DUPLEX RATIO TESTS AS DIAGNOSTIC TISSUE BIOMARKERS IN MALIGNANT MELANOMA

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Malignant melanoma is a tumour with tendency to metastasise early and with a rapidly increasing incidence. A minority of melanomas and their benign counterparts, naevi, prove difficult to diagnose on histopathology alone, which is currently the gold standard and a small percentage are genuinely histologically ambiguous. Chromosomal Instability is a well described feature of malignant tumours and melanomas have been shown to demonstrate typical patterns of chromosomal instability, in comparison to benign naevi which show minimal DNA copy number change. QPCR based assays called Duplex Ratio Tests (DRTs) have been developed by our laboratory for application on DNA from formalin fixed paraffin embedded samples of melanoma and naevi.

The reproducibility and accuracy of the DRTs has been demonstrated and appropriate correction factors for DNA quality have been calculated for the assays, based on the results of 108 diploid samples. As a panel, seven DRT assays were able to differentiate unambiguous cases of melanoma and naevi with sensitivity of 95% and specificity of 97.5% respectively on an opportunity sample of 20 naevi and 40 primary melanomas, when tested by logistical regression. When applied to independent true cohorts of 105 melanomas and 103 naevi the sensitivity of DRTs was 84% and specificity 88%. Logistical regression analysis of DRT scores for 20 non-metastasising primary melanomas and 20 unmatched metastasising primaries showed the DRT assays to correctly predict outcome in more cases (28/40) than clinical stage alone (24/40) and when combined with clinical stage, DRT scores predicted outcome with a sensitivity of 85% and specificity of 70%. BRAF mutation analysis of one series of cases indicated that 95% of the naevi and 27% of the primary melanomas tested showed the V600E mutation. Of the 5 metastasising primary melanomas with the mutation, 3 of the subsequent metastases were also V600E positive.

DRTs targeting chromosomal centromeres were also developed as part of this project, although testing these assays against a hybridoma cell line DNA panel demonstrated that centromeric alphoid repeat regions show homology between the targeted chromosomes not previously described, making these assays impractical to develop as part of the DRT panel at this stage.

DRT assays therefore show potential to act as molecular biomarkers of melanoma on FFPE specimens and DRTs may have applicability to prognosis in melanoma or to other tumour types if new DRTs to relevant loci are developed.

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Abbreviations

AE	Abdlrzag Ehdode
AJCC	American Joint Committee on Cancer
AQUA	Automated Quantitative Analysis
ARPC2	Actin related protein complex 2
ATF2	Activating Transcription factor 2
AUC	Area Under Curve
BLAST	Basic Local Alignment Search Tool
CDC	Centres for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic Acid
cDRT	Centromeric duplex ratio test
CGH	Comparative Genomic Hybridisation
Ch	Chromosome
COX-2	Cyclo-oxygenase enzyme 2
CRIS	Computerised Radiology Information System
CRUK	Cancer Research United Kingdom
CT	Cycle threshold
DASL	cDNA mediated Annealing, Selection, extension and Ligation
DB	Danielle Bury
DCN	DNA Copy Number
DFS	Disease free survival
DM	David Moore
DNA	Deoxyribonucleic Acid
DRT	Duplex ratio test
FDA	United States Food and Drug Administration
FFPE	Formalin Fixed Paraffin Embedded
FISH	Fluorescent In Situ Hybridisation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GS	Gerald Saldanha
H&E	Haematoxylin and eosin
IAP	Inhibitor of apoptosis

ICC	Intraclass correlation co-efficient
IHC	Immunohistochemistry
IMS	Industrial Methylated Spirit
JHP	Howard Pringle
LD	Lovesh Dyall
LP	Linda Potter
Met	Metastasis
MITF	Micropthalmia transcription factor
mm	millimetres
MT	Metallothionein
NCBI	National Centre for Biotechnology Information
NCI	National Cancer Institute
NHS	National Health Service
NICE	National Institute for Clinical Excellence
P+M	Primary melanoma with metastasis
PCR	Polymerase Chain reaction
P-M	Primary melanoma without metastasis
PRT	Paralogue Ratio Test
QPCR	Quantitative Polymerase Chain reaction
RGS1	Regulator of G-protein signalling
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristics
RTQPCR	Reverse Transcriptase Quantitative PCR
SD	Standard Deviation
SEM	Standard Error of the Mean
SNOMED	Systematized nomenclature of medicine clinical terms
SPP1	Osteoponin
STUMP	Spitzoid Tumour of Uncertain Malignant Potential
TMA	Tissue Microarray
UCSF	University California San Francisco
UHL	University Hospitals Leicester
UK	United Kingdom

US	United States
UV	Ultra Violet
WT2	Wilm’s Tumour 2
ZM	Zahir Mughal
ΔCT	Delta cycle threshold
ΔΔCT	Delta delta cycle threshold
µm	Micrometre

1. Introduction

1.1. Melanoma Background

Malignant melanoma is a tumour derived from melanocytes. Melanocytes are located at the base of the epidermal layer of the skin and are also found at some mucosal sites as well as in the meninges and inner ear. Melanocytes are embryologically derived from the neural crest and their main function is to synthesise melanin pigment. Melanin production is induced by ultra violet (UV) radiation from the sun and is taken up by keratinocytes to protect the skin from the effect of UV radiation. It is the presence of melanin which accounts for pigmentation of the skin, with different concentrations of melanin accounting for individual and racial variations in skin colour.

Malignant melanoma most commonly arises in the skin of lighter-skinned individuals and can occur at sun-exposed or non-sun-exposed sites. It also occurs rarely in mucosal sites such as the buccal or nasal mucosa and in the uvea. It is a tumour with a propensity to metastasise at an early stage of development; it has been reported that if a primary cutaneous melanoma achieves a volume of greater than 200mm^3 at the time of excision, the tumour is likely to have already metastasised (1). Fortunately many melanomas are detected at an earlier stage due to their notable appearance on the skin (described in 1.1.2), which causes patients to present to medical services.

The benign counterpart of melanoma is the naevus. Naevi are exceedingly common tumours which are often present in large numbers in those with types I and II skin (2). Skin types I and II represent the lightest skin of skin types I-VI described by Fitzpatrick

in 1975 and are typically seen in those of northern European ancestry (3). These lesions are typically smaller than melanomas, appearing uniform and well circumscribed macroscopically.

The name malignant melanoma is somewhat tautologous, since a melanoma is by definition a malignant tumour, with pre-malignant lesions being termed either melanoma-in-situ or lentigo maligna, depending upon the clinical features and histopathological appearances. The term 'melanoma' will be used throughout the remainder of this thesis to denote a malignant melanocytic tumour.

1.1.1 Melanoma Epidemiology

Melanoma is the third commonest and most deadly form of skin cancer in the both the UK and USA according to data published by Cancer Research UK (CRUK) and Centres for Disease Control and Prevention (CDC) respectively and in the UK melanoma is the fifth commonest form of cancer overall, excluding the non-melanoma skin cancers (4,5). There are over 10,000 new cases of melanoma diagnosed each year in the UK with those over the age of 65 most likely to be affected, though melanomas arise in adults of all ages (see figure 1-1). The incidence of melanoma in the UK has risen dramatically from 3.2/100,000 to 16/100,000 over 33 years from 1975 to 2008 – the most rapid increase over that period of any cancer type (6). This increase in the UK has been mirrored in the US (7).

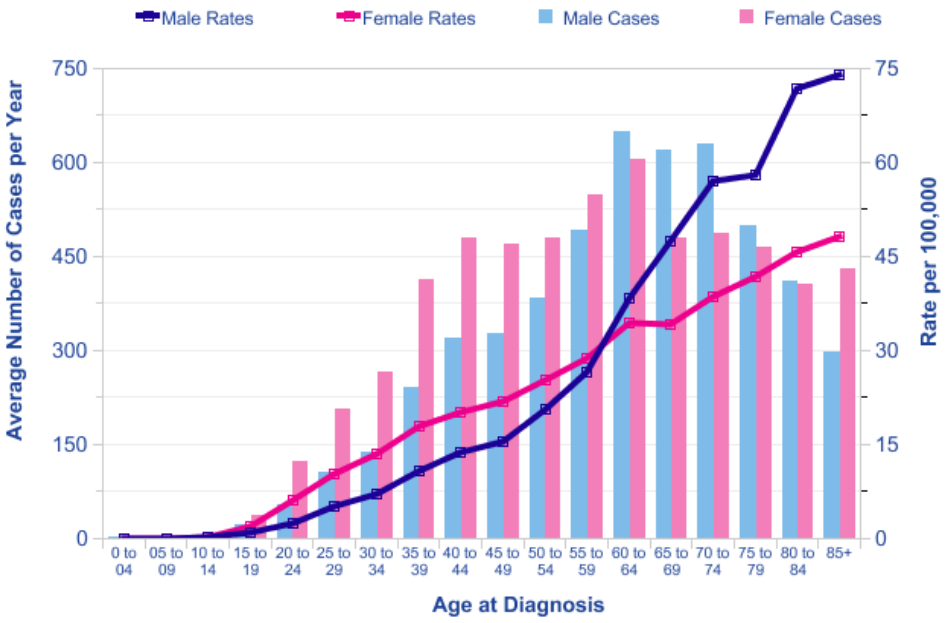

Figure 1-1 Age specific incidence rate for melanoma in the UK 2006-2008. (4)

Whilst most melanomas are cured with surgical resection, a significant proportion of cases will recur, either locally or in the form of metastatic spread after resection. Occasionally melanomas present with the symptoms of metastatic spread.

Prognosis for patients diagnosed with melanoma is largely dependent upon the American Joint Committee on Cancer (AJCC) disease stage, which is determined by tumour thickness (Breslow depth), mitotic count, ulceration and the presence or absence of local or distant disease spread. The basic staging system is outlined in table 1-1 (8). Metastasis often occurs early in tumour development and at present there is no effective systemic treatment once metastasis has occurred. The prognosis for those with disseminated disease is poor, with a 5 year survival of less than 10% (8).Over 2000 patients die each year from melanoma in the UK (9).

Stage I	Primary melanoma ≤ 1.00 mm in depth, or ≤2.00 mm if no ulceration. No distant spread.
Stage II	Primary melanoma with depth > 2.00 mm, or > 1.00 mm if ulcerated. No distant spread.
Stage III	Spread to local lymph nodes.
Stage IV	Distant metastasis.

Table 1-1 AJCC 2009 staging system for melanoma (8).

1.1.2 Melanoma Risk Factors

Risk factors for the development of melanoma can be either hereditary or environmental. Hereditary factors include skin type, hair colour, family history and the presence of multiple naevi on the skin. Some families harbour specific defects in genes such as the tumour suppressor gene *CDKN2A*. For these families all those with the affected gene are at increased risk of melanoma (10).

The most important environmental risk factor for melanoma is exposure to UV radiation (11). The vast majority of UV radiation exposure comes from direct sunlight although some individuals do subject themselves to artificial UV radiation in order to tan their skin. Epidemiological studies suggest that occasional high dose exposure to UV radiation sufficient to cause sunburn is much higher risk for the development of melanoma than prolonged UV exposure, whereas prolonged sun exposure is believed to be more important in the causation of non-melanomatous skin cancers such as basal cell carcinoma (12).

1.1.3 Melanoma Diagnosis

When a patient presents to medical services with a pigmented lesion, the lesion is examined to assess whether it may represent a melanoma. The classical clinical

features of melanoma are summarised in both the ABCDE criteria, (detailed in table 1-2) (13) and the Glasgow seven point checklist (detailed in table 1-3) (14), both of which have been developed as an aide to clinicians examining these lesions. A photograph of a typical melanoma is shown in figure 1.2.

A	Asymmetry
B	Border is irregular
C	Colour is variegated
D	Diameter greater than 5mm
E	Evolution over time

Table 1-2 ABCDE criteria for the clinical diagnosis of malignant melanoma (13).

Major features	**Minor features**
Change in size	Diameter > 7 mm
Irregular shape	Inflammation
Irregular colour	Oozing
	Change in sensation

Table 1-3 The Glasgow seven point checklist used to aid the clinical diagnosis of melanoma (14).

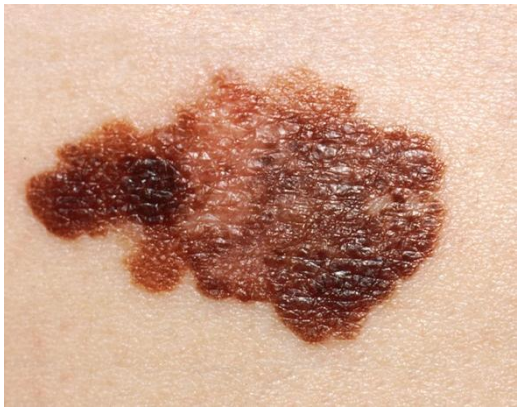

Figure 1-2 Typical macroscopic appearances of malignant melanoma. (Image sourced from the National Cancer Institute Visuals Online; image reference AV-8500-3850)

Many thousands of melanocytic lesions, benign and malignant, are excised and sent for histopathological analysis every year, to diagnose or exclude malignant or dyplastic lesions, for cosmetic reasons or because lesions are causing physical irritation.

Histopathological analysis is the gold standard for diagnosis of melanocytic tumours, as it is with the majority of tumour types. In the vast majority of melanocytic lesions a confident diagnosis of benign naevus or malignant melanoma can be made on a standard haematoxylin and eosin histology stained tissue section alone and the histological features of melanocytic lesions are well described and recognised (15). Within a small subset of melanocytic lesions however, it can be very difficult to differentiate between melanoma and naevus based upon light microscopy and even specialist dermatopathologists cannot agree over the classification of a subset of cases (16-19). As a result the misdiagnosis of melanocytic lesions accounts for a significant proportion of the litigation claims against histopathologists (20).

There are certain classes of melanocytic lesion which account for most of the difficult or erroneous diagnoses when distinguishing between naevi and melanoma. This includes Spitz naevi and Spitzoid melanoma, nevoid melanoma, traumatised naevi, mitotically active areas within congenital naevi and the differential diagnosis between severely dysplastic naevi and melanoma (21).

Diagnostically difficult melanocytic tumours may result in either misdiagnosis of a melanoma or naevus, or a patient being given the inconclusive label of a tumour with uncertain malignant potential. The medical and psychological consequences to the patient of an erroneous diagnosis of melanoma or naevus can be catastrophic. In those cases in which a definite diagnosis cannot be made this causes significant

uncertainty in planning treatment and predicting outcome, with potentially major social, psychological and financial repercussions for the patient. The difficulty posed by such lesions has led to attempts by expert dermatopathologists to produce diagnostic criteria based on histological features (in order to improve diagnosis) (22,23). Despite this, ambiguous lesions remain a problem and these lesions have prompted the search for an effective diagnostic biomarker for melanoma (24).

Data from the US indicates that despite an increasing incidence of melanoma (doubling over 15 years), the mortality rate has remained unchanged, even though only a minority of individuals are screened for melanoma by their dermatologist (7). In the UK the rate of melanoma has risen fivefold in 33 years (6), but with only a modest corresponding increase in mortality over the same period. This data raises the concern that either melanoma is being over-diagnosed by histopathologists, which may be partly due to medico-legal pressure not to 'miss a melanoma' (25), or that increased biopsy rates have led to more small, slow growing melanomas being diagnosed which fit the histological criteria for diagnosis of a melanoma, but are very low risk for metastasis. Possible slow growing low-risk melanomas might be analogous to the indolent, low grade prostate cancers which occur in elderly males, are asymptomatic and have long been described as being detected incidentally at post mortem (when an individual dies of other causes) (26). As melanomas, unlike most malignant tumours, are not given a grade of histological differentiation which is used to predict behaviour, it is not possible to determine whether the increased diagnosis rate is matched by an increase in the number of low-grade lesions being diagnosed, as the second explanation would suggest.

If either of these explanations for the notable recent increase in melanoma incidence hold any truth, then either a diagnostic biomarker (for the first explanation) or prognostic biomarker (for the second) would be of clinical value in redefining which patients diagnosed with primary melanoma are truly at long term risk from their disease.

1.1.4 Melanoma Prognostication

As mentioned above, the prognosis for patients diagnosed with malignant melanoma is currently based upon disease stage, additional histopathological features and some clinical information. Disease stage reflects the Breslow depth, surface ulceration, mitotic index and disease spread. Breslow depth, the presence of surface ulceration and local spread (tumour microsatellites) are determined by histopathological analysis of the tumour and the additional histopathological features which can influence prognosis to a lesser extent are evidence of regression and tumour infiltrating lymphocytes. Clinical information which is of potential prognostic importance is tumour site, patient age and gender. These features are included in the histopathology reports for melanoma and are used to define the stage of disease, based on the internationally recognised AJCC melanoma staging system (8). In the UK, all of the histopathological features used to determine disease stage plus comment on the presence or absence of tumour regression and tumour infiltrating lymphocytes are recommended for inclusion in the Royal College of Pathologists national dataset for skin cancer reporting (27)and are included in the American reporting guidelines produced by the College of American Pathologists (28).

1.2 Genetic Changes in Melanoma

All neoplasms are characterised by genetic changes and malignant tumours typically show a wide range of DNA alterations. Although each individual melanoma undergoes its own pattern of clonal events and shows an individual pattern of genetic alteration, as with most neoplasms, melanomas as a whole are characterised by certain somatic genetic alterations which characterise the tumour type. These changes include deletions, mutations and amplifications at certain loci, epigenetic alterations such as changes in DNA methylation and genome wide variations in DNA copy number (DCN) which occur as a result of chromosomal instability (29).

1.2.1 Chromosomal Instability in Melanoma

Genomic instability is a well-described feature of malignant tumours and has been studied for several decades (30,31). Changes in DCN can include whole chromosomal copy number loss or gain, formation of isochromosomes (where one chromosome arm is gained and the other arm is lost), and gains or losses of specific genes or regions within a chromosome. Studies using comparative genomic hybridisation (CGH) and array CGH have been used to investigate copy number profiles in malignant tumours since 1992 (32). It has subsequently been described that specific tumour types show typical patterns of somatic copy number alteration across their tumour genome and that these patterns vary between different tumour types (33,34). Array CGH is a technique in which DNA from a test sample and DNA from a diploid control sample are labelled with differing fluorescent tags before being co-hybridised to thousands of individual probes. The resultant hybridisation is detected by fluorescent fluorophores. The comparative fluorescence between the test and control sample is

used to establish any difference in DCN between the control and the test sample at the loci tested.

The array CGH method is an advancement on the original CGH technology. In the original CGH methods DCN change could be assessed for whole chromosomes or chromosome regions, but did not have the resolution to detect copy number changes in individual genes as with array CGH.

Genomic instability appears to indicate a worse prognosis for solid tumours (35) and has been described as conferring tumours with multidrug resistance (36). It is thought to occur as a result of several mechanisms including defects in the mitotic spindle assembly checkpoint (37). Translocations and deletions have also been described as occurring during repair of double stranded breaks in DNA or through the premature mitosis of cells with damaged DNA (38).

The CGH pattern of genomic instability seen in melanoma has been shown to vary from the changes seen in naevi, with the vast majority of melanomas showing a wide range of chromosomal aberrations (39-41). Naevi typically show little if any evidence of chromosomal instability, with one isolated region of common copy number gain described in Spitz naevi (42) (further described in section 1.3.2). The typical pattern of chromosomal instability as described by Baudis in a series of 99 primary melanomas is shown in figure 1.3 (33).

The largest scale studies using CGH to determine somatic copy number alteration in primary cutaneous melanoma were performed by Bastian et al (39,40) and Baudis(33).

These have detailed patterns of chromosomal copy number gain and loss in melanoma and the regions commonly lost or gained are summarised in table 1-4.

Study	Chromosomal regions of gain	Chromosomal regions of loss
Bastian et al 2003 (132 cases)	6p, 1q, 7p, 7q, 8q, 17q, 20q	9p, 9q, 10p, 10q, 6q, 11q
Curtin et al 2005 (106 cases)	6p, 7, 11q13, 8q, 17q, 20q	6q, 8p, 9p, 10, 11q, 13, 21q
Baudis 2007 (99 cases)	8q21, 6p, 1q, 7, 20q, 11q13	6q24, 10q22, 3, 9p, 13p, 1p 11q23

Table 1-4 Most common chromosomal regions of somatic copy number alteration as described by 3 large array CGH studies of primary cutaneous melanoma DCN variation. (33,39,40)

The study by Bastian et al also tested 54 benign naevi as part of the same study, demonstrating that these tumours show little somatic copy number alteration, with the exception of occasional gains in Chromosome (Ch) 11p and Ch 7 (39).

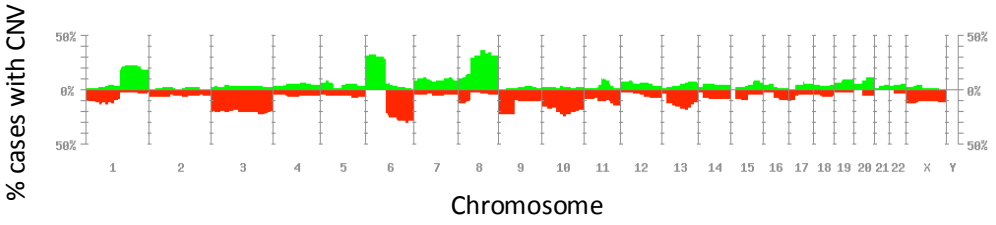

Figure 1-3 Imbalance profile showing regional frequency of genomic gain and loss, from CGH analysis of 99 malignant melanomas with gain shown as green and loss as red (reproduced from Baudis et al) (33).

1.2.2 Commonly altered genes in melanoma

There are several proto-oncogenes and tumour suppressor genes which are frequently altered in melanoma and characterise the genetic alterations seen in this tumour, their interactions are summarised in figure 1-4. The most well-characterised of these

are the proto-oncogene *BRAF* (43) and the tumour suppressor genes *CDKN2A* (44)and *PTEN* (45). BRAF is part of the Raf family of serine/threonine kinase enzymes which are involved in regulating the MAP kinase pathway and are therefore crucial for cell division. The *CDKN2A* genes codes for the tumour suppressor protein p16 plus several transcript variants. Protein p16 acts as a cell cycle checkpoint restriction factor by restricting the activity of the cyclin dependent kinases. PTEN inhibits the Akt signalling pathway and is therefore involved in regulating cell proliferation as well as having a role in the triggering of apoptosis.

Gene expression profiling and CGH analysis have identified a wider range of genes which are frequently altered in melanoma. Some of those which are believed to be important in the development of melanoma include the well-described tumour suppressor genes *RB* and *p53* (46,47). Changes in gene methylation of two further tumour suppressor genes, *APAF1* (48) and *APC* (49) have also been described as common events. The oncogenes *MITF* (50), *TBX2* (51) and *MYC* (52) are all found to be frequently amplified in melanoma, whereas *NRAS* and *Beta-catenin* may undergo activating mutations as part of melanoma development (53,54).

Genome wide sequencing analysis of melanoma cell line DNA has shown a wide range of mutations not previously described in melanoma and evidence of widespread DNA damage due to long term UV exposure (55). This has led to identification of further genes thought to be involved in the development of melanoma. Activating mutations in the gene *RAC1* were detected in 9.2% of melanomas sequenced by Krauthammer et al (56). *RAC1* codes for a signalling G-protein involved in regulation of the cell cycle and was also one of several novel genes found to be frequently mutated in a further

study by Hodis et al, which involved sequencing and permutation framework analysis of melanoma genomes (57).

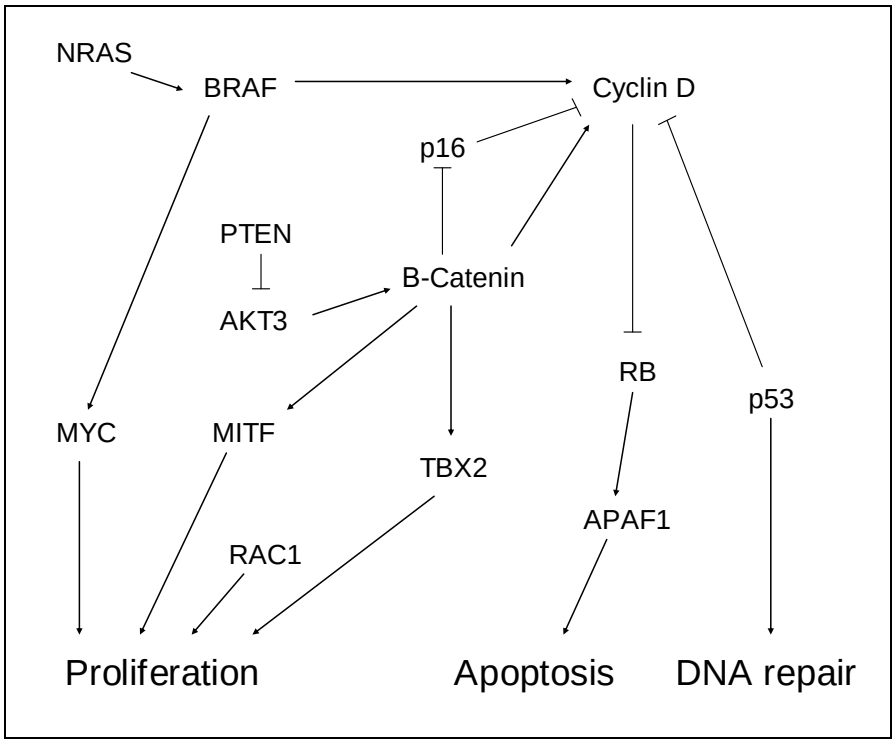

Figure 1-4 Summary of signalling pathways involving proteins known to be genetically altered in melanoma and their effect on cell proliferation, apoptosis and DNA repair.

1.3 Biomarkers in Melanoma

There are 4 important classes of tissue and serum biomarkers which could be of clinical use in the management of patients with melanoma. Diagnostic biomarkers have the potential for use in ambiguous melanocytic lesions to identify those which are truly malignant. Prognostic biomarkers are those which predict behaviour independently of the prognostic features used currently such as Breslow depth. Progression markers would have the ability to detect recurrent disease at an early stage as a circulating serum marker. Finally, predictive markers are those which predict response to a specific therapy.

This research project is focused upon the development of potential diagnostic and prognostic assays, and putative melanoma biomarkers in the diagnostic and prognostic classes described in the literature are therefore reviewed in sections 1.3.2 and 1.3.3.

1.3.1 The effect of formalin fixation and paraffin embedding in biomarker development

In order for melanocytic lesions to be reliably diagnosed and for optimum prognostic assessment of melanoma, these specimens are routinely formalin fixed in their entirety and, as most of the lesions are small, are then frequently embedded in paraffin in their entirety. Whilst this improves sampling and therefore aids diagnosis and prognostication using light microscopy, there is no opportunity for fresh tissue to be stored from the lesion. Formalin fixation causes cross linking and degradation of DNA and similarly alters RNA and although DNA can be retrieved from formalin fixed paraffin embedded (FFPE) tissue, it is typically of lower yield and quality than DNA extracted from fresh frozen samples. The size of the specimen along with the fixation period, storage period and storage conditions are all factors affecting the quality and yield of extracted DNA and RNA (58).

Due to the current histopathological laboratory practices discussed, any tissue biomarker developed for diagnosis or prognosis in melanoma must be applicable for use on DNA extracted from FFPE tissue.

1.3.2 Diagnostic Melanoma Biomarkers

Putative diagnostic melanoma biomarkers include those targeting characteristic changes in DNA seen in melanoma, those profiling RNA expression and proteins detected by immunohistochemistry (IHC) studies.

As mentioned in 1.2.1, a study investigating array CGH profiles of different melanocytic lesions reported that amongst benign naevi, only a small number showed specific gains on the short arm of Chromosome 11 and in the distal part of chromosome 7 (39). Comparisons of the copy number alterations between Spitz naevi and melanoma using this method have been widely reported in further studies and a specific isolated change at Ch11p is a well-recognised molecular characteristic of benign Spitz naevi (42,59). In a study by Ali et al, the pattern of copy number aberrations within a small series of Spitzoid tumours was predictive of tumour behaviour with the typical gains on Chromosome 11 and 7 seen in those behaving in a benign fashion and multiple chromosomal abnormalities seen in Spitzoid melanomas (59). There are case reports of borderline melanocytic lesions in which CGH analysis correctly predicted clinical outcome, despite in some cases a contrary histopathological diagnosis. These reports are suggestive as to the potential for copy number analysis to refine diagnosis (60,61).

Congenital naevi are potentially problematic melanocytic lesions for histopathological analysis. In a series of congenital naevi studied using CGH no significant aberration in DCN was identified in lesions without histological evidence of proliferation or those with conventional proliferative nodules. Those congenital naevi with proliferative nodules, which are benign but can ‘mimic’ melanoma histologically, displayed

chromosomal aberrations entirely different to those with nodular melanoma arising within a congenital naevus, which were of the same copy number pattern as conventional nodular melanoma (62). This is another area of problematic and borderline melanocytic pathology in which CGH may predict outcome more accurately than conventional histopathology.

In summary there is data supporting the use of CGH as a method for separating benign from malignant lesions in the borderline melanocytic tumours encountered in clinical practice. To further test its clinical applicability, the method warrants testing against a wide spectrum of benign, malignant and borderline melanocytic lesions. There are prohibitive factors for widespread use of CGH analysis at present however. These factors include the current cost of CGH analysis and the expertise and time required to analyse the large quantity of bioinformation produced. Array CGH is currently established in a handful of specialist centres such as University of California San Fransisco (UCSF) as the gold standard for DCN analysis in melanocytic tumours.

Using the information generated from CGH studies, Gerami et al have demonstrated that Fluorescent In Situ Hybridisation (FISH) can be used to differentiate between naevi and melanoma (63). The assay is a combination of 4 FISH probes (MYB, RREB1, CEP6 and CCND1). This is the only melanoma diagnostic biomarker to have been translated into clinical practice and to have become commercially available. Although the assay has shown good sensitivity and specificity in several studies comparing unambiguous melanoma and naevi, diagnostic accuracy has been less impressive when tested against genuinely ambiguous lesions with follow-up (64,65). A large scale recent study of this assay by an independent research group found that the sensitivity

and specificity of the FISH assay for detecting melanoma on straightforward cases were 85% and 90% respectively, although this was decreased to 45% and 80% when 90 ambiguous tumours with follow-up were tested (64).

Immunohistochemistry (IHC) biomarkers have the advantage of using an established technique which is widely used in routine practice. There are several IHC markers which have shown promise in differentiation between naevi and melanoma, either as single markers or as part of a diagnostic panel. Many of the markers have been tested only on histologically straightforward benign naevi and malignant melanoma, and although some have been tested on problematic melanocytic lesions, the number of cases tested is usually small. Few studies have tested a large set of borderline lesions.

The most widely studied proliferation marker is Ki-67 (66,67). Whilst naevi routinely express a proliferation fraction (cells positive for Ki-67) of less than 5%, compared to 13-30% for melanoma, the proliferation fraction is normally higher in Spitz naevi and dysplastic naevi (up to 15%) (67). Ki-67 has also been used in a panel in combination with the IHC mitosis marker phospho-Histone H3 (pHH3), and survivin, a protein encoded by a member of the inhibitor of apoptosis (IAP) family. Differentiation between Spitz naevus and melanoma within this series was dependent upon the pattern of staining for pHH3 in the superficial dermal component of the lesion for Spitz naevus compared with superficial and deep dermis in melanoma, with no study of inter-observer variability for this scoring. The subjective parameters used to differentiate the lesions in this study are further compounded by the small numbers of cases, with only 8 Spitz naevi studied (68).

Kashani-Sabet et al investigated an IHC assay using the several immunomarkers; ARPC2, FN1, RGS1, SPP1 and WT2. Actin related protein complex 2/3 subunit 2, (ARPC2) is a subunit of a protein complex implicated in actin polymerisation. Fibronectin (FN1) is a glycoprotein cell adhesion molecule. Regulator of G-protein signalling (RGS1) acts to attenuate G-protein signalling. Osteopontin (SPP1) is a protein known to have several functions including acting as an anti-apoptotic factor. Wilm's Tumour 2 (WT2) is a putative tumour suppressor gene implicated in Beckwith Wiedemann Syndrome. Using a scoring algorithm developed from a tissue microarray (TMA) training set, the panel was tested against a set of problematic and borderline lesions, correctly diagnosing 18 of 24 previously misdiagnosed melanocytic lesions (75%). As with the panel developed by Nasr et al, scoring with this panel was dependent upon judgement of where in the lesion (superficial or deep) the marker was expressed and again inter-observer variability for this method has not been described (69).

Expression of the protein S100A6, a member of the S100 family, has been investigated for use in the specific differential diagnosis between melanoma and Spitz naevi, showing a clearly different pattern of expression between melanoma and Spitz naevus in all of the cases studied, with diffuse strong staining for all Spitz naevi and patchy weak staining for melanoma (70). Hilliard et al demonstrated that expression of the tumour suppressor gene p16 differentiated between desmoplastic melanomas and desmoplastic Spitz naevi in a small set of cases (71).

In a tissue microarray study, expression of the kinase Cdc7 was found to differ between dyplastic naevi and melanoma, and to differ between Spitz naevi and

melanoma, but did not differentiate between atypical Spitzoid tumours and melanoma (72). As yet these results have not been replicated on full histological sections. IHC for the heat shock protein HSP105 in other tissues has shown increased expression in malignancy and a study by Park et al shows a clearly differing pattern of expression between melanoma and benign naevi, though this has not been studied in ambiguous or problematic lesions such as Spitz naevi (73). Expression of the cyclo-oxygenase enzyme (Cox-2), when studied using image analysis, was found to differentiate between melanoma and benign naevi, but again there has been no study of the of the pattern of expression in borderline lesions (74).

Whilst numerous IHC studies have demonstrated different patterns of protein expression across a range of melanocytic lesions, none of the putative IHC biomarkers have been shown to differentiate reliably between benign and malignant in a full range of borderline melanocytic lesions. These findings suggest that the most likely means of successfully developing a robust and clinically useful IHC biomarker would be through the development of an IHC panel, with a combination of antibodies providing a more robust test of benignity or malignancy than any single marker. Thus far Kashani-Sabet et al (69) have described the most successful panel for discriminating benign and malignant lesions, with good, though not definitive, accuracy in problematic and borderline lesions.

Methods used to quantify RNA expression in melanocytic lesions include RNA expression arrays, Reverse Transcriptase Quantitative Polymerase Chain Reaction (RTQPCR) and microRNA studies. RNA expression data using formalin fixed tissue has shown good, though not absolute, separation of melanoma from naevi using cluster

analysis from hybridisation to a DNA chip (75), though the sets of cases used in this study did not include problematic or borderline lesions. The use of expression microarrays on fresh frozen tissue may be less transferable to use in diagnosis, but these studies do have value for discovery of markers which differentiate between benign and malignant, such as PLAB and L1CAM (76) or those used by Kashani-Sabet et al described above (69). Most of these studies have focused on the identification of a prognostic marker for melanoma, rather than on the diagnosis of borderline lesions.

Lewis et al demonstrated that it was possible to classify melanomas using multiple targets for RTQPCR and performing a hierarchical cluster analysis, though the groupings identified have not been correlated with clinical outcome and the study was performed on frozen rather than fixed tissue. Whilst it would be of interest to identify how expression profiles vary across a range of borderline and problematic melanocytic lesions, the current technical difficulty of using this method on FFPE tissue would limit its clinical usefulness (77).

Research by Glud et al demonstrated that microRNA is well preserved in formalin fixed tissue from benign naevi, when compared with microRNA from fresh frozen tissue from the same specimens (78). Whilst there is no published data formally evaluating microRNA biomarkers for diagnosis in melanocytic lesions at the time of writing, Philippidou et al demonstrated the differing microRNA expression profiles between naevi and primary and metastatic melanoma (79). Given the suitability of this technique for use in FFPE tissue, microRNA RTQPCR could be developed into a successful diagnostic biomarker, especially within the cohorts of problematic and borderline tumours.

1.3.3 Prognostic Melanoma Biomarkers

Despite the efforts of histopathologists to stage tumours accurately and the wide array of information used to prognosticate those individuals diagnosed with a primary melanoma, melanomas which are morphologically similar often behave differently (80,81). This unpredictability makes accurate prognostication difficult, leading to uncertainty both for patients and for clinicians planning their management. This has led to a search for tissue markers which may improve the prognostic accuracy of the current AJCC criteria. As new systemic therapies for melanoma such as Vemurafenib are developed (82) it would be desirable to have a more accurate means of identifying which patients require more aggressive treatment.

Numerous IHC studies have described changes in expression of various cell cycle, cell signalling and cellular adhesion proteins thought to be involved in tumour progression in melanoma (24,83). Whilst this has identified many potential IHC biomarkers and has broadened the understanding of the cellular processes involved in disease progression, few have reported that expression is a significant risk factor for metastasis or for death when tested using multivariate analysis that includes the clinical data such as patient age and tumour Breslow thickness.

Mitotic count is currently one of the key prognostic factors in the AJCC criteria (8). Ladstein et al described that counting cells positive for the cell proliferation marker Ki-67 on IHC sections of nodular melanomas was a stronger prognostic indicator than mitotic count (84). Previously Ki-67 expression in thin melanomas (Breslow< 1mm) had been found to be a more accurate predictor of tumour behaviour than mitotic count (85,86). Studies in other tumour types reveal that mitotic count is a potentially

unreliable marker, due to a significant degree of inter-observer variability in both the counting and recognition of abnormal mitosis (87). There is therefore an argument that IHC for Ki67, a test which is readily available, could replace mitotic count as part of the AJCC criteria as a simple step to improve accuracy and uniformity in prognostication.

In the largest published prognostic IHC study using full histological sections, Weinlich et al used IHC to test for expression of Metallothionein (MT) I and II (88,89). These intracellular proteins with a high affinity for heavy metal ions were detcted using an antibody which reacts with a conserved epitope of both MT I and MT II isoforms. Over 1000 cases were included in the study with patients recruited between 1993 and 2004, and subsequent follow-up data was recorded. Over-expression of Metallothionein was identified as an independent prognostic factor on multivariate analysis. Validation of this large and statistically powerful study is yet to be published.

In smaller study also using full histological sections, Micropthalmia transcription factor (MITF) was investigated using IHC in a series of 63 intermediate thickness melanomas (1.0mm-4.0mm). This demonstrated that MITF staining pattern was an independent predictor of disease specific and overall survival on multivariate analysis, although inter-observer variability for scoring of the antibody was not described (90). Whilst no follow-up IHC studies have been done since publication of this study in 2000, MITF has been recognised as a strong potential biomarker in melanoma, targeted in several Quantitative Polymerase Chain Reaction (QPCR) tissue studies and used as a marker for detecting circulating tumour cells (77,91-93).

Wnt5a is a ligand in the Wnt signalling pathway, inhibiting B-catenin signalling. Research from our department has previously described Wnt5a expression as a prognostic marker in a series of 90 melanomas, with cytoplasmic Wnt5a expression found to be a marker of poor prognosis on multivariate analysis (94).

Kashani-Sabet et al developed a multimarker prognostic assay based on three IHC markers: NCOA3, RGS1, and SPP1 (69). The proteins RGS1 and SPP1 have been described in section 1.3.2. NCOA3 is a nuclear steroid receptor co-activator. The staining patterns for each of these markers were scored to give an overall multimarker index. Higher index scores were significantly predictive of reduced disease specific survival and the likelihood of positive sentinel lymph node biopsy, independent of the other predictive factors. This assay was first performed on a test set of 395 melanomas using a tissue microarray, and underwent validation on an independent set of 141 melanomas, using full histological tissue sections (95).

Gould-Rothberg et al tested a wide range of molecules involved in melanoma oncogenesis using the Automated Quantitative Analysis (AQUA) method for immunofluorescence-based IHC on a tissue microarray of melanomas with follow-up data (96). Five key markers were identified on the discovery set: Activating Transcription Factor 2 (ATF2), the cell cycle regulator p21, the tumour suppressor protein p16, β catenin, part of the Wnt signalling pathway, and Fibronectin, an extracellular glycoprotein. Expression thresholds were set using a discovery set of melanomas and an algorithm established to determine whether a melanoma was to be classified as high or low risk for metastasis. This method was then applied to a validation set of melanomas, to classify the tumours into low and high risk. The

validation set consisted of a further tissue microarray using 246 independent cases derived from the same archive as the discovery set. The high risk group were at increased risk of melanoma specific mortality on multivariate analysis (a 60% 10-year survival rate in the high risk group compared with 90% in the low risk group). Whilst this method has only been tested on a tissue microarray and would still require validation on full histological sections of melanoma samples, it does have the attractive advantage over conventional IHC of using an automated scoring method, thereby resolving the problem of intra- and inter-observer variability with IHC scoring, though access to the technology and expertise required may prove a barrier for the use of this method in routine practice.

A large scale study by Winnipennickx tested a set of melanomas, for which frozen tissue and follow-up data was available, using array technology to assess RNA expression (97). They were subsequently classified, using unsupervised hierarchical clustering, into two groups based on pattern of expression, with the different groups showing significantly differing survival profiles. Using this data, corresponding IHC markers were tested against a large set of melanomas to identify whether expression of the markers had an impact upon survival, with only staining for MCM4 and MCM6, two DNA replication licensing factors, showing a statistically significant impact on multivariate analysis (97).

Haqq et al investigated primary and metastatic melanomas using gene expression to perform hierarchical clustering and found that melanoma metastases could be separated into 'Type I' and 'Type II' based on the clustering (98). Whilst only small numbers were analysed (too small for statistical analysis), Type I metastases appear to

prove more lethal than Type II and these results suggest that gene expression profiling of metastases may be worthy of further investigation on a more extensive set of cases (98). This study identified several genes which varied in expression between naevi and melanoma or between primary and metastatic melanoma. The data was used to select the IHC biomarkers used in the multimarker panel described by Kashani-Sabet et al (above) (95).

Conway et al described the use of complementary DNA (cDNA) mediated Annealing, Selection, extension and Ligation (DASL) technology for the quantification of RNA in tumours (99). DASL technology quantifies expression of several hundred mRNAs and, crucially, it can be applied to FFPE tissue, thus bridging the gap between mRNA expression arrays and use of FFPE samples. This method examined the expression of 502 genes in a large series of melanomas, identifying that increased expression of the gene Osteopontin was most strongly associated with a reduction in relapse-free survival and verifying this on a validation set. On multivariate analysis however, Osteopontin expression was not a significantly independent factor when Breslow depth was included as a variable. Interestingly, Osteopontin was also assayed by Kashani-Sabet et al (see above) in their multimarker IHC assay where increased Osteopontin expression was associated with worse prognosis (95).

MicroRNA expression signature was investigated using array technology in a series of FFPE melanoma metastases by Segura et al (100). A predictor value was constructed from the microRNA signature which correlated to survival. When the method was applied to the same series, microRNA expression signature was found to be a significant post-recurrence survival factor independent of disease stage on Cox

proportional hazard regression analysis, although this prognostic assay has not been tested on primary melanomas.

Three microRNAs were identified as potential diagnostic markers by Satzger et al, using real-time (RT) PCR on a small series of FFPE melanomas and naevi. When tested on a larger set of 128 melanomas with follow-up data, upregulation of one of the microRNAs (miR-15b) was found to be a significant independent predictor of overall and disease free survival (DFS) on multivariate Cox analysis (101).

Jonsson et al (102) performed array CGH analysis on a series of 57 stage IV melanomas alongside gene expression analysis. Using the expression analysis data, these cases were categorised into 4 subtypes. The 'proliferative' subtype, in which loss of the 9p21 region was most common, was associated with a worse prognosis on univariate analysis. These findings were validated on melanoma gene expression data sets from other research groups.

Conway et al (103) described the use of Multiplex Ligation-Dependent Probe Amplification (MPLA) in FFPE melanoma samples, to identify the 'gene dosage' of loci in the Chromosome 9p21 region (including *CDKN2A*, *CDKN2B* and *MTAP*). This study identified that within a series of 75 vertical growth phase melanomas, reduced gene dosage of the 9p21 region overall and the specific regions coding for *CDKN2A* were associated with tumour recurrence on univariate analysis, though multivariate analysis was not reported for these findings.

At present there are no prognostic melanoma biomarkers that have been developed beyond the 'early' phase trials, as the few which have undergone robust testing and

validation on retrospective cohorts need to be tested in prospective studies. Details of the largest and most promising prognostic tissue melanoma markers are summarised in table 1-5 and the array based studies for melanoma prognosis are detailed in table 1-6.

biomarker name	feature associated with poor prognosis	assay type	FFPE or frozen tissue	discovery			validation					predicts outcome on MVA or UVA	which variables in multivariate analysis	outcome	effect size*	p value*
				size	cohort	TMA	size	cohort	TMA	independent	same archive as discovery					
Ki67 as alternative to mitotic count	Ki67 ↑	IHC	FFPE	202	Y	N						MVA	B M U	DSS	HR 3.1	0.003
Metallothionein	MT ↑	IHC	FFPE	1270	Y	N						MVA	B U S C A G	OS	RR 3.49	<0.001
MITF	MITF ↓	IHC	FFPE	63	N	N						MVA	B M U S H	OS	NSp	0.011
Wnt5a	cytoplasmic expression ↑	IHC	FFPE	94	N	N						MVA	B S G A	OS	HR 2.91	0.003
BRMS1	BRMS1 ↓	IHC	FFPE	137	N	Y						MVA	A G St	DSS	RR 0.51	0.02
MCAM (MUC18)	MCAM ↑	IHC	FFPE	120	N	Y	78	N	Y	Y	Y	MVA	B A U G	SNP	HR 14.8	0.01
SNF5	SNF5 ↓	IHC	FFPE	88	N	Y						MVA	A G B S U H	OS	RR 5.1	0.01
BCL6, Ki67, p16 & p21 mutlimarker	p21 ↓, p16 ↓, ki-67 ↑, BCL6 ↑	IHC	FFPE	60	Y	Y	72	N	Y	Y	Y	MVA	B, others NSp	OS	NSp	0.001
NCOA3, RGS1 & Osteopontin multimarker	NCOA3 ↑, RGS1 ↑, Osteopontin ↑	IHC	FFPE	395	Y	Y	141	Y	N	Y	N	MVA	B C U G S A	DSS	RR 1.34	0.01
ATF2, p21, β catenin, p16 & fibronectin multimarker	based on algorithm score	AQUA-IHC	FFPE	192	Y	Y	246	Y	Y	Y	N	MVA	B A G St Mi S	DSS	HR 2.7	0.03
MCM4	MCM4 ↑	IHC	FFPE	62	N	Y	176	N	Y	Y	N	MVA	B U A G	OS	HR 4.04	0.01
MCM6	MCM6 ↑	IHC	FFPE	62	N	Y	176	N	Y	Y	N	MVA	B U A G	OS	HR 7.42	0.003
Osteopontin	Osteopontin ↑	DASL	FFPE	156	Y	Y	198	N	Y	Y	N	MVA	A G S *(NS when B included)*	RFS	HR 1.67 *(1.24)*	0.006 *(0.32)*
miRNA expression signature	expression pattern	miRNA expression array	FFPE	59	N	N						MVA	St	OS	HR 3.16	0.0029
miR-15b	miR15-b ↑	miRNA RT-PCR	FFPE	128	N	N						MVA	A B U	OS	HR 0.41	0.013
Gene expression subtype	gene expression subtype	expression array	frozen	57	N	N	44	N	N	Y	Y	UVA		OS	NSp	0.04
INK4A	INK4A ↓	RT-PCR	FFPE & frozen	86	N	N						UVA (NS on MVA)		OS	NSp	0.006
9p21.3 gene dosage	9p21.3 ↓	MPLA	FFPE	75	N	N						UVA		relapse	NSp	0.04

*Based on validation set data where available. Key: A = age, B = Breslow thickness, C = Clark's level, DASL = cDNA mediated Annealing, Selection, extension and Ligation, DSS = disease specific survival, G=gender, FFPE= formalin fixed paraffin embedded, H= histological subtype HR = hazard ratio, IHC = immunohistochemistry, M= mitoses, Met = metastasis, Mi = microsatellite lesions, miRNA = microRNA, MPLA = multiplex ligation-dependent probe amplification, MVA = multivariate analysis, N = no, NA = nuclear area, NS = not significant, NSp = not specified, OS = overall survival, RFS = relapse free survival, RR = relative risk, S = site, SNP = sentinel node positivity, St = AJCC Stage, TMA = tissue microarray, U = ulceration, UVA = univariate analysis, Y = yes.

Table 1-5 Summary of prognostic tissue biomarker studies in melanoma, including detail of discovery and validation studies and outcome prediction for each marker.

author	assay type	number of cases in array (discovery / validation)	fixed or frozen tissue	validated on an independent set?	method used to identify biomarkers from array data	methods used to prevent false discovery and overfitting	biomarkers identified as a result of the array data
Winnipennickx et al	RNA expression array	58	frozen	N	class comparison and class prediction analysis	Benjamini and Hochberg method	MCM4 & MCAM6 IHC
Haqq et al	RNA expression array	NSp	frozen	N	multiclass significance analysis	SAM software	NCOA3, RGS1, Osteopontin multimarker IHC assay
Conway et al	mRNA - DASL	156 / 198	FFPE	Y	Cox proportional hazards ratio for each gene	Bonferroni method	Osteopontin
Segura et al	miRNA expression array	59	FFPE	N	Cox regression co-efficient	SAM software	miRNA expression signature
Jonsson et al	array CGH	57 / 44	frozen	Y	hierarchical clustering and two group SAM	SAM	9p21 homologous deletion

Key: CGH = comparative genomic hybridisation, DASL = cDNA mediated Annealing, Selection, extension and Ligation, FFPE = formalin fixed paraffin embedded, IHC = immunohistochemistry, miRNA = microRNA, N = no, NSp = not specified, SAM = significance analysis of microarrays, Y = yes.

Table 1-6 Summary of array-scale prognostic tissue studies in melanoma.

1.4 QPCR as a melanoma biomarker

Polymerase Chain Reaction (PCR) is a method first described by Kary Mullis et al in 1986 (104) and for which he subsequently received the Nobel Prize for chemistry. The principle of PCR is to amplify small quantities of DNA though a series of doubling reactions. PCR allows tiny amounts of DNA from a region of interest to be amplified up to the level at which it becomes detectable. A more recent development is real time, or Quantitative PCR (QPCR). This differs from PCR in that with each cycle a fluorescent dye allows the target DNA to be accurately quantified and therefore comparisons can be made between different samples or targets as to the amount of starting target DNA.

1.4.1 Paralogue Ratio Tests and Duplex Ratio Tests

Our laboratory has previously investigated the use of a QPCR-based assay called a Paralogue Ratio Test (PRT) (105) for the purpose of detecting DNA copy number (DCN) variations (106). PRTs target genetic paralogues. Paralogues are two genetic sequences at different loci which have identical or highly conserved sequences. Amplicons for PRTs are chosen such that the two paralogues are amplified using the same primers, but can be distinguished using two different fluorescent probes targeting areas of sequence difference. This compares the quantity of DNA at the 2 genomic sites occupied by the paralogues in the same reaction. The ratio between a pair of targets should be 1:1 in diploid tissue, assuming equal efficiency of the 2 reactions. When the relevant DCN changes are present, such as may occur in genomic instability, the ratio will differ from 1. Paralogue Ratio Tests were developed and tested by previous BSc and MSc students within the department (LD, DB and NH). In

the course of their work, the analytical validity of PRTs has been demonstrated by use on FFPE placental tissue from cases of trisomy, in order to demonstrate a paralogue ratio of 3:2 when a PRT targets one locus on the trisomy chromosome and another on a diploid chromosome, compared with a 1:1 ratio when two diploid loci are targeted (106).

The disadvantage of PRTs is the limited number of suitable paralogue sites in the regions of interest when studying chromosomal instability in melanoma. Therefore previous students in the department (AE and ZM) developed a similar series of assays called duplex ratio tests (DRTs). In these assays 2 different sets of primers and probes are used in the same reaction to compare DNA quantity at two separate loci which are not paralogues and again a ratio is created . Whilst this has the disadvantage of requiring 2 primer sets to be designed and synthesised, there is the advantage that a wider range of loci can be targeted.

Through the work performed by AE and ZM, a panel of 5 DRTs has thus far been shown to differentiate between histologically unambiguous cases of naevi and melanoma in a set of 20 cases presented by our research group (see Appendix 4). Following this small study, the five assays were modified and added to in order to create a final set of seven DRT assays by ZM. The details of the 7 assays are included in table 1-7 and a brief description of their targets in table 1-8.

Assay	Locus	Forward primer	Reverse primer	Probe
BRAF	7q34	5′-TCATGAAGACCTCACAGTAAAAATAGGT-3′	5′-ATCCAGACAACTGTTCAAACTGATG-3′	VIC-AATCTCGATGGAGTGGGTC-MGB
PTEN	10q23.3	5′-GCGACTGCGCTCAGTTCTCT-3′	5′-TCACAGCGGCTCAACTCTCA-3′	FAM-CTCTCGGAAGCTGC-MGB
RREB1	6p25	5′-TGTCCCAATGACGTCAAGTTC-3′	5′-CTACACTCATGACCGCCGAC-3′	FAM-GTTGATGGAAGATAGGTCT-MGB
MYB	6q22-23	5′-GCTTGTACAGAAATACGGTCCGA-3′	5′-GCCACCTCTCCCTACATTGTT-3′	VIC-TTGCCAAGCACTTAAA-MGB
SSR1	6p24.3	5′-CCTTAGATGCCTCATTCCGTTAT-3′	5′-CAGTGTTCAGAGGAAGAGCTGTG-3′	FAM-CAGGACTACCAGTTTTAT-MGB
PERP	6q24	5′-TACTCAGCGCCATCGCCT-3′	5′-AGCATTTCCACCACAGCGAG-3′	VIC-TTGCAGTCTAGCGACCAC-MGB
ASAP	8q24.1-24.2	5′-CAGGCTAAATCTGGAAAGTTCAATC-3′	5′-GTTTGTCATCCAGATCATCATCG-3′	VIC-AATCTTCGACAGGAGGAGATA-MGB
LZST1	8p22	5′-GTGACCACTCTTCTTTAAGCCATAGA-3′	5′-TGGAAAGCCACACCCTCTG-3′	FAM-CCTGGGCTGGGTGC-MGB
CCND1	11q13	5′-TGGTGAACAAGCTCAAGTGGAA-3′	5′-CGCCTCTGGCATTTTGGA-3′	FAM-CCGCACGATTTCATTGA-MGB
LDLRad3	11p13	5′-ACAACGTCAATAATGGCATCCA-3′	5′-GCCTACTTCCGACGCATTCT-3′	VIC-TTGCCAAGCACTTAAA-MGB
TBX2	17q23	5′-GGCCTAGACCGCGTGATAAA-3′	5′-GGTCTACACTGACTTCAGTCGTAACTG-3′	VIC-GGTTGAGGGATGCTGGA-MGB
HIC1	17p13.3	5′-TGTGCGACGTGATCATCGT-3′	5′-CACCACCAGGGACTTGAGGTAG-3′	FAM-AAGAGGGCGTTCTGCA-MGB
AKT3	1q44	5′-CTGGACATCACCAGTCCTAGCTC-3′	5′-ACCCTTGGCTGGTCTGGG-3′	VIC-ATAGCAGGGGCACCTT-MGB
MIB2	1p36.33	5′-CACCAAGCACCACTCCTTCTG-3′	5′-CAGCCGCTTCACTGTGTCAA-3′	FAM-CCGGGTCATCGGCGA-MGB

Table 1-7 The DRT targets for the series of 7 existing DRTs, including sequences for the primers and probes (target from region of gain shaded).

Gain	Gene information	Loss	Gene information
BRAF	Regulates the MAP kinase signalling pathway involved in cell division and differentiation.	**PTEN**	Tumour suppressor known to be frequently altered in melanoma.
RREB1	Transcription factor binding to RAS responsive elements. Locus gained in melanoma.	**MYB**	Transcriptional protein involved in controlling proliferation. Locus a region of DNA loss in melanoma.
SSR1	Endoplastic reticulum membrane receptor. Close to the RREB1 locus on chromosome 6.	**PERP**	Downstream target of the tumour suppressor p53 and close to the MYB locus on chromosome 6.
ASAP	Influences cell motility and is in a region of chromosome 8 known to be amplified in melanoma.	**LZST1**	Tumour suppressor gene in region of common DCN loss for several tumours.
CCND1	Part of the cyclin family involved in inhibition of tumour suppressor protein and regulates cell cycle.	**LDLRad3**	Lipoprotein receptor. Located in a region of DNA loss in melanoma.
TBX2	T-box transcription factor, located in common region of DNA gain in melanoma.	**HIC1**	Tumour suppressor gene frequently deleted in a range of cancers including melanoma.
AKT3	Promotes cell growth, survival and angiogenesis. Deregulated activity promotes melanoma.	**MIB2**	Within genomic region known to contain a putative tumour suppressor gene.

Table 1-8 Genetic relevance of the targeted loci used for the 7 existing DRTs.

DRTs and PRTs benefit from being relatively simple and cheap. A further advantage is that each sample can be tested with multiple assays, meaning that a wide array of DNA loci can be tested, providing a good sample of the relevant genomic alterations in melanoma. In comparison FISH assays are limited to assessing 4 loci. Array CGH is the gold standard, giving genome wide DCN analysis, but is expensive and requires specialist equipment.

1.4.2 Centromeric Repeats in Chromosomal Instability

Centromeric alphoid repeat sequences are non-coding sequences of DNA found in the centromere of each chromosome. The alphoid repeats typically consist of several monomers which show variable homology, with the full sequence then repeated across the centromeric region (107). For most of the chromosomes the described sequences are specific and can therefore be targeted to identify individual chromosomes (108), though there are chromosomes, such as Ch13, which share the alphoid repeat with other chromosomes (in this case Ch21 and Ch22) (107).

DRT and PRT assays utilise changes in single copy amplicons. This can be a problem if there is limited DNA which is of poor quality, as is frequently the case in DNA extracted from FFPE tissue. Centromeric repeats have the advantage that each one exists as multiple copies and should be easier to detect. The targeting of centromeric repeats therefore represents a possible alternative (or addition) to single copy target DRTs. If DRTs targeting centromeric sequences (cDRTs) could be successfully developed to target one frequently gained and one frequently lost chromosome, in which chromosome specific repeats are found, these assays could be used to detect

whole chromosomal genomic instability, potentially operating more successfully than conventional DRTs in cases where the quantity of DNA is lower.

1.5 Translation of Melanoma Biomarkers into Clinical Practice

In order to establish the steps which need to be taken for development of a biomarker in melanoma, we must consider how a biomarker makes its journey from initial discovery to translation into clinical practice and the factors which affect marker development (109). As with drug development, a biomarker must go through a series of study 'phases', each of which are defined by key research questions, in order to be established as a rigorous and well-tested marker. Careful study design is crucial to eliminate bias and also to reduce the possibility of chance findings. Markers must also be tested on a cohort of cases which are representative of the wider population, to ensure that the results of the study are applicable to routine clinical practice (the biomarker's 'generalisability'). In an attempt to standardise and improve the way biomarker studies are reported, structured guidelines for biomarker reporting have now been published.

1.5.1 Phases

In contrast to drug development where the phases of clinical trials are well established, in biomarker research the phases are still in proposal. One model for the phases of biomarker development has been described by Sackett (110). This sets out 4 phases : Phase I establishes that a marker differs between cases and controls (e.g. poor outcome melanomas versus good outcome melanomas). This is often described as the 'discovery phase' and is concerned with identifying scientifically validated

targets. Phase II determines the predictive power of the marker, by testing whether patients with a given result are more likely to have a certain disease or clinical outcome. These early phase studies are typically performed on unequivocal cases. Phase III studies examine whether a test distinguishes those with a disease from those without, in a group in whom the disease is suspected or the outcome is unknown. This is commonly the first instance in which a test is performed in a group of equivocal cases that are akin to those that would be tested in a real clinical setting and typically the sensitivity and specificity is less than that seen in the Phase I and II studies. Phase IV studies determine whether patients who undergo this test fare better in the long term than those who do not. This phase is carried out as a randomised controlled trial in which those suspected to have the disease are randomly assigned either to have the biomarker assay or not. A comparison of these two groups is used to determine whether the results of the biomarker test affect subsequent management and ultimately patient outcome. As later phase studies last for several years, the complete process of biomarker translation is likely to take 10 years or more from the point of initial discovery. No molecular tissue biomarkers that inform melanoma prognosis have, as yet, ascended this evidence ladder.

A different 5 stage model has been proposed by Pepe (111),and has been advocated by the National Cancer Institute (NCI) Early Detection Research Network. This is a more complex series of 'research questions' and subquestions. For both proposed models however the 'phases' of biomarker development are defined by the research questions rather than by study design. Individual markers ascend the evidence ladder by setting and answering each of the 'research questions'. Regardless of which

biomarker development model eventually becomes recognised as the standard, biomarkers must progress from 'early phase' trials, which involve cohorts of retrospective cases, onto prospective biomarker studies and finally onto randomised controlled trials which determine the impact upon clinical outcome.

1.5.2 Guidelines

Published biomarker studies vary widely in how they are reported, with differing degrees of detail provided regarding the cases used and how the results have been achieved. Following a recommendation from the NCI and The European Organisation for Research and Treatment, the REMARK guidelines have been established as a standard structure for the reporting of prognostic biomarker studies (112). Using the guidelines as a template for reporting biomarker studies can help to ensure that research is published with sufficient relevant information to allow for the quality and generalisability of the results to be confidently assessed by other research groups.

1.5.3 Study Design

It is important to realise that whilst the 'phases' of biomarker research outline the research questions and that 'guidelines' for prognostic biomarker research indicate how results should be reported, neither phases nor guidelines are directly concerned with study design. The real complexity of biomarker research lies in the nuances of study design.

Suitable study design is the most important factor in safeguarding the validity of a biomarker study's outcomes. The main threats to validity relate to chance findings, bias, confounding and the ability to generalise the results. These threats are controlled

by ensuring the use of appropriate samples; specifying the best biomarkers; using an assay that is accurate, reliable and cost-effective; by incorporating the results of the biomarker assay into the best algorithm, pattern or statistical model; by determining the best parameters of the algorithm, pattern or statistical model; and by specifying the best cut-off for assigning a patient to good versus poor prognosis category.

The effect of bias is a major problem in biomarker studies (109). Potential sources of bias in biomarker studies include specimen selection and the absence of blinding to outcome (for example, a researcher scoring an immunohistochemistry stain whilst being aware of clinical follow-up data). The use of tissue microarrays, used in many of the IHC biomarker studies, where only a small core of tumour is tested rather than full histological sections is another potential source of bias.

A common problem in discovery-driven 'omics' research, where vast numbers of molecules are screened at once for prognostic significance, is the potential for chance discovery of spurious associations. This occurs as a result of sampling variation and the use of inappropriate p-value cut offs. For example, in a study in which 25,000 targets are screened on a series of cases and controls, if 0.05 is the p-value cut off used to test whether each target shows a differential result between cases and controls, this will to lead to around 500 discoveries, of which only a small proportion are likely to represent 'true' biological markers which differentiate between cases and controls in the wider population. The true markers will therefore be swamped by the large number of false markers which are simultaneously 'discovered'. This problem can lead to 'overfitting' which occurs when cases are classified based on spurious chance associations. This problem is most acute where expression array data is used for class prediction analysis

and especially in studies where the cases used to define the classes are also included in the series on which the prediction analysis is tested. Statistical methods exist which can be applied to the data to reduce false discovery rate although Dupuy et al have reported the high rate of basic statistical flaws evident in published microarray studies and the potential therefore for false biomarker discovery (113).

A design for prospective (Phase III) biomarker studies has been described by Pepe et al as a 'prospective specimen collection, retrospective-blinded-evaluation' (PRoBE) study (114). This involves prospective collection of cases and controls from the same cohort. A true cohort comprises a chronological series of cases which meet certain inclusion and exclusion criteria and the use of a cohort is helpful to eliminate bias between the two groups and to ensure that the sample resembles the wider population as far as is possible. The study then involves the collection of follow-up data (e.g. regarding metastasis and survival), with blinded testing of the samples after they have been collected to identify whether the biomarker results correlate to the known outcome. Ideally, a similar method would be adapted to all biomarker studies since 'opportunity' samples do not reflect the target population well.

To ensure that a test is generalisable, it must be ensured that the population used to develop the test is representative of the population encountered in clinical practice. A biomarker may not be generalisable if the cases used to discover and validate a marker are not a true representation of the cross section of cases which are encountered in clinical practice. This is another factor which is influenced by study design.

For translation into clinical practice, biomarker assays need to demonstrate their reproducibility, specifically to determine whether the same results can be gained when the test is repeated on the same samples at different times by different operators. Assay reliability should increase with a standardised methodology and automated procedures. Finally, the cost of the technology needed for the assay to be performed must be affordable and the expertise required to process and interpret the assays must be easily available to diagnostic laboratories (115).

1.6 Aims and Objectives

As outlined earlier in the chapter, melanoma is frequently deadly and has a dramatically increasing incidence. One of the major challenges in melanoma pathology is distinguishing melanoma from benign naevi in a subset of histologically challenging lesions. Aside from the clinical details of the patient, prognostication of primary melanoma is based entirely on histological features seen on light microscopy and improving prognostication using molecular methods is another challenge in the development of melanoma management.

The hypothesis for this project was:

DCN variations in melanoma can be characterised using real-time PCR assays and these can be developed into clinically useful biomarkers.

The aims of this project were:

1. To **develop new DRT assays targeting chromosomal centromeres**, in order to assess whole chromosomal copy number changes in FFPE samples.

2. To determine whether the previously developed DRT assays (detailed in section 1.4.1) targeting specific genes can be shown to demonstrate **reliability and reproducibility**.

3. To determine whether previously developed DRT assays targeting specific genes show **discrimination between different types of melanocytic lesions** and therefore whether they have potential as either prognostic or diagnostic markers.

4. To further test the diagnostic and prognostic capability of these assays by **testing true cohorts of melanoma and naevi** with the DRTs.

The corresponding objectives for the research aims were as follows:

1. DRT assays targeting chromosomal centromeres

i. Selection of suitable chromosomal centromeric repeat regions for the targeting of cDRTs to demonstrate whole chromosomal copy number alteration in melanoma.

ii. Testing centromeric repeat DRT specificity and suitability using hybridoma cell line DNA.

2. Reliability and reproducibility of previously developed DRTs targeting specific genes

i. Testing DRT accuracy and reproducibility using samples with known copy numbers, matched samples and repeat experiments.

ii. Determining the optimal method for DNA extraction from FFPE tissues.

iii. Testing of the DRTs against a series of diploid FFPE samples and determining a normal range of values and calculating correction factors where required.

3. Discrimination between different types of melanocytic lesions in a small series of samples (primary series) to show proof of concept that DRTs are clinically useful.

i. Comparison of benign naevi and melanoma using DRT assays.

ii. Comparison with DRTs of melanomas with differing clinical outcomes to determine whether these assays have prognostic potential

iii. Correlation of DRT results to primary melanomas and the corresponding metastases for these lesions.

4. Testing true cohorts of melanoma and naevi

i. To further test any diagnostic or prognostic capabilities of the DRT assays in a larger cohort representative of incident melanoma cases.

ii. To develop a model for prognosis or diagnosis.

2. Methods

2.1 Clinical case selection

Cases of tonsil, benign skin, benign lymph node, naevus and primary and metastatic melanoma were identified from the histopathology diagnostic archive of the Histopathology Department of the University of Leicester Hospitals NHS Trust by searches conducted using the systematized nomenclature of medicine clinical terms (SNOMED) coding system, a system used to code all diagnostic specimens. A tissue (T) code is given to each specimen to denote the tissue of origin and a morphology (M) code given to define the pathological appearance (e.g. T = SKIN, M = MELANOMA). Searches can be made using the electronic archive of reports for specific codes over specific periods of time.

Where cases were identified from SNOMED code searches, the histopathology report was then accessed using the histopathology electronic archive, to identify those cases which were suitable for inclusion (based on the report). The original Haematoxylin and Eosin (H&E) stained histology was then reviewed by a histopathologist (GS or DM) to ensure that the lesion was of sufficient size for DNA extraction and to check that none of the remaining exclusion criteria were met (see tables 2-1 and 2-2). The cases which were selected on the basis of the original H&E section were then retrieved from the tissue block store of the Histopathology Department of the University of Leicester Hospitals NHS Trust (or the Human Tissue Authority Licenced storage area of the University Department, if the cases had been used in other research projects). The tissue blocks were then checked by a histotechnician (LP) to ensure that there was

sufficient tissue block thickness to cut the necessary sections for DNA extraction without cutting the archived material to extinction.

2.1.1 Reference range samples

In order to develop a robust normal range of results for the assays we proposed to test in benign tissue, a reference range of benign diploid tissue samples was developed. The aim was to select a series of samples with a wide range of DNA concentrations and quality, comprising 108 cases that included skin wide local excision (WLE) samples (n=26), reactive lymph nodes (n=23) and benign tonsil samples (n = 53), all of which were identified and selected using the clinical case selection methods detailed in 2.1. None of the 26 skin samples from wide local excisions for melanoma nor the 23 lymph nodes from lymph node dissections for melanoma contained melanoma. The only exclusion criteria for the benign lymph node and benign wide local excision skin sample cases were the presence of malignant cells in the section, a tissue section of less than 25mm^2 and insufficient block thickness for sections to be cut without the destruction of all remaining tissue. The samples selected were from cases received between 2005 and 2010.

An independent series of reactive tonsil specimens was selected to facilitate one series of experiments, in which matched fresh frozen and FFPE tissue from the same specimen was required for comparison. These cases were all taken from 2008.

DNA was extracted from a further 20 FFPE tonsil samples from the same archive which consisted of 10 specimens from male patients and 10 from female patients. These

samples were used to test the X/18 cDRT assay. These cases were also taken from 2008.

Finally, 10 separate tonsil samples were selected to act as plate controls for the DRT assays, also from the 2008 diagnostic archive. The results generated for these cases were used to normalise each PCR run. A full description of how this was calculated can be found in 2.8.4.

2.1.2 Primary series

An opportunity sample of 40 primary melanomas, 20 melanoma metastases and 20 benign common naevi were selected for use as a primary series of cases to identify whether DRT assays detected DCN differences between the different classes of lesions. These cases all originated from the diagnostic archive of the Histopathology Department of the University of Leicester Hospitals NHS Trust. A series of inclusion and exclusion criteria were established for both the naevi (see table 2-1) and melanomas (see table 2-2). The melanomas were selected to include 20 primary melanomas with more than 5 years disease free survival (DFS), henceforth referred to as Primary minus Metastasis or 'P-M', and 20 primary melanomas with histological evidence of metastasis, referred to as Primary plus Metastasis or 'P+M'. The 20 metastases were matched to the 20 P+M cases, such that each metastasis was derived from one of the P+M primary melanomas.

Inclusion	Exclusion
• Common naevus - compound or intradermal	• Equivocal diagnosis
	• Junctional naevus
• Sufficient DNA for use in the required number of PCR reactions	• Dysplastic (Clark's) naevus
	• Special type of naevus (e.g. Spitz, Blue, Congenital)
	• Sectional area < 25mm^2
	• Tissue block too thin
	• Naevus less than 5% sectional area

Table 2-1 Inclusion and exclusion criteria for naevi in the primary series.

Inclusion	Exclusion
• Breslow 2.0 mm or greater	• Equivocal diagnosis
• Primary melanoma	• Tissue block too thin
• Sufficient DNA for use in the required number of PCR reactions	• Melanoma less than 5% sectional area
	• Sectional area < 25mm^2

Table 2-2 Inclusion and exclusion criteria for melanoma in the primary series.

In order to define whether the assays are able to differentiate between a benign and malignant melanocytic lesion, they must be first shown to differentiate between unambiguous melanoma and naevi. For that reason, ambiguous melanocytic lesions and the problematic naevi types which give rise to most misdiagnosed and diagnostically challenging melanocytic lesions were excluded.

Previous work completed by other students in the laboratory indicated that DNA yield was much more likely to be adequate if the tissue sectional area was greater than

25mm^2 and (for melanomas) the lesions was greater than 5% of the sectional area (personal communication - not published). All melanomas selected were at least 2mm in Breslow depth. This criterion was set as melanoma thinner than 2mm are difficult to accurately micro-dissect (see 2.3.2).

This was an opportunity sample with no attempt made to 'match' the P-Ms and P+Ms for disease stage or any other clinical parameters, such as age or gender, and consisted of cases from the diagnostic archive which had already been used in other studies within the laboratory and were therefore easily accessible. For all 20 of the metastasising primary melanomas, the metastatic tissue was also retrieved from the diagnostic archive, making 4 classes of melanocytic lesions (80 in total): 20 benign naevi, 20 P-Ms, 20 P+Ms, 20 metastases matched to the P+Ms. All cases were less than 10 years old at the time of DNA extraction.

2.1.3 Melanoma and naevi cohorts

Following the testing of the primary series with the designed assays, a subsequent cohort of cases was developed to further compare the application of the assays in naevi and melanomas. To ensure that the cases selected for use in the subsequent series represented, as best as possible, the wider population of melanomas (as discussed in 1.5.3), true cohorts of melanoma and naevi were developed. The same inclusion and exclusion criteria used in the primary series were also used for the cohort of naevi (Table 2-1). The melanoma inclusion and exclusion criteria were as for the primary series (Table 2-2), with the exception that all melanomas greater than 1mm in thickness were included in an attempt to make the cohort as representative as possible.

From the 1st of January 2000 onwards, all melanomas and naevi sequentially diagnosed in University Hospitals Leicester meeting these criteria were included in the cohort, with the H&E histology reviewed and the paraffin embedded tissue block inspected in order to establish whether the criteria were met. The cohorts of melanoma and naevi extended up to the point where over 100 cases were included in each. All of the cases had been diagnosed more than 7 years before the date of data retrieval and were up to 11 years old at the time of DNA extraction. Due to the greater frequency of suitable naevi compared to melanoma, all naevi were cases from 2000, whereas the melanomas selected were from 2000 to 2003.

In total 111 naevi and 106 melanomas were selected from the diagnostic archive for use in this cohort.

2.1.4 Clinical information

For melanomas and naevi from both the primary series and the cohorts, clinical information including the patient's age, gender and the site of the lesion, were taken from the original histopathology report. The pathology system was also examined for each of these patients to identify whether there was any evidence of subsequent metastasis. This included identifying any whether any histology or cytology samples had been reported diagnosing metastatic melanoma, examining the clinical details from all samples sent after the diagnosis of primary melanoma (including microbiology, haematology and biochemistry) to identify any mention of metastatic melanoma in the clinical details and searching for mortuary entries to determine whether the patient had died (although death in itself was not taken as a marker of metastatic disease).

The cases for which there was no evidence of metastatic disease were then checked using UHL NHS Trust's Computerised Radiology Information System (CRIS). All radiology reports after the date of diagnosis were examined in order to identify any indication of metastatic disease. Where melanomas had no evidence of metastasis on these grounds, they were considered to have 5 years DFS. For the patients to be classified as having 5 years DFS, it was also necessary that the most recent entry for the patient required them to still have an address within Leicestershire or Rutland (the geographical area served by University Hospitals Leicester NHS Trust) and have pathology or radiology results extending 5 years beyond the date of the primary melanoma (to indicate that they were still in contact with local healthcare services 5 years from the initial diagnosis). This was to reduce the possibility that the patient may have died but not been brought to our mortuary, or the possibility that they may have moved to a different area where they had been treated for metastatic disease in another NHS Trust.

No cases of melanoma with the first recorded metastasis more than 5 years from initial diagnosis were included in the P-M versus P+M comparison.

2.1.5 London Dermatopathology Symposium series

Before testing of the primary series got underway, our research group leader (GS) was invited to contribute to a national meeting (London Dermatopathology Symposium 2011) using the DRT method. One of the sessions of the meeting was devoted to discussion of diagnosing ambiguous melanocytic lesions. To facilitate this a series of 5 melanocytic lesions which had been originally reported as being of uncertain malignant potential were collected. Prior to the meeting the lesions underwent review

of the histology by a panel of dermatopathology experts, DCN analysis using the FISH assay developed by Gerami et al (63), performed in the laboratory of Dr Daniela Massi, (University of Florence Medical School), and, thirdly, DRT testing using our assays. No clinical data regarding the lesions was made available to us and the results of all 3 assessments were presented at the meeting. This was performed before the primary series and cohort series on a limited timescale meaning that only a limited series of DRTs were tested with these cases. Although carried out earlier than the other series this is presented at the end of the results chapter as it represents a clinical application of the DRTs.

2.1.6 Ethical approval

This study was part of the research project 'Chromosomal instability as novel melanoma diagnostic marker', which was approved by the Derbyshire Research Ethics Committee (reference 08/H0401/77) and by the Research and Development office of the University of Leicester Hospitals NHS Trust (reference: REGPR00105). DM was listed as a co-investigator on this study with both bodies. The ethical approval allowed for the use of archived clinical specimens from the diagnostic archive of UHL histopathology department to be used to develop and test the PCR assays. It also allowed for DM and GS to access clinical information regarding the specimens used.

2.2 Hybridoma cell line DNA

The centromeric repeat DRTs (cDRTs) were tested against a panel of hybridoma cell line DNA (Corriel Institute, Camden, New Jersey) (116). Version 2 of the panel was used. The panel consisted of DNA from cell lines each containing one human

chromosome and the DNA of a mouse or Chinese hamster. Each of these cell lines is therefore specific for a human chromosome. The panel consists of 27 samples – one for each of the 24 different human chromosomes, 2 control samples consisting of hamster and mouse DNA only and a positive control containing all human chromosomal DNA. This hybridoma panel was acquired to test the specificity of the cDRTs for individual chromosomes.

2.3 Sample preparation

2.3.1 H&E staining and microscopy

Once the FFPE tissue blocks had been selected for inclusion as described in 2.1, at least 6 histological sections were cut from each case using a microtome and placed on conventional glass slides. All tissue sectioning was performed by LP. Tissue sections were then stained with Haematoxylin and Eosin (H&E). This was performed by first de-waxing the sections by immersing them in xylene (Genta Environmental Ltd.). The tissue sections then underwent rehydration through decreasing concentrations of industrial methylated spirit (IMS) (Genta Medical) and then running water. Sections were subsequently submerged in Mayer's Haematoxylin (Fisher Scientific) and then placed under running water. Sections were finally submerged in Eosin (BDH Laboratory Supplies) before being placed under running water. Dehydration was then performed by placing the sections in increasing concentrations of IMS and finally xylene, before being mounted with coverslips using a distyrene plasticizer xylene (DPX) resinous mountant (Sigma-Aldrich Life Sciences).

H&E sections were examined using light microscopy by GS or DM. In cases of naevi and primary or metastatic melanoma, the H&E section was used to mark the section for microdissection using an ultrafine permanent marker (Staedtler). This was done in order to enrich the subsequent DNA extract for tumour DNA and therefore limit the contamination with diploid DNA from surrounding non-tumour cells. For the benign diploid reference range samples the H&E sections were used to confirm that there were no malignant cells present in the tissue which would contaminate the diploid DNA and no marking of the section was required.

All photomicroscopy for the project was a performed using an E800 microscope (Nikon, Japan) connected to a Progres CF Scan digital camera (Jenoptik, Germany).

2.3.2 DNA extraction and quantification

Histological sections of 5µm from the selected cases were de-waxed using xylene and rehydrated in Industrial Methylated Spirit (IMS), before air drying. The tissue sections were then manually dissected using a 500µl pipette tip (by DM), using the H&E stained and marked section as a guide, and placed in 500µl of Tris(hydroxymethyl)aminomethane - HCl (pH 8.0)- 0.1% Sodium dodecyl sulfate (TRIS-SDS) solution. 25µl of 10mg/ml proteinase K digestion solution (Roche) was added to each sample before incubating at 55˚C overnight. For the majority of cases in this project the samples were kept at 55˚C for 72 hours with a second dose of proteinase K at 24 hours and a third at 48 hours (see below for details). Microdissected sections were stained with H&E (performed by LP) and examined (by DM) in order to ensure accurate microdissection and minimal contamination with non-tumour tissue (see figure 2-1).

Phenol Chloroform DNA extraction was performed following proteinase K digestion by adding 500µl Phenol/Chloroform/IAA (25:24:1, pH 8.0) (Simga Life Sciences) to the solution, vortexing the sample and microfuging at 14000 rpm for 3 minutes. The aqueous phase was then separated and placed in a sterile tube with a 500µl pipette and the process repeated in order to ensure removal of the protein content. The residual phenol was removed by adding 500µl Chloroform/IAA (24:1) and microfuging the sample. The resulting aqueous phase was mixed with 10µl 1M Sodium Chloride and absolute ethanol (both Fisher Scientific) at -20˚C. This was stored at -20˚C overnight and then microfuged at 14000 for 15 minutes to form a DNA pellet. The resulting DNA pellet was washed in 70% ethanol (Fisher Scientific) and was subsequently dried and re-suspended in 30µl of 1xTE (10mM TRIS-HCl, 1mM Ethylenediaminetetraacetic acid [EDTA] pH 8.0), for storage at 4˚C. The DNA concentration in the solution was quantified using the nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware). This was all performed as per the standard methodology used in our laboratory (106).

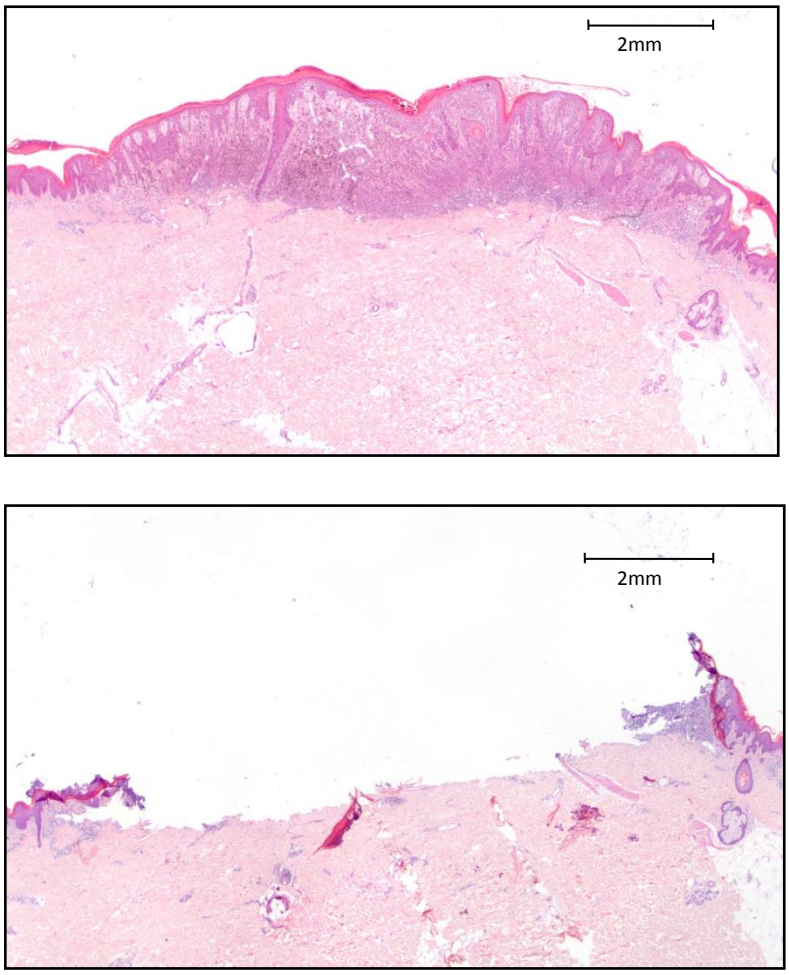

Figure 2-1 Malignant melanoma (above) and a histological section from the same lesion following manual microdissection (below).

In order to establish the best method for DNA extraction in skin and lymph node samples, where the DNA yield may be low, an experiment was designed in which single 4µm histological sections of skin samples and lymph node samples were either digested over 24 hours with 1 dose 25 µL of protein kinase (PK) (method 1), or digested over 72 hours with 3 doses (method 2). Following this comparison, the 3 day digestion method was tested using either 5 sections of tissue (method 3) or 1 section (method 2). Table 2-3 summarises the digestion methods. The samples were then tested using a cDRT and the mean cycle threshold (CT) value calculated. Mean CT

values were then compared between the 3 digestion methods using a paired t-test to determine whether differences between the groups were statistically significant.

Method	*4 µM sections used*	*PK doses*	*Digestion time*
1	1	1	24
2	1	3	72
3	5	3	72

Table 2-3 Summary of the three protein digestion methods used.

2.3.3 GAPDH analysis of DNA quality

Variability in the fixation of clinical specimens used can lead to differences in the quality of extracted DNA and this is not reflected in the quantification of the specimen using spectrophotometry described in 2.3.2 (117). It is therefore possible to extract DNA which is present in sufficient quantity to be detected by spectrophotometry but is degraded by FFPE and storage to the extent that it is not suitable for testing using QPCR. In order to test DNA quality for each specimen before applying a full panel of DRT assays, each specimen was tested using QPCR primers for the low copy promoter region of the gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which codes for an enzyme necessary for glycolysis and has widely been used to test DNA for QPCR. A set of GAPDH primers previously developed in our laboratory by Dr Karen Page for GAPDH (GAPDH100) (118) were used along with the SYBR Green probe (Applied Biosystems, Foster City, California, USA). CT values of over 34 cycles were taken to indicate poor quality of the DNA and these cases were excluded from the DRT assays.

2.4. Selection of regions of interest and specificity testing

2.4.1.Centromeric repeat sequences

Centromeric regions of interest were identified from the array CGH data made publicly available through the National Center for Biotechnology Information (NCBI) website (Jonsson, Curtin, Baudis). The selection of chromosomes for cDRT design was made on the basis of whole chromosomal copy number change. Alphoid repeat sequences specific to the selected chromosomes were identified through previously published data (119,120). The sequences for these repeats were available on the NCBI website.

In order to ensure that the regions selected for primer and probe design were specific for the gene or region of interest, specificity testing was performed. This involved testing each of the repetitive sections of the alphoid repeat sequences (monomers) using the NCBI Basic Local Alignment Search Tool (BLAST) against the human nucleotide database. This gave an indication of how specific each monomer was for the chromosome of interest. The monomers returned by BLAST as being most specific were then selected as targets for QPCR.

One of the chromosomes was selected for having frequent whole chromosome gain in melanoma and the other frequent whole chromosome loss in order to create a ratio of >1.

2.4.2 Regions of genomic instability in melanoma

The development of the non-centromeric DRTs was completed before this project as outlined in 1.4.1 and therefore analysis of the regions of instability in melanoma was performed by previous students (DB, AE, ZM, LD, NH). Their identification of regions of

frequent genomic instability in melanoma was completed using array CGH data published by Curtin et al (40), Jonsson et al (102) and Baudis et al (33), made publicly available via the NCBI. The array CGH plots were processed using the aCGH MultiExperiment Viewer (MEV) software (121,122) to identify common regions of somatic copy number alteration. The genomic regions were viewed using the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/) (123) and target genes were then tested using the NCBI BLAST application as described above. Following NCBI BLAST testing, the most specific regions from the gene of interest were selected as the loci for the amplicons.

The first gene in each pair was chosen because it was from a region frequently gained in melanoma whereas the second was from a region of frequent DNA copy number loss. Thus, the duplex ratio of DNA should be > 1 in those melanomas carrying a common pattern of somatic copy number alteration.

2.5 Design of DRTs and cDRTs

The online application Muplex was used to generate PCR primer pairs for the cDRTs and the DRTs, with amplicon lengths of 70-100 bases pairs, for use in a duplex QPCR reaction (124). Appropriate probes for the amplicons were then designed using the online application Primer3 (Howard Hughes Medical Institute, Chevy Chase, Maryland).

The cDRTs were all designed as part of this project by DM.

All the non-centromeric DRTs used in this project were previously designed by other students working in the department (DB, AE, ZM, LD, NH), under the supervision of

JHP. These previously developed DRT assays were designed using the same method described here. Details of the assay sequences can be found in tables 1-7 and 1-8.

2.6 Testing of DRT reaction kinetics

In order for QPCR reactions to work effectively it must be ensured that the designed primer and probe sequences have suitable reaction kinetics, by investigating the secondary structure formation and dimerization that may occur between the 2 pairs of primers and the 2 probes present in each reaction. Where the free energy of these dimers or structures is too great, this may prevent the PCR reaction from performing efficiently and 5 kcal/mol was considered to be the upper limit of acceptable free energy for a secondary structure or self-dimer in the assays. Reaction kinetics were predicted using two separate software applications; Primer Express (Applied Biosystems) and Beacon Designer (Premier Biosoft International, Palo Alto, California, USA) (http://www.premierbiosoft.com/QPCR/) (125).

These primers were checked (for annealing temperature and secondary structure) using Primer Express software (Applied Biosystems, Foster City, California) and the online software application NetPrimer (Premier Biosoft International, Palo Alto, California).

This was performed by DM for the cDRTs. The analysis had been performed previously for the non-centromeric DRTs by DB, AE, ZM and LD. Details of an example of reaction kinetics testing for a cDRT can be found in Appendix 1.

2.7. Testing of DRTs and quality

The designed primers were produced by Sigma-Aldrich Life Sciences (St Louis, Missouri, USA) and Taqman probes were produced by Applied Biosystems (Foster City, California, USA).

Primer sets were synthesised first and underwent testing using SYBR green (Applied Biosystems) probes. SYBR green binds to double stranded DNA produced in a PCR reaction and emits green light, but is not sequence specific. SYBR green testing was performed in order to test the viability of the assays before the corresponding probes were synthesised. For the cDRTs the primers were tested against the hybridoma cell line DNA panel to check chromosomal specificity (by DM). For the DRTs, the primers had been tested prior to this project on FFPE tonsil DNA (by DB, LB, ZM and AE). For those primer sets which were deemed successful the corresponding probes were then synthesised. Synthesis was performed in this order largely due to the cost of specific probes compared to the relatively cheap primer sets.

Further testing of the DRTs was performed by ZM prior to this project. This involved testing varying concentrations of a fixed tonsil derived DNA sample with the DRTs to plot standard curves of CT values for the 2 targets versus log (DNA concentration). These were performed and demonstrated equal efficiency changes with changing DNA concentration shown by parallel regression lines of the 2 targets for each DRT (personal correspondence ZM and JHP).

2.8 Quanitative PCR

As mentioned briefly in section 1.4, QPCR is an advanced form of PCR which allows for quantification of the product at each cycle of the reaction using a fluorescently labelled reporter probe. Probes can either be specific to the amplicon, such as the Taqman (Applied Biosystems) probe, or a non-specific probe, such as SYBR green, discussed above. The advantage of the Taqman probes is great specificity for a given target and the ability to differentiate between two products synthesised simultaneously (as with a DRT), but each Taqman probe must be designed and synthesised separately, making these probes more costly and time consuming to produce.

2.8.1 QPCR set up

QPCR was performed on 96 well QPCR plates (Applied Biosystems, Foster City, California, USA), using 10ng of DNA in each reaction.

For those reactions using SYBR green, 5 µl of SYBR Green (Applied Biosystems, Foster City, California, USA) master-mix was used in each reaction, along with 0.2µl of 10pM forward and reverse primers. 10ng of DNA was used each reaction and therefore DNA samples were diluted to give a concentration of 3.33ng/µl in order to give 10ng of DNA by adding 3µl of the diluted DNA solution into each reaction. For each reaction the volume was made up to 10µl with sterile H_20.

DRT reactions which used the Taqman probes were made up with 5µl of Genotyping Mastermix (Applied Biosystems, Foster City, California, USA), 0.3µl of 20pM forward and reverse primers and 0.2µl of both Taqman probes at 1:20 dilution. Again DNA

samples were diluted such that 3µl of sample gave 10ng of DNA in each reaction and all reactions were made up to 10µl with sterile H_20.

QPCR reactions were set up in triplicate on the 96 well plates. A no template control was run on each 96 well plate.

2.8.2 QPCR reaction

PCR Reactions were run on the Step One Plus QPCR machine (Applied Biosystems, Foster City, California, USA), with a thermal profiles outlined in table 2-4 for the SYBR green reporter reactions and the specifically designed Taqman probe reactions.

	Taqman Reactions	SYBR Green Reactions
	50˚C for 2:00	95˚C for 0:20
	↓	
	95˚ for 10:00	↓
	↓	
40 Cycles of:	95˚C for 0:15	95˚C for 0:03
	then 60˚C for 1:00	then 60˚C for 0:30

Table 2-4 Thermal profiles for the DRT reactions using the Taqman and SYBR green reporters.

2.8.3 QPCR data extraction

The cycle thresholds (CTs) for fluorescence from the reporter probes were set a ΔRN value of 0.05 which was selected as a level of fluorescence which is well above the background fluorescence which is seen before detectable amplification occurs, but

below the level when the DNA reaction becomes saturated with product. The ΔRN value is the fluorescence detected in the reaction normalised to the inert ROX dye which is present in the mastermix used to make up the reaction.

The standard deviation (SD) was performed on replicates for the ΔCT score and a range of values between -0.3 and 0.3 were accepted as precise replicate values, as previously described for the assessment of DCN variation with QPCR (126).

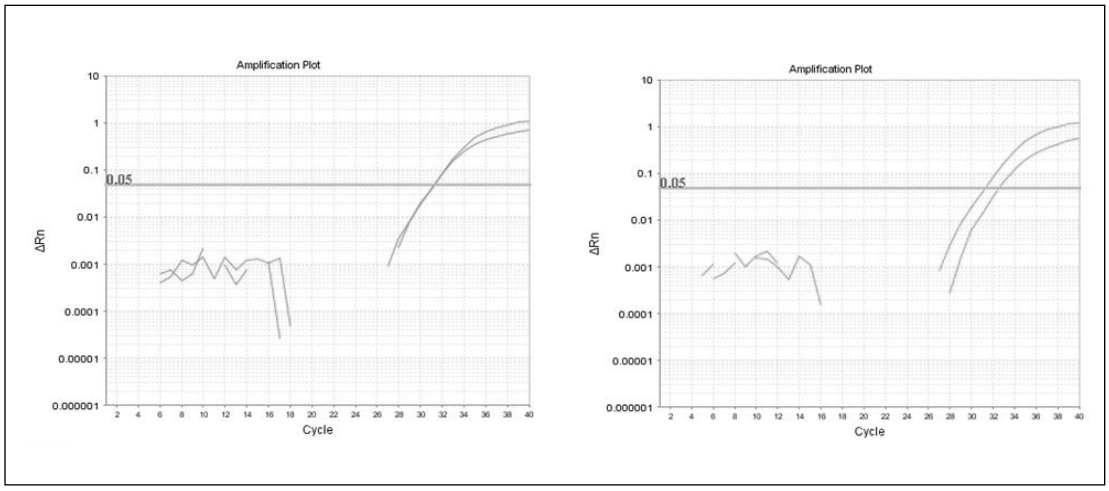

Figure 2-2 Amplification plots for the MYB.RREB assay for a naevus (left) and a melanoma (right). For the naevus plot the rate of amplification as reported by MGB probe fluorescence (ΔRN value) is equal at the cycle threshold (CT) of ΔRN = 0.05, giving a ΔCT of around 0. For the melanoma, the RREB1 probe shows gain with a lesser number of cycles then the MYB probe leading to a ΔCT of around 1.

2.8.4 Calculation of the DRT score from the raw QPCR data

The ΔCT score was calculated for each of the reactions using the equation: CT gene 1 – CT gene 2 = ΔCT, where gene 1 is the gene of common DNA gain and gene 2 that of common DNA loss. The mean ΔCT from the triplicate reactions was used for

subsequent analysis. A series of 10 benign diploid tonsil DNA samples (as described in 2.1.1) acted as control cases for each of the PCR runs using FFPE samples. From this series of 10 tonsils a mean tonsil ΔCT (for all 10 tonsil samples) was calculated. Normalisation was performed for each specimen by the following equation: ΔCT - mean tonsil ΔCT = ΔΔCT. Finally a DRT value was calculated using the equation: $2^{-\Delta\Delta CT}$ = DRT.

2.8.5 Testing of melanoma and naevus samples

The validated assays were tested on the London Dermatopathology Symposium series, the primary series and the cohort series of melanocytic lesions. Each experiment consisted of a 96-well QPCR plate containing 10 tonsil controls with only one DRT tested with each experiment. All samples on every plate were tested in triplicate. For the London Dermatopathology Symposium series the 10 plate controls, an extra 7 tonsil controls, 4 naevi, 4 melanomas and the 5 ambiguous cases were tested alongside a no template control (NTC) sample, totalling 31 sample and making 93 reactions on the plate due to triplication. The primary series was run with 10 plate controls, 5 naevi, 5 P-Ms, 5 P+Ms, 5 metastases and an NTC on each plate. The cohort series experiments were each run with 10 plate controls, 10 melanomas, 10 naevi and an NTC on each plate, apart from the 11^{th} plate of the cohort which contained 11 naevi, 6 melanomas and 2 Spitzoid tumours alongside the 10 plate controls and the NTC. The layout of the all the plates was kept standardised for each of the assays in order to facilitate pre-programmed data extraction tables developed in Microscoft Excel.

2.9 BRAF mutation analysis method

BRAF mutation analysis was performed using QPCR primers previously developed in our laboratory by JHP, based on work by Benlloch et al (127). The PCR primers target the region of exon 15 in the BRAF gene which contains the point mutation V600E which is the most common mutation in BRAF seen in melanoma. The primers do not include the mutation itself however and they will therefore amplify both the wild-type and mutated sequences. There are 2 minor groove binder (MGB) probes for the mutated and wild-type sequences. The details of the probes and primers can be found in table 2-5; the mutation is highlighted in bold on the mutation probe and the corresponding wild-type nucleotide also in bold.

	Nucleotide sequence
BRAF exon 15 forward primer	5'-TCATGAAGACCTCACAGTAAA-3'
BRAF exon 15 forward primer	5'-ATCCAGACAACTGTTCAAACT-3'
BRAF V600E mutation probe	FAM-CTACAG**A**GAAAT-MGB
BRAF wild-type probe	VIC-TAGCTACAG**T**GAAAT-MGB

Table 2-5 Primers and probes used in the BRAF mutation analysis experiments.

The set up method for this reaction was similar to that described in section 2.8.1: DRT reactions were made up with 5µl of Genotyping Mastermix, 0.6µl of 20pM forward and reverse primers and 0.2µl of both Taqman probes at 1:100 dilution. DNA samples were diluted such that 3µl of sample gave 10ng of DNA in each reaction and all

reactions were made up to 10µl with sterile H_20. The experiments were loaded onto 96 well plates and run on the Step One Plus QPCR machine using the Taqman settings set out in table 2-4.

2.10 Statistical Analysis

2.10.1 Z score analysis

Z scores for the naevi and melanoma were created using the mean and the SD of the naevi DRT values for all of the assays. This was used to determine which cases fell outside of the 99% normal range for naevi, with those outside this range being considered aneuploid for a given DRT assay.

2.10.2 Intraclass Correlation Coefficient

The Intraclass Correlation Coefficient (128) (ICC) was calculated using the SPSS statistical package Version 18.0.2 (SPSS Inc, Chicago, IL) PASW to assess the agreement between repeated assays using CT values for each DRT probe. The following parameters were used: Model= two-way random, type= Absolute agreement, Confidence Interval= 95%. Paired T-test analysis (two-tailed) was calculated using GraphPad Prism, version 5.02 to assess the systematic difference in repeated measures. Unpaired T-test analysis was also performed using GraphPad Prism version 5.02

2.10.3 Bland-Altman analysis

Bland-Altman analysis was performed using GraphPad Prism version 5.02 and was used to represent mean CT versus difference in CT. This analysis was also used to determine values for bias and limits of agreement.

2.10.4 Logistic regression analysis

SPSS statistical package Version 18.0.2 software (SPSS Inc, Chicago, IL) was used to perform binary logistic regression analysis using the corrected ΔΔCT values in order to determine the discriminatory sensitivity and specificity of the assays and to calculate a prediction probability value used to plot Receiver Operating Characteristics (ROC) curves. Malignancy was used as the binary dependent variable for when testing against naevi and melanoma and the corrected ΔΔCT values were entered as covariates. For testing of P-Ms and P+Ms, subsequent metastasis was used as the binary dependent variable and again the corrected ΔΔCT values were entered as covariates.

2.10.5 Normality testing

Testing of normalcy of the reference range CT values was performed in GraphPad Prism version 5.02 using the D'Agostino and Pearson omnibus normality test.

3. Results

3.1 DRT assays targeting chromosomal centromeres

3.1.1 Targeted centromeric regions

The following chromosomes were selected as candidate targets for the cDRT assays, owing to the frequency of these chromosomes showing whole chromosomal loss or gain in melanoma: X, 18, 9, 6, 8 and 10. In order for the DRT to show a ratio > 1, chromosomes with common copy number gain were paired with those showing common copy number loss. Three pairings were made (see table 3-1).

Pair	Loss	Gain
1	X	18
2	9	6
3	10	8

Table 3-1 Chromosome pairings. Three sets of pairs were established for the development of cDRT assays.

Several primer sets were made for most of the paired chromosomes, apart from chromosome 8, where only one appropriate amplicon could be generated from the relevant repeat sequence. The primer sets were first tested with SYBR Green before specific Taqman probes were synthesised. The primer sets were coded with a letter to differentiate the sets and for demonstration of their comparisons. Table 3-2 outlines the primer sets by target chromosome.

Pair 1		Pair 2		Pair3	
X	**18**	**9**	**6**	**8**	**10**
Xa	18a	9a	6a	8a	10a
Xb	18b	9b	6b		10b
Xc	18c	9c			
		9d			

Table 3-2 Primer sets designed for the paired cDRT chromosomes.

3.1.2 Testing of centromeric DRT assays with hybridoma cell line DNA

All of the centromeric repeat primers were tested individually against the hybridoma panel of cell line DNAs.

The mean CT value was taken as the output value for each sample and the various CT values for each case are presented in tables 3-3, 3-4 and 3-6. For chromosome X, primer pairs Xa, Xb and Xc all showed a high degree of specificity for that chromosome. For chromosome 18, 18b showed the greatest degree of specificity for this chromosome and therefore the set Xb/18b was selected and the appropriate probes designed.

	Primer pairs for X and 18					
	X			18		
Chromosome	Xa	Xb	Xc	18a	18b	18c
1	35.4	34.3	34.6	31.3	33.0	33.9
2	35.9	32.4	35.1	15.1	32.7	33.9
3	35.0	35.4	35.4	32.5	33.0	34.7
4	31.2	36.5	34.2	31.3	33.1	32.2
5	34.4	35.2	33.8	26.8	32.4	33.0
6	34.1	34.6	33.2	30.6	32.2	34.7
7	35.8	34.7	34.2	30.6	32.5	32.9
8	36.1	29.8	34.8	19.6	32.1	17.2
9	35.8	34.6	33.9	23.5	32.4	31.4
10	34.0	30.6	32.6	29.4	32.5	33.2
11	33.2	22.1	33.1	30.6	31.8	34.0
12	33.2	32.8	33.8	30.9	32.9	34.1
13	34.3	34.7	34.2	22.2	33.1	34.3
14	33.8	33.5	32.3	20.2	32.1	30.6
15	35.3	34.4	33.9	18.0	33.0	33.9
16	33.8	26.1	32.0	24.7	32.0	34.1
17	34.5	35.6	34.0	29.5	32.7	33.9
18	34.9	36.3	32.2	13.7	14.7	13.9
19	34.4	34.8	34.5	27.1	32.6	35.0
20	32.6	32.3	33.3	15.8	33.1	31.3
21	32.2	27.6	32.1	21.2	31.3	31.6
22	29.7	28.1	32.6	23.6	31.5	31.3
X	14.7	14.4	14.0	28.8	32.7	33.7
Y	34.9	35.2	34.9	17.8	19.5	19.4
mouse negative	35.0	36.5	32.6	31.0	32.0	34.1
hamster negative	34.4	35.9	33.1	31.9	29.6	33.8
no template control	37.0	37.5	35.6	33.4	33.0	36.4
positive human control	18.2	14.6	13.5	11.2	UD	13.8

Table 3-3 CT values for each of the primer pairs for chromosomes X and 18 tested against the full panel of hybridoma cell line DNA samples. The targeted chromosome is highlighted.

For chromosome 6, primer pairs 6a and 6b both showed a high degree of specificity.

For chromosome 9 however, neither 9a nor 9b showed a high degree of specificity and therefore alternative primers, which were tested for duplex PCR alongside 6a and 6b

using the appropriate software and online applications, were synthesised and labelled 9c and 9d.

	Primer pairs for 6 and 9					
	9				6	
Chromosome	9a	9b	9c	9d	6a	6b
1	UD	36.0	25.7	28.2	32.3	35.1
2	UD	22.1	24.4	26.4	32.0	35.2
3	UD	32.3	28.1	26.7	25.3	35.2
4	26.5	16.2	13.8	17.5	37.5	34.9
5	UD	30.5	24.4	27.5	30.7	36.4
6	UD	35.2	30.8	29.6	18.0	13.0
7	UD	29.7	24.3	28.7	35.2	34.8
8	36.0	28.0	28.6	27.5	31.4	33.0
9	22.6	14.2	12.8	12.8	34.0	35.2
10	UD	35.6	27.9	27.0	34.1	33.7
11	UD	31.9	29.8	25.4	29.3	36.8
12	UD	36.5	23.2	25.8	32.7	34.8
13	39.3	34.7	25.4	28.1	33.1	35.5
14	31.6	20.5	21.0	23.4	29.5	33.2
15	34.3	27.1	13.5	13.4	29.5	37.3
16	UD	25.5	23.2	25.9	32.1	30.3
17	UD	35.2	27.7	30.0	36.1	33.9
18	38.1	25.5	20.8	22.1	30.6	32.0
19	UD	30.1	25.8	28.4	29.1	35.2
20	31.6	23.5	22.5	26.9	32.4	35.3
21	36.6	33.0	22.7	27.6	32.3	32.2
22	34.4	20.9	23.2	23.9	29.9	33.4
X	28.5	31.8	26.2	27.8	34.4	35.7
Y	UD	18.9	16.1	19.4	31.7	33.4
mouse negative	UD	24.9	29.7	30.3	37.4	34.7
hamster negative	UD	38.0	30.1	31.3	38.6	35.2
no template control	UD	36.5	28.2	31.3	36.3	34.8
positive human control	21.3	13.6	11.9	11.8	16.1	13.7

Table 3-4 CT values for each of the primer pairs for chromosomes 9 and 6 tested against the full panel of hybridoma cell line DNA samples. The targeted chromosome is highlighted. UD = undefined where no product was detected with QPCR.

Regrettably, neither of the new chromosome 9 primers, 9c or 9d, showed greater specificity for chromosome 9, despite the primers being selected for regions where the repeat sequence is divergent from similar non-specific repeat sequences according to published data detailing the repeat regions (129).

The CT values for all of the primer sets 9a to 9d showed chromosomes other than 9 amplified by the primer pairs with CT values between 1 and 4 cycles greater than those for chromosome 9. The most specific was 9a, although a CT value of 22.6 was a much higher value than expected from fresh frozen DNA for a repeat sequence. The second most specific set was 9b which showed a difference of 2 cycles between CT values for chromosome 9 and chromosome 4. The Taqman probes were therefore synthesised for the 9b primer pair in an attempt to improve the specificity of the reaction for chromosome 9. If the repeat region on chromosome 9 (showing apparent homology with chromosome 4 using the primers and a SYBR green reporter) had a differing nucleotide sequence in the region binding with the MGB probe, this would improve specificity for chromosome 9 compared with the SYBR green assay. The result of testing the hybridoma panel with the MGB probe for primer set 9b was a CT of 14.8 for chromosome 9 and 16.2 for chromosome 4, compared to 14.2 for chromosome 9 and 16.2 for chromosome 4 using the SYBR green reporter, representing no increase (and indeed a decrease) in specificity with the addition of the MGB probe. Varying annealing temperatures were programmed into the QPCR machines whist testing the same samples in an attempt to improve the specificity (table 3-5), but again, varying the annealing temperature from the usual 60°C did not improve the specificity.

	Ch 9	Ch 4	Ch 14	Ch Y
Temp (˚C)	mean CT	mean CT	mean CT	mean CT
60	14.8	16.2	22.0	23.7
62	17.9	19.3	25.3	26.7
64	33.2	35.8	39.5	UD

Table 3-5 The effect of varying temperatures on QPCR CT values for the highest amplified chromosomes using the set 9b primers and probe. UD = undefined where no product was detected with QPCR.

Primers were designed for a chromosome 8 and 10 duplex reaction. Primer set 10b was found to have the greatest specificity for chromosome 10. The chromosome 8 primer set showed limited specificity. Due to lack of suitable regions within the repeat sequence for selection of an amplicon, it was not possible to generate further primers for the Chromosome 8 repeat.

	Primer pairs for 8 and 10		
	8	10	
Chromosome	8a	10a	10b
1	34.6	UD	30.7
2	32.6	UD	UD
3	34.3	UD	31.5
4	30.4	UD	32.2
5	31.7	UD	32.2
6	27.6	UD	31.7
7	23.5	UD	34.0
8	21.1	UD	31.8
9	33.4	UD	32.3
10	23.4	UD	19.6
11	32.1	UD	30.6
12	25.3	UD	36.5
13	33.8	UD	33.7
14	33.7	UD	33.4
15	34.7	UD	31.9
16	23.4	UD	31.3
17	34.5	UD	31.3
18	37.1	UD	33.2
19	25.6	UD	33.3
20	34.1	UD	29.4
21	34.8	UD	31.7
22	34.9	UD	30.8
X	26.0	UD	32.1
Y	34.3	UD	31.3
mouse negative	33.9	UD	32.0
hamster negative	32.9	UD	31.8
no template control	38.5	UD	32.6
positive human control	18.7	UD	UD

Table 3-6 CT values for each of the primer pairs for chromosomes 8 and 10 tested against the full panel of hybridoma cell line DNA samples. The targeted chromosome is highlighted.

3.1.3 Testing the accuracy and repeatability of the Xb/18b cDRT

The Xb/18b cDRT was applied to DNA extracted from 10 male and 10 female tonsils, to determine whether the assay could predict the sex (the ΔCT value should differ by a factor of 2, or one PCR cycle, between male and female, as females have 2 copies of the X chromosome).

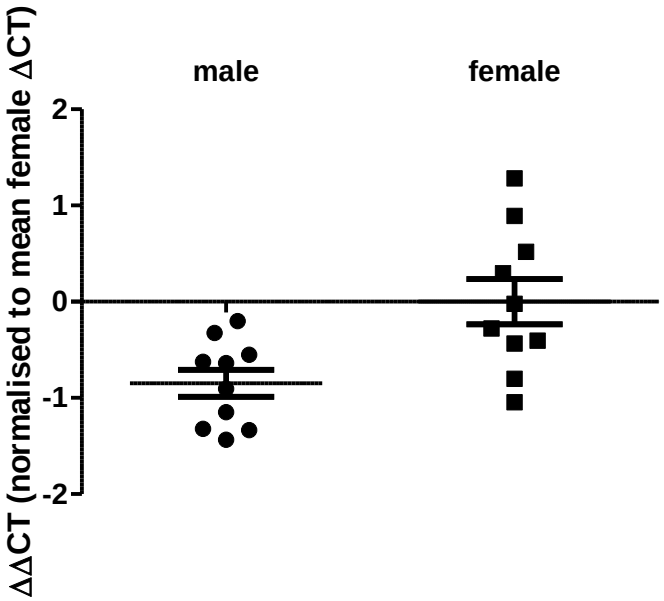

Figure 3-1 Male and female FFPE tonsil samples tested using the Xb/18b DRT. This plot demonstrates the overlapping spread of ΔΔCT values for male and female FFPE tonsil DNA when tested using the Xb/18b DRT. Note all values were normalised to the mean female ΔCT. Mean and Standard Error of the Mean (SEM) shown.

This showed a difference between the mean male ΔΔCT and the mean female ΔΔCT of 0.84, which is close to 1.0 (figure 3-1), though the spread of the results shows a large degree of overlap, not the clear separation which might be expected. In order to investigate whether this overlap was due to DNA quality in the FFPE tonsil samples or variations in repeat copy number between individuals, 5 male and female frozen tonsil samples were tested (figure 3-2). The assay was also repeated on the same samples on a different day, showing a high degree of reproducibility for the assay with a ΔΔCT correlation coefficient of 0.99 (figure 3-3).

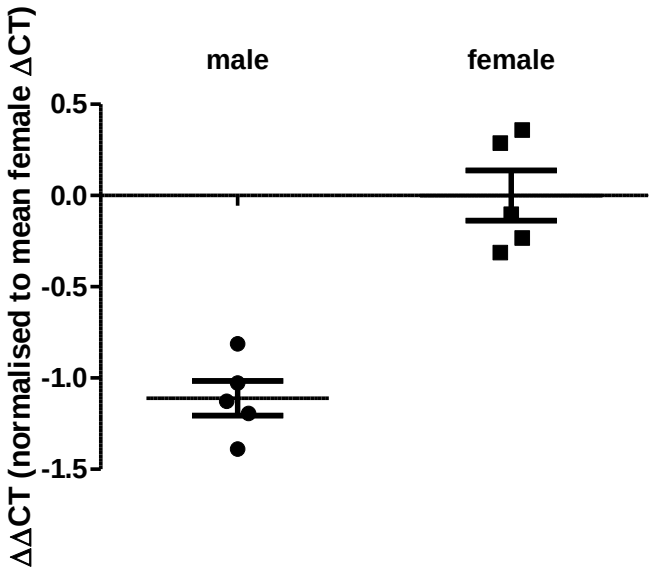

Figure 3-2 Male and female fresh frozen tonsil samples tested using the Xb/18b DRT. This plot demonstrates the clear separation of ΔΔCT values for male and female fresh frozen tonsil DNA when tested using the Xb/18b DRT. Note all values were normalised to the mean female ΔCT. Mean and SEM shown.

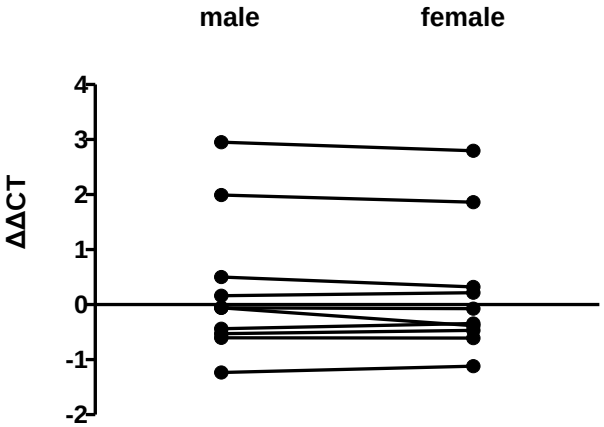

Figure 3-3 Reproducibility of the Xb/18b DRT. This plots the ΔΔCT values resulting from repeated testing of the same fresh frozen tonsil DNA when tested on two consecutive days (correlation co-efficient from all 10 cases = 0.99).

Testing the assay on frozen tonsil samples shows much clearer separation in this small cohort, suggesting that the poorer quality of DNA extracted from FFPE tissue does influence the cDRT accuracy.

To further investigate the accuracy of the assay, the set of ten pairs of matched diploid samples (skin and lymph node) were tested to identify whether the ΔCT between the 2 samples from the same patient were comparable. This was tested by calculating the ΔΔCT value between the skin and lymph node samples, with an expected ΔΔCT of 0 if both samples give the same result with the assay. The experiment revealed that for 7 of the 10 sample pairs the ΔΔCT value was less than 0.6. For the remaining 3 pairs, the ΔΔCT was between 1.0 and 3.0. The two highest values were also the cases in which the highest CT value was recorded. These 2 values were both WLE samples and were also those in which the original extraction gave the lowest yield of DNA. Therefore the 2 results concerned may represent those in which the DNA quality affected the accuracy of the assay. This further indicates that for the cDRTs to be useful assays, good DNA quality from FFPE samples is crucial. This therefore challenges the original hypothesis that targeting centromeric repeat sequences with cDRT assays would be a means of testing whole chromosome copy number changes in degraded DNA extracted from FFPE tissue with greater accuracy than DRTs targeting specific genes.

3.1.4 The value of centromeric DRTs as potential markers in this project

The initial aim of using cDRTs was to develop a range of assays which would identify chromosomal copy number change with greater sensitivity because they exist as multiple target sequences. However, when the CT values were compared between the cDRTs and 2 of the DRTs on the same samples at the same time, CT values were not

significantly lower for cDRTs than the DRTs. This issue, coupled with the problems in developing assays which were specific for individual chromosomes, revealed cDRTs to be more difficult to develop than DRTs and to appear less accurate in the assessment of somatic copy number alterations.

This work testing cDRTs combined with the work performed by previous students in our group testing DRTs suggested that DRTs represented more promising targets than cDRTs as tissue biomarkers. Due to the limited time for the project, the possibility of having limited amounts of DNA extracted from clinical specimens and limited funding for running assays and producing probes, DRTs became the focus for testing clinical specimens for the remainder of the project. This is discussed in sections 3.2 to 3.4.

3.2 Accuracy, reproducibility, method comparison and the development of normal ranges for DRTs

3.2.1 Testing DNA accuracy and reproducibility in DRTs

The CT and DRT values for 10 plate control samples which were tested every time an assay was performed were used to determine assay reproducibility. The CT values for the plate control samples on the first 2 occasions the DRTs were performed were used to determine an intraclass correlation co-efficient (ICC) for each DRT. The DRT values for these samples were used to determine bias, limits of agreement, co-efficient of variation and a paired t-test result. There were good agreement statistics for all 7 of the DRTs, with low levels of bias (i.e. low variation between the assay runs) and high (non-significant) p values on paired t-test comparison, meaning that there was no systematic bias between assays performed on different days (see table 3-7).

The difference in CT values for the same target over the 2 occasions was compared to the mean CT value over both experiments for Bland Altman comparison plots in figure 3-4. (NB the difference in CT is labelled ΔCT, but in this case is the difference of the same target CT on 2 occasions and not a comparison of the 2 different targets in a DRT on the same occasion). These show results clustered close to zero and on linear regression only one of the Bland-Altman plots (ASAP.LZST1) showed a significant trend (p=0.043) representing systematic differences between the first and second run.

Assay	*ICC*	*Bias*	*Limit of agreement*	*%CV*	*T test P value*
MYB	0.95	-0.00091	-0.21 to 0.21	8.4%	0.98
RREB1	0.95				
BRAF	0.95	-0.0010	-0.27 to 0.27	10.2%	0.98
PTEN	0.93				
SSR1	0.95	-0.0022	-0.22 to 0.22	8.2%	0.95
PERP	0.96				
ASAP	0.95	0.0045	-0.17 to 0.18	7.3%	0.88
LZST1	0.95				
TBX2	0.93	-0.0043	-0.31 to 0.30	12.9%	0.94
HIC1	0.94				
LDLRad3	0.96	0.0072	-0.28 to 0.30	11.0%	0.89
CCND1	0.96				
AKT3	0.98	0.0053	-0.18 to 0.19	7.7%	0.87
MIB2	0.97				

Table 3-7 Statistical analysis of the reproducibility of DRT values for the 7 DRTs. Intraclass correlation coefficient, bias, limit of agreement, %CV and t test p value is detailed for each of the 7 DRTs.

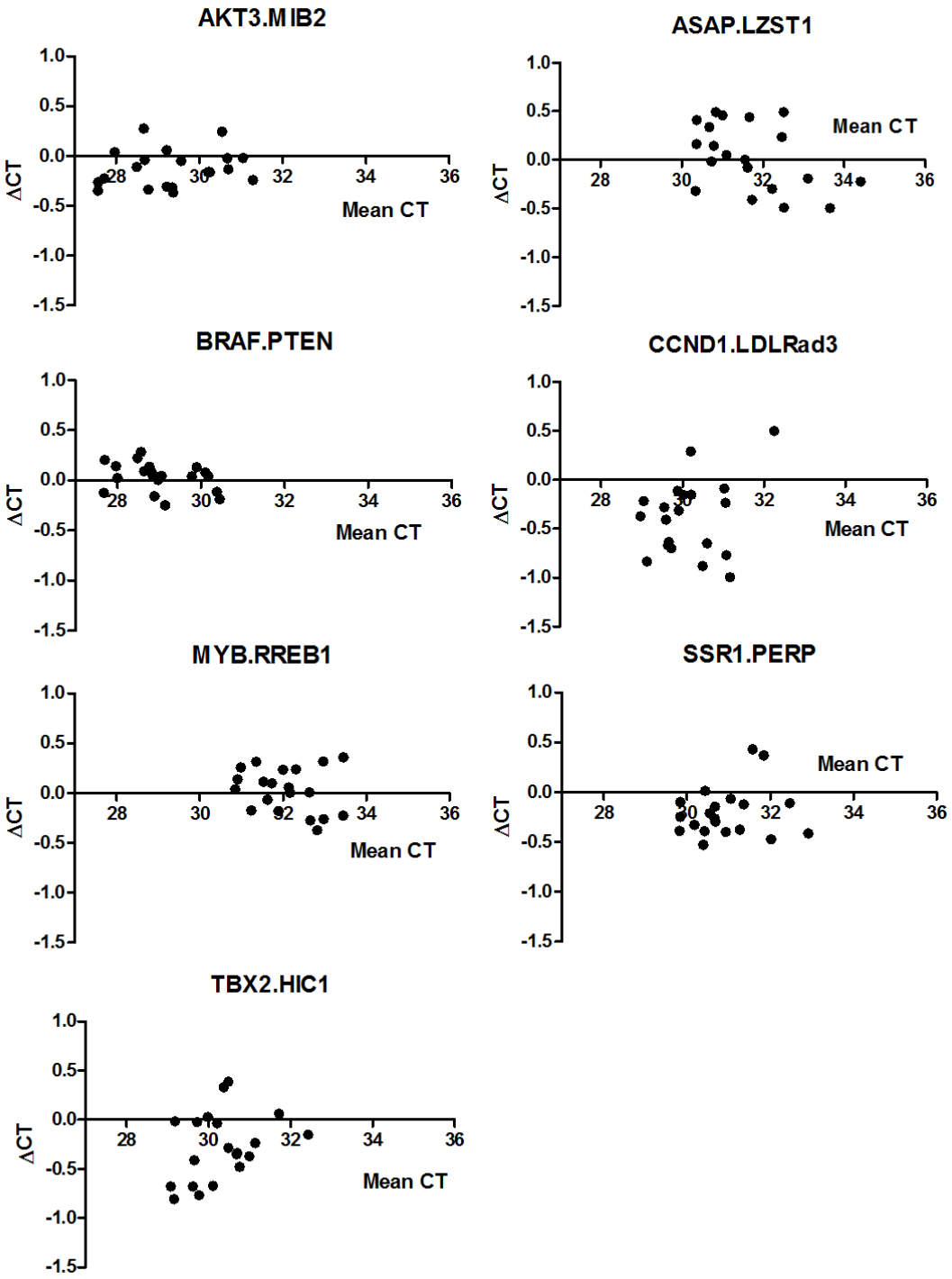

Figure 3-4 Bland Altman plots for the 7 DRTs using the control tonsil CT values. Only one of the plots (ASAP.LZST1) showed a significant trend on linear regression analysis.

Plots demonstrating the relationship between CT values for the same tonsil control cases using the same DRTs assays are shown in figure 3-5 and demonstrate the close association which is reflected in the statistical analysis of reproducibility (table 3-7).

Both the MYB.RREB1 and SSR1.PERP assays targeted loci on the gained p arm and loci on lost q arm of the Chromosome 6 isochromosome. The 80 naevi and melanoma DRT results were correlated for these 2 assays, returning an intraclass correlation co-efficient of 0.77. This gives a good indication that both assays are detecting the same changes in chromosome 6 and supports the accuracy of the DRTs.

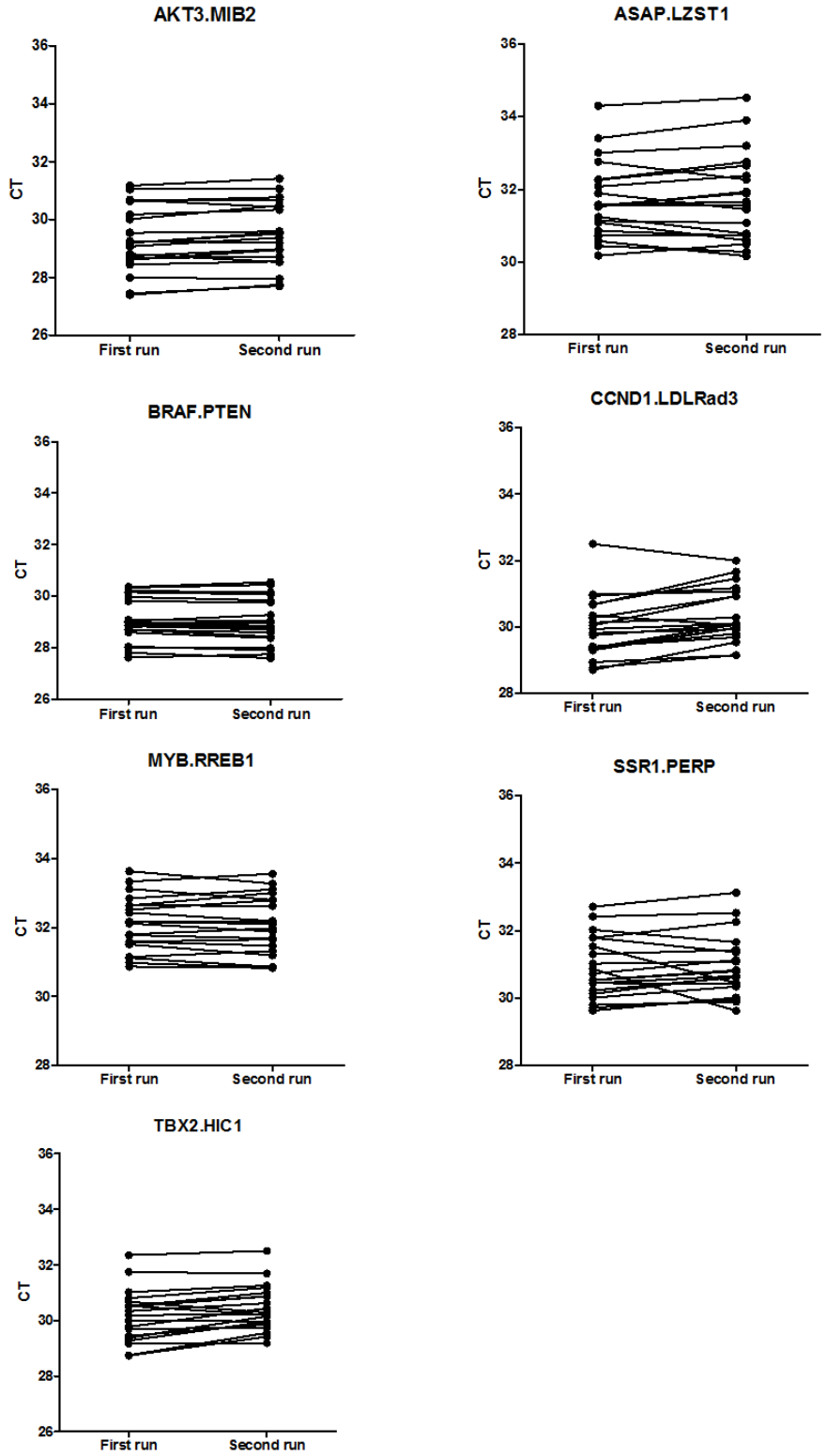

Figure 3-5 Correlation between CT results for the tonsil control samples in the first and second PCR runs, for all 7 of the tested DRTs. These values were used to calculate the correlation co-efficients quoted in table 3-7.

3.2.2 DNA extraction methods

As DNA quality appears to be crucial for the use of cDRT assays, 3 DNA extraction methods were compared (see table 2-3 in the previous chapter). The mean cDRT from the X/18 assay was used as a surrogate marker of DNA quality. The results in table 3-8 demonstrate that CT values are lower using method 3. Therefore longer digestion and the use of 5 histological sections rather than 1 for DNA extraction, appears to result in better quality DNA both for wide local excision specimens and lymph nodes. These differences are statistically significant ($p < 0.05$) for all comparisons between different methods.

Tissue	Method	cDRT mean CT
WLE	method 1	27.23
	method 2	25.56
	method 3	24.69
LN	method 1	24.35
	method 2	22.57
	method 3	22.34

Table 3-8 Results of comparison for protein digestion methods. Mean Xb/18b cDRT CT values resulting from the 3 different DNA extraction methods for a series of wide local excision (WLE) and lymph node (LN) specimens.

3.2.3 Testing DRTs against a series of diploid FFPE samples

The assays were tested against a reference range of 108 formalin fixed paraffin embedded benign tissue samples, as detailed in section 2.1.1, in order to determine the normal range of values in diploid tissue for each DRT.

The mean ΔΔCT values for the reference range, using each of the 7 DRTs, are summarised in table 3-9. When the ΔΔCT values for the diploid samples were

compared to the corresponding mean CT values, for 4 of the 7 assays a significant trend was detected (see figure 3-6). This indicates that the ΔΔCT result for these assays is dependent on the mean CT, which may indicate that the ΔΔCT is influenced by DNA quality, since a high CT for the same starting quantity of DNA reflects poor amplification. This suggests that as DNA quality changes, there is an unequal change in the reaction efficiency between the 2 PCR reactions present in each assay. As mean CT values may vary between different types of lesions (with smaller lesions such as naevi with lower DNA yields typically showing higher mean CTs), this represents a potential source of bias. In an attempt to eliminate any potential bias, for the 4 DRTs where a significant trend was identified between mean CT and ΔΔCT (see figure 3-6), a correction factor was calculated based upon the equation of the line (see table 3-9). This correction factor was applied to all ΔΔCT values for a given assay when the diploid, naevi and melanoma samples were tested, producing a 'corrected ΔΔCT' (cΔΔCT) for these 4 assays and these cΔΔCT values were used to calculate the DRT score.

In 2 of the 3 DRTs without a significant trend, the mean ΔΔCT was close to zero (<0.05). In those with a significant trend, applying the correction factor to the ΔΔCT values for all of the subsequent samples, producing a 'corrected ΔΔCT' (cΔΔCT) returned the mean value close to zero (see table 3-9).

Assay	Mean ΔΔCT	SD ΔΔCT	Significant trend CT vs ΔΔCT	Mean corrected ΔΔCT	SD corrected ΔΔCT
MYB.RREB1	0.162	0.285	Yes	-0.0197	0.269
BRAF.PTEN	0.220	0.478	Yes	<0.0001	0.461
SSR1.PERP	0.183	0.377	No		
ASAP.LZST1	0.163	0.263	Yes	0.0004	0.242
TBX2.HIC1	0.040	0.277	No		
LDLRad3.CCND1	0.016	0.443	No		
AKT3.MIB2	0.177	0.268	Yes	0.0002	0.245

Table 3-9 Correlation of mean CT and ΔΔCT values for the 7 DRT assays. Mean ΔΔCT, SD and the result of comparing the CT/ΔΔCT relationship are detailed from testing on the large reference range of DNA extracted from FFPE diploid samples.

Using the D'Agostino and Pearson omnibus normality test on the CT values for the reference range results, it was determined that of the seven DRTs, four showed a normal distribution of CT values: BRAF.PTEN, TBX2.HIC1, LDLRad3.CCND1, AKT.MIB2. For these four assays it can be presumed that the range of results on which any correction factor was calculated represents a normal range and is not skewed population. Both the MYB.RREB1 and ASAP.LZST1 assays show a non-Gaussian distribution of the results used to determine the correction factor.

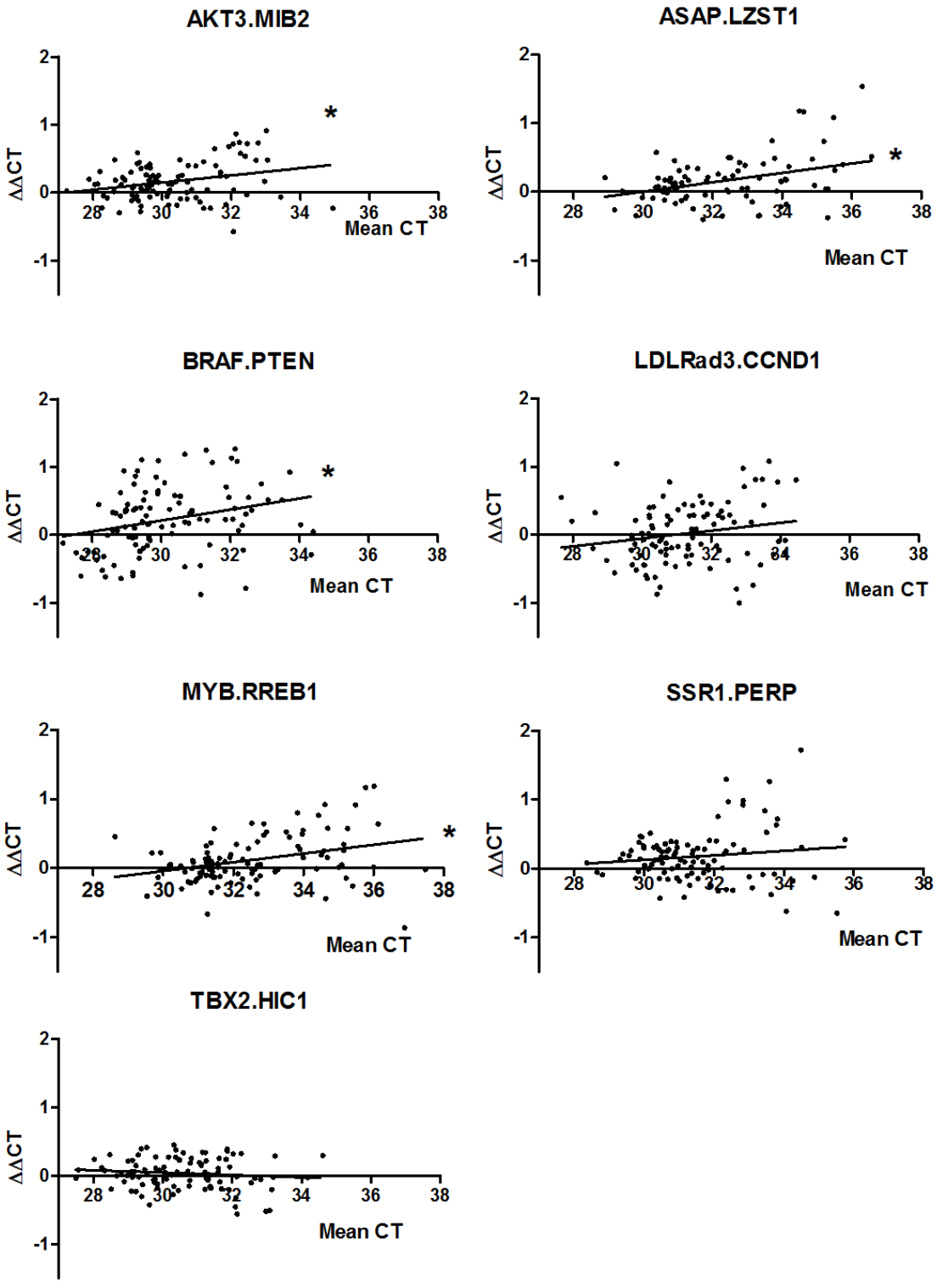

Figure 3-6 The relationship between CT and ΔΔCT for each of the DRTs. This demonstrates the mean CT to ΔΔCT relationship when applied to the reference range samples (* denotes significant trend line on logistic regression analysis).

The corrected ΔΔCT values were calculated from the equation of the trend lines as detailed for the 4 assays with a significant trend:

MYB.RREB1: corrected ΔΔCT = ΔΔCT – (0.05696 x CT) + 1.663

BRAF.PTEN: corrected ΔΔCT = ΔΔCT – (0.07697 x CT) + 2.102

ASAP.LZST1: corrected ΔΔCT = ΔΔCT – (0.05925 x CT) + 1.752

AKT3.MIB2: corrected ΔΔCT = ΔΔCT – (0.07818 x CT) + 2.186

3.3 Discrimination between different types of melanocytic lesions using the primary series of melanocytic lesions

3.3.1 Demonstration that DRTs are diagnostically useful – proof of concept on the primary series

The primary series of cases aimed to identify how the results for these DRT assays differ between 4 different classes of melanocytic lesions: benign naevi, primary melanomas without metastasis, primary melanomas with metastasis and melanoma metastases.

The 20 FFPE naevi samples were taken from 6 males and 14 females, with a mean age of 37.3 years and an age range from 12 to 67 years. The male to female ratio was 8:12 for the primary melanomas without metastasis (P-Ms) and 10:10 for the primary melanomas with metastasis (P+Ms). The mean age was 65 for the P-Ms and 69 for the P+Ms. The mean Breslow depth was 3.9 for the P-Ms and 5.4 for the P+Ms. The mean time from diagnosis of the primary tumour to the metastasis for the P+Ms was 16 months.

The results allow us to make 3 comparisons, that of naevi versus primary melanoma, P+M versus P-M and P+M versus their corresponding metastases, corresponding to potential value as diagnostic, prognostic and progression biomarkers. The DRT scores for the 7 assays on the 4 classes of melanocytic lesions are shown in fig 3.7-3.13, with the scores being corrected in those where a relationship existed between CT and ΔΔCT (MYB.RREB1, BRAF.PTEN, AKT3.MIB2, ASAP.LZST1 (see figure 3.6).

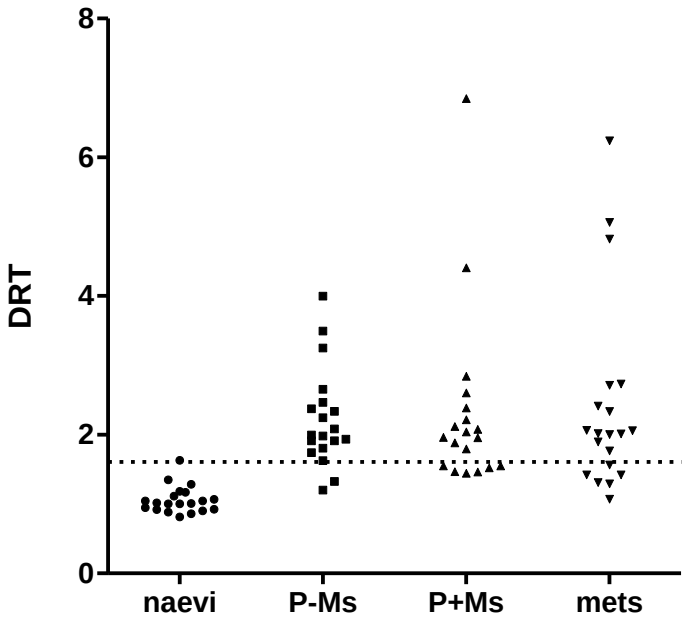

Figure 3-7 DRT values for the 4 classes of melanocytic lesions with the MYB.RREB1 DRT assay. This plot demonstrates the clustering of naevi scores around a value of 1. A z-score was created based on the naevi values and the 99% upper limit for these scores is indicated. Most of the melanomas of all 3 classes fall outside this limit.

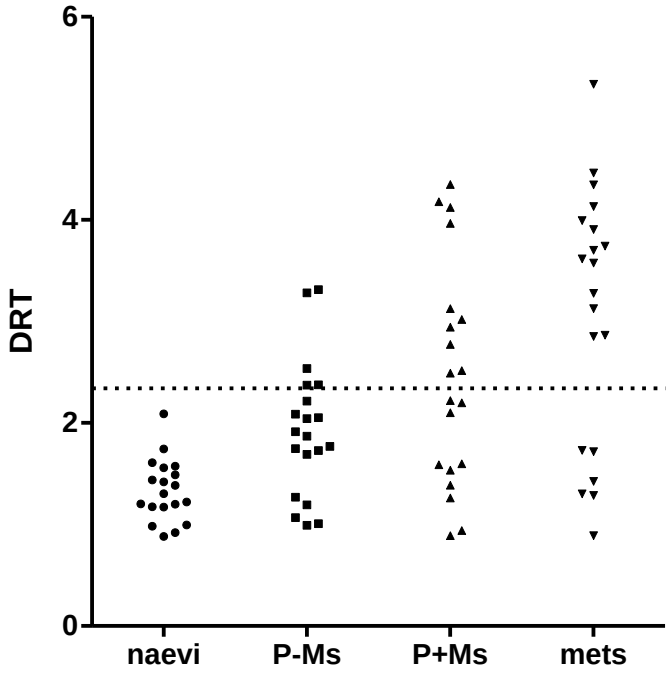

Figure 3-8 Plot of DRT values for the 4 classes of melanocytic lesions with the BRAF.PTEN DRT assay.

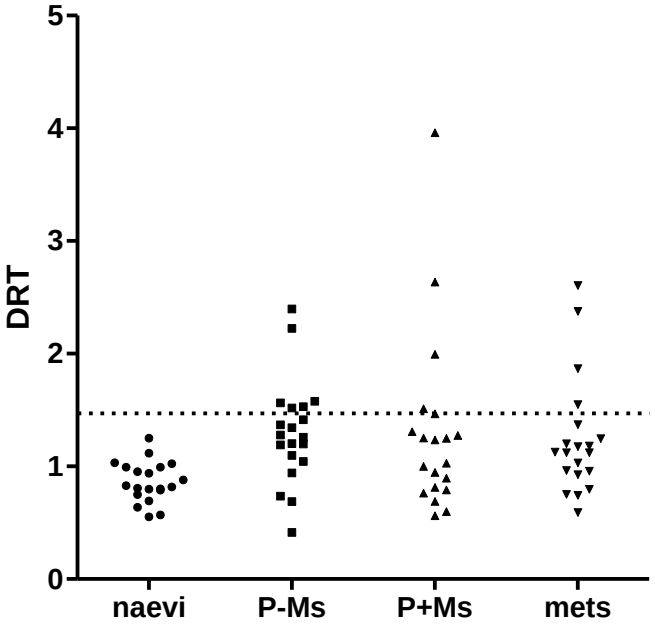

Figure 3-9 Plot of DRT values for the 4 classes of melanocytic lesions with the SSR1.PERP DRT assay.

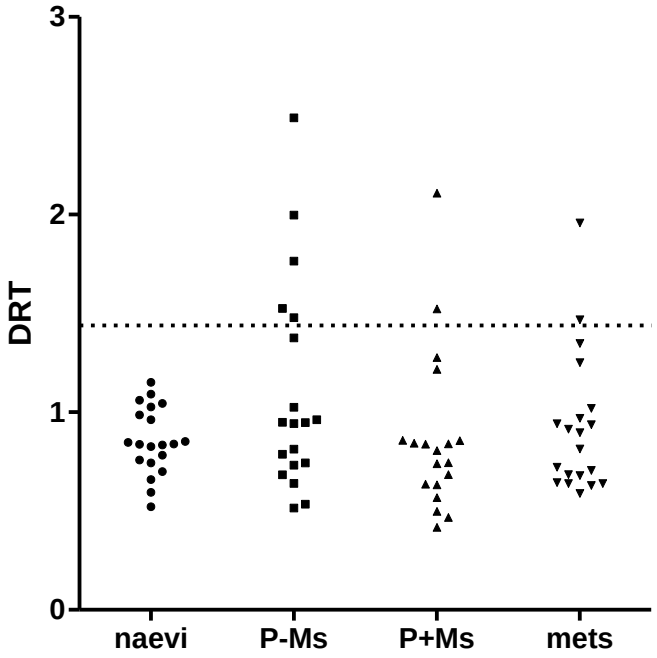

Figure 3-10 Plot of DRT values for the 4 classes of melanocytic lesions with the ASAP.LZST1 DRT assay.

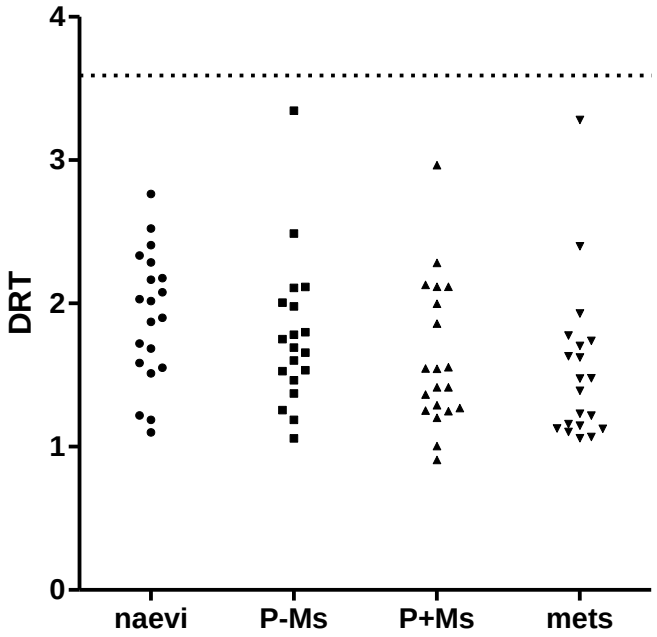

Figure 3-11 Plot of DRT values for the 4 classes of melanocytic lesions with the TBX2.HIC1 DRT assay.

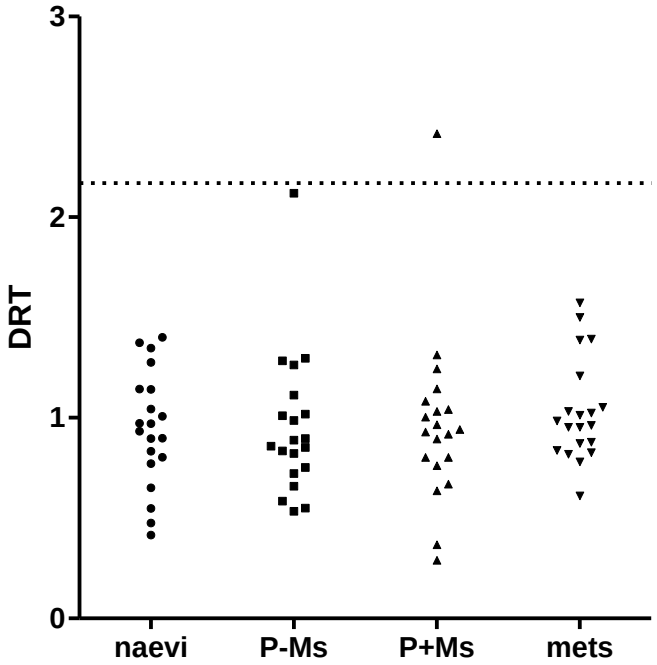

Figure 3-12 Plot of DRT values for the 4 classes of melanocytic lesions with the LDLRad3.CCND1 DRT assay.

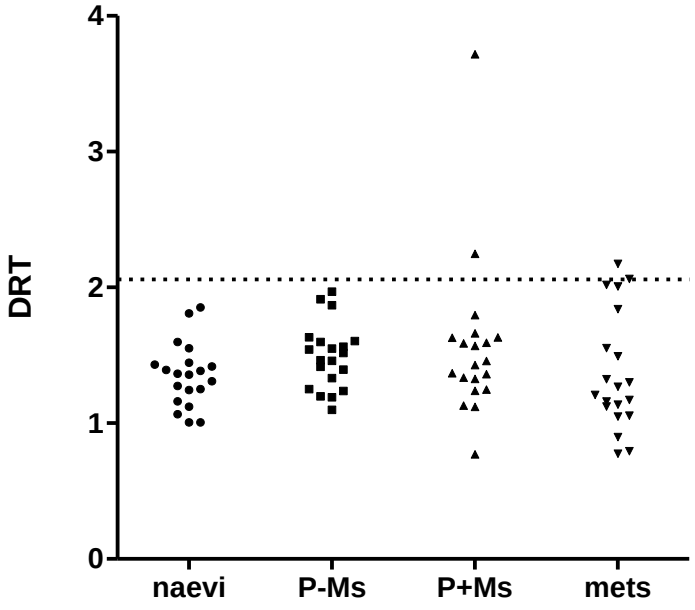

Figure 3-13 Plot of DRT values for the 4 classes of melanocytic lesions with the AKT3.MIB2 DRT assay.

3.3.2 Naevi versus melanoma comparison

With z-scores created based on the 20 naevi cases as the normal range, only one of the naevi returned a cΔΔCT value outside of the 99% normal range for one of the assays (MYB.RREB1). Of the primary melanomas, 38 of the 40 cases were detected with at least one of the DRTs. Of the 20 metastases, 19 were detected using the same method. All of the 7 assays detected at least one of the melanomas as being outside of this normal range (see table 3-10). This represents a sensitivity of 95% and a specificity of 95%.

A photomicrograph from the naevus which was classified as aneuploid using this method is shown in figure 3-14. Photomicrographs from the 3 melanomas which were not classified as aneuploid are shown in figure 3-15. All 3 of the 'negative melanomas' show a marked lymphocytic infiltrate which may have caused contamination of the extracted DNA. Further review of the histology from the 'positive naevus' by an experienced specialist dermatopathologist (GS) demonstrated no atypical features to call into question the original diagnosis and explain the aberrant result.

	naevi	P-Ms	P+Ms	mets
MYB.RREB1	1	17	14	14
BRAF.PTEN	0	5	10	14
SSR1.PERP	0	6	3	4
ASAP.LZST1	0	5	2	2
TBX2.HIC1	0	0	0	0
LDLRad3.CCND1	0	0	1	0
AKT3.MIB2	0	0	2	2
Detected with at least 1 DRT	1/20	19/20	19/20	19/20

Table 3-10 Number of cases from the primary series for which the cΔΔCT value was detected outside the 99% prediction band for naevi.

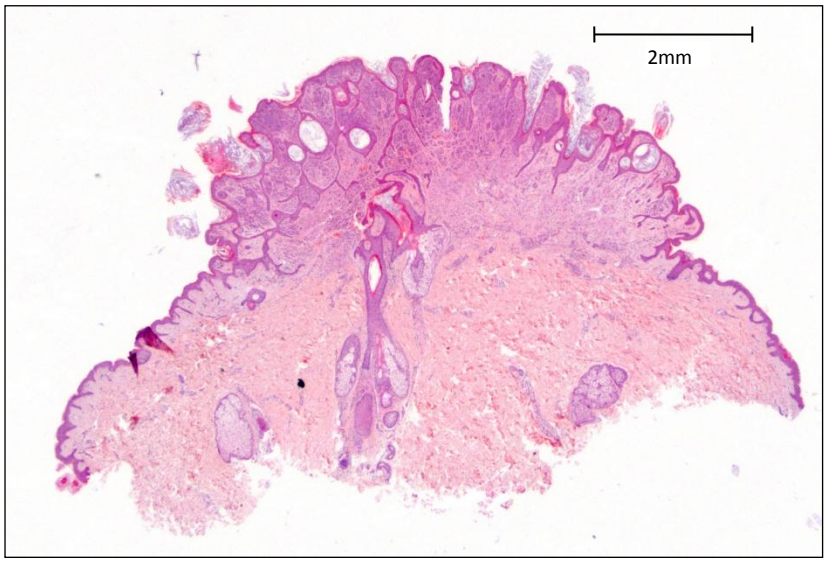

Figure 3-14 Photomicrograph (x1 objective lens) of the benign naevus which appeared aneuploid with the RREB1.MYB DRT assay. On further review, there were no atypical features identified and this was confirmed as a benign naevus.

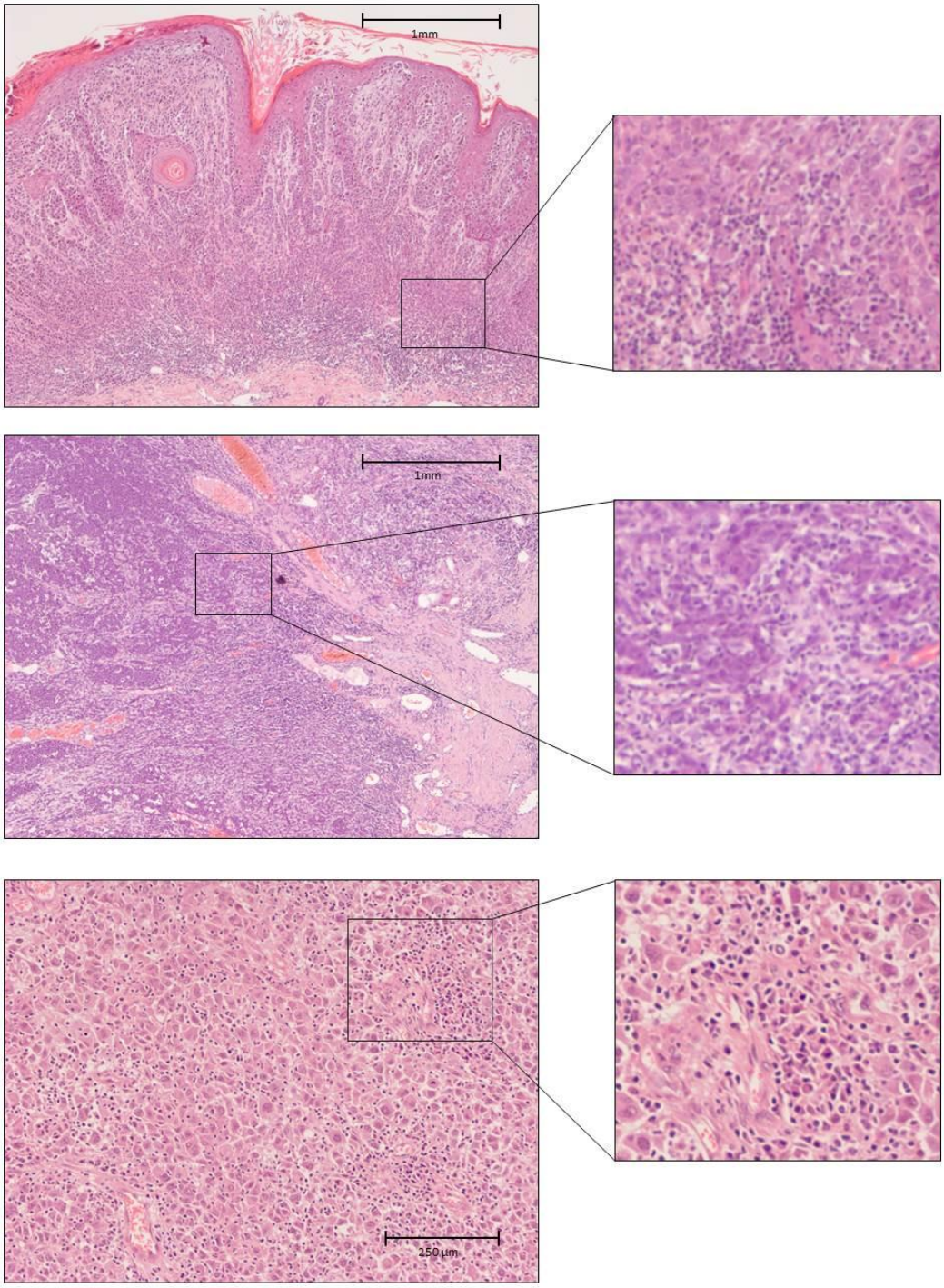

Figure 3-15 From top to bottom, photomicrographs from the P-M, P+M (both x10 objective lens) and metastasis (x20 objective lens) which were negative for aneuploidy with all DRT assays. Note the lymphocytic infiltrate in all 3 lesions.

Of the 7 assays, MYB.RREB1 detected the highest number of primary melanoma samples (30/40) as being outside of the 99% normal range (figure 3-7), followed by BRAF.PTEN (15/40) (figure 3-8). In combination, these 2 assays detected 36/40 primary melanoma cases using the 99% normal range as the cut-off. The 4 primary melanomas not detected with this method comprised 2 P-Ms and 2 P+Ms. Of these four cases, 2 were not detected with any of the other 5 DRTs, one was detected by two other DRTs (LDLRad3.CCND1 and AKT3.MIB2) and one was excluded from the MYB.RREB1 analysis due to poor replicate values but was detected by the SSR1.PERP DRT which also targets adjacent regions within 6p/6q. In fact of the 16 cases which were detected with the SSR1.PERP assay, this was the only case which was not also positive for MYB.RREB1 and this was only for the technical reason of poor CT replication.

The ASAP.LZST1 and the TBX2.HIC1 assay identified 9/40 and 0/40 total melanomas respectively. The ASAP.LZST1 assay did not however identify any melanomas which had not also been detected by either the BRAF.PTEN or MYB.RREB1 assay.

Binary logistic regression analysis using the 7 DRT values to compare naevi and primary melanoma demonstrated 97.5% sensitivity and 95% specificity for identifying malignancy. A Receiver Operating Characteristics (ROC) curve using predicted probability values derived from ΔΔCT values from all 7 DRT assays in the comparison between naevi and melanoma is presented in figure 3-16. This shows an area under the curve (AUC) value of 0.995. Using the 2 most discriminating assays alone (MYB.RRBE1 and BRAF.PTEN), gave a sensitivity and specificity of 92.5% and 90%

respectively, and the optimum values quoted above could only be achieved using all 7 assays.

When compared to the results from the diploid reference range, one of the DRTs (TBX2. HIC1) showed a significant difference in mean DRT between values for the reference range and the naevi ($p<0.0001$) with no significant difference in results between naevi and primary melanoma (figure 3-11). This unexpected finding was followed up with further work outlined in 3.3.5.

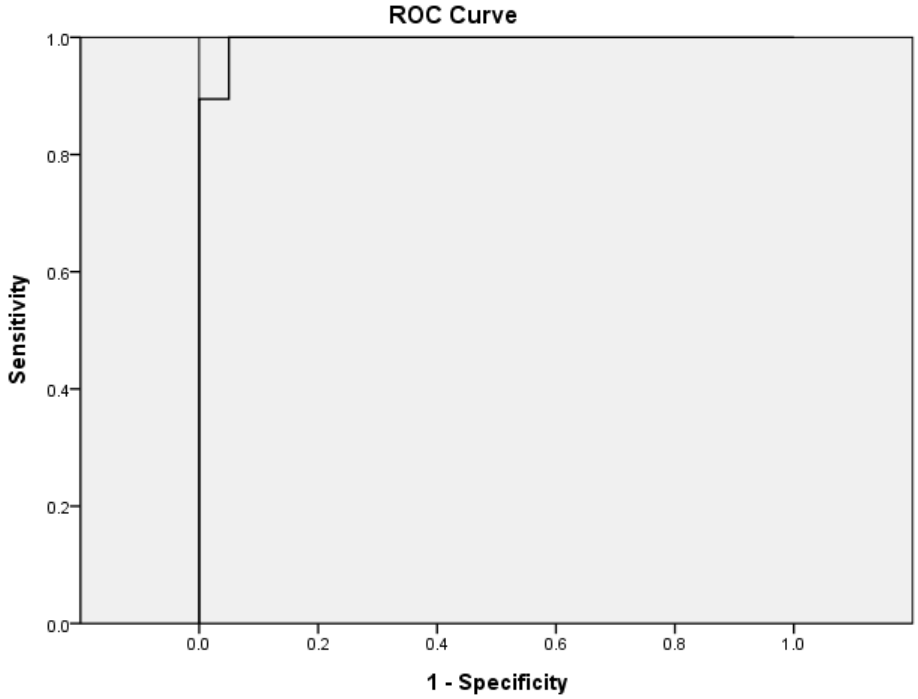

Figure 3-16 ROC curve demonstrating the specificity and sensitivity of the full panel of 7 DRTs to differentiate between melanoma and naevi. This was determined using binary logistic regression analysis of the 7 DRT scores on the 20 naevi and 40 melanomas from the primary series of cases. AUC = 0.995

3.3.3 Comparison of primary melanomas with or without metastasis

There were 20 melanomas with and 20 melanomas without metastasis selected in the primary series and the demographic data pertaining to these cases is detailed in 3.3.1.

There was no statistically significant difference between the DRT values for the P-Ms and P+Ms with any of the 7 DRTs on unpaired t-test analysis. Binary logistic regression analysis using the results of all 7 DRTs on the 40 primary melanomas was performed to determine the sensitivity and specificity for separation of P+Ms and P-Ms and the percentage correctly predicted. The same analysis was performed using clinical stage alone as a predictor of subsequent metastasis and the combination of clinical stage and DRT values. The results of this analysis are presented in table 3-11. The results indicate that there may be some, limited, discriminating difference in genetic instability between P+Ms and P-Ms at the loci targeted. The discrimination between metastasising and non-metastasising lesions with DRTs is not as successful as the discrimination between naevi and melanoma on this series. This might be expected, as the DRT targets were selected from genomic regions of somatic copy number alteration common to all primary melanomas in general.

	Sensitivity	*Specificity*	*% correctly predicted*
Clinical stage alone	40%	80%	60%
DRT results alone	70%	65%	70%
Clinical stage and DRT results combined	85%	70%	77.5%

Table 3-11 Sensitivities and specificities for prediction of subsequent metastasis in 40 melanomas (20 P-Ms and 20 P+Ms) using DRT assay results and clinical stage separately and combined, with logistic regression analysis.

3.3.4 Correlation between BRAF.PTEN DRT result and BRAF mutation status

The cases of naevi and melanoma for which there was sufficient remaining DNA from the primary series were subsequently tested for the V600E BRAF mutation. This was to investigate whether there was any association between the DRT score for BRAF.PTEN and the BRAF mutation status. DNA copy number alteration at the BRAF locus has not yet been described as being associated with BRAF mutation. This revealed that 18/19 naevi, 5/20 P-Ms, 5/17 P+Ms and 4/20 metastases harboured the mutation (see figure 3-17).

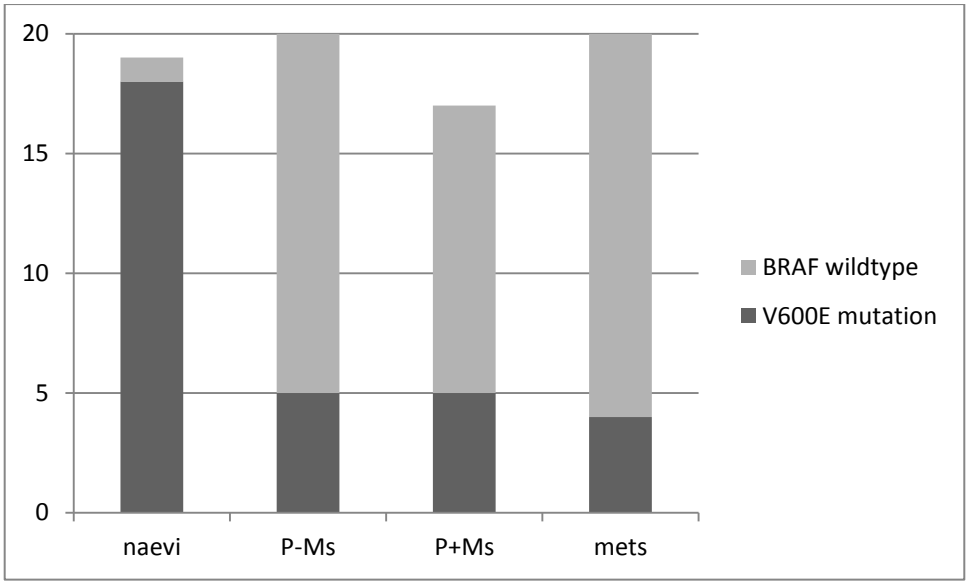

Figure 3-17 Proportion of primary sample cases tested demonstrating the BRAF V600E mutation on QPCR mutation analysis.

Of the 5 P+Ms which were V600E positive, 3 had a mutation positive matched metastasis, one had a matched metastasis with wild type genotype and one had no matched metastasis due to a lack of remaining DNA. The frequency of V600E BRAF mutations in this set of naevi was 95%. The rate of mutation in the primary melanomas was 27%. There was no significant correlation between the mutation status and DRT score, even when naevi were excluded from this analysis (p=0.86), though as the DRT result reflects the copy number status of PTEN as well as BRAF, it is difficult to draw firm conclusions about the relationship between BRAF copy number status and the V600E mutation from these results.

3.3.5 Comparison between primary melanomas and the corresponding metastasis

There was a significant association between the DRT values for the P+M cases and the corresponding metastases with all of the DRTs apart from LDLRad3.CCND1 when correlation co-efficients were calculated (see table 3-12). Further comparison of the

scores by paired t-test analysis revealed the BRAF.PTEN assay, but no other DRT pair, showed a significant difference between the set of P+Ms and the matched metastases (BRAF.PTEN paired t-test p=0.016). A significant correlation co-efficient and a lack of any significant difference between the 2 groups suggests that regions of somatic copy number alteration are conserved as primary tumour progresses to metastasis and that there is not a major increase in the DNA copy number alterations at these loci during this progression.

DRT	Correlation coefficient	Correlation coefficient P value
MYB.RREB1	0.663	0.002
BRAF.PTEN	0.520	0.019
SSR1.PERP	0.753	0.0001
ASAP.LZST1	0.532	0.019
TBX2.HIC1	0.675	0.001
LDLRad3.CCND1	-0.137	0.565
AKT3.MIB2	0.654	0.002

Table 3-12 Correlation coefficients and corresponding p values for comparison of DRT values derived for P+Ms and their corresponding metastases using all 7 DRTs.

3.3.6 Further investigation of the TBX2.HIC1 scores across melanocytic lesions in the primary series

As demonstrated in figure 3-11, the results for the TBX2.HIC1 assays using the range of melanocytic lesions suggests that naevi and melanoma may exhibit the same degree of aneuploidy relative to the TBX2 and HIC1 loci on Chromosome 17. If so this would represent a copy number change in benign naevi of either gain at the TBX2 locus or

loss at the HIC1 locus, neither of which have been previously reported. As it has been described that naevi show very few, highly specific alterations in DCN, it is most likely that if the results of the TBX2.HIC1 assay were to represent a genuine somatic alteration in DCN in naevi, this would only be seen at one of the loci. There was no significant relationship between CT and ΔΔCT when TBX2.HIC1 was tested on the reference range series of diploid cases, indicating that this phenomenon is not likely to be due to variations in DNA quality (and therefore CT value) between naevi and tonsil controls.

In order to determine which of the loci targeted was the most likely to represent DCN change in naevi, a locus was identified (Glucagon) from a region of Ch2 which appears to be genetically stable in previous CGH studies of melanocytic lesions. On testing of this locus using the Wellcome Trust Sanger Institute's CONAN application (130) no high level amplifications or homozygous deletions were identified on 51 melanoma cell line profiles. An amplicon was identified in the Glucagon gene which was highly specific for that region when tested against the human genome using the NCBI BLAST software and for which it was possible to design primers and probes which did not cross react with the primers and probes from either the TBX2 or HIC1 assays. This allowed the scores for melanoma, naevi and plate control tonsils to be tested with 3 different assays: TBX2.HIC1, TBX2.Ch2 and HIC1:Ch2. The objective of making this 3 way comparison was that if DRT scores for one of the assays involving Ch2 were to show a similar pattern of results to TBX2.HIC in suggesting aneuploidy, whilst the other Ch2 assay showed naevi with diploid DRT scores, this would indicate which of TBX2 or HIC1 was the likely candidate for copy number change.

This 3 way comparison was made using DNA extracted from, firstly, the 20 primary series naevi compared to 10 plate control tonsils (figure 3-18) and, secondly the first 20 cases of the naevi cohort compared to the 10 plate control tonsils (figure 3-19). The results demonstrated the same pattern with both sets of cases. In each the HIC1.Ch2 assay showed naevi clustered around 1, albeit with a wider range of values than the tonsil controls but, crucially, with no significant difference between the values for naevi and controls on t-test analysis. For both of the assays using TBX2 the naevi values were significantly different to the control values (see table 3-13).

Assay	Primary series cases	Cohort series cases
TBX2.HIC1	0.001*	<0.0001*
TBX2. Ch2	0.005*	0.0024*
Hic1.Ch2	0.886	0.06

Table 3-13 P values for the t-test comparison between naevi and tonsil DRT scores for the three assays involving TBX2, HIC1 and Ch2 (* denotes statistical significance). On both series of cases the assays involving TBX2 show a clearly significant difference.

There was a positive correlation between the results of individual cases with the TBX2.HIC1 and TBX2.Ch2 assays, with a correlation co-efficient of 0.77 (suggesting some correlation between those which show greatest evidence of chromosomal instability with the 2 assays involving TBX2). All of these results suggest that there may be an increase in the DCN of TBX2 in naevi.

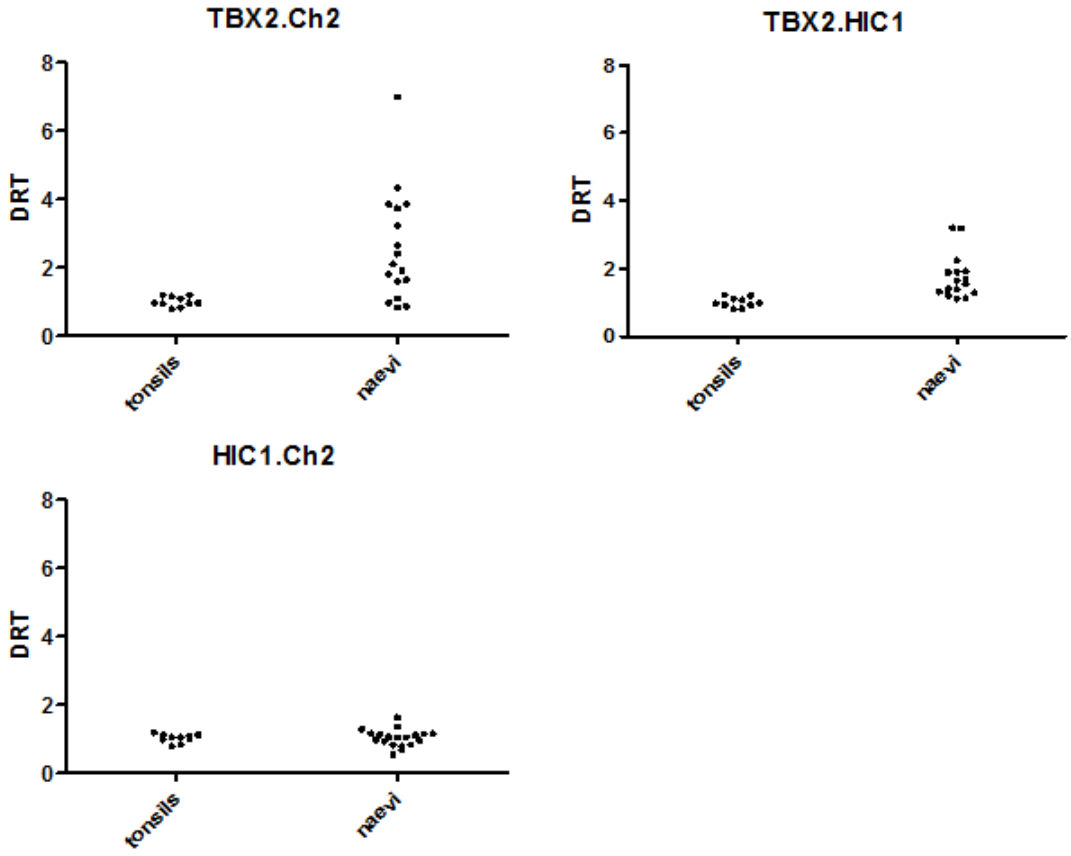

Figure 3-18 Results of the 3 assays testing the TBX2 HIC1 and Ch2 (Glucagon) loci using tonsil controls and naevi from the primary series of cases. Note the similar pattern of naevi scores compared to benign controls between the TBX2.HIC1 and TBX2.Ch2 loci. The HIC1.Ch2 naevi scores cluster around 1.

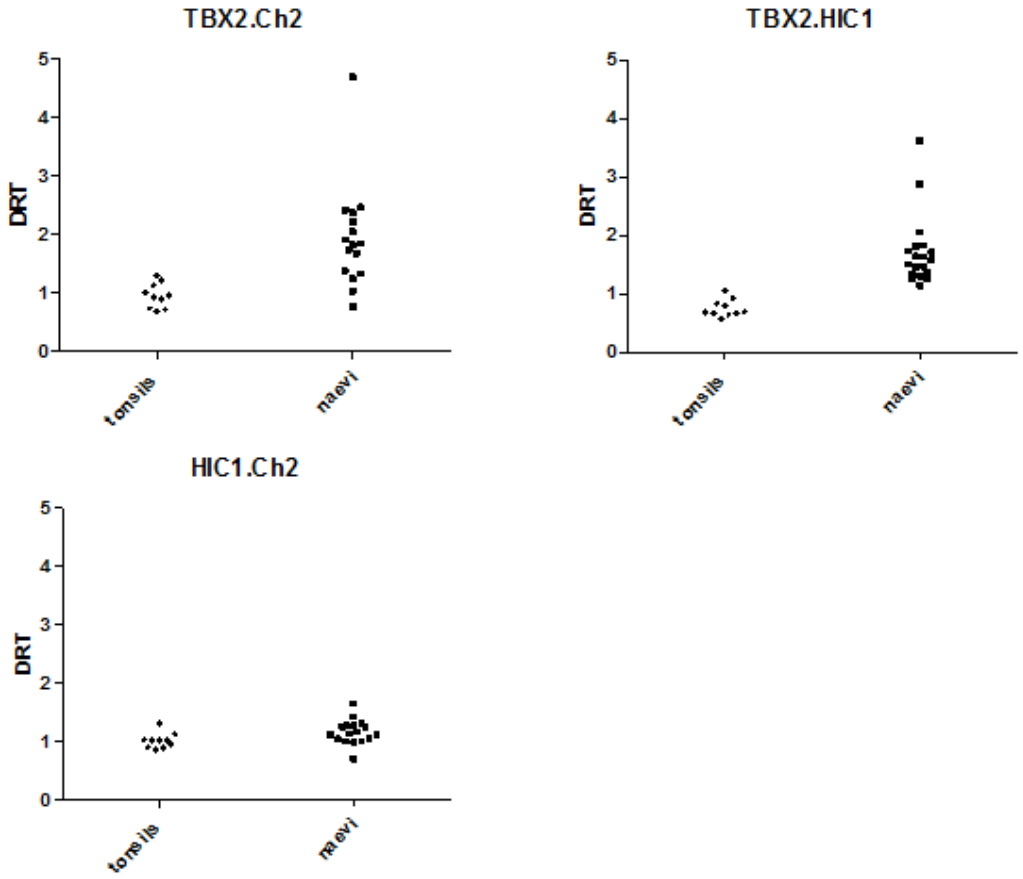

Figure 3-19 Results of the 3 assays testing the TBX2 HIC1 and Ch2 (Glucagon) loci using tonsil controls and a selection of naevi from the cohort series of naevi. Again, there is a similar pattern of naevi scores compared to benign controls between the TBX2.HIC1 and TBX2.Ch2 loci. The HIC1.Ch2 naevi scores cluster around 1.

3.4 Testing cohorts of melanoma and naevi

3.4.1 Cohort demographics

In development of the melanoma cohort, 660 melanoma reports were reviewed. 5 cases were excluded as their original reports indicated that they were of ambiguous histology and therefore not suitable for testing as a series of unambiguous cases. 52 cases were metastases rather than primaries. A further 407 were excluded as the melanomas were not of sufficient Breslow thickness for inclusion in the study or the

cases were in situ (non-invasive) melanoma. 34 cases were excluded as there was insufficient cross-sectional area to meet the inclusion criteria. Finally, 56 cases were excluded as the relevant tissue blocks were either unavailable or were not of sufficient thickness for sections to be cut for DNA extraction. This led to a cohort of 106 melanomas from which DNA was extracted. This comprised all suitable melanomas in the UHL pathology archive from 1st January 2000 to 29th of December 2003.

304 naevi were reviewed in the development of the corresponding naevus cohort. 1 case was excluded as it was a special type naevus listed in the exclusion criteria (table 2-1), though most of the special type of naevi would be excluded by the original pathology database search. Two cases were excluded as their reports expressed an equivocal or discursive diagnosis of benign naevus. 189 cases were excluded as there was insufficient sectional area of tissue or lesion (most of these were tiny lesions). All cases which had insufficient tissue block thickness were also of insufficient lesion cross sectional area. The remaining 111 naevi comprised all suitable naevi in the archive from 1st January 2000 to the 3rd of March 2000.

Of the 111 naevi and 106 melanomas selected for use in the cohort phase of the study, DNA extracted from 8 of the naevi and 1 of the melanomas did not amplify to the required CT of 34 using GAPDH primers on two separate occasions. On both occasions the cases were tested alongside tonsil plate controls which amplified at the expected CT. These 9 cases were therefore excluded from the study and the final analysis included 103 naevi and 105 melanomas.

The final 103 naevi in the cohort were taken from 50 males and 53 females, with a mean age of 33.6 years and from a range of ages 9 to 92.

The final 105 melanomas in the cohort came from 45 males and 60 females, age range 31 to 94. The mean age was 71.4 years. The mean Breslow depth was 4.3mm. Only 15 of the cases had evidence of metastasis on histology, cytology or radiology according to the pathology and radiology records of the UHL NHS Trust.

The principal aim of the cohort phase of the study was to test whether the differences between melanoma and naevi seen on the primary series could be reproduced in a true cohort of melanoma and naevi. Since the results of the primary series suggested that DRTs have less prognostic than diagnostic potential, the cohort was designed to test DRTs as diagnostic biomarkers.

3.4.2 Cohort analysis for melanoma versus naevi

The naevi versus melanoma comparison showed a less obvious separation between the 2 groups using the panel of DRTs than had been seen on the primary series. In particular the MYB.RREB1 assay did not discriminate between the majority of cases as successfully as previously, though there was still a statistically significant separation between the groups for 5 of the assays on unpaired t-test analysis. The TBX2.HIC1 and CCND1.LDLRad3 DRTs showed no significant difference between the two groups (p values of 0.22 and 0.69 respectively). All other p values were < 0.005. The lack of differentiation between melanoma and naevi on TBX2.HIC1 assay may be expected given the previous findings discussed in detail in section 3.3.6. In the primary series only one of 60 malignant melanoma specimens was outside the naevi 99% z-score range, therefore it is unsurprising that there was no statistically significant difference between melanoma and naevi in the cohort series of cases with this assay.

Alongside the cohorts, 2 available Spitzoid melanomas were also included as a small sample to determine whether these cases would be classified as malignant along with the conventional melanomas. These were specimens H242 and H51 and their histological appearances are shown in figure 3-20. Case H51 was originally diagnosed as being a Spitzoid Tumour of Uncertain Malignant Potential (STUMP) whilst case H242 was diagnosed as Spitzoid melanoma. Both subsequently metastasised, confirming their malignant nature. These are the type of cases which are sometimes histologically challenging to classify as benign or malignant and it is envisaged that the assay would ultimately be applied to these types of cases.

These lesions are both classified as being within the melanoma group on binary logistic regressions analysis of the results. The box and whisker plots in figures 21-27 show them having a high DRT value for several of the assays, with one of the cases showing a value above all the naevi values for ASAP.LZST1 and the other case showing a value above all the naevi values for AKT3.MIB2.

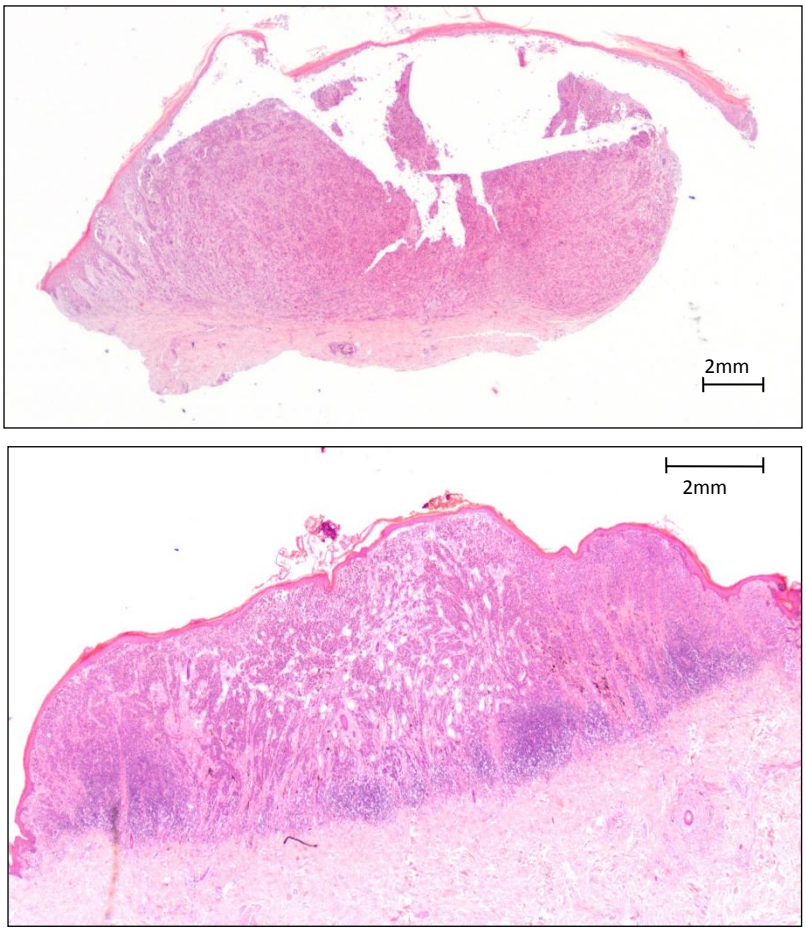

Figure 3-20 Photomicrographs of the two Spitzoid lesions tested, (both taken with x1 objective lens). Top image shows specimen H242, bottom image shows specimen H51.

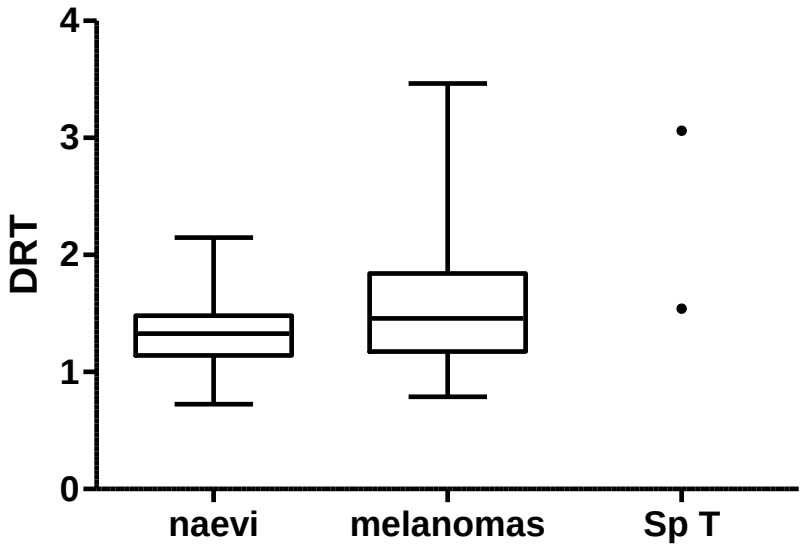

Figure 3-21 Box and whisker plot for the AKT3.MIB2 assay comparing the cohorts of naevi and melanoma. The results for the 2 Spitzoid tumours (Sp T) are included as scatter points.

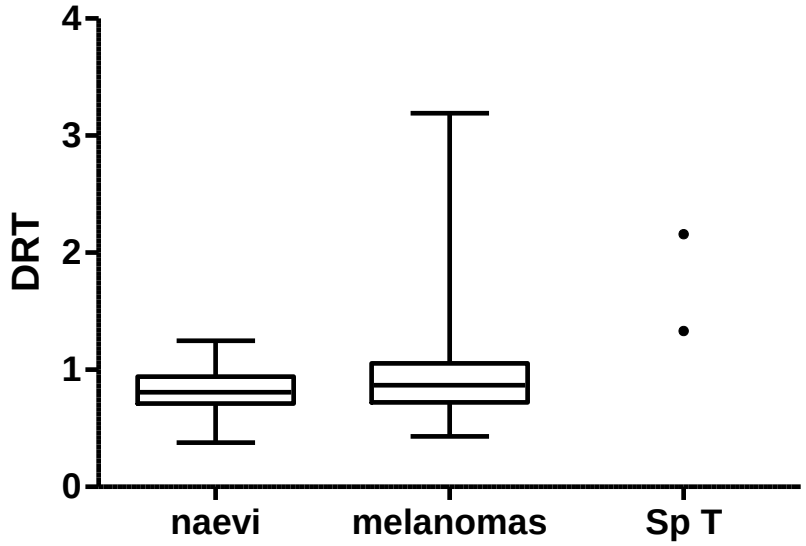

Figure 3-22 Box and whisker plot for the ASAP.LZST1 assay comparing the cohorts of naevi and melanoma. The results for the 2 Spitzoid tumours (Sp T) are included as scatter points.

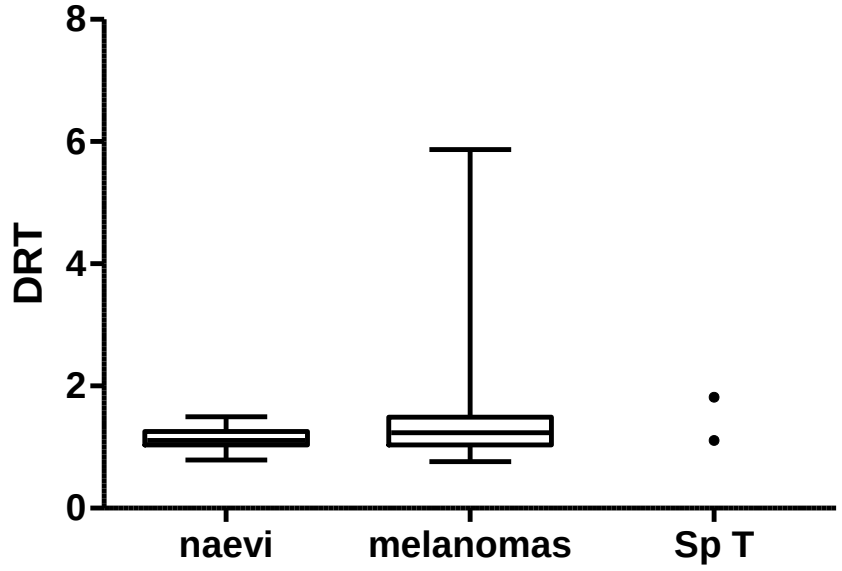

Figure 3-23 Box and whisker plot for the BRAF.PTEN assay comparing the cohorts of naevi and melanoma. The results for the 2 Spitzoid tumours (Sp T) are included as scatter points.

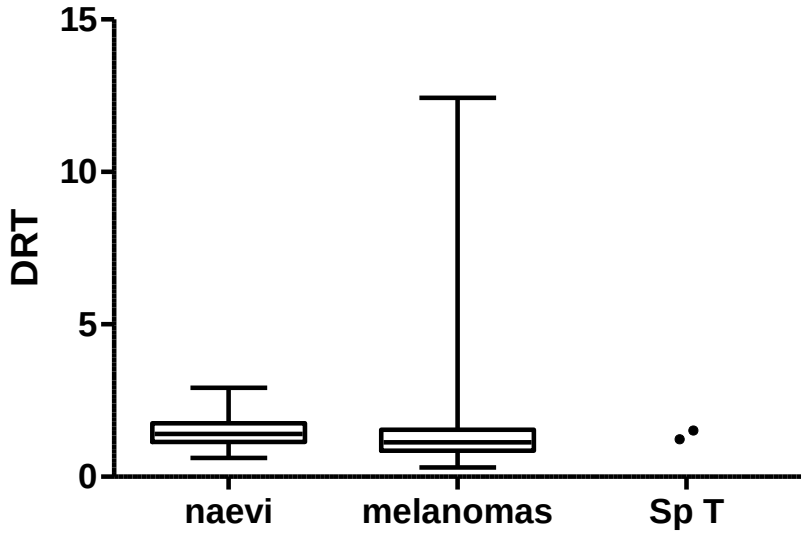

Figure 3-24 Box and whisker plot for the CCND1.LDLRad3 assay comparing the cohorts of naevi and melanoma. The results for the 2 Spitzoid tumours (Sp T) are included as scatter points.

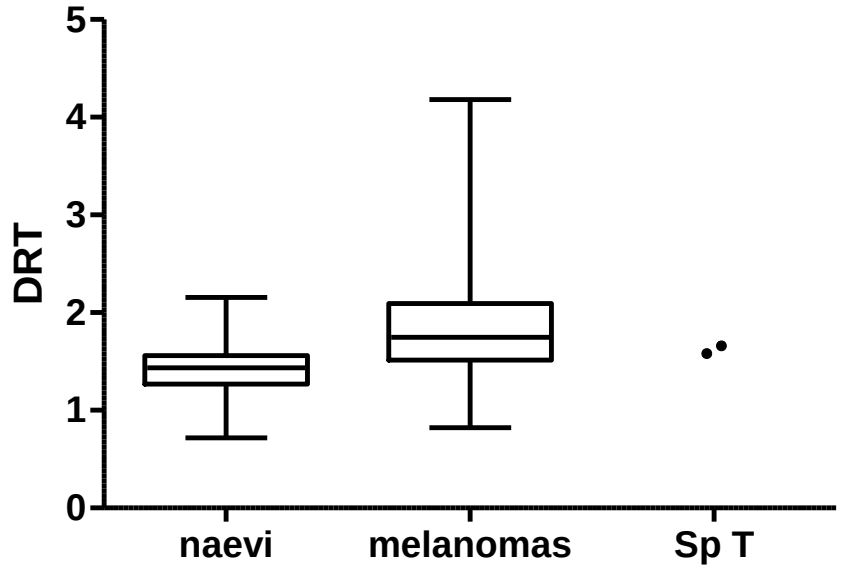

Figure 3-25 Box and whisker plot for the MYB.RREB1 assay comparing the cohorts of naevi and melanoma. The results for the 2 Spitzoid tumours (Sp T) are included as scatter points.

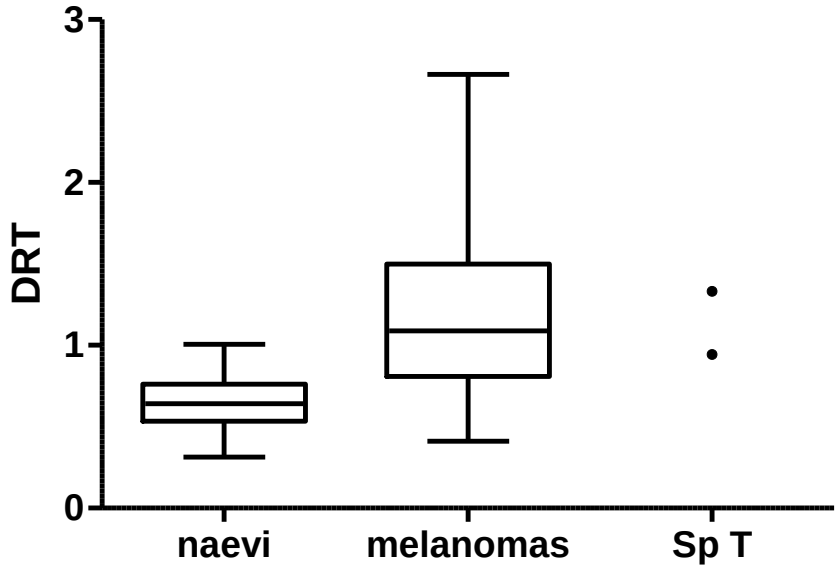

Figure 3-26 Box and whisker plot for the SSR1.PERP assay comparing the cohorts of naevi and melanoma. The results for the 2 Spitzoid tumours (Sp T) are included as scatter points.

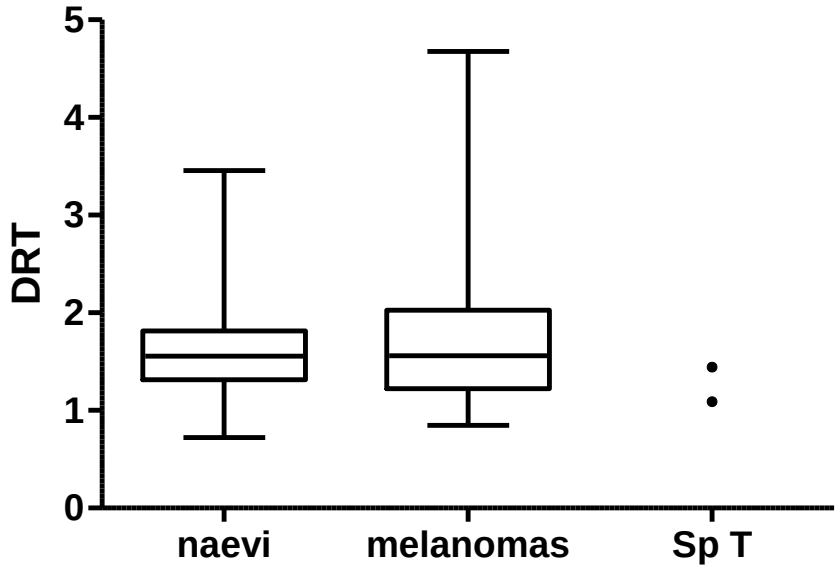

Figure 3-27 Box and whisker plot for the TBX2.HIC1 assay comparing the cohorts of naevi and melanoma. The results for the 2 Spitzoid tumours (Sp T) are included as scatter points.

Binary logistic regression analysis using the ΔΔCT results of all 7 DRTs to compare the naevi and melanoma cohorts showed the assays were able to predict lesions as malignant with a sensitivity of 84% and a specificity of 88%, with 86.0% of cases correctly predicted as melanoma or naevus. Separation between naevi and melanoma using predicted probability values developed from all 7 assays is represented in the ROC curve (Figure 3-28), with an AUC value of 0.923. As described previously, the TBX2.HIC1 assay showed minimal difference between the naevi and melanoma, although exclusion of the TBX2.HIC1 results from the logistic regression analysis resulted in the same sensitivity (84%), but a marginally lower specificity (87%), with 85.5% of cases corrected predicted as melanoma or naevus.

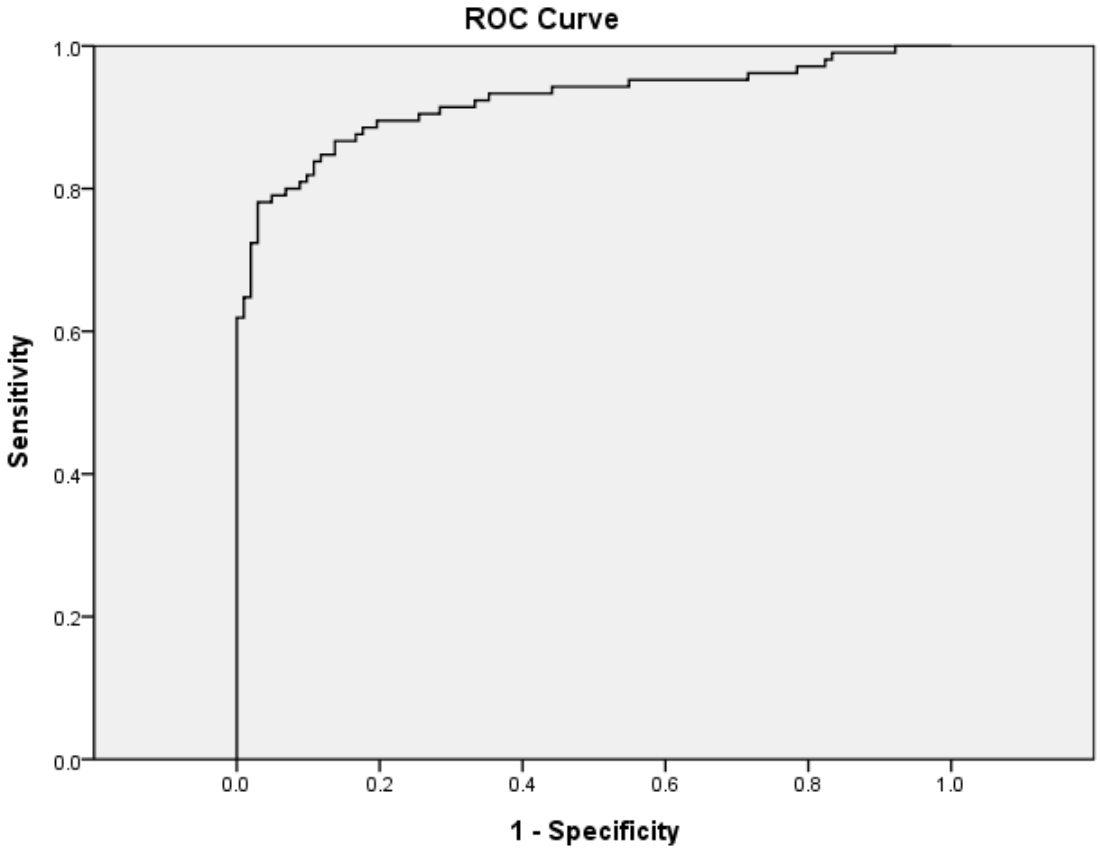

Figure 3-28 ROC curve demonstrating the specificity and sensitivity of the full panel of DRTs to differentiate between melanoma and naevi from the large cohort series using the ΔΔCT scores. AUC = 0.923.

When the naevi cohort z-score method of identifying chromosomal instability was used as in the primary series, with the 99% cut off used to define aneuploidy, the SSR1.PERP assay was the most discriminating between melanoma and naevi, followed by the MYB.RREB1 and BRAF.PTEN assays. Details of which cases were returned as being outside this 99% z-score range can be found in table 3-14. The sensitivity using this method on the cohort was much lower (67% compared to 95%) than on the primary series and the proportion of naevi detected was increased (8% compared to 5%), with a specificity of 92%.

Although the use of the z-score method of classifying aneuploidy was useful in the primary series, in the cohort study, binary logistic regression showed far greater sensitivity as a statistical method on the cohort series (84% compared with 67%). Specificity was marginally improved with the z-score method (88% for binary logistic regression and 92% for the z-score method).

	Naevi	Melanomas
MYB.RREB1	1	24
BRAF.PTEN	0	18
SSR1.PERP	1	39
ASAP.LZST1	1	15
TBX2.HIC1	3	4
LDLRad3.CCND1	1	12
AKT3.MIB2	1	10
Detected with at least 1 DRT	8/102	71/105

Table 3-14 Number of cases from the melanoma and naevi cohort studies for which the cΔΔCT value was detected outside the 99% prediction band for naevi.

3.5 London Dermatopathology Symposium Cases

Due to the limited time and tissue available for this analysis (which was actually performed prior to the primary and cohort series of cases), a limited series of DRT assays were applied to DNA extracted from the 5 ambiguous lesions for discussion at the Symposium. Photomicrographs of the H&E histological sections for each, all taken with x4 objective lens, are shown in figures 3-29 to 3-33**.** The cases were tested alongside the standard series of plate tonsil controls, extra tonsil controls and a small

number of benign naevi and melanomas. Although the numbers were too small for meaningful statistical analysis, figure 3-34 below shows all of the 5 cases appearing to show evidence of aneuploidy with the BRAF.PTEN DRT, more typical of melanoma than naevi. One of the cases showed a value typical of melanoma with ASAP.LZST1 (case 2) and the results for the MYB.RREB1 assay lay between the naevi and melanoma values. On the basis of the BRAF.PTEN assay alone, it was suggested that all 5 of the lesions had evidence of aneuploidy typically seen in melanoma rather than benign naevi. In comparison, only 2 of the cases (case 3 and case 4) were positive using the FISH assay. Expert review by a panel of experts led to a range of opinions and floor discussion with no consensus opinion on any of the cases (personal communication with GS). The lack of consensus between dermatopathology experts over difficult cases of this nature has been described previously (16-18)(19) and reiterates the inadequacy of conventional light microscopy for diagnosis in ambiguous melanocytic lesions. Regrettably, there was no follow-up data for these cases to indicate how they behaved clinically.

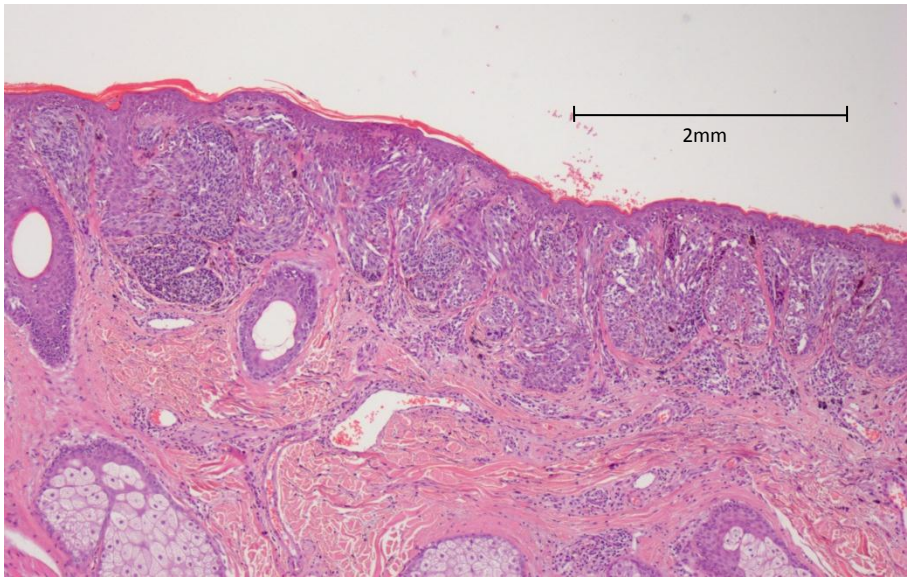

Figure 3-29 Photomicrograph of case 1 from the London Dermatopathology Symposium set.

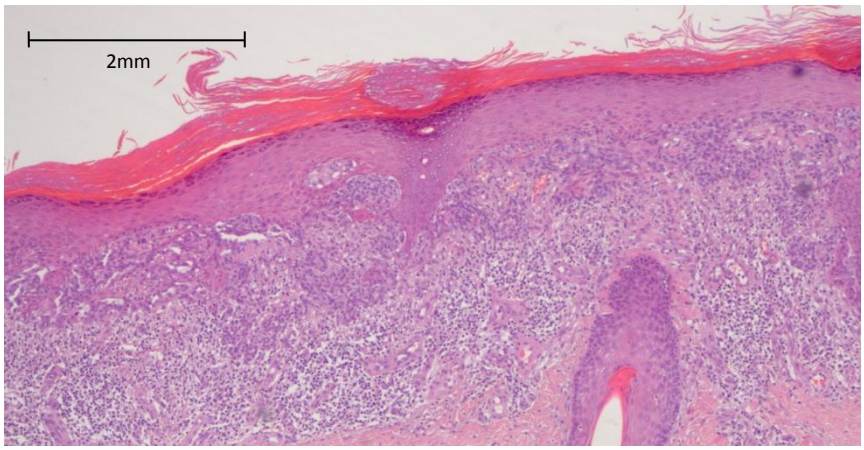

Figure 3-30 Photomicrograph of case 2 from the London Dermatopathology Symposium set.

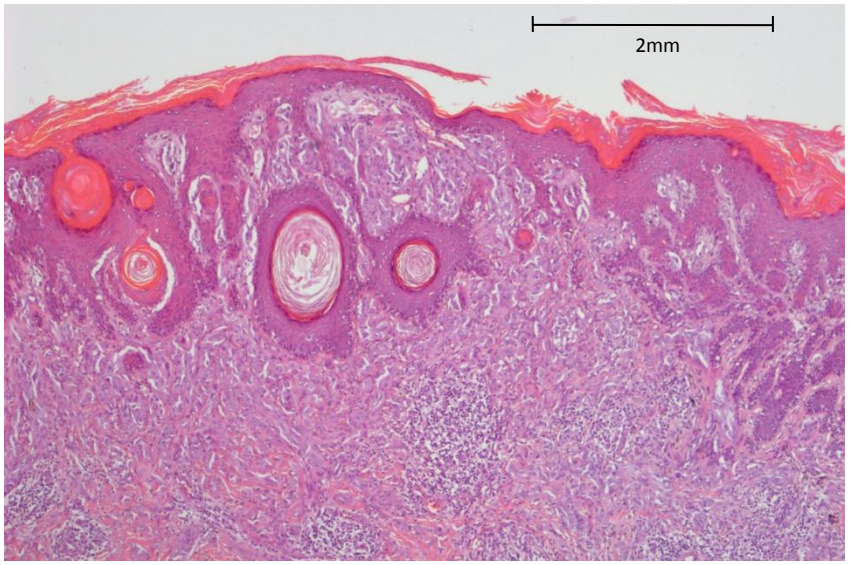

Figure 3-31 Photomicrograph of case 3 from the London Dermatopathology Symposium set.

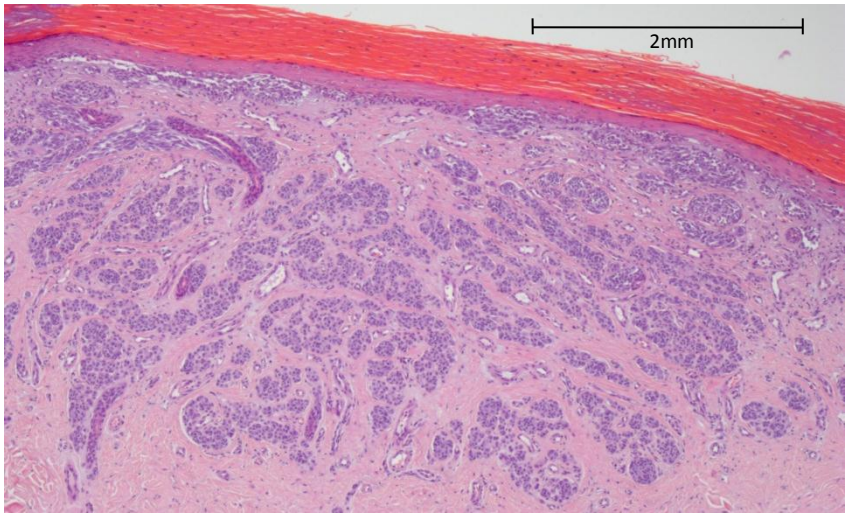

Figure 3-32 Photomicrograph of case 4 from the London Dermatopathology Symposium set.

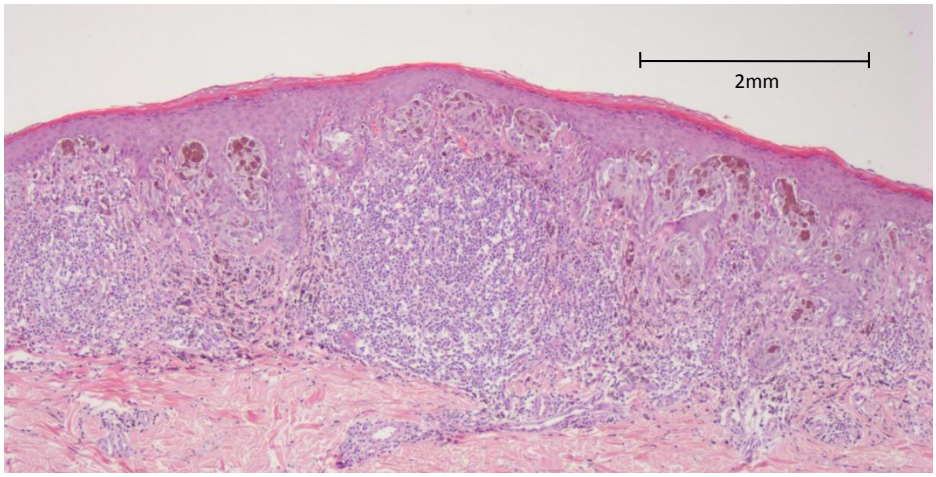

Figure 3-33 Photomicrograph of case 5 from the London Dermatopathology Symposium set.

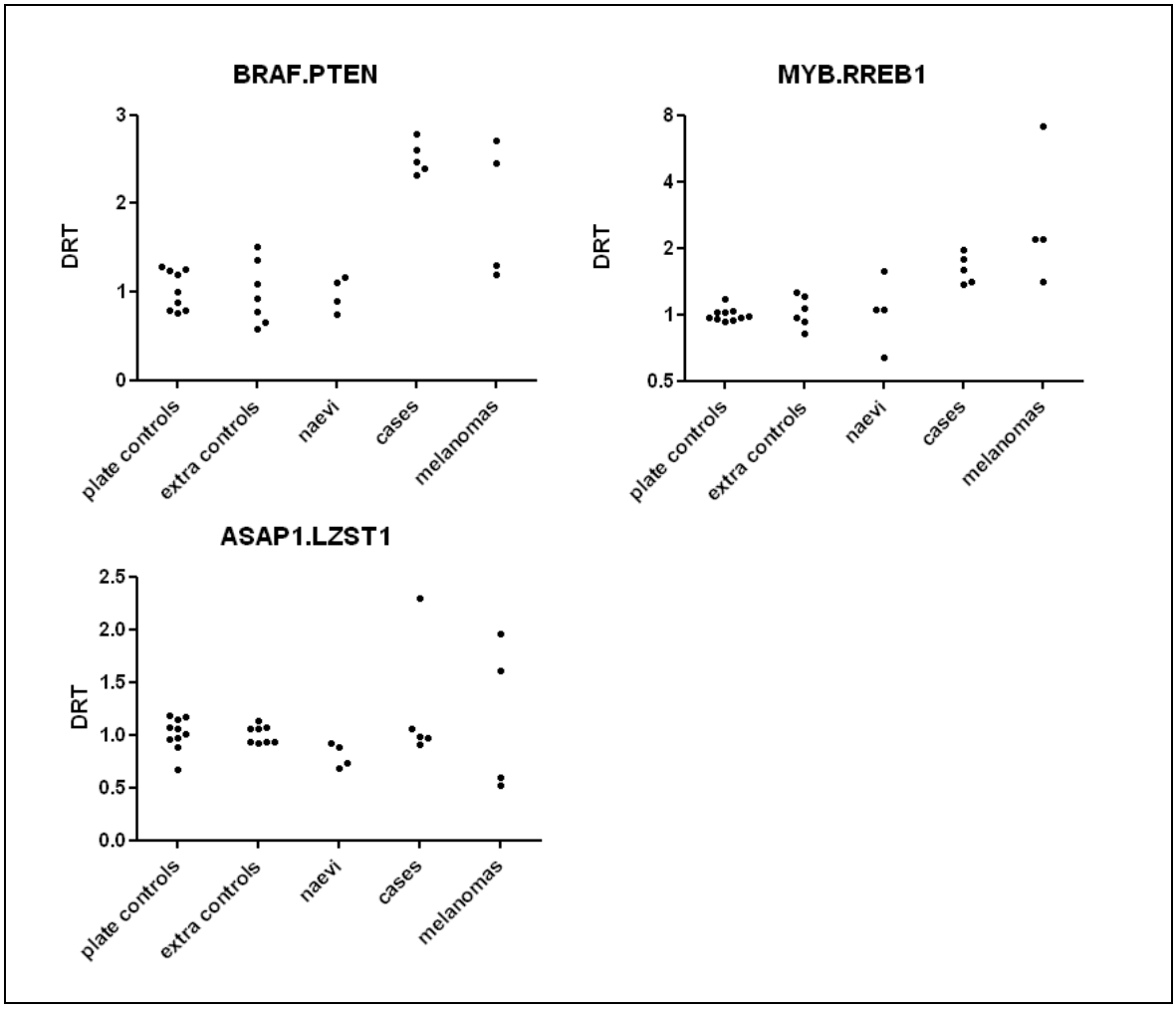

Figure 3-34 DRT scores for the 5 cases submitted for the London Dermatopathology Symposium, using 3 of the DRT assays. The controls, naevi and melanoma were tested at the same time.

4. Discussion

Numerous assays have been studied in an attempt to develop an effective molecular biomarker to, firstly, differentiate between melanoma and naevi for cases of diagnostic difficulty and, secondly, to aid prognostication. As yet only one diagnostic FISH assay (63) and no prognostic assays have been translated into clinical practice. The imbalance between the wide array of 'discovered' biomarkers and the lack of any prospective biomarker validation in this field may simply reflect that discovery and validation of a biomarker on small existing cohorts or opportunity samples of cases with follow-up data is a more viable task for research groups than a prospective study which involves a greater degree of organisation, requires more funding and takes several years to complete. Nevertheless, this means that despite the large body of research on the subject, there has been little practical benefit to patients with melanocytic tumours.

Work performed in our laboratory (carried out by AE and ZM and based upon some assays developed by LD and DB), comparing results from a small sample of melanoma and naevi suggests that QPCR may be developed into a useful diagnostic assay in the form of Duplex Ratio Tests (DRTs) (data presented at NCRI meeting 2011 – see Appendix 4).

The DRT method benefits from the simplicity of using a standardised technique and being relatively cheap to run (compared to array technologies or FISH). Before DRTs can be further validated however, the method needs to show a clear differentiation between melanoma and naevi on a wider range of samples.

4.1 Centromeric DRTs

Despite the huge volume of published genomic data regarding DNA sequences and their identified variations, limited information has been published characterising the alphoid repetitive sequences found in the centromeric region. The attempt to identify chromosome specific regions within these repeats described in section 3.1 has suggested that a great deal of homology exists between repeats on different chromosomes. This was identified when attempts were made to generate specific primer sets targeting chromosomes 8 and 9. It appears that chromosome 8 has homology with chromosome 10 and that chromosome 9 has homology with chromosome 4, presuming the hybridoma cell lines contain no centromeric cross-chromosomal contamination (and there is no evidence to suggest that they do). This makes it difficult to develop assays which show specificity for one chromosomal target and to use repeat sequences from the chromosomes thought to have the greatest degree DCN alteration in melanoma. This therefore poses problems for the potential use of centromeric repeat sequence 'cDRTs' in diagnostic or prognostic assays.

Furthermore, the specific repeats on different chromosomes appear to vary widely in the frequency of their repeats per unit of DNA, from our RT-PCR data, although varying efficiencies of simultaneously performed PCR reactions may be an explanation for this variation. Nevertheless, a useful DRT relies upon the paired targets to be roughly equal in number in normal tissue in order to give a ratio of 1:1 in diploid cases. If there is a large difference in DCN between the two targets in diploid tissue, subsequent aneuploidy variations in DCN are less easily detectable than if there is normally an equal number of both targets in normal tissue.

Initial results using the X/18 cDRT showed that in a minority of cases gender could not be predicted by the ΔCT (where females would be expected to have 2 copies of each and males one copy of X and two copies of 18). This raised the possibility that alphoid repeats may vary significantly in copy number between individuals or may harbour more frequent polymorphisms than was previously thought. Neither of these explanations are described in the literature however. When frozen tissue rather than FFPE was used for DNA extraction, gender could be accurately predicted based on the ΔCT and the assays showed a high degree of reproducibility. This informs us on two fronts: firstly, it is unlikely that there is a large degree of alphoid repeat variation or polymorphisms between individuals and secondly that even for high frequency sequences, DNA from fresh frozen tissue provides material which provides greater accuracy for amplifying alphoid repeat sequences. This is in contrast to data which has been derived from single copy target assays, where testing on DNA derived from FFPE tissue and fresh frozen tissue has shown Paralogue Ratio Tests (PRTs) and DRTs to be accurate and precise on both (106) (and see appendix 4). Although DNA degradation by formalin is well described (131), there is no clear explanation as to why this appears to represent a greater problem in centromeric DNA, but may be related to the mechanism by which DNA degradation occurs. In the case of an assay involving Chromosome X this may be related to the effect of formalin fixation on the inactivated Barr body in female cells. Although no evidence can be found in the literature to support this explanation, it is suggested by the wide range of formalin fixed female ΔΔCT values compared to corresponding to the male values (see figure 3-1).

Analysis of paired samples (high yield tonsil versus low yield skin) from the same patient indicates that in some cases individuals did have varying ΔCT values (i.e. ΔΔCT of >0.5 or <-0.5) between their two specimens. This appears to be related to the average CT value (and is likely to be a reflection of the quality of DNA present). This suggests that QPCR for alphoid repetitive sequences may be prone to a lack of specificity when DNA yield is low or poor quality, in much the same manner as QPCR for single copy sequences. cDRTs may therefore have no advantage over single copy DRTs for detection of genomic stability in small FFPE specimens other than detecting whole chromosomal losses and gains, although the experiment testing protein digestion times and the number of histological sections has shown that greater section numbers and longer digestion time reduced the CT values. At present, although the specific 18/X cDRT has been developed as a proof of concept for the cDRT method, despite repeated redesigning of the assays, neither the 6/9 or the 8/10 cDRT have been fully developed into assays which specifically compare the ratio of the two chromosomes.

There appears to be a lack of full chromosome specificity for alphoid repeat assays and their accuracy seems to be particularly affected by the use of DNA derived from FFPE tissue. At present this makes these assays unattractive candidates to be part of an algorithm which attempts to identify clinically useful changes in DCN. There is the possibility however that these assays as they currently stand may be useful in copy number evaluation, as we do not fully understand how the ratios produced on the hybridoma panel translate to the ratios found in the FFPE human DNA. Nevertheless, within the scope of this project, in which time, research funding and extracted DNA

samples were limited, it was decided that further testing of QPCR DCN assays should focus on non-centromeric DRTs for the remainder of the project. There is certainly potential for cDRTs to be of use as simple tests for whole chromosome copy number change if they can be optimised on the appropriate starting material and clinically validated, perhaps as an alternative to karyotyping or as a means of screening for genomic instability in tumours.

4.2 Analytical validity of DRT assays as potential biomarkers, method comparison and normal range development

Any biomarker which has the potential for clinical translation must demonstrate accuracy and reproducibility, as discussed in section 1.5. Accuracy indicates that the test is identifying a genuine change (in this case, genomic instability) and reproducibility confirms that the test will give the same (or very similar) results when applied to the same sample on different occasions.

4.2.1 Accuracy

The accuracy of the assays is firstly supported by the diploid samples showing copy number ratios clustering around 1:1 and the good agreement between the two DRT assays targeting 6p.6q across a range of samples.

The accuracy is somewhat further supported by the detection of aneuploidy in the majority of the melanomas tested and the lack of evidence of aneuploidy in the vast majority of benign specimens.

It is difficult to further test the accuracy of the assays without employing an additional method such as FISH or CGH analysis on the same samples. This was outside of the

remit of this project but would be required for full translation of a melanoma biomarker and is discussed in the further work section (4.7).

When a series of paralogue ratio tests (PRTs) were developed by our group prior to this project (see 1.4.1), paralogues from different chromosomes were tested within one assay. Accuracy of these assays could then be tested using placental tissue with known DCN variations in chromosomes X (varying gender), 18 and 13 (both common trisomy chromosomes) (106). As all but one of the DRTs detect 2 targets on the same chromosome, similar tests for accuracy with this series of DRT assays are not possible. The one assay targeting loci on different chromosomes is BRAF.PTEN, where the loci targeted are 7q34 and 10q23.3. Trisomy 7 is very rare and usually exists as a mosaic and Trisomy 10 is also very rare, only affecting the distal part of chromosome 10, with a breakpoint located between q22 and q25 (either side of the 10q23.3 PTEN locus). A deletion syndrome does occur affecting chromosome 1p36 which is the locus of MIB2, tested in the AKT3.MIB2 assay (the 1p36 deletion syndrome, occurring in approximately 1/10000 live births), although again the breakpoint is variable and can be either side of the exact MIB2 locus (1p36.33) targeted in the AKT3.MIB2 assay. There is therefore less opportunity to test the accuracy of the DRT in the same manner as PRTs through use of placental tissue.

As an alternative, the DRTs were tested, (prior to this project, by AE), using melanoma cell line DNA, demonstrating that the ΔCT scores were very close to those which would be predicted from the Sanger cancer genome project array data for these cell lines, (data previously presented - see Appendix 4).

4.2.2 Reproducibility

The reproducibility of the assays is demonstrated by the agreement statistics for the DRTs which show all of the assays to be broadly reliable on FFPE tissue. All of the values for calculated bias were similar, with the LDL.Rad3 assay showing a slightly higher value than the other assays and along with TBX2.HIC1, a somewhat wider limit of agreement and higher co-efficient of variation than the other assays.

The limits of agreement were widest for the TBX2.HIC1 assay (0.31 to -0.30), which is just beyond the margins of what is considered acceptable for a new technology used in clinical diagnosis (0.3 to -0.3) (132). All other assays were within these limits. The TBX2.HIC assay also revealed unexpected results when applied to the primary series of melanoma and naevi and the possible technical explanations are expanded in 4.3.3.

The MYB.RREB1 assay showed some of the best agreement statistics of all the DRTs in the assessment of reproducibility, yet the actual CT values were some of the highest for any of the DRTs (clustering between 31-34 for tonsil DNA specimens). This indicates that the mean CT value, which reflects starting DNA and reaction efficiency, is not the only determinant of reproducible results for DRTs and reaction kinetics may also be of relevance.

Only one of the Bland Altman plots comparing the difference between CT values on 2 occasions and the mean CT showed a significant trend on linear regression analysis with a borderline p value of 0.043. This further indicates that for the DRT assays as a whole there appears to be minimal systematic difference between separate experiments under the conditions and protocols used in our laboratory.

4.2.3 Optimisation of DNA extraction methods

Testing of the DNA extraction methods reported in section 3.2.2 show that prolonged protein digestion and greater volumes of FFPE tissue used as starting material have a statistically significant impact upon CT values for a given specimen. Prolonged protein digestion presumably has an impact on CT by breaking down more of the tissue to allow the DNA to be more readily extracted from the cells present. The results of the comparison of tissue section numbers indicates that the quantity of FFPE melanoma tissue used to prepare the DNA tested is a rate-limiting step with these assays. Although, on the basis of these findings, we used 5 histological sections as the material for extraction of DNA, the amount of DNA extracted from any tissue section is dependent on a number of factors such as the thickness of the section, which can be controlled by microtomy, and several factors which are not controllable when using retrospective samples. These include the length of fixation of the clinical specimen before embedding, the cross sectional area of the melanoma, the cellularity of the melanoma and the degree of DNA degradation during storage of the tissue. When using specimens which vary in nature it is likely that different specimens have somewhat differing requirements for protein digestion and DNA extraction. For instance a WLE specimen typically contains a fibrous scar and may require greater volumes of proteinase K for digestion of the dense area rich in protein than a small lymph node. No definite figure can therefore be given as the ideal number of sections to give optimum DNA quantity, but within the field of molecular testing of FFPE melanoma tissue, multiple 5 µm sections seem to be preferable. This correlates to

CGH studies on FFPE tissue which have used multiple sections to elute the required amount of DNA for analysis (41).

4.2.4 The diploid reference range

The series of 108 formalin fixed diploid samples used to develop the DRT score reference range showed for several of the DRTs there was a significant correlation between ΔΔCT and mean CT value. This indicates that for these assays changes in CT have an unequal effect on the two paired reactions and suggests that one of the reactions has lower efficiency at a higher CT than another. This might be expected, given that each is a separate reaction, although if uncorrected this might lead to a systematic bias if one class of lesions typically showed a lower yield of good quality DNA and therefore higher CT values overall. In three of the assays there was no significant trend, indicating that for these DRTs any systematic difference is undetectable with over 100 diploid samples. It is possible that small differences may exist for these assays but that there is insufficient statistical power with 108 diploid samples to determine this.

If the assays are to be developed into clinically useful biomarkers, normalisation may also need to be re-examined, in order to ensure that the reference range CT values all show a Gaussian distribution of CT values before correction factors are calculated, ensuring that the correction factors are not based on a skewed set of DNA samples. Application to a wider reference range sample, perhaps representing a diploid sample cohort rather than an opportunity sample, may be indicated.

4.3 Potential of DRTs to differentiate between classes of melanocytic tumours on the primary series of cases.

4.3.1 Differentiation between melanoma and naevi using DRTs on the primary series

Results from the primary series of cases demonstrate that this panel of DRTs can distinguish unambiguous cases of melanoma from naevi when using DNA extracted from FFPE tissues, with 97.5% sensitivity and 95% specificity for identifying malignancy using logistic regression analysis with all 7 DRTs.

The MYB.RREB1 and BRAF.PTEN DRTs showed the greatest separation between melanoma and naevi and these 2 assays alone returned a sensitivity and specificity of 92.5 and 90% respectively. The MYB.RREB1 targets 2 of the loci also utilised for the commercially available FISH assay (63) and the efficacy of these loci for testing melanoma specific copy number aberrations with both FISH and QPCR supports the accuracy of the DRTs.

One naevus was classified as aneuploid using both the z-score method and binary logistic regression and this showed no histological features to explain the aberrant results. The classification of the lesion as melanoma was on the basis of only one of the DRT assays (MYB.REB1), with the others showing a pattern typical of a diploid lesion. This raises the possibility of aneuploid contaminant DNA in this sample, although the classification of other benign naevi as aneuploid in the cohort series indicates that this is likely to be more than an isolated spurious result. There were two primary melanoma and one metastasis identified as diploid using the z-score cut offs. All of these lesions demonstrated a marked lymphocytic infiltrate within the tumour

on review of the histology, possibly causing contamination. This represents a disadvantage of this technique compared with FISH where the correct area within the tumour can be visualised. A possible solution to this problem would be the use of laser-capture microdissection for these samples, rather than crude pipette-tip microdissection using a pen-marked slide as a guide, although this would increase the case by case workload for this method.

The primary series was developed as an opportunity sample of both naevi and melanomas and there is therefore likely to be selection bias and a lack of generalisability with this series of cases, highlighting the need for cohort studies to indicate the likely sensitivity and specificity values which would be found in the wider population.

Discussion around the comparison of the z-score and binary logistic regression methods for separating melanoma and naevi can be found in section 4.4.

4.3.2 Differentiation between differing classes of melanoma using DRTs

Comparison of P-Ms and P+Ms using the DRTs was less discriminatory than comparison of melanoma and naevi, although when combined with clinical stage, the 7 DRT scores correctly separated P-Ms and P+Ms in 77.5% of cases.

It might have been reasonably expected that the naevus versus melanoma comparison would produce greater separation than P-M versus P+M, since widespread genomic instability is a feature of malignancy rather than just a marker of metastatic potential and also because the loci selected as targets for the assays were identified from array CGH studies of melanomas in general and not specifically those which were

metastasising primaries. There is no publically available array data comparing P-Ms with P+Ms and if this data were generated, this may highlight genomic regions in which there are copy number differences between the 2 groups. There is, however, array data comparing primary melanoma and metastatic tumours (133). Some of the loci identified in this study show greater copy number alteration in metastatic tumours and may identify candidate loci which would be valuable in prognostic DRTs. As it is not possible to identify when the alterations occurred in the development of the metastases, and given that metastases frequently show differing clones developing from the primary tumour (134), it is impossible to know if these loci are of true prognostic relevance for testing primary tumours.

Although there is a clearer reason why a method targeting genomic instability is applicable for diagnosis in melanocytic lesions (diploid naevi being compared with aneuploid melanoma), any clinically translated prognostic marker would impact on far greater numbers of patients, since the number of melanomas diagnosed far outweighs ambiguous melanocytic lesions. Indeed the diagnostic FISH assay developed by Bastian et al has now been tested as a prognostic marker (135) and studies in other solid tumours have identified that genomic instability is a marker of worse prognosis as discussed in section 1.2.1. (35).

The good correlation between P+Ms and matched metastasis with most of the DRTs suggests that the DCN changes are present in the primary tumour at the time of metastasis and persist as the tumour develops at a distant site. It is not possible to speculate on this small series of cases whether the correlation between the primary and metastasis with these assays would be of use in determining whether a new

malignant lesion from a patient with a history of melanoma represented a separate primary or a cutaneous metastasis.

The results of the BRAF.PTEN assay showed a significant difference between the melanomas with and without subsequent metastasis and this difference indicates that there may be DNA copy number change at one of these loci which occurs within a tumour clone causing propensity for the clone to metastasise. Similarly the lack of correlation between the LDLRad3.CCND1 DRT scores for the P+Ms and their metastases indicates that a copy number change may occur at either or both of these loci within the metastasising clone before the tumour spreads.

4.3.3 London Dermatopathology Symposium Cases

The London Dermatopathology Symposium Cases were a series of 5 ambiguous melanocytic lesions, all of which showed evidence of aneuploidy using our method and for which the overall analysis is limited by the lack of clinical information. They do however raise two interesting issues which are fundamental to this project. Firstly they represent the first comparison between the DRT method and the established FISH assay developed by Gerami et al (63). The comparison showed that the FISH assay detected aneuploidy in 2 of the 5 cases, compared to all 5 with DRTs. Although a tiny sample of cases, the results suggests that the DRT method shows either greater sensitivity for detecting melanoma than the FISH assay, or shows much lower specificity for melanoma than the FISH assay, or possibly a combination of the two. One of the advantages of a QPCR method is that a greater number of sites can be tested for chromosomal instability than with a FISH assay. This is because numerous different DRTs assays can be tested on the same sample, provided sufficient DNA can

be extracted from the tissue. In comparison FISH is performed using fluorescent probes on a tissue section with microscopic analysis and only a limited number of probes (usually up to a maximum of 4) can be used at one time. This may account for any improvement in sensitivity seen in comparison. There is counter-argument that an increased number of targets leads to an increased risk of false positive results and therefore where several assays are used the development of a robust prediction model is crucial.

The second interesting issue to arise from this series of ambiguous cases regards the problem of testing a biomarker on ambiguous lesions. Lesions are classified as ambiguous on the basis of their histology indicating that dermatopathologists cannot give a confident diagnosis of either a naevus or a melanoma, as demonstrated by the panel opinion and floor discussion at the symposium. The only way to identify whether they were definitely a melanoma or naevus on follow-up is to identify those lesions which subsequently metastasise (provided there is not a separate primary melanoma in the same patient). We know however, that only a proportion of excised melanomas will metastasise and the stage at which they metastasise is somewhat unpredictable (80,86). Of the ambiguous cases which do not metastasise, some may still therefore represent melanoma. Testing putative biomarkers on ambiguous lesions with subsequent metastasis used as the distinguishing feature of malignancy effectively means that the marker is being tested for its prognostic rather than diagnostic efficacy. A compromise solution for markers targeting chromosomal instability may be to test ambiguous lesions which have undergone other DCN analysis

such as array CGH or FISH to develop a 'molecular consensus diagnosis' before evaluating each of the individual markers in turn.

4.3.4 Interpreting results of the TBX2.HIC1 DRT assay in melanocytic lesions

The TBX2.HIC1 assay raised the unexpected possibility that naevi may show aneuploidy at this site. This was based on DRT scores in both the melanoma and naevi which were higher than the controls and suggested aneuploidy. The analysis of ΔΔCT versus CT indicated no significant trend to explain the results might be due to varying DNA quality. In order to further investigate this a third marker was developed for a region on chromosome 2 described as showing relative genomic stability in all melanocytic lesions. Three-way assays using the three loci showed that the change only occurred in the assays including TBX2 and that the pattern was also seen in an independent set of naevi.

If this does indicate a true region of genomic instability in benign naevi, this would represent a novel finding. No amplification has been identified in the Chromosome 17q23 region previously in the limited CGH studies involving naevi (39), although it is possible that any amplification may be highly specific and in a region not probed by CGH analysis. Using a FISH probe for the TBX2 locus would be the next step in establishing whether there is true copy number change and a TBX FISH probe has been identified and kindly made available to our laboratory by Prof Anne Kallioniemi (Institute of Biomedical Technology, Tampere, Finland). The probe has previously been used to investigate TBX2 amplification in breast cancer (136). Although there was no further time within this project to optimise the FISH assay and apply it to benign naevi

to answer the question about TBX2 copy number, it hoped that subsequent students will be able to take on this research and answer this question.

4.3.5 BRAF mutation analysis in primary series

Numerous oncogenic mutations in *BRAF* have been described, of which the V600E mutation is the most common and promotes early melanoma development (43). The BRAF V600E mutation describes a point mutation of T to A at nucleotide 1799, changing the associated codon from GTG to GAG. Around 50 % of melanomas have been reported to express BRAF mutations and of these over 90% are the V600E mutation, giving a V600E mutation rate in the studies reported elsewhere of around 45% (137). BRAF mutations are most commonly found in melanomas arising at sites of UV exposure (138).

The trend in BRAF.PTEN DRT scores increasing from P-Ms to P+Ms to metastases indicates that this assay may target a locus in which DNA copy number change is associated with progression of melanoma. This assay may therefore be suited to development as a serum marker of circulating tumour DNA in recurrent disease. The frequency of the V600E BRAF mutation (27%) in the primary melanomas is lower than reported elsewhere (137) although as this was an opportunity sample of only 40 primary tumours, the BRAF mutation rate may be influenced by the fact that all lesions in this primary series were 2mm or more in the thickness, introducing a selection bias. It is thought that thicker melanomas are more likely to demonstrate a NRAS mutation and therefore not be dependent on BRAF as a driver mutation (139). The rate of BRAF mutation detected in the metastases was only 20%. As the metastases were paired to the P+Ms, any bias involved in selecting the P+Ms would

also affect the subsequent metastases. In the single metastasis which had a BRAF mutated primary, but was wild type itself, there is the possibility that a clone has developed within the tumour which has lost the original driver mutation. This can occur in tumours which are heterozygous for a driver mutation and then undergo loss of heterozygosity as the tumour develops. Some of the subsequent clones are then homozygous for the mutation and others homozygous wild type and if the latter metastasises, the driver mutation may not be seen.

Although increased somatic copy number alterations have been described in melanoma with BRAF mutation in comparison to NRAS mutated tumours (140), no relationship has been described between BRAF mutation status and copy number alteration at the *BRAF* locus itself. The small sample of cases tested with the BRAF.PTEN DRT also shows no relationship between BRAF mutation status and the DRT score.

The issue of BRAF mutation has therapeutic relevance to melanoma, as in 2011, the BRAF inhibitor Vemurafenib was approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma and was approved by the UK's National Institute for Clinical Excellence (NICE) in 2012 for the treatment of advanced melanoma.

The rate of V600E mutations in the benign naevi was 95%. This is higher than the rate reported elsewhere which has been anywhere between 39 and 86% (141-145). As with the melanomas tested, use of an opportunity sample is likely to have led to selection bias, possibly affecting the BRAF mutation rate.

4.4 Separation of melanoma and naevi on the cohort study

The cohorts of naevi and melanoma were developed in order to test with greater certainty whether DRTs were able to differentiate between melanoma and naevi.

As the separation of P-Ms and P+Ms with the DRTs was less impressive than that between melanoma and naevi on the primary series, the cohort series was designed to test the diagnostic rather than prognostic capability of the DRTs. Once the melanoma and naevi cohorts were developed the relevant clinical data was extracted which identified that the cohort of melanomas included only 15 cases with confirmed metastasis. As this represents only a small proportion of the total number of melanomas in the cohort (14%) this was not considered suitable for testing as a prognostic cohort. Another reason for not using this cohort for prognostic analysis is that whilst the primary series cases were selected such that presence or absence of clinically confirmed metastasis was clearly demonstrated, the cohort was a true inclusive series of all melanomas which met the inclusion and exclusion criteria in a given period and many of the cases included in the cohort were ambiguous for metastasis. This includes those cases for which there was no follow-up on pathology or radiology after diagnosis of the primary melanoma (many of the patients treated for primary melanoma are subsequently followed up at local district general hospitals). Also many of the patients may have either died or moved out of the area with no record of this on the pathology and radiology systems used for clinical information.

More detailed investigation of the patients' follow-up would be required to determine with a reasonable degree of accuracy which cases had developed metastasis. This

would require, at least, access to the hospital notes system and the General Register Office which notes all deaths in England and Wales (for those patients who die out of hospital or move out of the local area). Accessing these sources of information for the purposes of this study was not covered under the ethics application for this project.

Two methods were used throughout the project to differentiate naevi and melanoma. Firstly the z-score was created for naevi and the 99% limit of the naevi used as the limit of a normal diploid sample. This method shows good separation between the melanoma and naevi on the primary series. Secondly, logistic regression analysis was performed. This gives greater statistical 'weight' to certain assays which seem to be more discriminatory (such as the score of the MYB.RREB1 assay). Although this shows very similar sensitivity and specificity values to the z-score method on the primary series of cases, in the more representative cohort series, the logistical regression prediction model seems a more robust method of processing the DRT scores to determine diagnosis as this allows the appropriate weighting of each the 7 values to be combined to calculate a prediction probability value.

The results from the cohort analysis demonstrated that in combination the method was able to diagnose melanoma using the results of six of the seven DRT assays (TBX2.HIC1 excluded) with a sensitivity of 84% and a specificity of 88%. Although not as impressive as the results seen in the opportunity primary series, this result still indicates good diagnostic ability of the assays, with values very close to those reported for the FISH assay developed by Bastian et al (63) when tested on unambiguous cases of melanoma and naevi by an independent study group (sensitivity 85% and specificity 90%)(64).

Cohort analysis is less susceptible to selection bias and these results have greater generalisability to the wider population than an opportunity sample. Despite this there are inevitable biases which occur, with potential sources of bias in this cohort series being the exclusion of thin tissue blocks and thin Breslow thickness tumours, both of which were due to technical limitations described in 2.1.3.

There may be other technical sources of bias from the cohort analysis. Due to large numbers of samples tested the DNA was extracted in large batches (20-30 cases at a time) and the DNA was then run as repeated large scale QPCR experiments involving 30 cases. In clinical practice it would be more likely than one or two ambiguous tumours would be extracted and tested at once. Performing the techniques in batches was necessary given the large numbers involved and limited time and resources available, but this does raise the possibility of DNA or reagent degradation occurring due to the longer periods taken to set up and load large scale experiments, or even an increased risk of sample contamination given the large number of cases being processed on the bench simultaneously.

Alongside the cohort analysis an opportunity sample of two Spitzoid tumours which subsequently metastasised, confirming their malignant nature, was included. Only one of these was diagnosed as representing melanoma on histopathology. These were both classified as melanoma using the DRTs and although this is a tiny sample, too small for statistical testing, it does support the idea that DRTs may be of diagnostic utility in problematic and genuinely ambiguous lesions.

4.5 Further Work

The clear differentiation between naevi and melanoma using a panel of DRTs is strong grounds for extending the cohort of unambiguous cases of naevi and melanoma. This is the next step of biomarker development (as outlined in 1.5.1) to develop a prediction algorithm and cut off points for naevi and melanoma. The next step would be then to test the algorithm on a new series of cases including unambiguous naevi and melanoma, alongside ambiguous lesions and rare variants of melanoma and naevi. A prediction algorithm would be developed using logistic regression analysis of the cohort data. Given that DRTs represent a putative diagnostic melanoma biomarker it would be of interest to test a series of melanoma and naevi samples with both the DRTs and the FISH assay developed by Gerami et al (63), to compare these two methods which both target DCN alteration as a marker of malignancy.

Although these DRTs do show some separation between P-Ms and P+Ms, the panel of DRT assays show only limited additive value in predicting melanoma behaviour when analysed alongside the disease stage. It is possible that an alternative panel of DRT assays could be developed to act as prognostic markers by performing array CGH studies on matched P-Ms and P+Ms. This may identify patterns of copy number change which could predict metastasis (no such CGH data sets are currently publicly available). This would allow for new DRTs to be designed specifically targeting the loci which differentiate between the two classes of lesions.

Another possibility is that the DRTs could be used as a means of detecting abnormal DCN profiles in circulating tumour DNA. It has been shown that circulating tumour DNA can be detected even from tumours of small size and at an early stage of

development (146). Whilst there is little clinical need for biomarkers to detect primary tumours in melanoma as a general screening tool as early primary lesions can be detected by clinical examination, or self-examination (147), detection of metastatic disease at an early stage may be beneficial to the patient. Metastatic melanoma commonly spread firstly to the local lymph nodes and early detection of metastatic spread using a serum progression marker may allow for the removal of the local lymph node groups. However, a randomised controlled trial to determine whether this actually improved survival would be required if this route of biomarker development was taken and early detection of metastasis demonstrated.

Difficulties in testing ambiguous cases with diagnostic biomarkers have already been discussed in section 4.3.1, although a further limitation is the scarcity of ambiguous lesions for which there is spare tissue readily available for testing. As the lesions are uncommon, they must be sourced from different institutions in order to develop sets large enough for statistical analysis. Furthermore, as the cases are of histopathological and scientific interest, there is often demand for the limited tissue available for both for histology slide sets and molecular testing. Typically these lesions are in the region of 10mm in maximal dimension and again this limits the tissue available for research without exhausting the diagnostic material. Inclusion of ambiguous cases in a study set also involves the agreement around their diagnosis as being ‘ambiguous’ by dermatopathology experts. As noted previously, achieving consensus on these cases can be difficult (17).

The unexpected results of the TBX2.HIC1 assay in benign naevi have been discussed at length in section 4.3.4. The continuation of this work using the recently acquired FISH

probe for TBX2 in benign naevi would be of considerable interest, as a positive result may provide further insight into the process of naevogenesis.

A possible technical improvement in the design of the assays would be the titration of primer concentrations within the assays such that the parallel regression lines for two targets in one assay with varying DNA concentration, described in 2.7, were overlapping. This would mean that the raw ΔCT result would be a closer reflection of the absolute DNA copy number difference of the 2 targets and less reliant upon normalisation against diploid controls to indicate aneuploidy.

Although the development of effective centromeric DRTs was not achieved as part of this project, the results of the design phase of experiments raises scientific questions regarding these repeat sequences. Despite repeat sequences apparently showing specificity for individual chromosomes on testing of the NCBI genome database, the assays developed for these sequences show reactivity with several other chromosomes in practice. This suggests that a limited amount is known about the homology of centromeric alphoid repeat sequences. As these represent regions of non-coding DNA it may be argued that this homology is of little clinical relevance, but, with new molecular biomarkers constantly being developed, this as yet undescribed homology may influence the development and accuracy of future PCR or FISH assays which target the centromeres.

5. Conclusion

DRTs have been demonstrated to be accurate and reliable and show impressive capability for differentiating between unambiguous cases of melanoma and naevi using DNA derived from FFPE tissue, with sensitivity and specificity similar to that reported for the FISH assay developed by Gerami et al (63); currently the only diagnostic melanoma biomarker to be clinically translated.

Centromeric DRTs do not seem to improve sensitivity when compared to DRTs which target coding DNA, and suffer from the problem of targeting centromeric regions which appear to show homology with other chromosomes.

New DRTs are easily developed and may have potential in melanoma prognosis if further information regarding DNA copy number changes which differentiate between metastasising and non-metastasising melanomas can be generated. There may also be applications for DRTs in other tumours for diagnosis or prognosis.

Appendix

Appendix 1 – Example of free energy testing for primer design of X.18 cDRT

The following tables and dimerisation representations detail the predicted dimers and hairpins with their free energies for a X.18 cDRT (ID 9410). This information was collated for several potential assays for X.18 and the most promising primer pairs were synthesised. The collated information for 10 different potential assays can be found in a table at the end of appendix 1.

July 21, 2010

Name: ID9410

Description: x

Assay Type: TaqMan®

Reaction Conditions:

Nucleic Acid Concentration (nM)	0.25	**Monovalent Concentration (mM)**	50
Free Mg++ Concentration (mM)	5	**Total Na+ Concentration (mM)**	332.84

Sense Primer: AAACGGAAGCATTCTCAGAA

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
20	53.3	40	1	-2.4	-1.8	-0.6

Anti-sense Primer: AAAGGCATGTTCAGCTCTGT

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
20	55.7	45	1	-2.4	-3.0	0.0

TaqMan®: TTCTTTGTGATGATGGAGTTTCA

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
23	55.11	34.78	1	With Sense -1.8 With Anti-sense -1.7	-0.9	-0.9

Secondary Structures for Sense Primer

Dimer:-

```
5' AAACGGAAGCATTCTCAGAA 3'
              |||| ¦¦¦¦
          3' AAGACTCTTACGAAGGCAAA 5'
```

-1.8

```
 5' AAACGGAAGCATTCTCAGAA 3'
         |||   ¦¦¦
3' AAGACTCTTACGAAGGCAAA 5'
```

-0.6

Hairpin:-

```
/TCTTACGAAGGCAAA 5'
C |||
\AGAA 3'
```

-0.6

```
/GAAGGCAAA 5'
C |||
\ATTCTCAGAA 3'
```

-0.6

Secondary Structures for Anti-sense Primer

Dimer:-

```
5' AAAGGCATGTTCAGCTCTGT 3'
               ||||
        3' TGTCTCGACTTGTACGGAAA 5'
```

-3.0

```
      5' AAAGGCATGTTCAGCTCTGT 3'
              ||||
3' TGTCTCGACTTGTACGGAAA 5'
```

-2.3

```
5' AAAGGCATGTTCAGCTCTGT 3'
             |||  ¦¦¦
         3' TGTCTCGACTTGTACGGAAA 5
```

-1.1

Hairpin:-

Not Found

Secondary Structures for Taqman®

Dimer:-

```
5' TTCTTTGTGATGATGGAGTTTCA 3'
            |||       ¦¦¦
          3' ACTTTGAGGTAGTAGTGTTTCTT 5'
```

-0.9

```
5' TTCTTTGTGATGATGGAGTTTCA 3'
          |||   ¦  ¦   ¦¦¦
       3' ACTTTGAGGTAGTAGTGTTTCTT 5'
```

-0.9

Hairpin:-

```
/GGTAGTAGTGTTTCTT 5'
A   |||
\GTTTCA 3'
```

-0.9

```
/GTAGTAGTGTTTCTT 5'
| ¦   |||
\GAGTTTCA 3'
```

-0.9

Cross Dimer

Cross Dimer between Sense Primer and Anti-sense Primer:-

```
5' AAACGGAAGCATTCTCAGAA 3'
                  ||||
             3' TGTCTCGACTTGTACGGAAA 5'
```

-2.4

```
5' AAACGGAAGCATTCTCAGAA 3'
         |||       ¦
   3' TGTCTCGACTTGTACGGAAA 5'
```

-1.8

```
        5' AAACGGAAGCATTCTCAGAA 3'
            |||       ¦
3' TGTCTCGACTTGTACGGAAA 5'
```

-0.7

```
5' AAACGGAAGCATTCTCAGAA 3'
                ¦ |||
         3' TGTCTCGACTTGTACGGAAA 5'
```

-0.6

```
   5' AAACGGAAGCATTCTCAGAA 3'
       ¦ ¦ |||     ¦¦
3' TGTCTCGACTTGTACGGAAA 5'
```

-0.6

```
  5' AAACGGAAGCATTCTCAGAA 3'
     ¦ ¦    ¦ ||| ¦
3' TGTCTCGACTTGTACGGAAA 5'
```

-0.5

Cross Dimer between Sense Primer and Probe Sequence:-

```
  5' AAACGGAAGCATTCTCAGAA 3'
     ||||   ¦ ¦¦¦    ¦  ¦
3' ACTTTGAGGTAGTAGTGTTTCTT 5'
```

-1.8

```
   5' AAACGGAAGCATTCTCAGAA 3'
      ¦¦    ¦      ¦  ||||
3' ACTTTGAGGTAGTAGTGTTTCTT 5'
```

-1.8

```
5' AAACGGAAGCATTCTCAGAA 3'
            ¦  |||
        3' ACTTTGAGGTAGTAGTGTTTCTT 5'
```

-1.0

```
5' AAACGGAAGCATTCTCAGAA 3'
       ¦¦¦ ¦    |||  ¦
    3' ACTTTGAGGTAGTAGTGTTTCTT 5'
```

```
-0.9

5' AAACGGAAGCATTCTCAGAA 3'
           ¦¦¦  ||| ¦¦
 3' ACTTTGAGGTAGTAGTGTTTCTT 5'

-0.9

5' AAACGGAAGCATTCTCAGAA 3'
                   |||
               3' ACTTTGAGGTAGTAGTGTTTCTT 5'

-0.6

              5' AAACGGAAGCATTCTCAGAA 3'
                 ¦ ¦  |||
3' ACTTTGAGGTAGTAGTGTTTCTT 5'

-0.6

            5' AAACGGAAGCATTCTCAGAA 3'
               ¦     ||| ¦
3' ACTTTGAGGTAGTAGTGTTTCTT 5'

-0.5

                5' AAACGGAAGCATTCTCAGAA 3'
                   |||
3' ACTTTGAGGTAGTAGTGTTTCTT 5'

-0.1
```

Cross Dimer between Anti-sense Primer and Probe Sequence:-

```
                5' AAAGGCATGTTCAGCTCTGT 3'
                   ||||
3' ACTTTGAGGTAGTAGTGTTTCTT 5'

-1.7

5' AAAGGCATGTTCAGCTCTGT 3'
           ¦  ¦ |||  ¦
         3' ACTTTGAGGTAGTAGTGTTTCTT 5'

-1.0

5' AAAGGCATGTTCAGCTCTGT 3'
    ¦  ¦    ||| ¦ ¦
3' ACTTTGAGGTAGTAGTGTTTCTT 5'

-0.9

   5' AAAGGCATGTTCAGCTCTGT 3'
      ¦¦   ¦¦¦  |||
3' ACTTTGAGGTAGTAGTGTTTCTT 5'
```

-0.9

```
      5' AAAGGCATGTTCAGCTCTGT 3'
                |||    ¦
3' ACTTTGAGGTAGTAGTGTTTCTT 5'
```

-0.5

```
  5' AAAGGCATGTTCAGCTCTGT 3'
     |||  ¦        ¦   ¦
3' ACTTTGAGGTAGTAGTGTTTCTT 5'
```

-0.1

July 21, 2010

Name: ID9410

Description: 18

Assay Type: TaqMan®

Reaction Conditions:

Nucleic Acid Concentration (nM)	0.25	**Monovalent Concentration (mM)**	50
Free Mg++ Concentration (mM)	5	**Total Na+ Concentration (mM)**	332.84

GAGCAGTCCTGAAACACTCTT

Sense Primer:

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
21	56.16	47.62	1	-1.1	-1.1	-1.0

ACCTTAGCCCTGAAAGCG

Anti-sense Primer:

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
18	54.36	55.56	3	-1.1	-0.5	-0.5

TCTGGAACTGGACTTTTGGAG

TaqMan®:

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
21	55.76	47.62	1	With Sense -4.3 With Anti-sense -1.7	0.0	0.0

Secondary Structures for Sense Primer

Dimer:-

```
       5' GAGCAGTCCTGAAACACTCTT 3'
             |||  ¦¦¦
3' TTCTCACAAAGTCCTGACGAG 5'
```

-1.1

```
  5' GAGCAGTCCTGAAACACTCTT 3'
     |||   ¦ ¦ ¦ ¦   ¦¦¦
3' TTCTCACAAAGTCCTGACGAG 5'
```

-1.0

```
5' GAGCAGTCCTGAAACACTCTT 3'
    ¦  |||  ¦  ¦  ¦¦¦  ¦
 3' TTCTCACAAAGTCCTGACGAG 5'
```

-0.8

Hairpin:-

```
/CCTGACGAG 5'
T  ¦   |||
\GAAACACTCTT 3'
```

-1.0

```
/GTCCTGACGAG 5'
| ¦  |||  ¦
\AAACACTCTT 3'
```

-0.8

Secondary Structures for Anti-sense Primer

Dimer:-

```
5' ACCTTAGCCCTGAAAGCG 3'
     |||        ¦¦¦
3' GCGAAAGTCCCGATTCCA 5'
```

-0.5

Hairpin:-

```
/CCGATTCCA 5'
|    |||
\CTGAAAGCG 3'
```

-0.5

Secondary Structures for Taqman®

Dimer:-

Not Found

Hairpin:-

Not Found

Cross Dimer

Cross Dimer between Sense Primer and Anti-sense Primer:-

```
   5' GAGCAGTCCTGAAACACTCTT 3'
         |||  ¦¦ ¦
3' GCGAAAGTCCCGATTCCA 5'
```

-1.1

```
5' GAGCAGTCCTGAAACACTCTT 3'
                  ¦ |||
               3' GCGAAAGTCCCGATTCCA 5'
```

-0.5

Cross Dimer between Sense Primer and Probe Sequence:-

```
   5' GAGCAGTCCTGAAACACTCTT 3'
       ¦  ||||| ¦   ¦¦
3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-4.3

```
        5' GAGCAGTCCTGAAACACTCTT 3'
           ¦  |||| ¦   ¦
3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-2.5

```
5' GAGCAGTCCTGAAACACTCTT 3'
         |||  ¦¦   ¦  ¦¦
```

```
     3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-1.3

```
         5' GAGCAGTCCTGAAACACTCTT 3'
                  ||| ¦¦
3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-1.3

```
              5' GAGCAGTCCTGAAACACTCTT 3'
                    |||
3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-1.1

```
5' GAGCAGTCCTGAAACACTCTT 3'
                  |||
               3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-1.0

```
5' GAGCAGTCCTGAAACACTCTT 3'
          ¦   |||    ¦
       3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-0.1

```
5' GAGCAGTCCTGAAACACTCTT 3'
           ¦  |||  ¦   ¦
      3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-0.1

Cross Dimer between Anti-sense Primer and Probe Sequence:-

```
5' ACCTTAGCCCTGAAAGCG 3'
          ¦ ¦  ||||
       3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-1.7

```
5' ACCTTAGCCCTGAAAGCG 3'
           ¦   |||
        3' GAGGTTTTCAGGTCAAGGTCT 5'
```

-0.1

	Xmon	18mon	x forward primer	x reverse primer	x probe	structural problems
9365	8	4	TCGTATAGGAAGGAACTTCATA	CTCCATCATCACAAAGAATATT	TAAAAGGCAAACGGAAGCAT	dimers at true 3 end of RP
9373	8	5	CTACGGTCGTATAGGAAGG	TGAGTGAAACTCCATCATCA	TAAAAGGCAAACGGAAGCAT	cross dimers at 3 end of FP
9374	8	6	GGTCGTATAGGAAGGAACTT	AAACTCCATCATCACAAAGAA	TAAAAGGCAAACGGAAGCAT	hairpin involving 3 end of FP and cross dimers of RP, FP and probe
9376	11	4	TTGGAAACGGGAATATTT	GTGGCTGGAACACAAACA	TGGAAACGGGAATATTTCCA	FP and probe have hairpins and dimers involving 3 end
9377	11	5	AAACTGAAACATTCTCAGAAAC	AAAGCAATGTTAAACTCTGTG	ATGTTTGTGTTCCAGCCACA	multiple hairpins at 3
9378	11	6	CGGGAATATTTCCACAGA	TGTGGCTGGAACACAAAC	ATGTTTGTGTTCCAGCCACA	hairpins involving 3
9392	5	6	TGCGATGACTGCATTCAA	TCCAAAGAAAGAGGGTTTC	TGGAGCAGTTTTGAAACCCT	hairpin and dimers involving the true RP 3 end
9396	12	6	TCTTCACAGAAAGACGAGAGA	AGTTGAATGCAATCATCACA	CGAGAGAGAAGCATTGTCAGAA	good - little involving 3 end
9400	6	5	TTTGTATTCAACTCCCAGAGT	AAGAGTGTTTCATAGCTGCTC	TTTCCTTTTGAAAGAGCAGCT	hairpins at 3 end of RP and FP
9401	6	6	AACTAAACAGAAGCATTCTCG	AACTCTGGGAGTTGAATACAA	AAACAGAAGCATTCTCGGAAA	cross dimers at true 3 end of RP only

ID	Xmon	18mon	18 forward primer	18 reverse primer	18 probe	structural problems
9365	8	4	CATAGAGCAGGTTTGAATCAC	TTTCCAACATAGGCCTGA	GCTTTCAGGCCTATGTTGGAAA	probe shows 4 dimers at 3 end
9373	8	5	GAATCTGCAAAGTGGATATTT	TATATGTATTCCGTTCCAGC	GATTTCGCTGGAACGGAATA	forward primer hairpin with bonds at 3 end
9374	8	6	TTGAACACTCCCTTTCATAG	TCCAGTTCCAGATACTACAAA	CCCTTTCATAGAGCAGTCCTG	dimers at 3 end of probe
9376	11	4	CTGAGAAACATCTTTGTGATG	GGAGTGATTCAAACCTGCT	CCCTTTCATAGAGCAGTCCTG	hairpin at 3 on the FP
9377	11	5	AGAGAATTGAACCACCGTT	CAAATATCCACTTTGCAGAT	CACCGTTTTGAAGGAGCAGT	hairpin at 3 on the FP and cross dimer at 3 on the probe
9378	11	6	TGAACACTCCCTTTCATAGAG	AAAGTCCAGTTCCAGATACTAC	CACCGTTTTGAAGGAGCAGT	dimers close to 3 ends on FP and RP
9392	5	6	TGCTTTCTGATGTTTGCAT	GAGTGTTTCAGGACTGCTCTA	CCCTTTCATAGAGCAGTCCTG	dimers and hairpins close to 3 on FP at dimers at 3 on probe
9396	12	6	ATAGAGCAGTCCTGAAACACT	CACCTTAGCCCTGAAAGC	GACTTTTGGAGCGCTTTCAG	FP hairpin at 3, probe dimers at 3
9400	6	5	TAAGAGAATTGAACCACCG	AGCTAGCCAAATATCCACTT	CACCGTTTTGAAGGAGCAGT	good - little close to 3 ends
9401	6	6	TCTGAGAAACTGCTTTCTGAT	GGACTGCTCTATGAAAGGGA	TTGAACCTTTCTTTTGATAGAGCA	some dimers close to 3 end, but better

best structures

pairs with the best structures

The results of free energy studies for ten potential DRT assays for the X.18. The ChX primers and probe are detailed at the top, the Ch18 primers and probe at the bottom. On the basis of these calculations, assays 9365 and 9401 appeared the most attractive and these primers were synthesised.

Appendix 2 – Documentation pertaining to research funding

Pathological Society
Understanding Disease

Dr DA Moore
8 Uplands Road
Oadby
LEICESTER
LE2 4NS

19th July 2010

Dear Dr Moore,

Pathological Society Small Grants Scheme grant application – 01 April 2010
Grant Reference No: SGS 2010/04/01
I am writing to inform you that the sum of **£10 000** has been paid directly into the University of Leicester's bank account today, Monday 17th July 2010 to fund your project '***Translating Prognostic Biomarkers in Melanoma'***. The payment has been referenced RM60G0308 as requested.

It is a condition of the award that you submit a two page written report (approx 300 words) of the work you undertook **within one year** of the award. I have attached a document outlining the required layout of your report. Please ensure that your report is received no later than ***Friday 15 July 2011.***

Enjoy your project and I look forward to hearing from you
Yours sincerely

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Structured Report for the Pathological Society Awards: Small Grant Award

Grant Reference No: SGS 2010/04/01

Title: Translating Prognostic Biomarkers in Melanoma

Name & Address: Dr David A Moore, 8 Uplands Road, Oadby, Leicester, LE2 4NS

Background:

Melanoma is a form of skin cancer which frequently metastasises at an early stage. At present the prognosis for a patient diagnosed with primary melanoma is based upon the histopathological features.[1] Melanomas with the same histological features behave differently in different individuals however, making prognostication more difficult.[2, 3] Furthermore, misdiagnosis of melanoma as a benign naevus is one of the most common serious errors made in histopathology, with huge potential consequences for patients. A small subset of melanocytic lesions make up the majority of the diagnostically challenging lesions which account for these misdiagnoses.

It is known that DNA copy number changes occur in melanoma and that these changes vary between individual tumours.[4,5] If differences in copy number change can be demonstrated between naevi and melanoma or between melanoma which progress and those which don't, this could be developed into a diagnostic or prognostic test. A diagnostic test for melanoma has already been developed using FISH,[6] though this has shown variable sensitivity when tested on ambiguous lesions and there are practical drawbacks of this method.

Original Aims (copied from original application):

This study aims identify DNA copy number changes in melanoma which can be translated into prognostic and diagnostic markers for use on formalin fixed paraffin embedded tissue.

Results:

We have developed a range of real-time PCR assays each of which consist of a duplex PCR reaction targeting 2 loci in which there is known chromosomal instability in melanoma (one region of common gain and another of common loss). These assays are referred to as either paralogue ratio tests (PRTs) or duplex ratio tests (DRTs) depending upon whether or not the PCR primers for these 2 loci are the same.

The range of real-time PCR assays developed have subsequently been tested against melanomas and benign tissue, with a certain combination of assays being able to differentiate between benign tissue and melanoma. They have also been tested against over 100 benign

tissue samples with varying DNA yields in a series of experiments consisting of over 1000 assays. The results of these experiments represent a robust reference of the normal range for these assays in diploid tissue. Although the assays are still in the process of being tested against a further series of melanomas and naevi, initial results suggest they also differentiate between histologically unequivocal cases of melanoma and naevi (see figure 1 for an example of one of the assays) and may show a difference between those melanomas which metastasise and those which do not.

Conclusions:

The developed DRTs and PRTs are able to differentiate melanoma from benign tissue in the majority of cases and seem to be able differentiate melanoma form naevi (although our investigations are still ongoing). It will therefore be extremely interesting to test a series of ambiguous lesions using these assays to determine if the results of the assays predict the behaviour of the lesion. There is also the possibility of testing these DRTs against primary cases with follow-up data, to determine any possible prognostic value they may add.

How Closely Have the Original Aims been Met:

At the time of our grant application, we planned to use array CGH on a series of matched melanoma cases with differing outcomes to develop targets for our assays. However, the DNA yield we were typically able to acquire for each melanoma sample was insufficient for array CGH analysis and while we investigated the possibility of using whole genomic amplification to increase the DNA yield, it became apparent that due to the noise to signal ratio seen with this degree of amplification, many of the specific deletions and amplifications we were aiming to detect would have been lost. For this reason we redefined our strategy and decided to use publicly available array CGH data to identify regions of copy number gain and loss in melanomas and used these loci to develop multiple DRTs and PRTs.

The grant from the Pathological Society has allowed us to design and synthesise 13 real-time PCR assays from which we have been able to identify a select group of assays which can effectively differentiate melanoma from benign tissue in the majority of cases and have tested these against our reference range of over 100 samples. We have therefore met the original aim of identifying ‘DNA copy number changes in melanoma which can be developed into a prognostic or diagnostic markers for use on paraffin embedded tissue’ and are in the process of translating these markers. Once validated against an independent cohort, we plan to test this panel of assays against a series of less common types of naevi (including Spitz and Congenital naevi) and ultimately cases of diagnostic difficulty for which there is follow-up data, to determine their true diagnostic value.

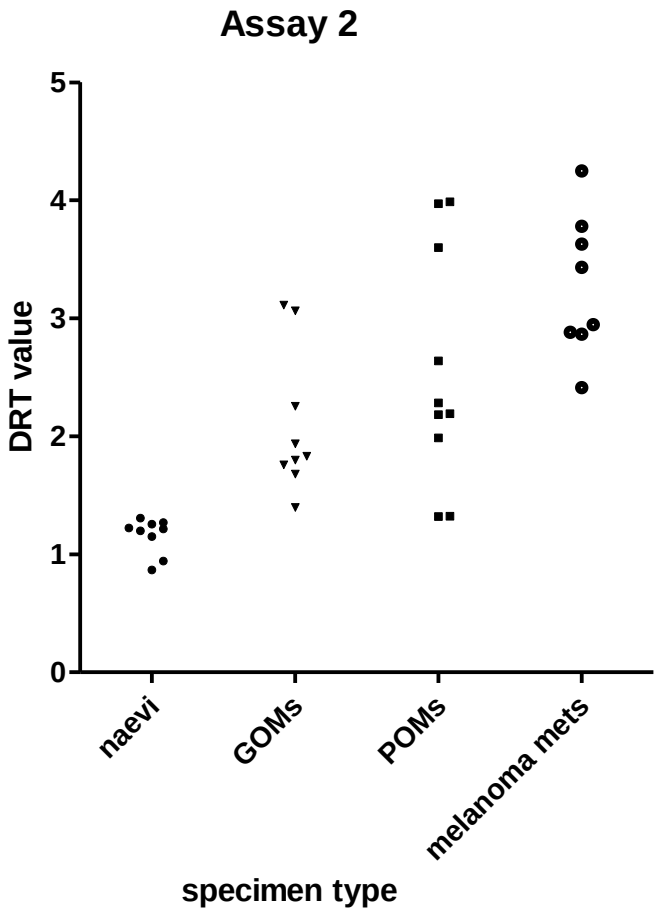

Figure 1. Comparison of DRT results naevi, good outcome melanomas (GOMs), poor outcome melanomas (POMs) and melanoma metastasis, using one of the DRT assays (BRAF/PTEN).

References:

(1) Balch CM, Gershenwald JE, Soong SJ, *et al*. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol*. 2009;**36**:6199-206.

(2) Kaur C, Thomas RJ, Desai N, *et al*. The correlation of regression in primary melanoma with sentinel lymph node status. *J Clin Pathol*. 2008;**61(3)**:297-300.

(3) Gimotty PA, Guerry D, Ming ME, *et al*. Thin primary cutaneous malignant melanoma: a prognostic tree for 10-year metastasis is more accurate than American Joint Committee on Cancer staging. *J Clin Oncol*. 2004;**22(18)**:3668-76.

(4) Bastian BC, Olshen AB, LeBoit PE, Pinkel D. Classifying melanocytic tumors based on DNA copy number changes. Am J Pathol 2003 Nov;163(5):1765-1770.

(5) Bauer J, Bastian BC. Distinguishing melanocytic nevi from melanoma by DNA copy number changes: comparative genomic hybridization as a research and diagnostic tool. Dermatol Ther 2006 Jan-Feb;19(1):40-49.

(6) Gerami P, Jewell SS, Morrison LE, Blondin B, Schulz J, Ruffalo T, et al. Fluorescence in situ hybridization (FISH) as an ancillary diagnostic tool in the diagnosis of melanoma. Am J Surg Pathol 2009 Aug;33(8):1146-1156

(7) Vergier B, *et al*. Fluorescence in situ hybridization, a diagnostic aid in ambiguous melanocytic tumors: European study of 113 cases. Mod Pathol. 2011 May;24(5):613-23.

Appendix 3 – Research Ethic Committee Approval

NHS

National Research Ethics Service

Derbyshire Research Ethics Committee
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25 July 2008

Dr G S Saldanha
Consultant Dermatopathologist
Dept of Cancer Studies and Molecular Medicine
Level 3 Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
PO Box 65
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Dear Dr Saldanha

Full title of study: **Chromosomal instability as a novel melanoma diagnostic marker**
REC reference number: 08/H0401/77

The Research Ethics Committee reviewed the above application at the meeting held on 15 July 2008.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to submit the Site-Specific Information Form to any Research Ethics Committee. The favourable opinion for the study applies to all sites involved in the research.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

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Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Application	2952/2930/1/638	17 June 2008
Investigator CV		17 June 2008
Protocol	2	01 July 2008
Covering Letter		18 June 2008
Peer Review		11 February 2008
Student researcher's CV		

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Continued/

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H0401/77	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Mr Peter Korczak
Chair

Email: jenny.hancock@derwentsharedservices.nhs.uk

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments
"After ethical review – guidance for researchers" SL-AR-2

Copy to: R& D Office for University Hospitals of Leicester - NHS trust
Miss N Hathiari via email

Appendix 4 – Presentations and Publications arising from this work

1. The following abstract was accepted for a poster presentation at the National Cancer Research Institute Meeting in Liverpool, November 2011:

Accurate detection of copy number changes in DNA extracted from formalin fixed paraffin embedded tissue using duplex ratio tests

A minority of melanocytic lesions cannot be confidently classified as benign or malignant on histopathological examination, causing significant diagnostic uncertainty. DNA copy number (DCN) changes can be used to distinguish benign from malignant cases, though DNA extracted from formalin fixed paraffin embedded (FFPE) is of poor quality and more difficult to analyse for DCN assessment than DNA from fresh frozen tissues. In this study DCN assays called duplex ratio tests (DRT) were developed using a duplex real-time polymerase chain reaction designed to compare two target loci, the first commonly amplified or gained in cancer, the second commonly deleted or lost. The purpose of this study was to evaluate whether DRTs are analytically valid for detecting aneuploidy in cancer when applied to degraded DNA extracted from FFPE histopathological samples.

Five DRT assays to loci with common DCN changes in melanoma were designed and tested for the following genes BRAF/PTEN; TBX2/CDKN2A; CCND1/LDLRAD3; RREB1/MYB; SSR1/HDDC2. These were evaluated using DNA extracted from archived FFPE samples microdissected from 10 of each: melanoma, benign naevi, diploid control tonsil tissue and 5 melanoma cell lines.

The assays proved accurate when DNA extracted from fresh and FFPE melanoma cell lines were compared, with an interclass correlation coefficient of 0.99. The DRT results from the melanoma cell lines correlated to the SNP6 array data from the Sanger cancer genome project. The assays gave precise results when repeated though the precision was marginally reduced once the cycle threshold value for 10ng of FFPE DNA increased above 32 cycles reflecting the importance of DNA quality. In application the combined values from the 5 markers distinguished between all the naevi and melanomas.

This technique shows the diagnostic potential to distinguish genetically unstable tumours such as malignant melanoma from benign genetically stable lesions such as naevi in FFPE tissues.

2. The following abstract was accepted for a poster presentation at the Pathological Society of Great Britain and Ireland's Winter Meeting in London, January 2012:

Duplex Ratio Tests Differentiate Between Naevus and Melanoma Using DNA Extracted From Formalin Fixed Paraffin Embedded Surgical Specimens

Melanoma misdiagnosis is a common cause of litigation against histopathologists due to the difficulty of distinguishing benign from malignant lesions within a subset of melanocytic tumours. This has led to the search for an effective diagnostic biomarker for melanoma which is suitable for use on formalin fixed paraffin embedded (FFPE) tissue. Genomic instability is a well characterised feature of malignancy and the typical DNA copy number changes seen in melanoma are well described. The DNA copy number changes seen in benign naevi generally occur much less frequently in highly specific regions of the genome.

We have developed a series of duplex real-time PCR assays (Duplex ratio Tests, or DRTs) to compare regions of frequent DNA copy number gain and loss, which are applicable for use in FFPE tissue. These included a Chr:7p BRAF versus Chr:10q PTEN DRT and a Chr:6p RREB1 versus Chr:6q MYB DRT. These DRTs have been tested against DNA extracted from a series of 20 naevi, 20 melanomas without recurrence, 20 melanomas with recurrence and 20 matched melanoma metastases (MMets).

The DRT values for the BRAF/PTEN assay showed a significant difference between the 20 naevi and all 40 primary melanomas ($p=0.0004$) and between all 40 primary melanomas and the 20 Mmets ($p=0.0017$). DRT values for the RREB1/MYB assay showed a significant difference between naevi and all melanomas ($p=0.0001$). When the 20 naevi samples were compared to the 40 melanoma samples using logistic regression analysis, the combination of these 2 assays had a sensitivity of 93% and a specificity of 95% for predicting malignancy in this set of melanocytic lesions, with ROC curve analysis showing an area under the curve of 0.97.

These results strongly support the further development of DRT-based diagnostic assays and their eventual application to FFPE ambiguous melanocytic lesions.

3. A review article by DM, GS and JHP was published in *Histopathology* in April 2012. The title and abstract are presented below; the full article follows:

Prognostic Tissue Markers in Melanoma

Prognosis for patients diagnosed with cutaneous melanoma is currently based upon histopathological features alone, although tumours which are morphologically similar can behave differently. Numerous putative biomarkers have been identified in an attempt to aid prognostication for primary melanoma, using methods which include immunhistochemistry, PCR, array CGH and gene expression arrays. Despite this wide body of research, no biomarkers for prognosis in melanoma have been translated or are close to translation into clinical practice. In this review selected prognostic biomarkers are evaluated and the factors influencing successful biomarker translation including phases of biomarker development and study design are explored, in an attempt to highlight the current gap between prognostic melanoma biomarker research and clinical translation.

Bibliography

(1) Friedman RJ, Rigel DS, Kopf AW, Grin CM, Heilman E, Bart RS, et al. Volume of malignant melanoma is superior to thickness as a prognostic indicator. Preliminary observation. Dermatol Clin 1991 Oct;9(4):643-648.

(2) Ford D, Bliss JM, Swerdlow AJ, Armstrong BK, Franceschi S, Green A, et al. Risk of cutaneous melanoma associated with a family history of the disease. The International Melanoma Analysis Group (IMAGE). Int J Cancer 1995 Aug 9;62(4):377-381.

(3) Fitzpatrick T. Soleil et peau. J Med Esthet 1975(2):33034.

(4) http://www.cancerresearchuk.org/cancer-info/cancerstats/types/skin/incidence/.

(5) http://www.cdc.gov/media/releases/2011/p1019_melanoma_skincancer.html.

(6) http://info.cancerresearchuk.org/cancerstats/types/skin/incidence/#Trends.

(7) Welch HG, Woloshin S, Schwartz LM. Skin biopsy rates and incidence of melanoma: population based ecological study. BMJ 2005 Sep 3;331(7515):481.

(8) Balch CM, , Gershenwald JE, Soong SJ, Thompson JF, et al. Final version of 2009 AJCC melanoma staging and classification.; Biologic and prognostic significance of dermal Ki67 expression, mitoses, and tumorigenicity in thin invasive cutaneous melanoma. - J Clin Oncol.2009 Dec 20;27(36):6199-206.

(9) http://info.cancerresearchuk.org/cancerstats/types/skin/mortality/.

(10) Goldstein AM. Familial melanoma, pancreatic cancer and germline CDKN2A mutations. Hum Mutat 2004 Jun;23(6):630.

(11) Armstrong BK, Kricker A. How much melanoma is caused by sun exposure? Melanoma Res 1993 Dec;3(6):395-401.

(12) Weinstock MA, Colditz GA, Willett WC, Stampfer MJ, Bronstein BR, Mihm MC,Jr, et al. Nonfamilial cutaneous melanoma incidence in women associated with sun exposure before 20 years of age. Pediatrics 1989 Aug;84(2):199-204.

(13) Thomas L, Tranchand P, Berard F, Secchi T, Colin C, Moulin G. Semiological value of ABCDE criteria in the diagnosis of cutaneous pigmented tumors. Dermatology 1998;197(1):11-17.

(14) Keefe M, Dick DC, Wakeel RA. A study of the value of the seven-point checklist in distinguishing benign pigmented lesions from melanoma. Clin Exp Dermatol 1990 May;15(3):167-171.

(15) Urso C, Saieva C, Borgognoni L, Tinacci G, Zini E. Sensitivity and specificity of histological criteria in the diagnosis of conventional cutaneous melanoma. Melanoma Res 2008 Aug;18(4):253-258.

(16) Shoo BA, Sagebiel RW, Kashani-Sabet M. Discordance in the histopathologic diagnosis of melanoma at a melanoma referral center. J Am Acad Dermatol 2010 May;62(5):751-756.

(17) Lodha S, Saggar S, Celebi JT, Silvers DN. Discordance in the histopathologic diagnosis of difficult melanocytic neoplasms in the clinical setting. J Cutan Pathol 2008 Apr;35(4):349-352.

(18) Barnhill RL, Argenyi ZB, From L, Glass LF, Maize JC, Mihm MC,Jr, et al. Atypical Spitz nevi/tumors: lack of consensus for diagnosis, discrimination from melanoma, and prediction of outcome. Hum Pathol 1999 May;30(5):513-520.

(19) Farmer ER, Gonin R, Hanna MP. Discordance in the histopathologic diagnosis of melanoma and melanocytic nevi between expert pathologists. Hum Pathol 1996 Jun;27(6):528-531.

(20) Troxel DB. Error in surgical pathology. Am J Surg Pathol 2004 Aug;28(8):1092-1095.

(21) Elder DE, Xu X. The approach to the patient with a difficult melanocytic lesion. Pathology 2004 Oct;36(5):428-434.

(22) Barnhill RL. The Spitzoid lesion: rethinking Spitz tumors, atypical variants, 'Spitzoid melanoma' and risk assessment. Mod Pathol 2006 Feb;19 Suppl 2:S21-33.

(23) Okun MR, Edelstein LM, Kasznica J, Kirkham N, Slater DN. What criteria reliably distinguish melanoma from benign melanocytic lesions? Histopathology 2000 Nov;37(5):464-472.

(24) Carlson JA, Ross JS, Slominski AJ, Carlson JA, et al. New techniques in dermatopathology that help to diagnose and prognosticate melanoma.; Molecular diagnostics in melanoma. - Clin Dermatol.2009 Jan-Feb;27(1):75-102.

(25) Glusac EJ. The melanoma 'epidemic', a dermatopathologist's perspective. J Cutan Pathol 2011 Mar;38(3):264-267.

(26) Edwards CN, Steinthorsson E, Nicholson D. An autopsy study of latent prostatic cancer. Cancer 1953 May;6(3):531-554.

(27) http://www.rcpath.org/resources/pdf/skincancers2802.pdf.

(28) http://www.cap.org/apps/docs/committees/cancer/cancerprotocols/2011/SkinMelanoma_11protocol.pdf.

(29) Bennett DC. How to make a melanoma: what do we know of the primary clonal events? Pigment Cell Melanoma Res 2008 Feb;21(1):27-38.

(30) Yunis JJ. The chromosomal basis of human neoplasia. Science 1983 Jul 15;221(4607):227-236.

(31) Wolman SR. Karyotypic progression in human tumors. Cancer Metastasis Rev 1983;2(3):257-293.

(32) Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 1992 Oct 30;258(5083):818-821.

(33) Baudis M. Genomic imbalances in 5918 malignant epithelial tumors: an explorative meta-analysis of chromosomal CGH data. BMC Cancer 2007 Dec 18;7:226.

(34) Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, et al. The landscape of somatic copy-number alteration across human cancers. Nature 2010 Feb 18;463(7283):899-905.

(35) Walther A, Houlston R, Tomlinson I. Association between chromosomal instability and prognosis in colorectal cancer: a meta-analysis. Gut 2008 Jul;57(7):941-950.

(36) Lee AJ, Endesfelder D, Rowan AJ, Walther A, Birkbak NJ, Futreal PA, et al. Chromosomal instability confers intrinsic multidrug resistance. Cancer Res 2011 Mar 1;71(5):1858-1870.

(37) Cahill DP, Lengauer C, Yu J, Riggins GJ, Willson JK, Markowitz SD, et al. Mutations of mitotic checkpoint genes in human cancers. Nature 1998 Mar 19;392(6673):300-303.

(38) Paulovich AG, Toczyski DP, Hartwell LH. When checkpoints fail. Cell 1997 Feb 7;88(3):315-321.

(39) Bastian BC, Olshen AB, LeBoit PE, Pinkel D. Classifying melanocytic tumors based on DNA copy number changes. Am J Pathol 2003 Nov;163(5):1765-1770.

(40) Curtin JA, Fridlyand J, Kageshita T, Patel HN, et al. Distinct sets of genetic alterations in melanoma. - N Engl J Med.2005 Nov 17;353(20):2135-47.

(41) Bastian BCLeBoit PE, Hamm H, Brocker EB, et al. Chromosomal gains and losses in primary cutaneous melanomas detected by comparative genomic hybridization. - Cancer Res.1998 May 15;58(10):2170-5.

(42) Bastian BC, Wesselmann U, Pinkel D, Leboit PE. Molecular cytogenetic analysis of Spitz nevi shows clear differences to melanoma. J Invest Dermatol 1999 Dec;113(6):1065-1069.

(43) Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature 2002 Jun 27;417(6892):949-954.

(44) Gray-Schopfer VC, Cheong SC, Chong H, Chow J, Moss T, Abdel-Malek ZA, et al. Cellular senescence in naevi and immortalisation in melanoma: a role for p16? Br J Cancer 2006 Aug 21;95(4):496-505.

(45) Aguissa-Toure AH, Li G. Genetic alterations of PTEN in human melanoma. Cell Mol Life Sci 2012 May;69(9):1475-1491.

(46) Yang G, Rajadurai A, Tsao H. Recurrent patterns of dual RB and p53 pathway inactivation in melanoma. J Invest Dermatol 2005 Dec;125(6):1242-1251.

(47) Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat 2007 Jun;28(6):622-629.

(48) Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, et al. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 2001 Jan 11;409(6817):207-211.

(49) Worm J, Christensen C, Gronbaek K, Tulchinsky E, Guldberg P. Genetic and epigenetic alterations of the APC gene in malignant melanoma. Oncogene 2004 Jul 1;23(30):5215-5226.

(50) Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, Ramaswamy S, et al. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature 2005 Jul 7;436(7047):117-122.

(51) Vance KW, Carreira S, Brosch G, Goding CR. Tbx2 is overexpressed and plays an important role in maintaining proliferation and suppression of senescence in melanomas. Cancer Res 2005 Mar 15;65(6):2260-2268.

(52) Treszl A, Adany R, Rakosy Z, Kardos L, Begany A, Gilde K, et al. Extra copies of c-myc are more pronounced in nodular melanomas than in superficial spreading melanomas as revealed by fluorescence in situ hybridisation. Cytometry B Clin Cytom 2004 Jul;60(1):37-46.

(53) Omholt K, Platz A, Kanter L, Ringborg U, Hansson J. NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. Clin Cancer Res 2003 Dec 15;9(17):6483-6488.

(54) Chen D, Xu W, Bales E, Colmenares C, Conacci-Sorrell M, Ishii S, et al. SKI activates Wnt/beta-catenin signaling in human melanoma. Cancer Res 2003 Oct 15;63(20):6626-6634.

(55) Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. Nature 2010 Jan 14;463(7278):191-196.

(56) Krauthammer M, Kong Y, Ha BH, Evans P, Bacchiocchi A, McCusker JP, et al. Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. Nat Genet 2012 Sep;44(9):1006-1014.

(57) Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, et al. A landscape of driver mutations in melanoma. Cell 2012 Jul 20;150(2):251-263.

(58) von Ahlfen S, Missel A, Bendrat K, Schlumpberger M. Determinants of RNA quality from FFPE samples. PLoS One 2007 Dec 5;2(12):e1261.

(59) Ali L, Helm T, Cheney R, Conroy J, Sait S, Guitart J, et al. Correlating array comparative genomic hybridization findings with histology and outcome in spitzoid melanocytic neoplasms. Int J Clin Exp Pathol 2010 Jun 28;3(6):593-599.

(60) Vanison C, Tanna N, Murthy AS. Comparative genomic hybridization for the diagnosis of melanoma. Eur J Plast Surg 2010 Feb;33(1):45-48.

(61) Takata M, Maruo K, Kageshita T, Ikeda S, Ono T, Shirasaki F, et al. Two cases of unusual acral melanocytic tumors: illustration of molecular cytogenetics as a diagnostic tool. Hum Pathol 2003 Jan;34(1):89-92.

(62) Bastian BC, Xiong J, Frieden IJ, Williams ML, Chou P, Busam K, et al. Genetic changes in neoplasms arising in congenital melanocytic nevi: differences between nodular proliferations and melanomas. Am J Pathol 2002 Oct;161(4):1163-1169.

(63) Gerami P, Jewell SS, Morrison LE, Blondin B, et al. Fluorescence in situ hybridization (FISH) as an ancillary diagnostic tool in the diagnosis of melanoma. - Am J Surg Pathol.2009 Aug;33(8):1146-56.

(64) Vergier B, Prochazkova-Carlotti M, de la Fouchardiere A, Cerroni L, Massi D, De Giorgi V, et al. Fluorescence in situ hybridization, a diagnostic aid in ambiguous melanocytic tumors: European study of 113 cases. Mod Pathol 2011 May;24(5):613-623.

(65) Senetta R, Paglierani M, Massi D. Fluorescence in-situ hybridization analysis for melanoma diagnosis. Histopathology 2012 Apr;60(5):706-714.

(66) Li LX, Crotty KA, McCarthy SW, Palmer AA, Kril JJ. A zonal comparison of MIB1-Ki67 immunoreactivity in benign and malignant melanocytic lesions. Am J Dermatopathol 2000 Dec;22(6):489-495.

(67) Ohsie SJ, Sarantopoulos GP, Cochran AJ, Binder SW. Immunohistochemical characteristics of melanoma. J Cutan Pathol 2008 May;35(5):433-444.

(68) Nasr MR, El-Zammar O. Comparison of pHH3, Ki-67, and survivin immunoreactivity in benign and malignant melanocytic lesions. Am J Dermatopathol 2008 Apr;30(2):117-122.

(69) Kashani-Sabet M, Rangel J, Torabian S, Nosrati M, Simko J, Jablons DM, et al. A multi-marker assay to distinguish malignant melanomas from benign nevi. Proc Natl Acad Sci U S A 2009 Apr 14;106(15):6268-6272.

(70) Ribe A, McNutt NS. S100A6 protein expression is different in Spitz nevi and melanomas. Mod Pathol 2003 May;16(5):505-511.

(71) Hilliard NJ, Krahl D, Sellheyer K. p16 expression differentiates between desmoplastic Spitz nevus and desmoplastic melanoma. J Cutan Pathol 2009 Jul;36(7):753-759.

(72) Clarke LE, Fountaine TJ, Hennessy J, Bruggeman RD, Clarke JT, Mauger DT, et al. Cdc7 expression in melanomas, Spitz tumors and melanocytic nevi. J Cutan Pathol 2009 Apr;36(4):433-438.

(73) Park HS, Park CH, Choi BR, Lim MS, Heo SH, Kim CH, et al. Expression of heat shock protein 105 and 70 in malignant melanoma and benign melanocytic nevi. J Cutan Pathol 2009 May;36(5):511-516.

(74) Chwirot BW, Kuzbicki L. Cyclooxygenase-2 (COX-2): first immunohistochemical marker distinguishing early cutaneous melanomas from benign melanocytic skin tumours. Melanoma Res 2007 Jun;17(3):139-145.

(75) Koh SS, Opel ML, Wei JP, Yau K, Shah R, Gorre ME, et al. Molecular classification of melanomas and nevi using gene expression microarray signatures and formalin-fixed and paraffin-embedded tissue. Mod Pathol 2009 Apr;22(4):538-546.

(76) Talantov D, Mazumder A, Yu JX, Briggs T, Jiang Y, Backus J, et al. Novel genes associated with malignant melanoma but not benign melanocytic lesions. Clin Cancer Res 2005 Oct 15;11(20):7234-7242.

(77) Lewis TB, Robison JE, Bastien R, Milash B, et al. Molecular classification of melanoma using real-time quantitative reverse transcriptase-polymerase chain reaction. - Cancer.2005 Oct 15;104(8):1678-86.

(78) Glud M, Klausen M, Gniadecki R, Rossing M, Hastrup N, Nielsen FC, et al. MicroRNA expression in melanocytic nevi: the usefulness of formalin-fixed, paraffin-embedded material for miRNA microarray profiling. J Invest Dermatol 2009 May;129(5):1219-1224.

(79) Philippidou D, Schmitt M, Moser D, Margue C, Nazarov PV, Muller A, et al. Signatures of microRNAs and selected microRNA target genes in human melanoma. Cancer Res 2010 May 15;70(10):4163-4173.

(80) Gimotty PA, Guerry D, Ming ME, Elenitsas R, et al. Thin primary cutaneous malignant melanoma: a prognostic tree for 10-year metastasis is more accurate than American Joint Committee on Cancer staging. - J Clin Oncol.2004 Sep 15;22(18):3668-76.

(81) Kaur C, Thomas RJ, Desai N, Green MA, et al. The correlation of regression in primary melanoma with sentinel lymph node status. - J Clin Pathol.2008 Mar;61(3):297-300.

(82) Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med 2011 Jun 30;364(26):2507-2516.

(83) Gould Rothberg BE, Bracken MB, Rimm DL. Tissue biomarkers for prognosis in cutaneous melanoma: a systematic review and meta-analysis. - J Natl Cancer Inst.2009 Apr 1;101(7):452-74.

(84) Ladstein RG, Bachmann IM, Straume O, Akslen LA, et al. Ki-67 expression is superior to mitotic count and novel proliferation markers PHH3, MCM4 and mitosin as a prognostic factor in thick cutaneous melanoma. - BMC Cancer.2010 Apr 14;10:140.

(85) Gimotty PA, Van Belle PElder DE, Murry T, et al. Biologic and prognostic significance of dermal Ki67 expression, mitoses, and tumorigenicity in thin invasive cutaneous melanoma. - J Clin Oncol.2005 Nov 1;23(31):8048-56.

(86) Frahm SO, Schubert C, Parwaresch R, Rudolph P, et al. High proliferative activity may predict early metastasis of thin melanomas. - Hum Pathol.2001 Dec;32(12):1376-81.

(87) Barry MSinha SK, Leader MB, Kay EW, et al. Poor agreement in recognition of abnormal mitoses: requirement for standardized and robust definitions. - Histopathology.2001 Jan;38(1):68-72.

(88) Weinlich G, Bitterlich W, Mayr V, Fritsch PO, et al. Metallothionein-overexpression as a prognostic factor for progression and survival in melanoma. A prospective study on 520 patients. - Br J Dermatol.2003 Sep;149(3):535-41.

(89) Weinlich G, Eisendle K, Hassler E, Baltaci M, et al. Metallothionein - overexpression as a highly significant prognostic factor in melanoma: a prospective study on 1270 patients. - Br J Cancer.2006 Mar 27;94(6):835-41.

(90) Salti GI, Manougian T, Farolan MShilkaitis A, et al. Micropthalmia transcription factor: a new prognostic marker in intermediate-thickness cutaneous malignant melanoma. - Cancer Res.2000 Sep 15;60(18):5012-6.

(91) Kitago M, Koyanagi K, Nakamura T, Goto Y, et al. mRNA expression and BRAF mutation in circulating melanoma cells isolated from peripheral blood with high

molecular weight melanoma-associated antigen-specific monoclonal antibody beads. - Clin Chem.2009 Apr;55(4):757-64.

(92) Wang Y, Radfar S, Liu S, Riker AI, et al. Mitf-Mdel, a novel melanocyte/melanoma-specific isoform of microphthalmia-associated transcription factor-M, as a candidate biomarker for melanoma. - BMC Med.2010 Feb 17;8:14.

(93) Samija I, Lukac J, , Maric-Brozic J, Buljan M, et al. Prognostic value of microphthalmia-associated transcription factor and tyrosinase as markers for circulating tumor cells detection in patients with melanoma. - Melanoma Res.2010 Aug;20(4):293-302.

(94) Da Forno PD, Pringle JH, Hutchinson P, Osborn J, Huang Q, Potter L, et al. WNT5A expression increases during melanoma progression and correlates with outcome. Clin Cancer Res 2008 Sep 15;14(18):5825-5832.

(95) Kashani-Sabet M, Venna S, Nosrati M, Rangel J, et al. A multimarker prognostic assay for primary cutaneous melanoma. - Clin Cancer Res.2009 Nov 15;15(22):6987-92.

(96) Gould Rothberg BE, Berger AJ, Molinaro AM, Subtil A, et al. Melanoma prognostic model using tissue microarrays and genetic algorithms. - J Clin Oncol.2009 Dec 1;27(34):5772-80.Epub 2009 Nov 2.

(97) Winnepenninckx V, Lazar V, Michiels S, Dessen P, et al. Gene expression profiling of primary cutaneous melanoma and clinical outcome. - J Natl Cancer Inst.2006 Apr 5;98(7):472-82.

(98) Haqq C, Nosrati M, Sudilovsky D, Crothers J, et al. The gene expression signatures of melanoma progression. - Proc Natl Acad Sci U S A.2005 Apr 26;102(17):6092-7.

(99) Conway C, Mitra A, Jewell R, Randerson-Moor J, et al. Gene expression profiling of paraffin-embedded primary melanoma using the DASL assay identifies increased osteopontin expression as predictive of reduced relapse-free survival. - Clin Cancer Res.2009 Nov 15;15(22):6939-46.

(100) Segura MF, Belitskaya-Levy I, Rose AE, Zakrzewski J, et al. Melanoma MicroRNA signature predicts post-recurrence survival. - Clin Cancer Res.2010 Mar 1;16(5):1577-86.

(101) Satzger I, Mattern A, Kuettler U, Weinspach D, et al. MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. - Int J Cancer.2010 Jun 1;126(11):2553-62.

(102) Jonsson G, Dahl C, Staaf J, Sandberg T, Bendahl PO, Ringner M, et al. Genomic profiling of malignant melanoma using tiling-resolution arrayCGH. Oncogene 2007 Jul 12;26(32):4738-4748.

(103) Conway C, Beswick S, Elliott F, Chang Y, et al. Deletion at chromosome arm 9p in relation to BRAF/NRAS mutations and prognostic significance for primary melanoma. - Genes Chromosomes Cancer.2010 May;49(5):425-38.

(104) Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol 1986;51 Pt 1:263-273.

(105) Armour JA, Palla R, Zeeuwen PL, den Heijer M, Schalkwijk J, Hollox EJ. Accurate, high-throughput typing of copy number variation using paralogue ratios from dispersed repeats. Nucleic Acids Res 2007;35(3):e19.

(106) Saldanha G, Potter L, Dyall L, Bury D, Hathiari N, Ehdode A, et al. Detection of copy number changes in DNA from formalin fixed paraffin embedded tissues using paralogue ratio tests. Anal Chem 2011 May 1;83(9):3484-3492.

(107) Jorgensen AL, Bostock CJ, Bak AL. Homologous subfamilies of human alphoid repetitive DNA on different nucleolus organizing chromosomes. Proc Natl Acad Sci U S A 1987 Feb;84(4):1075-1079.

(108) Baumgartner A, Weier JF, Weier HU. Chromosome-specific DNA repeat probes. J Histochem Cytochem 2006 Dec;54(12):1363-1370.

(109) Ransohoff DF. How to improve reliability and efficiency of research about molecular markers: roles of phases, guidelines, and study design. J Clin Epidemiol 2007 Dec;60(12):1205-1219.

(110) Sackett DL, FAU - Haynes RB, Haynes RB. The architecture of diagnostic research. - BMJ.2002 Mar 2;324(7336):539-41.

(111) Pepe MS, Etzioni R, Feng Z, Potter JD, et al. Phases of biomarker development for early detection of cancer. - J Natl Cancer Inst.2001 Jul 18;93(14):1054-61.

(112) McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005 Aug 17;97(16):1180-1184.

(113) Dupuy A, Simon RM. Critical review of published microarray studies for cancer outcome and guidelines on statistical analysis and reporting. - J Natl Cancer Inst.2007 Jan 17;99(2):147-57.

(114) Pepe MS, Feng Z, Janes H, Bossuyt PM, et al. Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: standards for study design. - J Natl Cancer Inst.2008 Oct 15;100(20):1432-8.

(115) Harbour JW. Molecular prognostic testing and individualized patient care in uveal melanoma. Am J Ophthalmol 2009 Dec;148(6):823-9.e1.

(116) Drwinga HL, Toji LH, Kim CH, Greene AE, Mulivor RA. NIGMS human/rodent somatic cell hybrid mapping panels 1 and 2. Genomics 1993 May;16(2):311-314.

(117) Volkenandt M, Burmer GC, Schadendorf D, Koch OM, Wienecke R, Degitz K. The polymerase chain reaction. Method and applications in dermatopathology. Am J Dermatopathol 1993 Apr;15(2):118-126.

(118) Shaw JA, Brown J, Coombes RC, Jacob J, Payne R, Lee B, et al. Circulating tumor cells and plasma DNA analysis in patients with indeterminate early or metastatic breast cancer. Biomark Med 2011 Feb;5(1):87-91.

(119) Laursen HB, Jorgensen AL, Jones C, Bak AL. Higher rate of evolution of X chromosome alpha-repeat DNA in human than in the great apes. EMBO J 1992 Jul;11(7):2367-2372.

(120) Devilee P, Slagboom P, Cornelisse CJ, Pearson PL. Sequence heterogeneity within the human alphoid repetitive DNA family. Nucleic Acids Res 1986 Mar 11;14(5):2059-2073.

(121) Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, et al. TM4 microarray software suite. Methods Enzymol 2006;411:134-193.

(122) Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, et al. TM4: a free, open-source system for microarray data management and analysis. BioTechniques 2003 Feb;34(2):374-378.

(123) Wheeler DL, Barrett T, Benson DA, Bryant SH, Canese K, Chetvernin V, et al. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 2008 Jan;36(Database issue):D13-21.

(124) Rachlin J, Ding C, Cantor C, Kasif S. MuPlex: multi-objective multiplex PCR assay design. Nucleic Acids Res 2005 Jul 1;33(Web Server issue):W544-7.

(125) Thornton B, Basu C. Real-time PCR (qPCR) primer design using free online software. Biochem Mol Biol Educ 2011 Mar-Apr;39(2):145-154.

(126) D'haene B, Vandesompele J, Hellemans J. Accurate and objective copy number profiling using real-time quantitative PCR. Methods 2010 Apr;50(4):262-270.

(127) Benlloch S, Paya A, Alenda C, Bessa X, Andreu M, Jover R, et al. Detection of BRAF V600E mutation in colorectal cancer: comparison of automatic sequencing and real-time chemistry methodology. J Mol Diagn 2006 Nov;8(5):540-543.

(128) Shrout PE, Fleiss JL. Intraclass correlations: uses in assessing rater reliability. Psychol Bull 1979 Mar;86(2):420-428.

(129) Rocchi M, Archidiacono N, Ward DC, Baldini A. A human chromosome 9-specific alphoid DNA repeat spatially resolvable from satellite 3 DNA by fluorescent in situ hybridization. Genomics 1991 Mar;9(3):517-523.

(130) Forer L, Schonherr S, Weissensteiner H, Haider F, Kluckner T, Gieger C, et al. CONAN: copy number variation analysis software for genome-wide association studies. BMC Bioinformatics 2010 Jun 14;11:318-2105-11-318.

(131) Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002 Dec;161(6):1961-1971.

(132) Critchley LA, Critchley JA. A meta-analysis of studies using bias and precision statistics to compare cardiac output measurement techniques. J Clin Monit Comput 1999 Feb;15(2):85-91.

(133) Kabbarah O, Nogueira C, Feng B, Nazarian RM, Bosenberg M, Wu M, et al. Integrative genome comparison of primary and metastatic melanomas. PLoS One 2010 May 24;5(5):e10770.

(134) Katona TM, Jones TD, Wang M, Eble JN, Billings SD, Cheng L. Genetically heterogeneous and clonally unrelated metastases may arise in patients with cutaneous melanoma. Am J Surg Pathol 2007 Jul;31(7):1029-1037.

(135) North JP, Vetto JT, Murali R, White KP, White CR,Jr, Bastian BC. Assessment of copy number status of chromosomes 6 and 11 by FISH provides independent prognostic information in primary melanoma. Am J Surg Pathol 2011 Aug;35(8):1146-1150.

(136) Barlund M, Monni O, Kononen J, Cornelison R, Torhorst J, Sauter G, et al. Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. Cancer Res 2000 Oct 1;60(19):5340-5344.

(137) Ascierto PA, Kirkwood JM, Grob JJ, Simeone E, Grimaldi AM, Maio M, et al. The role of BRAF V600 mutation in melanoma. J Transl Med 2012 Jul 9;10:85-5876-10-85.

(138) Platz A, Egyhazi S, Ringborg U, Hansson J. Human cutaneous melanoma; a review of NRAS and BRAF mutation frequencies in relation to histogenetic subclass and body site. Mol Oncol 2008 Apr;1(4):395-405.

(139) Devitt B, Liu W, Salemi R, Wolfe R, Kelly J, Tzen CY, et al. Clinical outcome and pathological features associated with NRAS mutation in cutaneous melanoma. Pigment Cell Melanoma Res 2011 Aug;24(4):666-672.

(140) Lazar V, Ecsedi S, Vizkeleti L, Rakosy Z, Boross G, Szappanos B, et al. Marked genetic differences between BRAF and NRAS mutated primary melanomas as revealed by array comparative genomic hybridization. Melanoma Res 2012 Jun;22(3):202-214.

(141) Zalaudek I, Guelly C, Pellacani G, Hofmann-Wellenhof R, Trajanoski S, Kittler H, et al. The dermoscopical and histopathological patterns of nevi correlate with the frequency of BRAF mutations. J Invest Dermatol 2011 Feb;131(2):542-545.

(142) Karram S, Novy M, Saroufim M, Loya A, Taraif S, Houreih MA, et al. Predictors of BRAF Mutation in Melanocytic Nevi: Analysis Across Regions With Different UV Radiation Exposure. Am J Dermatopathol 2012 Oct 9.

(143) Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM, et al. High frequency of BRAF mutations in nevi. Nat Genet 2003 Jan;33(1):19-20.

(144) Yazdi AS, Palmedo G, Flaig MJ, Puchta U, Reckwerth A, Rutten A, et al. Mutations of the BRAF gene in benign and malignant melanocytic lesions. J Invest Dermatol 2003 Nov;121(5):1160-1162.

(145) Papp T, Schipper H, Kumar K, Schiffmann D, Zimmermann R. Mutational analysis of the BRAF gene in human congenital and dysplastic melanocytic naevi. Melanoma Res 2005 Oct;15(5):401-407.

(146) Guttery DS, Blighe K, Page K, Marchese SD, Hills A, Coombes RC, et al. Hide and seek: tell-tale signs of breast cancer lurking in the blood. Cancer Metastasis Rev 2012 Oct 30.

(147) De Giorgi V, Grazzini M, Rossari S, Gori A, Papi F, Scarfi F, et al. Is skin self-examination for cutaneous melanoma detection still adequate? A retrospective study. Dermatology 2012;225(1):31-36.