with the PCR product yield. Twenty six cases are shown, all extractions were performed on 10 sections. One µl of template DNA was used per PCR reaction with B-raf Fsh1+Rsh primers.

For all oligonucleotide primer pairings except B-raf exon 15, the DNA was extracted by phenol/ chloroform/ IAA with ethanol precipitation, to remove all protein from the extract. This had little effect on the quality of the PCR amplification, but was found to give considerably clearer results on SSCP analysis, with less background staining and consequently aberrantly migrating bands were easier to identify. This extraction method appeared to have a detrimental effect on the quality of PCR of B-raf exon 15 because in many cases the PCR reaction would subsequently fail whereas a PCR product could be obtained prior to Phenol/ chloroform/ IAA extraction. Furthermore, the Phenol/ chloroform/ IAA extracted DNA would amplify well at other loci, thus indicating that DNA had not been lost or significantly damaged during the process of extraction. An example of this is shown in Figure 6-5. The reason for this anomaly was unclear but it may have resulted from the loss of proteins that are important for the structural integrity of the B-raf gene specifically at exon 15, such as histones, during

the phenol/ chloroform/ IAA extraction. Consequently, DNA was extracted with phenol/ chloroform/ IAA following the completion of all B-raf exon 15 PCR reactions.

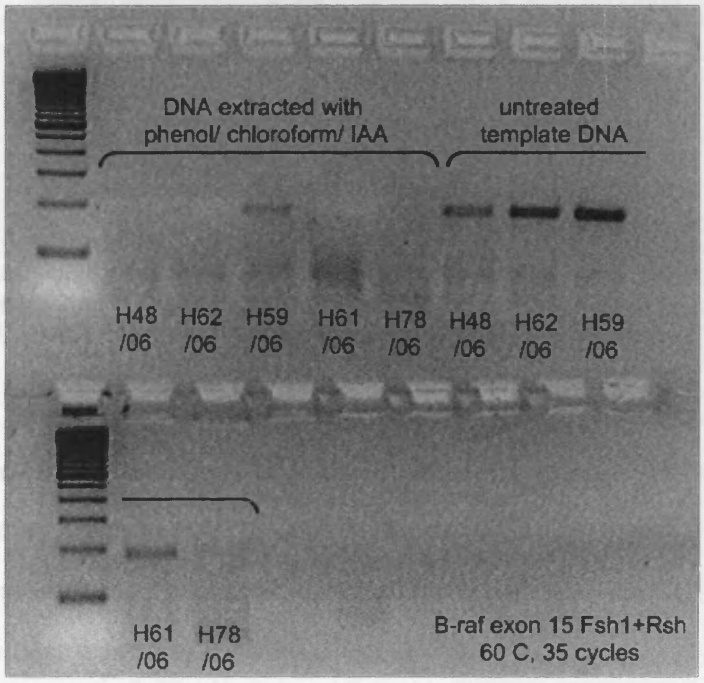


Figure 6-5Comparison of the quality of B-raf exon 15 PCR, before and after phenol/ chloroform/ IAA extraction.

6.3.2 PCR amplification of B-raf exon 15

A variety of forward and reverse oligonucleotide primers for B-raf exon 15 were available for PCR. Initially a combination of primers F and R were used in a small number of analyses. This combination gave good results when run on an SSCP gel (see samples H641 and H642 in Figure 3-9), but was frequently inconsistent when used with DNA extracted from formalin fixed tissue, with only a small percentage of cases giving a PCR product. An example of this inconsistency is shown in Figure 6-6.

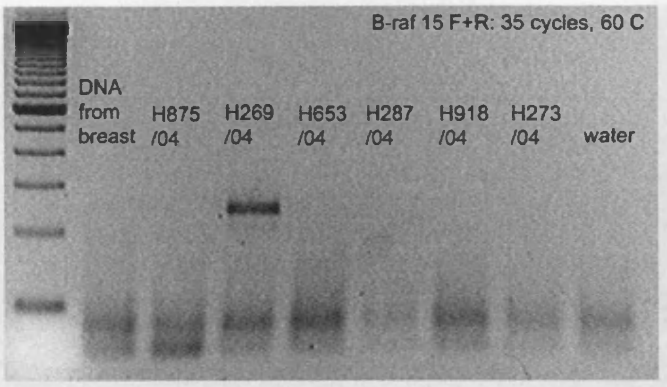


Figure 6-6 PCR with the primer combination B-raf 15 F+R. Infrequently cases would give a good PCR product yield but in many cases the PCR reaction would not work. The results did not improve after altering the annealing temperature or number of cycles used in the PCR reaction protocol. Five of the six cases shown subsequently amplified with the Fsh1+Rsh primers.

A variety of alterations to the primer annealing temperature and number of cycles used for the PCR protocol were tried in order to optimise the primers, but the results did not improve. Alternative primers were sought, and in order to determine which combination of the available primers gave the best results, all possible combinations of primers were used in a series of PCR reactions. Template DNA extracted from human blood and from paraffin sections of formalin fixed tissue were used for comparison. The reverse primer Rsh appeared to give a better PCR result than the other reverse primers while Fsh1 appeared to be the better forward primer, particularly with the DNA extracted from formalin fixed tissue. The results of this experiment are shown in figure Figure 6-7.

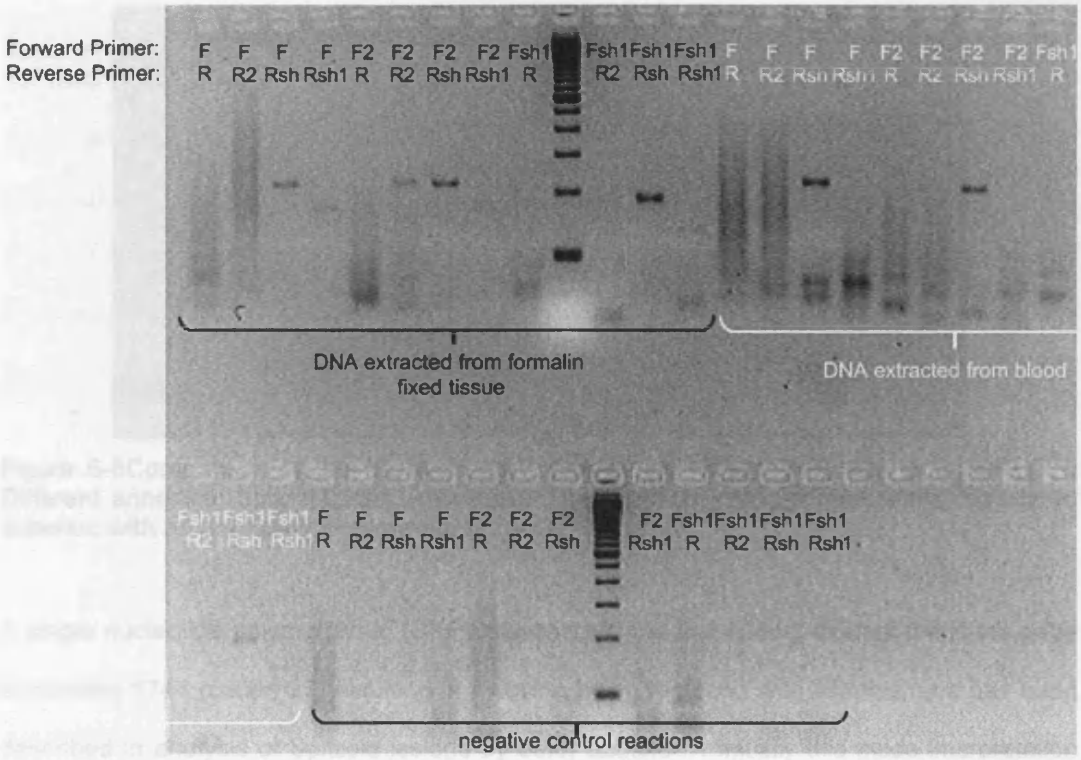


Figure 6-7 B-raf exon 15 PCR products from reactions run with template DNA extracted from formalin fixed paraffin embedded tissue and blood. All possible combinations of primers were used. The combination of Fsh1 & Rsh were considered optimal, particularly with the template DNA extracted from formalin fixed, paraffin embedded tissue.

6.3.3 PCR amplification and SSCP analysis of H-ras exons 1 and 2 and N-ras exons 1 and 2

No previously validated oligonucleotide primers were available for H-ras exon 1 therefore two sets of primers were designed (Hras1 F+R and Hras1shF+shR) using Primer 3 software. Both primer sets appeared to be suitable for amplification of H-ras exon 1 *in silico* and so they were compared via PCR, at different annealing temperatures. The results of this are shown in Figure 6-8. The Hras1shF+shR primers were considered superior, with an annealing temperature of 60°C.

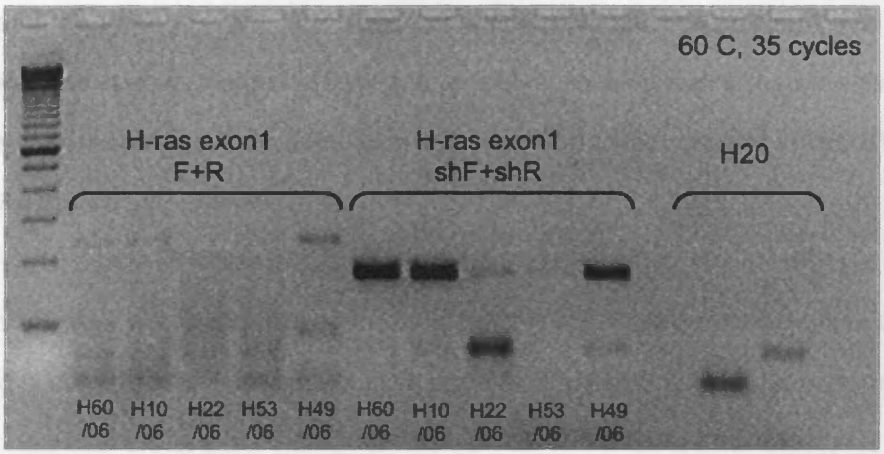


Figure 6-8Comparison of the oligonucleotide primer sets Hras1 F+R and Hras1shF+shR. Different annealing temperatures were used. The Hras1shF+shR primers were considered superior, with an annealing temperature of 60°C.

A single nucleotide polymorphism (SNP) is present in the first coding exon of the H-ras gene at position 1744 (codon 27) resulting in cytosine being replaced with thymine, and has been described in analysis of Spitzoid lesions by other authors[120]. Initially this made interpretation of SSCP gels of H-ras 1 PCR products difficult. However, once the appearance of wild type bands for 1744cytosine homozygotes, 1744thymine homozygotes and heterozygotes were appreciated, genuinely aberrantly migrating bands were identified without difficulty. An example of this is shown in Figure 6-9.

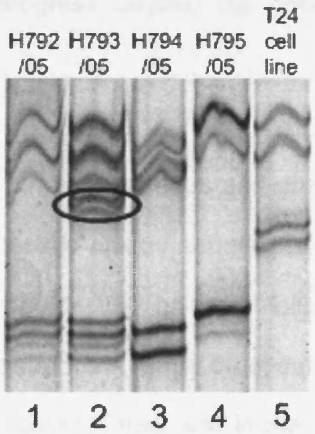


Figure 6-9SSCP gel illustrating the polymorphism at position 1744 of the H-ras 1 gene. The cases in lanes 3 and 4 were homozygous wild type and show the differing appearances of the two polymorphisms. The case in lane 1 was heterozygous wild type. The case in lane 3 was a G13R mutant in a heterozygous patient, the mutant bands are encircled.

For H-ras exon 2, two forward oligonucleotide primers (F and F2) were available for use with a reverse primer (R). The two primers were compared in a similar manner to the H-ras1

primers, and the pairing H-ras2 F2+R was considered optimal. The amplicon size from this combination was relatively large, 261 bp, whereas all other amplicon sizes were around 200 bp or less. Consequently a long duration of electrophoresis was required for SSCP analysis (20 hours at 350V).

N-ras primers were already available for exons 1 and 2 within the department, and these had been used successfully for PCR amplification and SSCP analysis in previous experiments.

6.4 Development of an H-ras probe for colorimetric in situ hybridisation

Measurement of DNA copy number has shown considerable differences between melanoma and benign naevi[158, 160, 218]. A specific alteration in Spitz naevi, amplification of chromosome 11p the locus of the H-ras gene, has also been described. This alteration allows the differentiation of Spitz naevi from CAN[140, 159]. Following on from this data, for the investigation of Spitzoid tumours, it was intended that probes would be developed for colorimetric *in situ* hybridisation (CISH) of B-raf, N-ras and H-ras and applied to the tumour series. Unfortunately this part of the study did not progress beyond the optimisation phase, but attempts at optimisation are described below.

DNA probes were developed by the simultaneous use of two inter-*Alu*-PCR oligonucleotide primers for the amplification of inter-*Alu* unique sequences. *Alu* repeats are a family of short interspersed elements that are roughly 280 bp long, followed by a poly-A tail of variable length[223]. They comprise the most abundant family of repeats in the human genome. Although *Alu*'s have no known biological function they are known to contribute to human genetic disease and can be used as markers to determine the evolutionary distance between and within species. The inter-*Alu* primers are designed to prime to two highly conserved regions of the *Alu* repeat family between bp 13-31 and 240-258. By using inter-*Alu* primers for the two conserved regions in the same reaction, the regions between the *Alu* segments, independent of their orientation along a segment of genomic DNA, are amplified.

Template DNA for the *Alu* PCR was derived from bacterial artificial chromosomes (BACs) that contained the regions of interest. BACs are extra chromosomal genetic elements that confer antibiotic resistance and other traits to their host. BACs can accept DNA inserts of up to 300kb. The BAC is inserted into a bacterial cell by electroporation and subsequent culture amplifies the BAC through cell division[190]. The relevant BACs were identified via the NCBI website[183] and obtained from the Wellcome Trust Sanger Institute (Hinxton, Cambridge, UK). These are shown in Table 6-1.

Region and gene of Interest	**BAC clone**
Chromosome 1p13.1 N-ras	RP5-1000E10
Chromosome 7q34 B-raf	RP4-726N20 RP4-813F11 RP5-839B19 RP11-304K20 RP11-416H18 RP11-543P6
Chromosome 11p15.5 H-ras	RP11-412M16 RP11-496I9 RP13-46H24

Table 6-1 Chromosomal regions and genes of interest within them, together with the relevant BAC clones that provided template DNA for the production of CISH probes using inter-*Alu* primers.

Single colonies from each BAC were cultured, grown and then harvested, with the DNA isolated using a QIAGEN plasmid midi kit. The isolated DNA was quantified and the inter-*Alu* PCR was performed. The PCR products were run on an agarose gel, which revealed amplicons of many different sizes that corresponded to the variably sized inter-*Alu* regions, as shown in Figure 6-10.

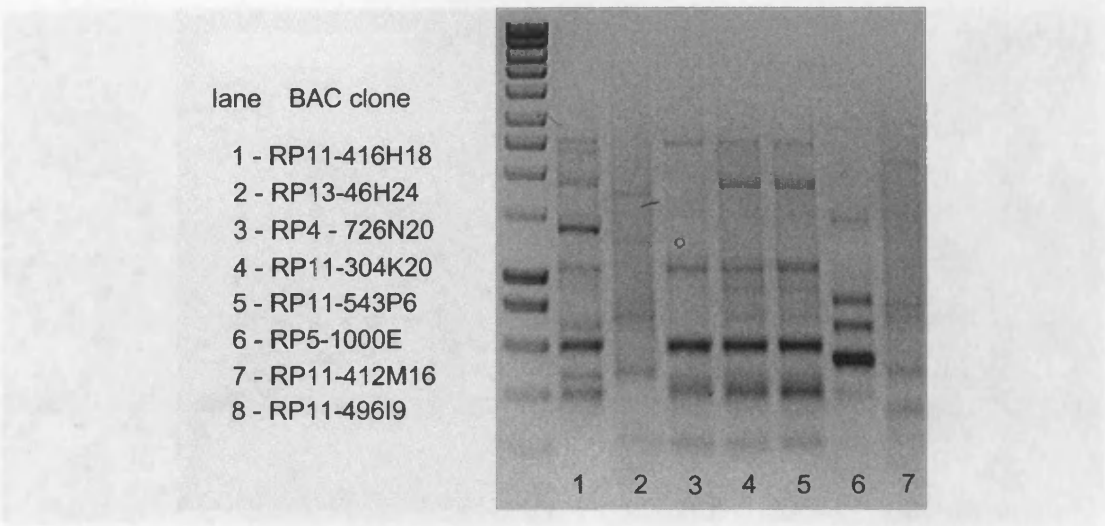


Figure 6-10 Inter-*Alu* PCR products run on a 1% agarose gel. Note that the PCR products in lanes 1,3,4 and 5, and 2 and7 appear similar because the template DNA was from BACs that had inserts from overlapping regions of the genome.

Following inter-*Alu* PCR, the products from different BAC clones that contained the same locus (i.e. 1p13.1, 7q34 and 11p15.5) were combined to ensure a total of 1µg of inter-*Alu* PCR product for the three loci of interest. This DNA was subsequently labelled with Digoxigenin to give a probe suitable for CISH. CISH was performed according to a protocol that had worked to good effect in the laboratory in previous investigations. Briefly, following de-waxing and rehydration, slides were incubated at 37°C in proteinase K, of varying concentrations for one hour, washed in ultrapure water, and placed in 0.4% paraformaldehyde at 4°C for 20 minutes. Next the tissue sections were incubated in pre-hybridisation solution at 42°C for 1 hour, followed by a hybridisation step for 10 minutes at 100°C and over night incubation at 42°C. Following further washes, detection of hybridised probe was via mouse anti-digoxigenin and secondarily biotinylated rabbit anti-mouse antibodies followed by streptavidin-biotin complex and visualisation with DAB/nickel.

The result of an initial experiment using the probe for 11p15.5, the H-ras locus, was that there was diffuse signal from the entire nucleus in all cells, as shown in Figure 6-11a. In order to validate the quality of the materials used, a probe for the centromere of the Y chromosome was used alongside the new probe. This worked satisfactorily as shown in Figure 6-11b.

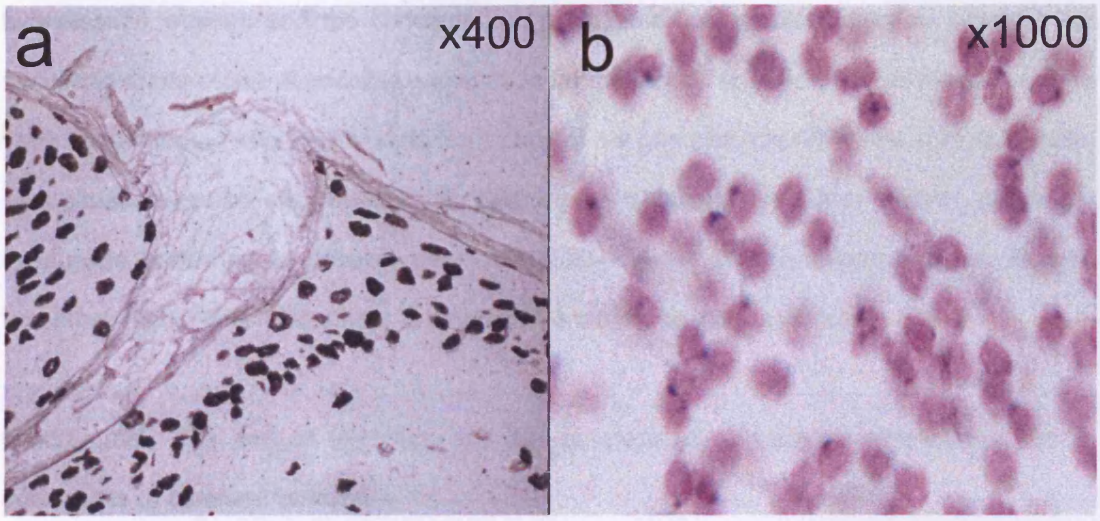


Figure 6-11 Optimisation of CISH: a) probe designed to hybridise to chromosome 11p15, the site of the H-ras gene. b) Centromeric probe for the Y chromosome. (tissue: normal skin)

From this experiment it became apparent that the probe lacked specificity and that it was necessary to block DNA repeat sequences with human Cot-1 DNA to prevent non-specific hybridisation. In subsequent experiments, human Cot-1 DNA excesses of 1000, 1500 and 2000 fold relative to the concentration of probe were used. The results of these experiments are shown in Figure 6-12.

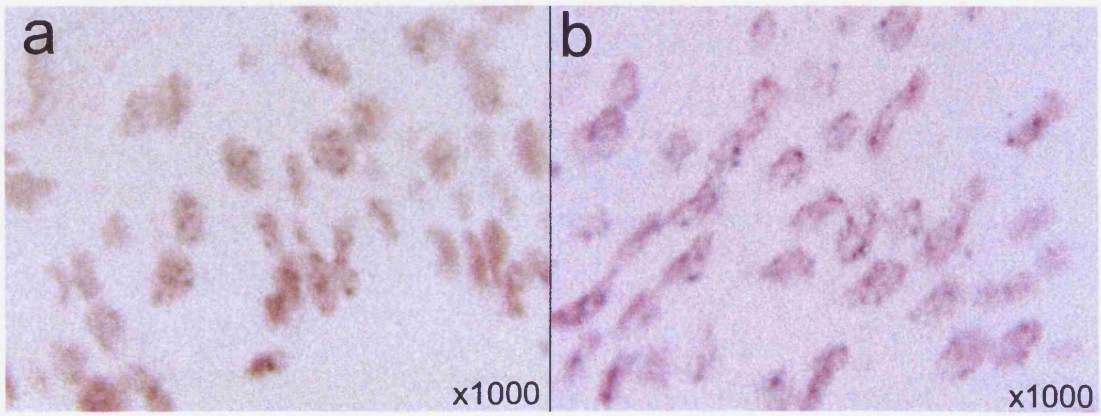


Figure 6-12 Optimisation of CISH: blocking of repeat sequences in the human genome with human cot-1 DNA at 1500 fold (a) and 2000 fold (b) excess relative to the probe concentration. As shown in both a and b hybridisation remained non-Specific. (tissue: normal skin)

As can be seen in Figure 6-12, blocking repeat sequences with human cot-1 DNA did reduce the amount of background nuclear signal but several focal of staining remained visible in most nuclei where only two would be expected in normal tissue. Altering the stringency of post

hybridisation washes and the concentration of enzyme for digestion failed to increase the specificity of the probe. A possible explanation for the lack of specificity is that the BAC clones were contaminated with DNA inserts from parts of the genome other than the area of interest. This situation can be encountered with BAC clones obtained from other centres, with some institutions preferring to develop their own BAC libraries (verbal communication, Maryou Lambros, Higher Scientific officer, Institute of Cancer Research, London)

Unfortunately this part of the study did not progress any further, predominantly due to constraints of time and resources.

7 Appendix 2: Complete clinicopathological and mutation analysis data

Clinicopathological data and complete mutation analysis data from characterisation of B-raf, N-ras and H-ras mutations in Spitzoid tumours. The tumours are grouped according to the review diagnosis. *This is a summary of the initial diagnosis which, in many cases would be descriptive or subsequently revised upon referral. +Total number of atypical features, out of 9, as assessed by two reviewers. m, male; f, female; df, disease free; wt, wild type; m, mutant; nd, no data.

Case	Sex	Age	Site	(Breslow) Depth	*Initial diagnosis	+Atypical features total	Clinical outcome	Follow up duration	B-raf 15	N-ras 1	N-ras 2	H-ras 1	H-ras 2
Spitz naevus													
H211/06	m	29	knee	1.1	atypical Spitz naevus	0	df	0.8	wt	wt	wt	wt	nd
H237/06	f	45	left arm	3.8	malignant melanoma	0	df	17.7	nd	wt	wt	wt	nd
H799/05	f	37	left thigh	1.5	Spitz naevus	0	df	13.2	wt	wt	wt	wt	wt
H464/05	m	57	left mastoid	2.7	Spitz naevus	0	df	4.1	wt	wt	wt	wt	wt
H58/06	f	22	left foot	3.5	Spitz naevus	1	df	17.8	wt	wt	wt	wt	wt
H793/05	f	19	right temple	2.4	Spitz naevus	0	df	13.8	wt	wt	wt	m G13R	wt
H460/05	m	32	thigh	in situ	Spitz naevus	0	df	11.7	m V600E	m G12C	wt	wt	wt
H61/06	m	24	not stated	1	Spitz naevus	1	df	18.4	wt	wt	wt	wt	wt
H444/05	f	28	shin	1.4	Spitz naevus	0	df	14.3	wt	wt	wt	wt	wt
H60/06	m	1	left leg	4	Spitz naevus	0	df	3.5	wt	wt	wt	wt	wt
H78/03	m	24	dorsum right foot	in situ	Spitz naevus	0	df	6.4	wt	wt	wt	wt	wt
H62/06	m	22	not stated	0.5	Spitz naevus	0	df	12.3	wt	wt	wt	wt	wt
H11/06	m	22	right thigh	0.7	features of	0	df	5.8	wt	wt	wt	wt	nd

Case	Sex	Age	Site	(Breslow) Depth	*Initial diagnosis	+Atypical features total	Clinical outcome	Follow up duration	B-raf 15	N-ras 1	N-ras 2	H-ras 1	H-ras 2
					Spitz naevus but most likely melanoma								
H57/06	f	35	left leg	1	Spitz naevus	1	df	9.8	wt	wt	wt	wt	wt
H196/06	f	41	left upper posterior thigh	0.4	STUMP	0	df	0.6	wt	wt	wt	wt	wt
H80/06	f	46	back	1.4	Spitz naevus	1	df	2.0	nd	wt	wt	wt	nd
atypical Spitz naevus													
H216/06	m	15	upper thigh	6.1	Spitzoid melanoma	3	df	4.3	wt	wt	wt	wt	wt
H140/06	m	10	posterior scalp	3.2	atypical Spitz tumour	2	sentinel lymph node metastasis	5.2	wt	wt	wt	wt	wt
H447/05	m	48	right upper arm	1.7	Spitz naevus	0	df	7.8	wt	wt	wt	wt	wt
H59/06	f	36	left calf	1.5	Spitz naevus	2	df	8.5	wt	wt	wt	wt	m Q61R
H236/06	m	43	left upper arm	1.3	malignant melanoma	0	df	17.3	nd	wt	wt	wt	nd
H451/05	m	59	not stated	0.6	Spitzoid lesion	1	df	4.5	wt (synonymous mutation)	wt	wt	wt	wt
H13/06	f	43	right thigh	0.7	features of Spitz naevus but most likely melanoma	1	df	5.4	m	m G13S	wt	wt	nd
H22/06	f	36	right ear	1.7	malignant melanoma	3	df	17.1	wt	wt	wt	wt	wt
H459/05	f	29	right shin	1.2	atypical Spitz	2	df	4.1	wt	wt	wt	wt	wt

Case	Sex	Age	Site	(Breslow) Depth	*Initial diagnosis	+Atypical features total	Clinical outcome	Follow up duration	B-raf 15	N-ras 1	N-ras 2	H-ras 1	H-ras 2
					naevus								
STUMP													
H133/06	m	22	left calf	2.2	probably Spitz naevus but treat as melanoma	0	df	5.0	wt	wt	wt	wt	nd
H795/05	f	19	right shoulder	3	atypical Spitz naevus	2	df	7.3	wt	wt	wt	wt	Q61L
H455/05	f	40	left elbow	2.1	Spitz naevus	3	df	3.0	wt	wt	wt	wt	wt
H51/06	f	36	back	4	malignant melanoma	2	axillary node metastases	3.8	wt	wt	m Q61K	wt	wt
H791/05	f	18	right outer thigh	1.3	Spitz naevus	2	df	11.6	wt	wt	wt	wt	nd
H137/06	m	25	n/a	2.3	atypical Spitz naevus	4	df	5.3	m	wt	wt	wt	nd
H202/06	f	33	left calf	10	Spitzoid melanoma	3	df	0.4	wt	wt	wt	wt	wt
H449/05	f	41	leg	2.8	Spitz naevus of uncertain behaviour	2	df	6.9	m V600E	m G12C	wt	wt	wt
H245/06	m	51	back	10	malignant melanoma	3	Left groin lymph node metastasis	7.9	nd	nd	nd	nd	nd
Spitzoid melanoma													
H132/06	f	34	left thigh	1.4	Spitzoid melanocytic lesion	2	df	2.0	wt	wt	wt	wt	wt
H212/06	f	22	n/a	1.2	Spitzoid melanoma	4	df	1.3	wt	wt	wt	wt	wt
H209/06	f	67	upper arm	1.6	Spitzoid melanoma	5	df	6.6	wt	wt	wt	wt	wt

Case	Sex	Age	Site	(Breslow) Depth	*Initial diagnosis	+Atypical features total	Clinical outcome	Follow up duration	B-raf 15	N-ras 1	N-ras 2	H-ras 1	H-ras 2
H213/06	m	60	right pinna	2.8	Spitzoid melanoma	3	df	6.5	wt	wt	wt	wt	wt
H215/06	m	16	thigh	2.5	Spitzoid melanoma	3	df	11.2	wt	wt	wt	wt	wt
H214/06	m	12	upper arm	3.3	Spitzoid melanoma	4	df	13.0	wt	wt	wt	wt	wt
H258/06	f	3	face	2.6	malignant melanoma	3	left neck lymph node metastasis	10.8	wt	wt	wt	wt	nd
H197/06	m	6	back	from digital image	Spitz naevus – melanoma upon referral	3	df	17.6	wt	wt	wt	wt	nd
H136/06	f	5	left buttock	8.5	severely atypical melanocytic lesion with Spitzoid component	5	df	5.3	m V600E	wt	wt	wt	wt
H796/05	f	18	left upper arm	0.8	worrying Spitzoid lesion, cannot exclude melanoma	3	df	4.1	wt	wt	wt	wt	wt
H142/06	m	12	right ankle	4.7	atypical Spitz tumour	4	sentinel lymph node metastasis	6.2	wt	wt	wt	wt	wt
H138/06	f	40	left cheek	3	atypical Spitz tumour	3	sentinel node metastasis	3.2	wt	wt	wt	wt	wt
H798/05	f	40	left thigh	0.3	malignant melanoma	5	df	6.6	wt	wt	wt	wt	wt
H794/05	f	21	right thigh	0.5	malignant melanoma	3	df	5.5	m V600E	wt	wt	wt	wt

Case	Sex	Age	Site	(Breslow) Depth	*Initial diagnosis	+Atypical features total	Clinical outcome	Follow up duration	B-raf 15	N-ras 1	N-ras 2	H-ras 1	H-ras 2
H462/05	m	32	right ear	2.1	Spitz naevus	4	df	4.8	wt	wt	wt	wt	wt
H242/06	f	76	left ankle	3.6	malignant melanoma	5	left foot skin metastasis	19.7	m V600K	wt	wt	wt	nd
H239/06	m	29	back	3.6	malignant melanoma	6	axillary lymph node metastasis	17.2	m V600E	wt	wt	wt	wt
H243/06	f	45	right calf	0.7	malignant melanoma	5	df	12.7	nd	wt	wt	wt	nd
H241/06	m	59	back	2.7	Spitzoid melanoma	3	df	14.6	wt	wt	wt	wt	wt
H12/06	m	14	neck	14.2	metastasising Spitz naevus upon referral	5	lymph node and lung metastases	26.2	wt	wt	wt	wt	wt
H240/06	f	59	not stated	2.3	malignant melanoma	5	df	17.5	wt	wt	wt	wt	nd
H238/06	m	64	face	1.9	malignant melanoma	4	df	11.1	wt	wt	wt	wt	wt
H458/05	m	51	right elbow	2.4	atypical Spitz naevus	4	df	2.5	wt	wt	wt	wt	wt
H415/03	f	2	right foot	1.6	atypical Spitz naevus	4	df	3.4	wt	wt	wt	wt	wt
H244/06	f	43	left wrist	5.5	Spitzoid melanoma	3	df	0.5	wt	wt	wt	wt	wt
H81/06	m	14	back	4.9	Spitz naevus	2	df	12.0	wt	m G12S	wt	wt	wt
H234/06	m		right calf	4	malignant melanoma	5	df	21.6	nd	wt	wt	wt	nd
non-Spitzoid melanoma													
H208/06	f	46	thigh	1.4	atypical Spitz tumour	N/A	df	10.9	wt	wt	wt	wt	nd
H135/06	m	1	right calf	1.6	Spitzoid melanoma	N/A	df	5.4	wt	wt	wt	wt	nd

Case	Sex	Age	Site	(Breslow) Depth	*Initial diagnosis	+Atypical features total	Clinical outcome	Follow up duration	B-raf 15	N-ras 1	N-ras 2	H-ras 1	H-ras 2
H54/06	m	16	right pinna	incomplete excision	malignant melanoma	N/A	df	6.2	m	wt	wt	wt	wt
H454/05	f	41	leg	2	malignant melanoma	N/A	df	7.2	wt	wt	m Q61R	wt	wt
H56/06	m	25	cheek	1.1	malignant melanoma	N/A	df	1.8	m	wt	wt	wt	wt
H792/05	f	18	right shoulder	0.7	malignant melanoma	N/A	df	8.8	m (V600E)	wt	wt	wt	wt
H461/05	m	51	elbow	in situ	malignant melanoma	N/A	df	4.7	wt	m G15E	wt	wt	wt
H52/06	m	15	leg	0.9	malignant melanoma	N/A	df	10.6	m	wt	wt	wt	wt
H55/06	m	21	leg	0.7	malignant melanoma	N/A	df	11.3	wt	wt	wt	wt	wt
H49/06	f	22	not stated	1.3	malignant melanoma	N/A	df	8.4	m	wt	wt	wt	wt
H445/05	f	29	shin	in situ	malignant melanoma	N/A	df	6.4	wt (synonymous mutation)	wt	wt	wt	wt
H53/06	m	24	back	3	malignant melanoma	N/A	left axillary lymph node metastases	11.3	m V600E	wt	wt	wt	wt
H463/05	m	59	back	6.1	malignant melanoma	N/A	df	8.2	m V600E	wt	wt	wt	wt
H14/06	m	25	right forearm	1.2	atypical Spitz naevus	N/A	axillary lymph node metastases	3.8	m	wt	wt	wt	wt
H246/06	f	36	thigh	1.3	malignant melanoma	N/A	df	2.7	wt	wt	wt	wt	wt
H199/06	f	47	knee	1.1	malignant	N/A	df	0.5	m	wt	wt	wt	wt

Case	Sex	Age	Site	(Breslow) Depth	*Initial diagnosis	+Atypical features total	Clinical outcome	Follow up duration	B-raf 15	N-ras 1	N-ras 2	H-ras 1	H-ras 2
					melanoma				V600E				
H198/06	f	61	left thigh	0.4	malignant melanoma	N/A	df	0.5	wt	wt	wt	wt	nd
H50/06	f	43	left thigh	6.9	malignant melanoma	N/A	left groin lymph node metastases	9.6	m	wt	wt	wt	wt
H247/06	f	100	eye brow	7	malignant melanoma	N/A	df	12.4	wt	wt	wt	wt	wt
H200/06	f	29	right thigh	0.6	malignant melanoma	N/A	df	0.2	wt	nd	wt	wt	wt
H249/06	f	91	dorsum right hand	5.5	malignant melanoma	N/A	df	12.1	wt	wt	wt	wt	wt
H10/06	m	25	right cheek	3.5	favour Spitz naevus but treat as melanoma	N/A	df	13.0	wt	wt	wt	wt	wt
H48/06	f	34	back	0.4	malignant melanoma	N/A	df	6.0	m	wt	wt	wt	wt
H248/06	m	71	left ear	3.1	malignant melanoma	N/A	df	12.9	wt	nd	m Q61R	wt	wt
H235/06	f	2	left fore arm	8	Spitzoid melanoma	N/A	df	1.7	nd	wt	wt	wt	wt

8 Appendix 3: Publications arising from this work

The following abstract was for an oral presentation to the Pathological Society of Great Britain and Ireland, Cambridge, January 4th - 6th 2006

Comparison Of Wnt5a Expression With Alterations Of p16 And B-raf In Cutaneous Melanoma Progression

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There is no effective therapy for metastatic cutaneous melanoma (CM); hence prognosis is poor. Molecular alterations during CM progression may yield therapeutic targets, but remain poorly understood. Preliminary studies suggest that expression of Wnt5a is important in CM progression. Our aim was to characterise Wnt5a expression in CM and compare this with changes of p16 and B-raf, in the context of Clark's progression model.

Wnt5a and p16 expression was determined in 173 melanocytic tumour samples via immunohistochemistry. Radial growth phase (RGP), vertical growth phase (VGP) and metastatic CM samples were examined for B-raf mutation.

Trend analysis showed increasing Wnt5a expression with progression ($p=0.013$), while p16 expression was reduced ($p<0.001$). B-raf mutation was frequently a pre-metastatic event that usually correlated with the matched metastasis. Alterations of Wnt5a, p16 and B-raf appear to be non-random events and may be incorporated in a progression model of CM.

There were significant changes in expression between different phases of the progression model. Differential expression between RGP tumours and RGP-like areas surrounding VGP tumours (RGPA) was shown. Researchers regard RGP and RGPA as equivalent in CM progression models, however, our results suggest they are different and that RGPA progression data should be interpreted with caution.

The following abstract was for a poster presentation at Perspectives in Melanoma X and the 3rd International Melanoma Congress 14th – 16th September 2006, Noordwijk, Netherlands.

The Radial Growth Phase Alters During Cutaneous Melanoma Progression

Clark's model of cutaneous melanoma (CM) recognises three clinicopathological progression phases, the radial growth phase (RGP) where the tumour has minimal metastatic potential, the vertical growth phase (VGP) where the risk of metastasis is significant, and metastatic CM. Therefore, determining whether a primary lesion is in the RGP or VGP is of prognostic significance. Occasionally included in the model is the common acquired naevus (CAN), which appears to be a benign precursor of CM in some cases. Many VGP tumours have residual RGP tumour from which the VGP apparently arose (the RGPA). It is unknown whether the RGP and RGPA are equivalent however, some similarities between the RGPA and VGP have been demonstrated (Omholt *et al,* 2002; Demunter *et al*, 2001).

The purpose of this study was to better characterise the molecular alterations that underlie Clark's progression model, particularly the relationship between the RGP and RGPA. This was achieved by using three events known to be important during CM progression: B-raf mutation (Davies *et al,* 2002), reduced $p16^{ink4a}$ expression (Hussussian *et al,* 1994) and increased Wnt5a expression (Bittner *et al,* 2000). We examined 173 tumour samples comprising CAN, RGPCM, and VGPCM, 38(64%) of which had an RGPA component, and corresponding metastases. Wnt5a and $p16^{ink4a}$ expression was determined via immunohistochemistry. B-raf exon 15 mutations were determined via SSCP analysis.

Assessment of these three markers revealed statistically significant differences during CM progression. Trend analysis showed increasing Wnt5a expression and decreasing $p16^{ink4a}$ expression with progression ($p=0.013$ and $p=0.006$ respectively), changes that were highly significant, while B-raf mutation was largely a pre-metastatic event. These findings mirror previous reports, supporting the validity of these methods for analysis of progression. Most

notably, these 3 markers showed that RGP and RGPA differed significantly, with a reduction in $p16^{ink4a}$ and increase in Wnt 5a expression ($p<0.001$ and $p=0.9$, respectively).

In conclusion, changes in Wnt5a, $p16^{ink4a}$ and B-raf appear to be non-random events that may be incorporated in a step-wise progression model of CM. Most importantly, the results show that RGP and RGPA are biologically different, which suggests that RGPA may represent a distinct entity from RGP and VGP. This implies that a re-appraisal of RGPA may be necessary in terms of its biological significance, for example whether residual RGPA following tumour excision is likely to behave more aggressively than an RGP tumour. Furthermore the utility of RGPA in progression studies as a surrogate for pre-existing RGP must be questioned and results from previous studies should be interpreted with some caution.

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Understanding spitzoid tumours: new insights from molecular pathology

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malignant melanoma, mitogen-activated protein kinase pathway, molecular pathology, Spitz naevus, tumour progression

Conflicts of interest
None declared.

Summary

Spitzoid tumours are a morphologically diverse group of lesions that share histological similarity to the Spitz naevus, a benign melanocytic skin tumour. Distinguishing classic Spitz naevi from cutaneous malignant melanoma is usually achievable on standard histology sections, but occasionally equivocal lesions are encountered that show features intermediate between these two entities and consequently generate considerable clinical and histopathological concern. The nomenclature and diagnostic criteria for spitzoid lesions are not standardized and this article begins by considering the adverse effect this has on our understanding of spitzoid tumour biology. Investigations of some of the hallmark features of cancer and neoplasia in spitzoid tumours are described, and the contribution of these studies to our understanding of spitzoid tumour biology is considered, along with their potential diagnostic utility. These studies compare spitzoid tumours with better-characterized melanocytic lesions, and from such comparisons assumptions concerning the biological nature of different spitzoid tumours can be made. In contrast, investigations of the mitogen-activated protein kinase (MAPK) pathway and DNA gains and losses have suggested that Spitz naevi may be genetically distinct from other melanocytic tumours. The studies that led to this conclusion are reviewed, as well as subsequent work examining whether the same applies to all spitzoid tumours. Possible explanations for the considerable inconsistencies within some of these data are explored. Finally, potential pathways of tumour progression within spitzoid lesions are considered, with an emphasis placed upon insights gained from investigations of MAPK genes and DNA gains and losses.

The Spitz naevus is a benign, usually acquired, melanocytic tumour that is characteristically composed of spindle or epithelioid melanocytes, or a mixture of these two cell types. Criteria to discriminate Spitz naevi from the major differential diagnosis of cutaneous malignant melanoma were first published in 1947 by Sophie Spitz.[1] In this seminal study it was reported that these lesions were a juvenile variant of cutaneous melanoma in which the prognosis was frequently excellent. This opinion was revised in a subsequent study, which concluded that 'juvenile melanoma' was a benign tumour.[2] In 1960 Kernen and Ackerman published a series of 27 cases and concluded that the title 'spindle/epithelioid cell naevus' was more befitting for a lesion which they regarded to be a variant of the benign naevus.[3] While the concept that such tumours were benign naevi became central dogma, the eponym of Spitz naevus has been preferred by most. Nearly 60 years have passed since criteria to help differentiate Spitz naevi from cutaneous melanoma were first published and, while these have been considerably refined, there is no consensus opinion on specifically which criteria define the entity of Spitz naevus. There is disagreement among expert dermatopathologists concerning the biological potential of spitzoid tumours (lesions with a Spitz-like histological morphology),[4–6] and cutaneous melanomas misdiagnosed as Spitz naevi constitute a large proportion of dermatopathology malpractice claims.[7,8] The terms 'atypical Spitz naevus', 'atypical spitzoid tumour' and 'spitzoid tumour of uncertain malignant potential (STUMP)' are all used for these clinically troublesome lesions, but there is considerable ambiguity concerning criteria for their diagnosis. Similarly, the entity of spitzoid melanoma lacks well-specified diagnostic criteria; however, it implies a combination of features of a classical Spitz naevus and 'common' melanoma (i.e. cutaneous melanoma without spitzoid features).

The aim of this article is not to review literature concerning the histological diagnosis of spitzoid lesions, as for this there are many excellent articles already available,[9–12] but to review research into molecular alterations in spitzoid tumours which may provide insights into their pathogenesis. For some time, in an effort to improve diagnosis, ancillary techniques have been sought to assist in the identification of aggressive spitzoid lesions. Typically these techniques detect common hallmarks of cancer such as increased proliferation,[13] without regard to the underlying molecular changes such as disruption of signalling pathways. By contrast, genetic studies, particularly those examining the ras-raf-mek-erk mitogen-activated protein kinase (MAPK) pathway and DNA copy number, have provided information concerning the underlying mechanisms by which spitzoid tumours arise, and how they might differ from those of 'common' cutaneous melanoma. From these investigations, it is hoped that further genetic characterization may refine diagnosis in spitzoid lesions and inform our knowledge of the genetic basis of melanocytic neoplasia in general. Spitzoid tumours have been investigated using a variety of molecular techniques, which can make direct comparisons between studies difficult. Analyses of DNA gains and losses have proven especially informative, and various methods are summarized in Table 1. It is now timely to review this research and in doing so we will address the following questions: (i) What effect does the absence of standardized diagnostic criteria have on research into spitzoid tumours? (ii) Do spitzoid tumours show a neoplastic phenotype? (iii) Are spitzoid tumours genetically distinct from other melanocytic tumours? (iv) Does the histological spectrum of spitzoid tumours represent a progression from Spitz naevus to malignant melanoma?

What effect does the absence of standardized diagnostic criteria have on research into spitzoid tumours?

There are numerous histological features described in classic Spitz naevi, but these are often nonspecific and, as a consequence, diagnosis requires the interpretation of a constellation of criteria.[4,14–17] The relative importance placed upon these features by different pathologists varies considerably. The lack of diagnostic clarity is greater still in tumours that deviate from the classical appearance and show features more commonly associated with the main differential diagnosis, malignant melanoma. Atypical Spitz naevus, atypical Spitz tumour, STUMP and spitzoid melanoma are entrenched terms for the classification of lesions that are neither classic Spitz naevus nor 'common' melanoma;[18–20] however, their use is staunchly resisted by some.[10,12] The application of these terms lacks reproducibility, because there is little guidance on what features delineate 'classic' Spitz naevi from nonclassic spitzoid lesions and nonclassic spitzoid lesions from cutaneous melanoma.[4,9,10,15,17,21] A basic PubMed search revealed 60 review articles published since 1986 addressing the question of diagnosis in spitzoid lesions (date of search February 2007, search terms: Diagnosis of Spitz Naevus), which surely reflects, and contributes to, the variation in diagnostic practice.

Poor histological reproducibility adversely affects research into spitzoid tumours. It is difficult to compile well-defined, standardized tumour series and therefore experimental differences between types of spitzoid tumour may go unnoticed. Furthermore, when comparing data from different studies, direct comparisons between supposedly identical types of

Table 1 Comparison of technical considerations and diagnostic utility of the molecular techniques commonly used to determine DNA gains and losses (adapted from van Dijk *et al.*[99])

Technique	Flow cytometry	CGH	Array-CGH	(F)ISH	LOH	Multiplex ligation-dependent probe amplification
Starting material needed	Isolated nuclei from tissue sections	500 ng DNA	1 μg DNA	Isolated nuclei or tissue sections	50 ng DNA	50 ng DNA
Resolution of copy number change	Changes of ploidy	10–20 Mb	100–200 bp	100–500 bp	10–100bp	1–40 bp
Number of loci investigated per experiment	–	Whole genome	Up to 35 000 loci	1–3 loci	1–5 loci	Up to 45 loci
Discrimination between gains and losses	–	+	+	+	–	+
Discriminatory between Spitz naevi and cutaneous melanoma	+/–[86,87]	+[91,92]	+[93]	+[89]	+[88]	+[90]
Technique applied to atypical spitzoid tumours	–	+ (three case reports)[100,110]	–	–	+ (found not to be diagnostically useful)[99]	+ (may have value when used alongside other molecular techniques)[83]

CGH, comparative genomic hybridization; (F)ISH, (fluorescence) in situ hybridization; LOH, loss of heterozygosity.

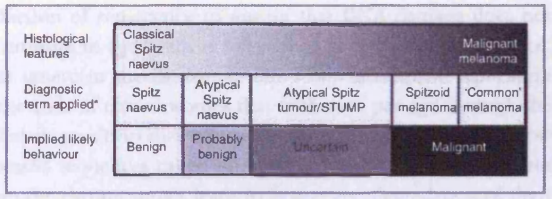

Fig 1. The spectrum of histological features, diagnostic terminology and clinical behaviour encountered in Spitz naevi, malignant melanoma and intermediate lesions. *Of these entities only Spitz naevus and 'common' melanoma (i.e. nonspitzoid melanoma) have widely accepted histological criteria.

lesions may be invalid due to variations in the criteria applied to acquire them.[5,6] Categorizing lesions as unequivocally benign, of uncertain malignant potential or unequivocally malignant may be the most reproducible way of comparing different data sets; however, this risks oversimplifying this diverse group of lesions. Figure 1 indicates how we consider the various diagnostic terms used within the literature may relate to the spectrum of histological features and the risk of malignancy within spitzoid tumours, but we accept that others may disagree with our summation. For the purpose of this review all nonclassic spitzoid lesions, apart from spitzoid melanoma, will be termed 'atypical spitzoid tumours' when not referring to a specific series of published cases. With the advent of online publishing, one way to remove some ambiguity might be to encourage authors to submit digital images of the tumours they investigate as on-line addenda, together with clearly stated criteria for diagnosis. This would enable the reader to be certain about the types of lesions examined, how they were classified, and facilitate more accurate comparisons between studies.

Do spitzoid tumours show a neoplastic phenotype?

Examination of cell functions that are commonly altered during carcinogenesis supports the notion that Spitz naevi are benign, while similar investigations in atypical spitzoid tumours are consistent with tumour progression. Many of these observations come from characterizing hallmark features of neoplasia[13] in spitzoid tumours and comparing the findings with those of better-characterized lesions such as common acquired naevi and 'common' melanoma. Some of this research is described below.

Proliferation

Increased cell proliferation is a fundamental element of the neoplastic process and can be measured by detection of Ki-67, a protein expressed by cells that have entered the cell cycle from late G_1 phase onwards.[22] Kapur *et al.* found Ki-67 expression to increase significantly from Spitz naevi, to atypical Spitz naevi, to cutaneous melanoma,[23] while Chorny *et al.* found that expression of Ki-67 in Spitz naevi and common acquired naevi was significantly lower than that seen in a group of 'minimum deviation' melanomas that included spitzoid lesions.[24] In addition, Kanter *et al.* and Rudolph *et al.* found that proliferation indices increased from unequivocally benign melanocytic lesions, to borderline lesions to melanoma.[25,26] This trend of increasing proliferation from Spitz naevus to atypical spitzoid tumour to melanoma indicates that progression towards malignancy may occur in spitzoid tumours.

Detection of Ki-67 has been reported to have diagnostic utility;[27–31] however, the overlap that exists between the proliferation indices of Spitz naevi and cutaneous melanoma means that occasional false positive and false negative results occur. Consequently Ki-67 expression may not be a sufficiently robust predictor of behaviour in spitzoid tumours to replace haematoxylin and eosin diagnosis on an individual case basis,[23,27,28,32] although it may have value when used in conjunction with other histological criteria.

Evading apoptosis

Apoptosis is a process of programmed cell death that is vital for preventing genetic damage being passed on to cell progeny. The tumour suppressor gene p53 plays a vital role in apoptotic regulation, in particular by promoting apoptosis in cells with genetic damage. The mutant form of the protein is detected immunohistochemically, but the wild-type form is generally undetectable. Expression of p53 in Spitz naevi is found at levels similar to that of common acquired naevi, which is significantly lower than that present in cutaneous melanoma.[28,33–36] Loss of functional p53 appears to be a late change in melanoma progression and for this reason it has been suggested that immunohistochemical detection of p53 may not suitable for differentiating between benign spitzoid tumours, including Spitz naevi, and primary cutaneous melanoma,[34,36] while others believe it may be of use in conjunction with assessment of Ki-67 expression.[37]

Apoptosis has been shown to be dysregulated in Spitz naevi via immunostaining for Fas and Fas ligand,[38] which initiate the apoptotic process, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling, a measure of apoptotic activity.[38,39] Deregulation of apoptotic pathways, for example via loss of p53, is a common feature of human cancer and therefore investigations of apoptosis in atypical spitzoid tumours would be of interest, but are yet to be published. Dysregulation of apoptosis has also been observed in cutaneous melanoma,[38–40] while alterations of various components of apoptosis can also be demonstrated such as Apaf-1 expression and tumour necrosis factor-related apoptosis inducing ligand receptor expression.[38,41,42]

Immortalization

Senescent cells are in a permanently nondividing state that prevents any accumulated DNA damage, which could potentially induce malignancy, being inherited by daughter cells. The

induction of senescence to ensure that DNA damage does not accumulate in aged cells is referred to as replicative senescence. This senescent mechanism results from incomplete replication at the ends of chromosomes that with each passage through the cell cycle results in their shortening and the gradual loss of short repeated sequences called telomeres. The loss of the telomeres from the chromosomes leads to fusion and degradation of their termini, which signals a growth check point and allows the cell to become senescent.[43] This mechanism essentially places a limit on the number of replications a cell may undergo but is overridden in some instances, for example in germ cells and stem cells, by activation of the enzyme telomerase, which stabilizes the telomeres by adding to the ends of chromosomes.[44] Telomerase activation is also a common feature of malignant cells and is required for immortalization. Telomerase activity has not only been shown to discriminate between classic Spitz naevi and cutaneous melanoma,[45–47] but expression has also been shown to increase with progression from common acquired naevi to dysplastic naevi to malignant melanoma.[48] Despite these intriguing preliminary data, there have been no investigations of telomerase activity in a series of atypical spitzoid tumours to date.

Aside from replicative senescence, cells may undergo premature senescence in response to cellular events that are unrelated to cell ageing. A premature senescent mechanism called oncogene-induced senescence has been proposed in common naevus cells, whereby activating mutations of B-raf induce wild-type p53 and $p16^{INK4a}$ expression, which inhibit cell division.[49–52] In the minority of common acquired naevi that progress to malignancy it is thought that these tumour suppressor genes become functionally altered, which allows progression to melanoma. In the context of Spitz naevi, two oncogene-induced senescent mechanisms have been proposed. Bastian *et al.* have correlated H-ras activation (which is found in around a fifth of Spitz naevi) with cytological features of malignancy such as marked nuclear pleomorphism and prominent nucleoli.[53] They postulate that this is a manifestation of partial transformation towards malignancy, but crucially malignancy does not develop because the H-ras-induced hyperproliferation drives these lesions into senescence via increased expression of cell cycle inhibitory proteins. Alternatively, Denoyelle *et al.* suggest that in the context of H-ras activation, senescence results from a stress-induced response of the endoplasmic reticulum, termed the unfolded protein response.[54] The unfolded protein response produces structural alterations within the endoplasmic reticulum that may account for the morphological changes observed in H-ras-activated Spitz naevi by Bastian *et al.* Further work is needed in this area because the senescent mechanisms in non-H-ras-activated Spitz naevi are yet to be described and it remains to be shown whether dysregulation of the above mechanisms occurs in atypical spitzoid tumours and subsequently in spitzoid melanoma.

The investigations described above examine hallmark features of neoplasia and common end points of carcinogenesis. There is good evidence that Spitz naevi are benign tumours while atypical spitzoid tumours appear to show some features more commonly associated with malignancy, which implies they may be undergoing neoplastic progression. These changes are not specific to spitzoid tumours and, as detailed above, can be seen in other melanocytic tumours. By contrast, investigations of MAPK pathway genes and DNA gains and losses have shown alterations that do appear specific to spitzoid tumours. Distinct sets of genetic alterations have been demonstrated in other melanocytic tumours,[55] indicating that they comprise discrete genetic subtypes. This provides a rational basis for postulating that spitzoid tumours could also be a distinct subset of melanocytic lesions, as is discussed below.

Are spitzoid tumours genetically distinct from other melanocytic tumours?

Spitz naevi

Key genetic alterations in melanocytic tumours were described as early as the 1980s with the identification of N-ras mutations in a proportion of melanomas.[56] The subsequent discovery of a high frequency of B-raf mutations in cutaneous melanoma nearly 20 years later indicated that dysregulation of the MAPK pathway was likely to be an important event in cutaneous melanoma development[57] (Table 2). MAPK pathway signalling promotes entry of cells into the cell cycle and hence activating mutations of oncogenes within the pathway enables cells to become self-sufficient in growth signals (Fig. 2). Such acquired growth signalling autonomy is a consistent feature of cancers and neoplasms in general.[13]

Around 90% of B-raf mutations in cutaneous melanoma are V600E mutations in exon 15. Mutations of N-ras typically involve codons 12 and 13 of exon 1 (the first coding exon of the gene) or codons 59 and 61 of exon 2.[58] Most sporadic cutaneous melanomas appear to have an N-ras or B-raf mutation, with mutation frequencies of approximately 20% and 70%, respectively.[14,57,59] This contrasts with a low mutation frequency in comparatively rare lesions from nonsun-exposed acral and mucosal surfaces,[60,61] and the desmoplastic variant of cutaneous melanoma which is most commonly encountered in skin with chronic sun exposure.[62] Importantly, B-raf and N-ras mutations are frequently mutually exclusive, but a small number of double-mutant cases has been reported.[63–66] The role of mutant B-raf as an early and fundamental feature of melanocytic neoplasia was confirmed with the identification of an even higher frequency of B-raf mutations (approximately 80%) in common acquired naevi and dysplastic naevi[67] (Table 2).

With such a high frequency of B-raf or N-ras mutations in benign and malignant melanocytic tumours, reports in Spitz naevi of a complete absence of B-raf mutations and a very low frequency of N-ras mutations was surprising for a lesion considered by many to arise via mechanisms similar to cutaneous melanoma and common acquired naevi[66,68,69] (Table 2). Several authors have subsequently confirmed this observation,[20,57,63,67,70–83] and so distinctive is the absence of these mutations within the context of melanocytic tumours that one

Table 2 Comparison of B-raf and/or N-ras mutation frequency in published studies of melanocytic tumours

Study	Common acquired naevi	Spitz naevi	Atypical Spitz naevi	Suspected for melanoma/STUMP	Primary spitzoid melanoma	Spitzoid melanoma metastasis	'Common' melanoma
Palmedo *et al.*[71]	–	0/21	–	–	2/6	–	–
Gill *et al.*[18]	–	0/10	–	–	0/9	–	–
Lee *et al.*[19a]	8/11	–	–	–	1/33[a]	0/2	8/12
van Dijk *et al.*[20]	–	0/14	0/16	8/23	30/36	6/7	–
Fullen *et al.*[84]	–	5/23	5/25	0/7	2/13	–	–
Takata *et al.*[83]		0/12		2/16[b]			15/24
Other studies[20,57,63,66–82,85]	249/319	9/212 (eight mutant cases reported in one study)	–	–	–	–	281/534
Total	257/330 (78%)	14/292 (5%)	5/41 (12%)	8/30 (27%)	35/97 (36%)	6/9 (67%)	304/570 (53%)

The studies summarized in detail have analysed atypical spitzoid tumours or spitzoid melanomas, while studies examining common acquired naevi, Spitz naevi or common melanomas are grouped together. Note that the mutation frequencies for atypical spitzoid tumours and spitzoid melanoma are based on a comparatively small number of samples and show considerable variation between studies. STUMP, spitzoid tumour of uncertain malignant potential. [a]Authors concede some cases might be diagnosed as suspected for melanoma/STUMP by other dermatopathologists. [b]Cases not specifically categorized and excluded from column totals.

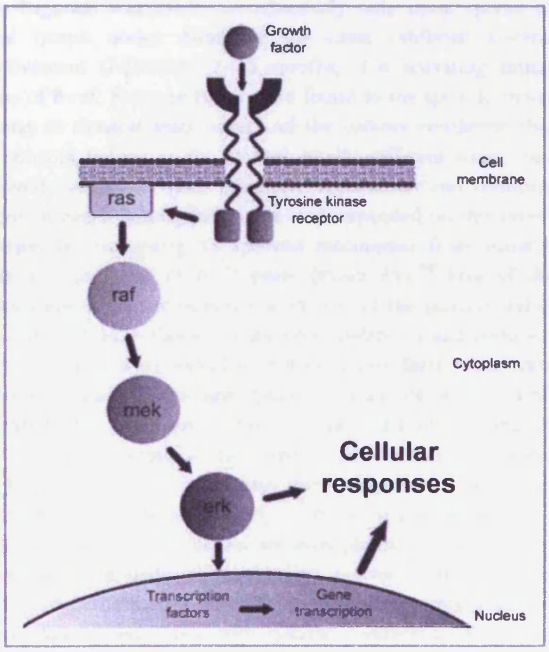

Fig 2. The mitogen-activated protein kinase signal transduction pathway. Activating mutations of ras or raf result in constitutive activation of the pathway enabling cellular responses, such as cell proliferation, to occur independently of external growth signals.

might consider the presence of a B-raf or N-ras mutation incompatible with a true Spitz naevus. It should be noted, however, that two studies have described B-raf-mutant Spitz naevi. Fullen *et al.*[84] reported B-raf mutations in 10 of 48 Spitz naevi and in support of this is the finding of B-raf mutations in a small series of Spitz naevi by La Porta *et al.*[85] These are most surprising results when one considers that no mutations of B-raf have been reported in seven previous studies that have analysed Spitz naevi, on a total of 204 cases, using different methods of mutation detection.[20,66,68–72] It is possible that differences of diagnostic criteria, as described earlier, may account for these outlying cases; for example, Fullen *et al.* state that some of the lesions classified in their series as Spitz naevi had atypical features. The authors do not confirm their mutations via alternative methods and the lesions they analysed are not explicitly described or illustrated. Takata *et al.* have proposed that the lesions investigated by Fullen *et al.* may have been dysplastic naevi, where mutations of B-raf are common.[83] It will be interesting to see if other researchers provide evidence of a B-raf mutant subset of classic Spitz naevi, but for now it appears that B-raf or N-ras mutations in these lesions are extremely uncommon.

Characterization of DNA gains and losses in Spitz naevi has also shown clear differences from cutaneous melanoma and common acquired naevi. One of the first techniques employed to examine for genomic imbalances in melanocytic tumours was analysis of ploidy via flow cytometry. This was shown to have some discriminatory power between Spitz naevi and cutaneous melanoma[86,87] but failed to show significant specificity for routine diagnostic use. Subsequently, studies of chromosome 9p via *in situ* hybridization (ISH) and loss of heterozygosity (LOH) also demonstrated an ability to differentiate between classic Spitz naevi and 'common' melanoma.[88,89] A study using multiplex ligation-dependent probe amplification also demonstrated multiple copy number aberrations in cutaneous melanoma but minimal alterations in Spitz naevi.[90] Comparative genomic hybridization (CGH) shows that Spitz naevi, along with other benign naevi, have virtually no alterations of DNA copy number, while in melanoma these changes can be numerous.[91–93] Bastian *et al.* showed that around 20%

of Spitz naevi have a characteristic increase of chromosome 11p copy number, the site of the H-ras gene, which in 67% of amplified tumours is also concurrently mutated.[53,92] No such amplifications were seen in other naevus types, while H-ras mutations are extremely uncommon in cutaneous melanoma.[94]

The evidence from B-raf and N-ras mutations and H-ras amplification has made for a compelling argument that most Spitz naevi arise via different mechanisms from common acquired naevi and cutaneous melanoma. The identification of these important differences has led to speculation that nonclassical spitzoid lesions may also differ from common naevi and 'common' melanoma.[9,10] Genetic changes in spitzoid melanomas will be considered first.

Spitzoid melanoma

Gill *et al.* examined nine spitzoid melanomas, alongside 10 age-matched 'typical' Spitz naevi, for mutations of B-raf, N-ras and H-ras[18] (Table 2). All cases were from children aged 10 years or younger. Upon initial histological assessment none of the cases was reported as unequivocally malignant; the diagnosis was made retrospectively only upon spread to local lymph nodes. None of the cases exhibited visceral involvement (follow-up 2–78 months). No activating mutations of B-raf, N-ras or H-ras were found in the spitzoid melanomas or classical Spitz naevi and the authors concluded that all spitzoid lesions might be biologically different from nonspitzoid melanomas (i.e. 'common' melanoma) and common acquired naevi. Subsequently, Lee *et al.* expanded on this investigation by examining 33 spitzoid melanomas from patients with an age range of 6–71 years (mean 35).[19] Five of the cases were metastatic tumours with two of the patients dying from their disease. Cases of cutaneous melanoma and common acquired naevi were included, but no classic Spitz naevi were examined. They found one spitzoid melanoma with a B-raf mutation, but no N-ras mutations. The mutation frequencies in the common acquired naevi and cutaneous melanoma were in keeping with those reported previously in the literature. They concluded that their study, along with that of Gill *et al.*, indicates that spitzoid lesions are biologically separate entities from common acquired naevi and conventional cutaneous melanoma. These data provide a convincing argument that classic Spitz naevi along with spitzoid melanoma comprise a distinct subtype of melanocytic tumour in which mutations of B-raf or N-ras are very rare (Table 2). In stark contrast to these conclusions, however, other studies indicate that there is some genetic heterogeneity within spitzoid tumours.

Palmedo *et al.* described two B-raf-mutant spitzoid melanomas in a series of six, but no mutations in 21 'classical' Spitz naevi.[71] They concluded that while Spitz naevi appear genetically different, spitzoid melanomas have a mode of tumour progression similar to other cutaneous melanomas. A larger investigation by van Dijk *et al.*[20] concurs with this finding. They examined 36 primary spitzoid melanomas and seven spitzoid metastases and found a B-raf or N-ras mutation frequency of 83% but no mutations of H-ras. By contrast, they found no N-ras or B-raf mutations in 14 Spitz naevi or 16 atypical Spitz naevi, but within these lesions mutations of H-ras were found. They concluded that Spitz naevi and atypical Spitz naevi are genetically distinct from spitzoid melanomas. In keeping with this, Fullen *et al.* reported B-raf mutations in two of 13 spitzoid melanomas.[84]

The conflicting conclusions of these studies could have arisen out of variation in the diagnostic criteria employed for case selection. Gill *et al.* and Lee *et al.* may have had highly specific diagnostic criteria and consequently assembled a discrete series of 'true' spitzoid melanomas that have the Spitz signature of wild-type B-raf/N-ras. In contrast, Palmedo *et al.* and van Dijk *et al.* may have applied more inclusive criteria and examined some lesions that are spitzoid in certain aspects of their appearance, but genetically similar to nonspitzoid melanomas. Indeed, van Dijk *et al.* classify spitzoid melanoma as any cutaneous melanoma composed entirely of spindle and/or epithelioid cells, even if lateral radial growth is present (verbal communication). Using these criteria, some cases of superficial spreading melanoma or nodular melanoma (i.e. 'common' melanoma) might be called spitzoid melanoma. This situation is inevitable and understandable due to the lack of reproducible diagnostic criteria for spitzoid lesions. Importantly, Lee *et al.*[4,95] and van Dijk *et al.*[96,97] take their criteria for diagnosis from different sources which makes it possible that like has not been compared with like. However, the precise reason for differences between the studies remains to be established.

Aside from possible differences of case selection, van Dijk *et al.* suggest that different age distributions might explain the conflict between their data, predominantly derived from adults, and those of Gill *et al.*, derived entirely from children.[20] This may at first seem an unlikely explanation because Lee *et al.* reach similar conclusions to Gill *et al.*, and included adult tumours in their series. However, the age distribution of patients with primary spitzoid melanoma in Lee *et al.* differs considerably (age range 6–71 years, mean 35) from that of van Dijk *et al.* (age range 10–88 years, mean 52). This suggests that age could have some bearing on the likelihood of B-raf or N-ras mutation in a spitzoid lesion. Classic Spitz naevi are more frequently encountered in young people and perhaps this extends to their malignant 'true' spitzoid (i.e. B-raf or N-ras wild-type spitzoid melanoma) counterparts, if such lesions exist. Conversely, tumours with a similar histological appearance but found in older patients may be unrelated to such 'true' spitzoid lesions by virtue of a B-raf or N-ras mutation and share their pathogenesis with 'common' melanoma. Our own unpublished data would not appear to support this hypothesis, however, because we have identified mutations of B-raf and N-ras in a minority of spitzoid melanomas, but have found no preponderance for such lesions in older patients. Indeed, we have encountered B-raf-mutant spitzoid melanomas in children as young as 5 years of age.

Data concerning DNA gains and losses in Spitz naevi have not been expanded to include spitzoid melanomas. Despite

this one might speculate that, because genomic instability is a common feature of malignancy, such lesions would show frequent changes of DNA copy number like any other type of melanoma.

Atypical spitzoid tumours

There are limited data concerning the frequency of MAPK pathway gene mutations in atypical spitzoid tumours and those that are available are contradictory (Table 2). Van Dijk *et al.*[20] and Fullen *et al.*[84] have examined a number of intermediate lesions that appear to fit into this category. Both authors analysed atypical Spitz naevi, while van Dijk *et al.* looked at 23 cases of 'suspected spitzoid melanoma' and Fullen *et al.* at seven 'atypical Spitz tumours of uncertain biological potential'. The latter two groups comprised lesions showing atypia beyond that of an atypical Spitz naevus but not sufficient for a diagnosis of spitzoid melanoma and which therefore might be considered STUMPs (see Fig. 1). The results conflict considerably, with van Dijk *et al.* finding mutations in roughly a third of their 'suspected spitzoid melanoma' group but no mutations in atypical Spitz naevi, while Fullen *et al.* found mutations in a fifth of their atypical Spitz naevi but none in 'atypical Spitz tumours of uncertain biological potential' (see Table 2). Takata *et al.* examined 16 cases of 'ambiguous spitzoid lesions' for mutations of B-raf, N-ras and H-ras alongside classic Spitz naevi and 'common' melanoma.[83] They employed a panel of two dermatopathologists to diagnose the tumours independently and disagreements between the two were common, with a variety of diagnostic terms used. Two mutant lesions were found. The first case was a mutation of B-raf in a lesion regarded as 'melanoma' by one reviewer and 'atypical Spitz tumour (favours melanoma)' by the other. The second case involved an N-ras mutation in a lesion regarded as malignant by both reviewers. The authors conclude that most atypical Spitz tumours are probably not different from conventional Spitz naevi. Clearly, further work is needed to examine the nature of atypical spitzoid tumours but, as a result of their considerable histological ambiguity, they may prove the most challenging spitzoid lesions to characterize.

Investigations into DNA gains and losses in atypical spitzoid tumours are also limited but some interesting reports have emerged. De Wit *et al.* found that the frequency of aberrations of chromosome 1, as detected via ISH, were significantly different between Spitz naevi and nodular melanoma but, when applied to metastatic lesions originally diagnosed as Spitz naevi (and hence possibly STUMPs), the frequency of aberrations varied considerably.[98] Similarly, examination of LOH shows features intermediate between Spitz naevi and cutaneous melanoma in the context of atypical spitzoid tumours.[99] Using CGH, Bauer and Bastian examined two lesions that might be classified as STUMP in so much as histological assessment could not assign them as being unequivocally benign or malignant. They found CGH to be diagnostically useful in this setting, with one lesion demonstrating no genomic aberrations, as in classic Spitz naevi, and consequently being considered benign, while the other lesion had many genetic aberrations, similar to 'common' melanoma, and was consequently considered malignant.[100] Neither case showed increased chromosome 11p copy number, as previously described in a minority of Spitz naevi, and crucially no follow-up data on the patients were given. Mihic-Probst *et al.* describe a lesion initially diagnosed as a Spitz naevus that was rediagnosed as melanoma upon metastasizing. Using CGH they demonstrated a number of genetic aberrations including loss of chromosome 9p (which contains the p16 tumour suppressor gene locus), and concluded that if CGH had been used for the initial diagnosis, the malignant nature of the lesion would have been revealed.[72] Takata *et al.*, in addition to the mutation analysis described above, analysed the same series of 'ambiguous' spitzoid lesions for DNA copy number aberrations.[83] They found that a tumour with a B-raf mutation also showed multiple copy number alterations, which suggested that this lesion was in fact 'common' melanoma. No aberrations were found, however, in an N-ras-mutant tumour with clinical evidence of distant metastases. Loss of the *CDKN2A* gene, which encodes p16, was found in three 'ambiguous' spitzoid tumour cases and was not seen in 12 Spitz naevi. The authors concluded that the loss of this tumour suppressor gene may account for the clinical and histological deviations of these tumours from typical Spitz naevi. With so few cases of atypical spitzoid tumours in the literature, no significant patterns of DNA copy number change can be identified to show similarity to or differences from Spitz naevi, but from a diagnostic standpoint these reports are encouraging.

In summary, therefore, the bulk of evidence suggests that classic Spitz naevi are indeed a distinct type of melanocytic tumour, arising via B-raf- or N-ras-independent mechanisms, with a subset of these having amplified H-ras, which is in stark contrast to 'common' melanoma and common acquired naevi. This conclusion cannot, however, be extended to other spitzoid lesions because the molecular findings are contradictory, possibly due to inherent difficulties in establishing criteria for case selection.

Does the histological spectrum of spitzoid tumours represent a progression from Spitz naevus to malignant melanoma?

The progression model of Clark *et al.*,[101] which proposes that common acquired naevi progress to dysplastic naevi and then to melanoma, is accepted by many as a model of stepwise carcinogenesis in melanocytic tumours (however, the model is not accepted by all and most cutaneous melanomas are thought to arise *de novo*[102,103]). This has led to speculation that a similar or indeed identical process occurs in spitzoid tumours, whereby the Spitz naevus becomes an atypical spitzoid tumour and finally melanoma.[104,105] In support of this, atypical spitzoid tumours show a spectrum of histological features intermediate between classic Spitz naevi and cutaneous melanoma, and while most atypical spitzoid tumours do not

behave like melanoma, there is unquestionably a small percentage that does and is referred to retrospectively, by some, as spitzoid melanoma.[10,18–20] Alternatively, the broad histological spectrum of spitzoid tumours might simply comprise a dichotomy of Spitz naevi and 'common' melanomas that look spitzoid. If this hypothesis were correct, atypical spitzoid tumours would merely represent a mix of Spitz naevi and cutaneous melanoma that could not be separated by current pathological techniques. In this setting, Spitz naevi would never progress to malignancy and lesions that had a spitzoid appearance and showed aggressive behaviour would actually be 'common' melanoma mimicking the Spitz naevus phenotype.

It will not be possible to distinguish between these two scenarios by analysis of B-raf or N-ras mutation status alone, because a mutation could be interpreted as both evidence that a tumour is progressing towards malignancy or that the lesion is actually malignant *ab initio*. If Spitz naevi and atypical spitzoid tumours do progress to malignancy by acquiring B-raf or N-ras mutations, in a manner similar to Clark's progression model, one might expect the frequency of these mutations to increase with progression, as was found by van Dijk *et al.*[20] (Table 2). In further support of this model, a study by the same authors found an increasing frequency of LOH from atypical Spitz naevi (33%) through lesions suspicious of melanoma (65%), to unequivocal spitzoid melanomas (88%).[99] The alternative situation in which there is a dichotomy of Spitz naevi and cutaneous melanomas with no borderline lesions has been fiercely championed by some authors.[12,95,106] Whether this dichotomy exists is in part a semantic argument independent of the underlying molecular events. Some dermatopathologists believe that once a spitzoid lesion ceases to be of classic type, the term Spitz should be discarded and melanoma applied, with no scope for intermediate lesions, and would therefore call for a revision of taxonomy rather than biology.

A third possibility is that there is a separate spitzoid progression pathway, distinct from the 'common' melanoma pathway of Clark's progression model. In this case the whole family of spitzoid tumours would be a distinct subset of melanocytic tumours. Investigations that found an absence of B-raf or N-ras mutations in spitzoid melanoma suggest this to be a likely hypothesis.[18,19] In this scenario, if Spitz naevi progress to spitzoid melanoma, it should be possible to detect genetic alterations of Spitz naevi that were acquired early during spitzoid progression, for example H-ras mutations and focused amplifications at the H-ras locus, in the later stages of progression, i.e. spitzoid melanoma. This is yet to be described, but H-ras mutations have been reported in atypical Spitz naevi and a single spitzoid tumour suspected of melanoma.[20] The possibility of a distinct spitzoid pathway is made more likely by the increasing body of evidence supportive of alternative melanoma progression pathways in the various melanoma subtypes.[55,76] The fact that some reports find B-raf and N-ras mutations in spitzoid melanoma[19,20,71,84] would argue against spitzoid tumours being a distinct subset.

Reports of improved prognosis in spitzoid melanomas in young people is regarded by some as supportive of a separate spitzoid progression pathway.[21,107,108] The oxymoronic concept of the 'malignant/metastasizing' Spitz naevus, a lesion histologically in keeping with a Spitz naevus, that shows local lymph node metastasis but no spread beyond this, is a concept that polarizes opinion. It appears reasonable to regard metastasis as a highly specific but poorly sensitive marker of malignancy, yet with the advent of the 'malignant/metastasizing' Spitz naevus even the specificity of metastasis for malignancy is questioned. It should be noted that patients with regional nodal metastases (stage 3 disease) from 'common' melanoma show greater than 30% 15-year survival, but these tumours would not be regarded as benign.[109] Perhaps the most robust criteria for malignancy in a young person would be widespread metastasis, but fortunately such instances are rare. Due to the limited number of published cases, often with minimal follow-up, the 'malignant/metastasizing' Spitz naevus cannot currently be considered robust evidence in support of spitzoid tumours being a distinct group. The most forceful opponents of this entity tend to advocate the dichotomy of benign Spitz naevi and malignant melanoma and do not acknowledge the possibility of intermediate lesions (Mones and Ackerman provide a comprehensive review and fierce critique of this concept[106]).

There are many hypothetical pathways of progression for spitzoid tumours, but as yet there is little evidence to substantiate them, and that which is available is often contradictory. Nevertheless, research in this area must continue because determining pathways of progression within melanocytic tumours may offer novel methods of diagnosis and treatment in cutaneous melanoma.

Conclusions

It was perhaps to be expected, in an area of histopathology notorious for diagnostic difficulty and polarized opinions that at the molecular level spitzoid tumours would be equally challenging to characterize. Clear differences between classic Spitz naevi and 'common' melanoma have been demonstrated by various techniques but similar investigations in nonclassic spitzoid tumours are inconclusive. By building on data from Spitz naevi, it was hoped that molecular pathology might provide some unequivocal conclusions concerning the pathology of atypical spitzoid tumours and spitzoid melanoma to improve our understanding of spitzoid lesions and melanocytic neoplasia in general. Unfortunately such conclusions are yet to emerge and, in some cases, data conflict considerably. Despite these disparate findings, some speculation concerning the molecular biology of atypical spitzoid tumours and spitzoid melanoma is possible, and this research will hopefully provide a basis for further investigations. Molecular pathology has advanced phenomenally in recent years, and is likely to assist research into spitzoid tumours in the future, but much more work is required before fundamental questions regarding the pathogenesis of these lesions can be answered. Importantly, we wish to highlight how future research in this field

must attempt to address the current lack of emphasis on diagnostic criteria and the paucity of photomicrographs accompanying published data. We feel that any publication in this field must place emphasis on the diagnostic criteria used to ensure that, between different studies, like is compared with like.

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9 Appendix 4: Ethics approval

Leicestershire, Northamptonshire and Rutland
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Ethics Administration
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04 June 2003

6926 Please quote this number on all correspondence

Dr Gerald Saldanha
Senior Clinical Lecturer/Honary Consultant in Histopathology
Level 3
Clinical Sciences Building
LRI

Dear Dr Saldanha

Re: An investigation into the role of the mitogen activated protein kinase pathway in cutaneous melanoma with respect to initation, progression and therapy, ethics ref: 6926

The Chair of the Leicestershire Local Research Committee (Committee One) has considered the amendments submitted in response to the Committee's earlier review of your application on 07 February 2003 as set out in our letter dated 18 February 2003. The documents considered were as follows:

Your letter dated 28/5/03
PIS version 2, 28/5/03
Consent form version 2, 28/5/03

In particular, not contacting those patients who are no longer being followed up is approved, and I accept this justification for access to these patients' notes.

The Chair, acting under delegated authority, is satisfied that these accord with the decision of the Committee and has agreed that there is no objection on ethical grounds to the proposed study. I am, therefore, happy to give you the favourable opinion of the committee on the understanding that you will follow the conditions set out below:

RESEARCH & DEVELOPMENT
10 JUN 2003
LIHL NHS TRUST

Conditions

- You do not recruit any research subjects within a research site unless favourable opinion has been obtained from the relevant LREC.
- You do not undertake this research in an NHS organisation until the relevant NHS management approval has been gained as set out in *the Framework for Research Governance in Health and Social Care.*

- You do not deviate from, or make changes to, the protocol without prior written approval of the LREC, except where this is necessary to eliminate immediate hazards to research participants or when the change involves only logistical or administrative aspects of the research. In such cases the LREC should be informed within seven days of the implementation of the change.

- You complete and return the standard progress report to the LREC one year from the date on this letter and thereafter on an annual basis. This form should also be used to notify the LREC when your research is completed and in this case should be sent to this LREC within three months of completion.

- If you decided to terminate this research prematurely you send a report to this LREC within 15 days, indicating the reason for the early termination.

- You advise the LREC of any unusual or unexpected results that raise questions about the safety of the research.

- The project must be started within three years of the date on which LREC approval is given.

- You should be able to assure the Ethics Committee that satisfactory arrangements have been made for the labelling, safe storage and dispensation of drugs and pharmaceutical staff are always willing to provide advice on this.

Your application has been given a unique reference number. Please use it on all correspondence with the LREC.

Yours sincerely

pp Dr PG Rabey
Chairman
Leicestershire Local Research Ethics Committee One

(N.B. All communications related to Leicestershire Research Ethics Committee must be sent to the LREC Office at Leicestershire, Northamptonshire and Rutland Health Authority. If, however, your original application was submitted through a Trust Research & Development Office, then any response or further correspondence must be submitted in the same way).

SPECIAL NOTE

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Metropolitan Multi-centre Research Ethics Committee

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Chair – Dr H M Biggs

16th October 03

Dr Gerald Saldanha
Senior Lecturer/Honorary Consultant
University of Leicester
Department of Pathology
Level 3, RKCSB,
Leicester Infirmary
Leicester
LE2 7LX

Dear Dr Saldanha

Full title as detailed in question A2 of the application form: **An investigation into the relationships between benign and malignant melanocytic tumours. - *NO LOCAL RESEARCHER***

MREC reference number: **MREC/03/11/085**

The Chair of the Metropolitan MREC has considered the amendments submitted in response to the Committee's earlier review of your application on 5th September 03 as set out in our letter dated 19th September 03. The documents reviewed were as follows:

MREC Application Form	(dated 5/8/03)
Protocol and Flow chart	(dated 3/8/03, version1)
Chief Investigator's CV	(not dated)

The Chair, acting under delegated authority, is satisfied that these accord with the decision of the Committee and has agreed that there is no objection on ethical grounds to the proposed study. I am, therefore, happy to give you our approval on the understanding that you will follow the conditions of approval set down below. A record of the review undertaken by the MREC is contained in the attached MREC Response Form. The project must be started within three years of the date on which MREC approval is given.

While undertaking the review of your application the MREC noted the research involves the establishment of a new disease or patient database for research purposes / the use of an existing database collected for previous research or other purposes with no patient contact patient. For this reason you are not required to notify any LRECs when undertaking this research.

LREC Involvement
When undertaking the review of your project the MREC observed that there 8is limited patient contact involving the performance of technical procedures or additional data collection as described in the MREC approved protocol/initial contact by a local clinician for purposes of recruitment. It is felt that these tasks appear well within his/her routine

professional competence and adequate facilities for such procedures are available as part of his/her normal professional practice.

For this reason you are asked to only inform the appropriate LREC of the project by sending a copy of this letter and also **giving the name and contact details of the local clinician involved and what procedures will be undertaken by this person.** If (unusually) the LREC has any reason to doubt that the local clinician is competent to carry out the tasks required, it will inform the clinician and the MREC that gave ethical approval giving full reasons.

When such tasks are performed by centrally based researchers it should be assumed that the MREC has reviewed their competence to undertake the tasks and it is not necessary to inform the LREC of the contact details, but only that the research will take place.

You are not required to wait for confirmation from the LREC before starting your research.

Local NHS Management
The local clinician must inform his/her NHS organisation of their co-operation in the research project and the nature of their involvement. Care should be taken to ensure with the NHS organisation that local indemnity arrangements are adequate.

Legal and Regulatory Requirements
It remains your responsibility to ensure in the subsequent collection, storage or use of data or research sample you are not contravening the legal or regulatory requirements of any part of the UK in which the research material is collected, stored or used. If data is transferred outside the UK you should be aware of the requirements of the Data Protection Act 1998.

ICH GCP Compliance
The MRECs are fully compliant with the International Conference on Harmonisation/Good Clinical Practice ((ICH GCP) Guidelines for the Conduct of Trials Involving the Participation of Human Subjects as they relate to the responsibilities, composition, function, operations and records of an Independent Ethics Committee/Independent Review Board. To this end it undertakes to adhere as far as it consistent with its Constitution, to the relevant clauses of the ICH Harmonised Tripartite Guideline for Good Clinical Practice, adopted by the Commission of the European Union on 17 January 1997. The Standing Orders and a Statement of Compliance were included on the computer disk containing the guidelines and application form and are available on request or on the Internet at **www.corec.org.uk**

Yours sincerely,

DR HAZEL BIGGS
CHAIR
METROPOLITAN MREC

Enclosures MREC Response Form16th October 03

List of members present and members who submitted written comments.

10 Appendix 5: Bibliography

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