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Development of a blood test for the detection of cutaneous malignant melanoma

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Development of a blood test for the detection of Cutaneous Malignant Melanoma

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ABSTRACT

Cutaneous Malignant Melanoma is a highly aggressive form of skin cancer that has a tendency to metastasise. In its early stages, the 5-year survival rate of patients is greater than 90% following surgical excision of an in situ tumour. However, following metastasis of the tumour, the 5-year survival rate declines to 5-35%. Due to the correlation between metastasis and declining survival, studies have attempted to identify potential metastasis as early as possible. Molecular markers of melanoma cells in the peripheral blood of CMM patients have been investigated, as the invasion of tumour cells into the blood is one of the first events in melanoma metastasis. Genetic markers *PAX3d, MITF, MART1, SFRP5* and*TGFβ2* are involved in the development, proliferation and migration of melanocytic cells, and are aberrantly expressed in melanoma. Therefore, the aim of this study was to develop an assay that can be used for CMM diagnosis and prognosis and to assess *PAX3d, MITF, SFRP5, MART1* and *TGF-β2* expression in peripheral blood of CMM patients relative to healthy volunteers.

In this study, lymph node tissue and peripheral blood samples were taken from CMM patients and blood was donated from healthy volunteers. Total RNA was extracted from all samples and following the analysis of RNA quality, samples were Reverse Transcribed. The resulting cDNA was analysed by PCR for the expression of the housekeeping gene *GAPDH,* following which samples expressing this gene were analysed by PCR for the presence of each of the genetic markers: *PAX3d, MITF, MART1, SFRP5* and *TGFβ2.*

Statistical analysis of marker expression showed that CMM patients were significantly more likely than healthy volunteers to express one or more markers, as well as significantly higher expression of *MITF, TGFβ2* and *MART1* in patients relative to healthy volunteers. The number of markers shown in each sample was statistically analysed and showed that a significantly higher frequency of *TGFβ2* expression correlated with primary tumours of >2.00mm in CMM patients, as well as a significantly lower frequency of *MART1* expression in patients with a tumour depth of 0.01-2.00mm relative to patients with in situ

melanoma or >2.00mm tumour depth. Frequent expression of all markers in CMM patients even after more than one year since diagnosis suggests the longevity of melanoma cells in the blood. The results presented here show that *MITF, MART1* and *TGFβ2* are sensitive and useful markers of melanoma cells in the peripheral blood of CMM patients even after more than one year since diagnosis.

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Signed. .....

Date 21-12-07

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Development of a blood test for the detection of
Cutaneous Malignant Melanoma

CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

1. INTRODUCTION

1.1. Cutaneous Malignant Melanoma

Cutaneous Malignant Melanoma (CMM) is a form of skin cancer that arises from cutaneous melanocytic cells. Although a rare form of skin cancer, it is responsible for almost 80% of skin cancer related deaths due to a strong metastatic tendency (Lewis *et al.*, 2005).

The American Joint Committee on Cancer (AJCC) has provided a classification system for the stages of melanoma development based on tumour thickness and ulceration as well as lymphatic and systemic metastasis (Balch *et al.*, 2001). According to this classification system, prognosis of a patient is closely related to metastasis of CMM; patients undergoing the surgical excision of a thin, non-metastatic melanoma have a greater than 90% five-year survival rate, while patients with metastasis of a thicker tumour have a far poorer prognosis and a 5-35% five-year survival rate (Balch *et al.*, 2001; Wascher *et al.*, 2003).

Metastasis is commonly detected in sentinel lymph nodes as a result of an invasive biopsy or a Positron Emission Tomography (PET) scan (Holder *et al.*, 1998). Recent studies have attempted to use circulating melanoma cells in the blood stream as an indicator of spread of the disease. Invasion of tumour cells into the blood is one of the first events in melanoma metastasis and screening the blood for melanoma markers allows early identification of potential metastasis (Wascher *et al.*, 2003). A blood test for CMM would also act as an important prognostic tool as earlier detection of metastatic spread may suggest that a more appropriate treatment relative to disease progression is required.

1.2. Molecular Markers for CMM haematological spread

There are many potentially suitable molecular markers currently being assessed in several research labs. This research involves screening of CMM patient blood for melanoma markers. These markers range from genes involved in

melanocyte production, such as *Paired-Box transcription factor (PAX3)* and *Microphthalmia-Associated Transcription Factor (MITF)*, to melanin protein genes such as tyrosinase, and to genes encoding melanoma-associated antigens like Melanoma Antigen Recognised by T-cells (MART1). Other genes, such as those thought to be involved in the proliferation of melanoma cells and disease progression like *Transforming Growth Factor Beta (TGF-β)* and *Secreted Frizzled-Related Protein 5 (SFRP5)* have not been assessed as yet. Markers involved in these various pathways have been trialled in this study and their presence in peripheral blood of CMM patients has been compared with presence in blood from healthy volunteers.

One potential marker, PAX3, encodes a Paired-Box transcription factor, and is expressed early in embryonic development to direct melanogenesis. PAX3-c and -d alternative isoforms are known to be aberrantly expressed in melanomas and their expression is thought to be restricted to more malignant metastatic cells (Vachtenheim and Novotna, 1999). Due to the presence of PAX3 in metastatic cells, this transcription factor may be an important marker of melanoma cells circulating in blood.

MITF regulates genes encoding the pigment producing enzymes Tyrosinase (TYR), Tyrosinase Related Protein 1 (TYRP1) and Dopachrome Tautomerase (DCT) as well as genes responsible for cell proliferation and survival (Wagner and Fisher, 2005). Expression of the *MITF* gene is associated with melanoma progression and can therefore be used as a marker of melanoma cell migration in the blood (Carreira *et al.*, 2005).

MART1 is a melanoma-associated antigen involved in melanosome biogenesis. It is a specific marker used in biopsies to differentiate melanoma cells from those of other skin malignancies (Wascher *et al.*, 2003). MART1 has been used in this study to mark migrating melanoma cells in peripheral blood as it is the most commonly used marker of melanoma.

SFRPs are upstream regulators of the signaling pathway that controls MITF production. *SFRP* gene expression would therefore be expected to be a marker

of early undifferentiated melanoma cells. To date, this marker has not been used to detect melanoma cell migration, although low levels of expression of the genes have been associated with other forms of cancer (Jones and Jomary, 2002). In this project *SFRP-5* expression was trialed as a marker of migrating melanoma cells in peripheral blood.

TGF-β is a multifunctional cytokine involved in many pathways that regulate cell function. By activating many different transcription factors, TGF-β controls cell proliferation, differentiation, apoptosis and neogenesis (Reed *et al.*, 1994). Thus, it is expected that alteration in the level of expression of the gene may allow abnormal growth and proliferation of melanoma cells. For these reasons, *TGF-β* gene expression was trialled as one of the markers of melanoma cell migration in the bloodstream of patients with CMM.

1.3. Significance of the Study

With the incidence of CMM rising yearly and relatively little progress being made in the treatment of this terminal disease, the development of a test for screening CMM patients, so as to identify the spread of melanoma cells, is rapidly becoming a necessity (Lewis *et al.*, 2005). As the morbidity of most CMM patients is not due to the primary tumour, but to metastatic disease, it is important to identify the likelihood of metastasis as early as possible.

Recent studies have identified gene expression in melanoma cells as useful for identification of primary and secondary tumours removed from melanoma patients, as well as in the detection of migrating melanoma cells in peripheral blood (Palmieri *et al.*, 1999; Wascher *et al.*, 2003). Moreover, several studies have recognised the necessity of using multiple markers in RT-PCR assays in order to improve assay sensitivity (Hoon *et al.*, 2000; Koyanagi *et al.*, 2005). The particular suite of markers that would give optimal correlation to disease stage and progression remains to be clarified.

In this project, the presence of mRNA transcripts indicative of gene expression of *Paired-Box Transcription Factor (PAX3), Microphthalmia-Associated Transcription Factor (MITF), Secreted Frizzled-Related Proteins (SFRPs), Melanoma Antigen Recognised by T-cells (MART1)* and *Transforming Growth Factor Beta (TGF-β)* have been tested in blood and tumour samples from patients diagnosed with differing tumour depths, as well as in the peripheral blood of healthy volunteers. Results from this study have allowed us to determine which of these markers should be included or excluded in an optional suite of markers for the detection of migrating melanoma cells. Results have been compared with those obtained from previous studies.

The detection of malignant melanoma cells in patient sera has a diversity of applications including disease detection, disease staging, assessing the probability of disease recurrence and analysis of treatment efficacy. Accurate staging of a patient may be possible by comparing the number and suite of markers present in the patient blood relative to a set of markers known to be associated with melanoma tissue samples. Such a test would assist in providing appropriate adjuvant treatment (Hoon *et al.*, 2000; Palmieri *et al.*, 1999). All of these applications of marker detection in peripheral blood samples would aid in the early diagnosis and staging of CMM, and would assist with disease management and treatment possibly leading to improved five-year survival rates of patients with the more advanced stages of disease.

2. REVIEW OF THE LITERATURE

2.1. Melanocytes

Melanocytes are specialised pigment producing cells found in the epidermal and dermal layers of the skin (Dupin and LeDouarin, 2003). These cells differentiate from melanoblasts which originate from neural crest cells and subsequently migrate to the skin during early embryonic development (Blake and Ziman, 2005).

Melanocytes synthesise melanin pigment by the activities of melanin-secreting proteins; tyrosinase (TYR), tyrosinase related protein (TYRP1) and dopachrome tautomerase (DCT) in response to transcriptional signaling within the cell (Hearing, 2000; Wagner and Fisher, 2005). The purpose of melanin pigment is to provide photoprotection and thermoregulation of the melanocyte and surrounding keratinocytes. These two cell types have a symbiotic relationship in which keratinocytes control melanocyte growth, and melanocytes produce melanin to protect mitotic keratinocytes from the ionising effect of UV irradiation (Haass *et al.*, 2005).

2.2. Melanocyte development

The development of melanocytes from their neural crest origin is closely regulated by several transcription factors. These factors affect the migration, specification and proliferation of melanoblasts as they migrate to the skin, as well as their terminal differentiation into melanocytes once they arrive there (Blake and Ziman, 2005). A very complex and intricate network of overlapping signaling pathways regulate these processes.

2.2.1. *MITF* expression in melanocyte development

The microphthalmia-associated transcription factor (MITF) acts as a master regulator of melanocyte development, having direct involvement in the differentiation, proliferation and survival of these cells (Wagner and Fisher, 2005).

The *MITF* gene has a multi-promoter organization which allows alternative splicing to produce several MITF isoforms (Hershey and Fisher, 2005), with the M promoter selectively expressed in melanocytes (Fuse *et al.*, 1996; Udono *et al.*, 2000). MITF-M binds to an M-box sequence (CATGTG or AGTCATGTG) found in the promoter regions of MITF responsive genes such as those involved in melanogenesis (*TYR, TYRP1* and *DCT*) and apoptosis (*BCL2*) and is known to regulate the expression of these genes (Figure 2.1b) (Wagner and Fisher, 2005).

2.2.2. *MITF* signaling pathways

Expression of the *MITF-M* gene is regulated by an array of transcription factors that bind to its promoter (Figure 2.1a). These include the paired box transcription factor (PAX3), sex determining region Y-box 10 (SOX10), the lymphoid enhancer factor (LEF1) and the cAMP responsive element binding transcription factor (CREB) (Vance and Goding, 2004).

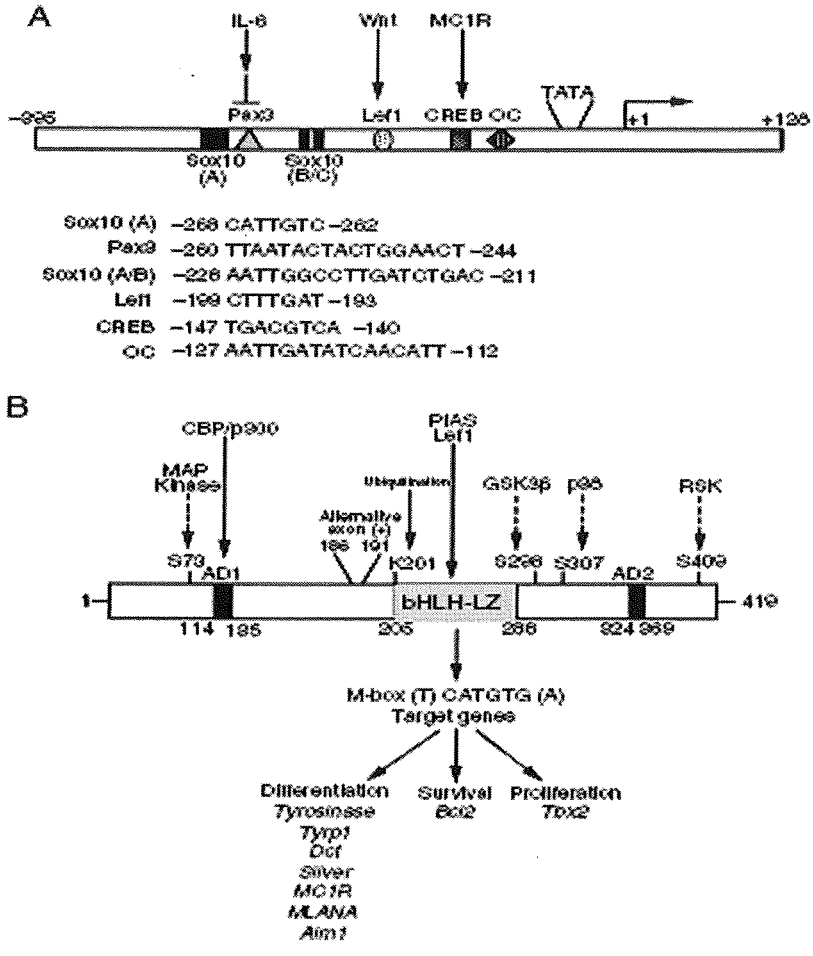


Figure 2.1. Regulation of *Mitf* expression and activity. (a) The *Mitf-M* promoter showing the binding sites for transcription factors known to regulate *Mitf* expression and the signal transduction pathways regulating their activity. (b) Features of the Mitf protein. Phosphorylation sites and locations of regions interacting with co-factors or other transcription factors are indicated above, while those genes known to be regulated by Mitf are shown below (adapted from Vance and Goding, 2004).

2.2.2.1. *PAX3* regulation of MITF

PAX3 can activate *MITF* expression by binding to recognition sites located upstream of the *MITF-M* transcript initiation site (Bondurand *et al.*, 2000; Watanabe *et al.*, 1998). PAX3 can bind independently, or in synergy with SOX10 to activate *MITF* expression (Figure 2.2) (Lang *et al.*, 2005).

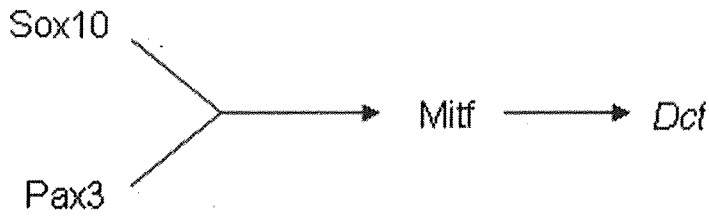


Figure 2.2. Pax3 binds to *Mitf* promoters either directly or in synergy with Sox10 to up-regulate *Mitf* leading to up-regulation of MITF responsive gene *Dct* (Adapted from Lang *et al*, 2005).

2.2.2.2. LEF1 regulation of *MITF*

The LEF1 transcription factor interacts with β-catenin via the WNT signaling pathway to regulate *MITF* expression (Larue *et al.*, 2003; Saito *et al.*, 2003). The WNT protein binds to Frizzled receptors, inactivating glycogene synthase kinase 3β (GSK-3β), a kinase that inhibits β-catenin. Thus, by inactivating GSK-3β, WNT signaling allows accumulation of β-catenin, which translocates into the nucleus and binds with LEF1 to co-activate transcription of *MITF* (Figure 2.3, pathway a.) (Wagner and Fisher, 2005). MITF is then able to induce the expression of genes involved in pigmentation, proliferation and survival (Figure 2.3, pathway b.).

2.2.2.3. CREB regulation of *MITF*

The two signaling pathways that lead to the activation of *CREB* are the melanocyte stimulating hormone (MSH) pathway and the endothelin (END) pathway (Wagner and Fisher, 2005). MSH binds to the melanocortin receptor (MC1R) on the surface of the cell and leads to the production of cyclic AMP (cAMP). Similarly, END binds to the endothelin 3 receptor (ENDRB) and leads to production of cAMP. The cyclic AMP then liberates the catalytic subunit of protein kinase A (PKA) allowing it to phosphorylate CREB (Steingrimsson *et al.*, 2004), which is then able to bind to the cAMP responsive element (CRE) DNA binding site on the *MITF* promoter, thus activating MITF (Figure 2.3, pathway c.).

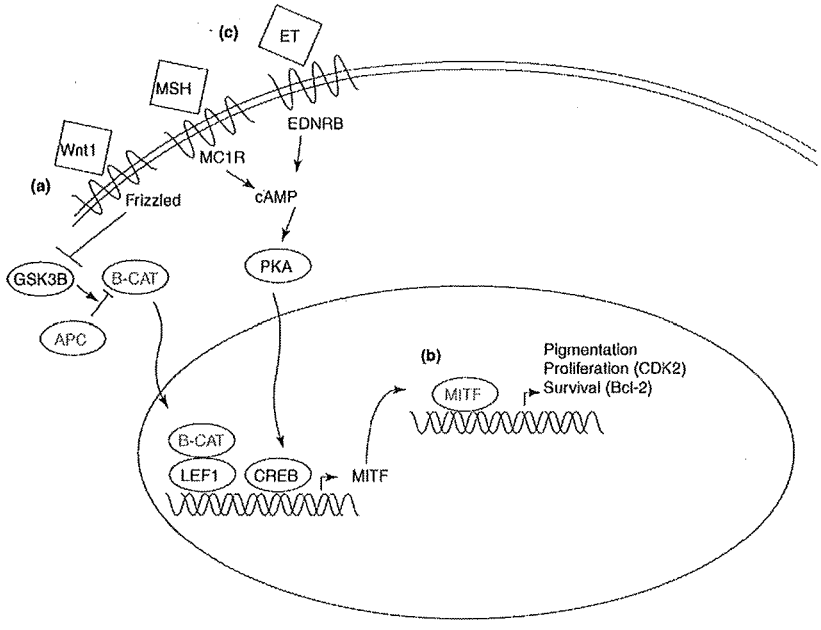


Figure 2.3. Normal melanocyte signaling pathways. (a) Stimulation of the Wnt signaling pathway leads to inactivation of *GSK3B*, which inhibits β-catenin. In this manner, Wnt signaling leads to enhanced transcription of MITF by allowing β-catenin to translocate to the nucleus and cooperate with LEF-1 on the *MITF* promoter. (b) In melanocytes, MITF induces expression of genes involved in pigmentation, proliferation and survival. (c) Stimulation of the MSH or endothelin receptors leads to production of cAMP which in turn activates protein kinase A (PKA). Phosphorylation of CREB by PKA induces DNA-binding and enhances transcription of the *MITF* promoter (Adapted from Wagner and Fisher, 2005).

2.2.2.4. SFRP association with *MITF* expression

Secreted Frizzled-Related Proteins (SFRPs) are secreted glycoproteins, positioned upstream of WNT in the WNT cascade (Jones and Jomary, 2002). They contain a cysteine-rich domain homologous to the WNT binding site of Frizzled receptors (Uren *et al.*, 2000).

At high levels, SFRP acts as an antagonist to WNT signaling by preventing WNT interaction with Frizzled receptors (He *et al.*, 2005). Structurally, it resembles a Frizzled receptor, allowing competitive binding with WNT ligands to prevent signaling through the Frizzled receptor. This leads to a decrease in the intracellular levels of β-catenin (Wissmann *et al.*, 2003). As seen previously in Figure 2.3, β-catenin is required for LEF1 binding of the *MITF* promoter. Thus, by blocking WNT/Frizzled interaction, SFRP represses the expression *MITF*. At

low levels however, SFRP can augment WNT/Frizzled interaction, consequently up-regulating *MITF* (Jones and Jomary, 2002).

2.2.3. *PAX* genes in melanocyte development

The Paired Box (*Pax)* genes are a family of transcription factors with essential and distinct roles in embryonic development (Ziman and White, 2006). Within this family of genes, *Pax3* is associated with melanoblast proliferation and differentiation (Hornyak *et al.*, 2001).

As described above PAX3 activates *MITF* transcription leading to the production of DCT. At the same time, PAX3 competes with MITF for occupancy of the *DCT* enhancer to repress DCT production, unless displaced from the DNA by β-catenin (Figure 2.4).

By repressing *DCT* expression, PAX3 holds the melanoblast in an undifferentiated state while MITF accumulates. The cell is primed for rapid expression of downstream genes once the PAX3 mediated repression is relieved by β-catenin. Thus PAX3 acts as a molecular switch allowing the undifferentiated melanoblast to proliferate and migrate, or to differentiate once it reaches the dermis and PAX3 is down-regulated (Lang *et al.*, 2005).

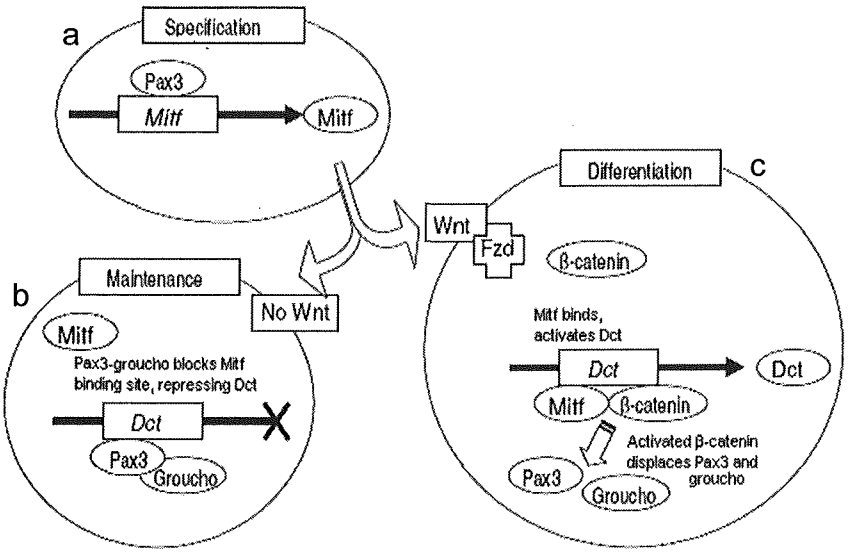


Figure 2.4. The role of Pax3 in the specification and differentiation of melanocytes. a) Pax3 binds and activates expression Mitf during specification of melanoblasts from neural crest cells. b) Pax3 complexes with groucho, repressing the differentiation gene *Dct*, thus maintaining cells in an undifferentiated state. c) Wnt signaling activates β–catenin which displaces Pax3 and groucho, allowing Mitf to bind and activate *Dct* expression, stimulating melanocyte differentiation (Adapted from Ziman and White, 2006).

The pathways described above are thought to be re-expressed in melanoma (Scholl *et al.*, 2001), leading to aberrant expression of genes controlling proliferation, migration and cell survival in melanoma cells.

2.3. Cutaneous Malignant Melanoma

Cutaneous Malignant Melanoma (CMM) is a type of skin cancer that is thought to arise from melanocytes present in the epidermis. While it accounts for only 4% of skin cancers, it is responsible for almost 80% of skin cancer related deaths due to its aggressive tendency to metastasise (Chang *et al.*, 1998; Lewis *et al.*, 2005).

Australia has the world's highest incidence of melanoma (MacLennan *et al.*, 1992). In Western Australia, melanoma is the third most common cancer in men and women over 39 years of age and it is the most common cancer diagnosed in the 15-39 year-old age group and it is increasing in incidence by 3% per year (Threlfall and Thompson, 2002).

2.3.1. Formation and metastasis

The first event in the formation of a melanoma is thought to be the proliferation of structurally normal melanocytes leading to a benign nevus. Next the aberrant growth develops to form a dysplastic nevus, within a benign nevus or at a new site. At this early stage the tumour is classified as in-situ and is thought to be inaccessible to blood and lymphatic vessels (Meier *et al.*, 1998; Pantel *et al.*, 1999).

There are then 2 stages of melanoma growth: radial and vertical. During the radial growth phase, cells aquire the ability to proliferate within the epidermis. In the vertical growth phase, cells acquire the ability to invade the dermis and form an expansive nodule, widening the papillary dermis with extensions into the reticular dermis and fat layers (Miller and Mihm Jr, 2006). At this stage tumours are classified as invasive. It is at this stage that the initial risk of metastasis exists increasing as the tumour invades the deeper layers (Pantel *et al.*, 1999).

Following dermal invasion, metastatic cells can infiltrate the blood and lymphatic systems and circulate in the body to new sites where they can adhere to the walls of a capillary and invade a new tissue to form a micrometastasis (Pantel *et al.*, 1999). Once the CMM reaches the subcutaneous fat tissue, the risk of metastasis is high and systemic metastases are likely to occur. This process is shown diagrammatically in figure 2.5 below.

For metastases to be formed, metastatic cells must undergo delamination, migration through tissues and adhesion at secondary sites, processes which they undergo while in a relatively undifferentiated state (Ziman and White, 2006).

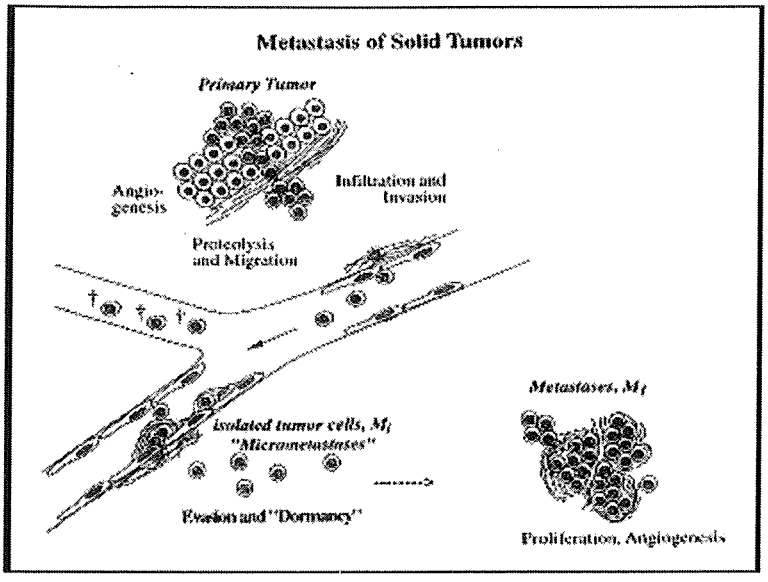


Figure 2.5. Diagram of tumour cell metastasis. Cells from the primary tumour leave the tumour as a result of proteolysis, infiltrate and invade the circulatory system and migrate to a new site where they adhere to the walls of the capillary and invade a new organ. At these sites micrometastases, isolated tumour cells or small clusters of tumour cells, undetectable by conventional tumour detection procedures can survive for several years. At some later stage, the cells become proliferative, stimulate angiogenesis, and begin to form a metastatic tumour (adapted from Pantel et al, 1999).

2.3.2. Causes of CMM

Most melanomas are caused by Ultra-violet (UV) radiation damaging the DNA within the epidermal cells. The UV light causes molecules to become excited which leads to the formation of a covalent link between adjacent pyrimidines on a single strand of DNA. While most faults are repaired by DNA repair enzymes, occasionally this link may lead to a point mutation in DNA. If the affected DNA sequence happens to fall within the regulatory region of a gene controlling cell proliferation or apoptosis, it leads to the uncontrolled production of cells, and a tumour (King, 1996; Pantel *et al.*, 1999).

Only 10% of CMM cases are hereditary (Fountain *et al.*, 1990). Dysplastic Nevus Syndrome (DNS) is an inherited syndrome thought to increase susceptibility to CMM. Affected patients possess 10-100 large nevi or moles which are at higher risk of becoming tumourigenic than normally pigmented skin (Bale *et al.*, 1986; Greene *et al.*, 1985).

Another precursor of CMM is a congenital nevus, present at birth as a brown or black, round or oval plaque. Statistics show that 40% of CMM cases seen in children arise from a giant congenital nevus (Dellon *et al.*, 1976; Quaba and Wallace, 1986).

2.3.3. Classification and prognosis

Clinicians use several methods for staging of CMM, to compare prognosis with disease severity. Breslow's system of classification of CMM was developed in 1975 and is still used as a major prognostic tool. This classification system is based on the vertical depth of the lesion as shown below in table 2.1 (Breslow, 1975).

Table 2.1. Breslow's system of tumour grading/prognosis (Breslow, 1975).

Total thickness of tumour	Five year survival rate
0.00-0.76 mm	98-99%
0.76-1.49 mm	85%
1.50-2.49 mm	84%
4.00 mm	44%

Another important system of classification was developed by Clark et al (1975). In this classification system a tumour is graded according to depth of invasion of atypical cells or inflammatory infiltrates in relation to cutaneous histologic structures. Prognosis is correlated to the anatomical level of local invasion as seen in table 2.2 below.

Table 2.2. Clark's system of tumour grading/prognosis (Clark *et al.*, 1975)

Clark' classification	Anatomic level of local invasion	Five year survival rate
Level I	Lesion involves only epidermis (also called **in-situ** melanoma)	98-100%
Level II	Lesion invades the papillary dermis, but does not reach the papillary-reticular dermis	72-96%
Level III	Invasion fills and expands the papillary dermis, but does not penetrate the reticular dermis	46-90%
Level IV	Lesion invades into the reticular dermis, but not into the subcutaneous tissue	31-67%
Level V	Invasion spreads through the reticular dermis into the subcutaneous tissue	12-48%

More recently the American Joint Committee for Cancer (AJCC) has outlined a classification system which takes into account the micro staging of the primary melanoma as well as clinical and radiological evaluation of regional lymph nodes and pathological evaluation of distant metastases (Balch et al, 2001). Prognosis of a patient is closely related to the clinical stage of melanoma development as seen in table 2.3 below.

Table 2.3. AJCC system of tumour grading/prognosis (Balch *et al.*, 2001).

AJCC classification	Details of classification	Five year survival rate
Stage 0	Tumour in situ AND no regional lymph metastasis, no distant metastasis.	>95%
Stage IA	Tumour ≤ 1.0mm thick & Clark level I or II with no ulceration AND no regional lymph metastasis, no distant metastasis	>95%
Stage IB	Tumour ≤ 1.0mm thick & Clark level IV or V with ulceration OR tumour >1.0mm but ≤ 2.0mm thick with no ulceration AND no regional lymph metastasis, no distant metastasis	89-91%
Stage IIA	Tumour >1.0mm but ≤ 2.0mm thick with ulceration OR tumour > 2.0mm but ≤ 4.0mm thick with no ulceration AND no regional lymph metastasis, no distant metastasis	77-79%
Stage IIB	Tumour > 2.0mm but ≤ 4.0mm thick with ulceration OR tumour > 4.0mm thick with no ulceration AND no regional lymph metastasis, no distant metastasis	63-68%
Stage IIC	Tumour >4.0mm thick with ulceration AND no regional lymph metastasis, no distant metastasis.	44-46%
Stage III	Tumour any thickness with or without ulceration AND metastasis in 1 or more regional lymph nodes, no distant metastasis	25-70%
Stage IV	Tumour any thickness with or without ulceration AND metastasis in 1 or more regional lymph nodes AND distant metastasis	5-10%

These staging systems draw attention to the fact that once a tumour has metastasised, the five-year survival rate of the patient significantly decreases. As a result, studies involving the early identification of melanoma spread are of great importance in this field.

2.4. Melanoma detection

2.4.1. Detection of primary tumours

In order to reduce the incidence of melanoma, national Australian health campaigns have urged citizens to understand the ABCD method of melanoma detection; that is to observe any **A**symmetry, and changes in the **B**order, **C**olour or **D**imension of a skin lesion or mole. This is a clinical guide for the diagnosis of early stage melanoma which is important as the early excision of a thinner lesion leads to a better prognosis for the patient (Bono *et al.*, 1999).

Once a lesion has been identified as possibly being at an early clinical stage melanoma, a biopsy is performed and examined macro- and microscopically for morphological evidence of melanoma (Holder *et al.*, 1998). Immunohistochemistry is performed on the sample to confirm the presence of tumourigenic melanoma cells.

Primary antibodies used in immunohistochemistry assays include antibodies to melanoma specific markers such as MART-1/Melan-A (A103, M2-7C10, M2-9E3 antibodies) and gp100 (gp100-recognizing antibodies are HMB45 and NK1-beteb), and occasionally Tyrosinase (T311 antibody) or MITF (C5+D5 antibodies) (Steingrimsson *et al.*, 2004).

2.4.2. Detection of circulating melanoma cells

Once CMM cells become invasive, they have the ability to infiltrate surrounding lymph and blood vessels. At early stages of metastasis, malignant melanoma cells are found in the sentinel lymph nodes, a factor which significantly decreases five-year survival rates for patients (Balch *et al.*, 2001).

Interestingly, malignant melanoma cells can be detected in patient sera at all stages of the disease, but the level of spread may be dependent on both the quantity of circulating cells, their ability to evade the immune system, and their

ability to invade tissue (Ulmer *et al.*, 2004). Techniques have been developed that can quantify the number of tumour cells present in patient peripheral blood and it is thought that this may be used as an indicator of disease spread (Hoon *et al.*, 2000; Palmieri *et al.*, 1999). However this remains to be fully elucidated as the tendency of these cells to become metastatic remains largely unexplored. The possibility that particular cells have a greater ability to metastasise than others is a crucial issue that remains to be resolved by a sample blood test.

2.4.3. Marker detection techniques

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is a procedure which creates cDNA from mRNA (in this case isolated from cells in peripheral blood) followed by amplification of a sequence within a specific gene of interest. Using RT-PCR to target genes specific to melanoma cells, detection of the cells in peripheral blood is possible. RT-PCR has been used in laboratory research to detect melanoma cells in this way since 1991 (Smith *et al.*, 1991). The sensitivity of this technique has been well demonstrated; one tumour cell can be detected amongst 10^6-10^7 normal cells, but quality control for diagnostic use remains a problem due to the potential for both false positive and false negative results (Ghossein and Bhattacharya, 2000).

Since melanomas are highly heterogeneous with respect to tumour-related gene expression, multi-marker RT-PCR has been developed (Medic *et al.*, 2007). This procedure uses the simultaneous detection of several markers to allow for the expression of a variety of mRNA transcripts in CMM cells (Hoon *et al.*, 2000; Reynolds *et al.*, 2003). Multi-marker RT-PCR potentially overcomes the problem of false negative results as a variety of markers can be chosen that reflect different biochemical and pathological events characteristic of melanoma cells in general (Hoon *et al.*, 1995). As a result, identification of melanoma cells is more frequent in multi-marker analysis than in individual marker trials (Reynolds *et al.*, 2003). When combined with RT-PCR, multi-marker analysis

can be used to compare marker expression between patients possibly providing an estimate of tumour progression.

Circulating melanoma cells can be detected in patient sera at all stages of the disease, but the ability of these cells to metastasise may depend on the number of circulating cells (Ulmer *et al.*, 2004). Recent studies utilising multi-marker RT-PCR indicates that a higher number of markers are detected in peripheral blood of patients with more advanced stages of CMM. In addition, low risk of disease recurrence is associated with an absence of detectable markers in the peripheral blood of CMM patients who have undergone treatment (Hoon *et al.*, 2000; Palmieri *et al.*, 1999; Wascher *et al.*, 2003). Alternately, markers may be identified in the future, that are characteristic of a more metastatic cell.

2.5. Genetic markers of melanoma formation

Survival and differentiation of normal melanocytic cells is tightly regulated by several signaling pathways. In melanoma however, aberrant increase or decrease in expression of any of the genes in these pathways may lead to tumour progression (Wagner and Fisher, 2005). Also, the invasion and spread of melanoma cells is associated with changes in cell adhesion as well as the increased ability of cells to proliferate (Miller and Mihm Jr, 2006).

Markers of great interest in the detection of circulating melanoma cells in peripheral blood include genes involved in melanocyte differentiation and adhesion. Similarly, genes controlling cell proliferation and survival are required for the growth of a tumour to the invasive stage, making these genes possible markers of melanoma cell once they have entered the blood. Markers trialled in this study included those involved in melanocyte biology, development and survival such as *PAX3d, SFRP5, MART1* and *MITF* and those regulating cell proliferation and invasive potential like *TGFβ2*.

2.5.1. *MITF* expression in melanoma

MITF is a master regulator of melanocyte differentiation, proliferation and survival. As a result, abnormal expression of this transcription factor may result in the uncontrolled replication of immortal melanoma cells, possibly leading to tumour progression (Wagner and Fisher, 2005).

MITF contributes to melanocyte survival by increasing the expression of the anti-apoptotic gene *BCL2*. It does this by occupying the M-Box sequence located in the *BCL2* promoter region (Figure 2.6) (McGill *et al.*, 2002). In melanoma cell lines, a decrease in BCL2 protein causes cell death, indicating that the survival of melanoma cells is dependent on BCL2 (Banerjee, 2002) and consequently on MITF.

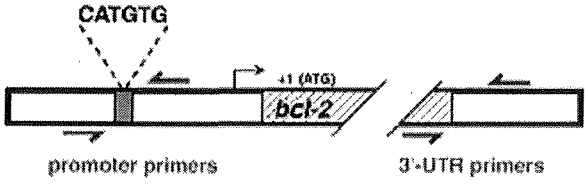


Figure 2.6. MITF M-Box sequence in the promoter region of *BCL2* (Adapted from McGill *et al*, 2002).

MITF also acts as a pro-proliferation factor by up-regulating cyclin-dependent kinase 2 (CDK2) and t-box transcription factor 2 (TBX2) in melanoma. CDK2 is required for cell proliferation, and is specific to melanoma (Du *et al.*, 2004). TBX2 is required to maintain proliferation and suppress senescence via down-regulation of p21 in melanoma (Vance *et al.*, 2005). Thus MITF may be an important marker of melanoma progression.

2.5.2. *PAX3* expression in melanoma

For melanoma cells to metastasise, they need to migrate and adhere to distant sites to form micrometastases. PAX genes have been observed to affect cell surface molecules and thus have a role in the regulation of cell motility and adhesion (Blake and Ziman, 2005; Scholl *et al.*, 2001; Ziman and White, 2006).

During development PAX3 regulates proliferation and migration of melanoblasts, while maintaining the cells in an undifferentiated state. Once the cells reach their destination in the dermis, the cells differentiate and PAX3 is down-regulated, and remains down-regulated in the adult (Lang *et al.*, 2005). In melanoma cells however, *PAX3* expression is de-regulated possibly causing the de-differentiation of melanocytes while promoting cell proliferation and migration (Lang *et al.*, 2005; Scholl *et al.*, 2001). This function of PAX3 may be implicated in melanoma metastasis (Blake and Ziman, 2003).

PAX3 is highly expressed in melanoma; but not in normal tissue or normal skin aside from skin stem cells (Scholl *et al.*, 2001). During development, *PAX3-c* and *PAX3-d* transcripts control melanocyte differentiation and migration (Blake and Ziman, 2005). Interestingly, CMM cells predominantly express the *PAX3d* transcript indicating that it may be *PAX3d* aberrant expression that is important in melanomas (Barr *et al.*, 1999; Medic, 2006). Thus PAX3d would make a good marker of melanoma cells in peripheral blood.

2.5.3. *MART1* expression in melanoma

Melan-A/MART1 (hereafter referred to as MART1) is an antigen produced by the *MLANA/MART1* gene (hereafter referred to as *MART1*). It is involved in melanosome ontogeny; however its precise function is not known (Medic *et al.*, 2007). *MART1* is transcriptionally regulated by MITF, with MITF consensus recognition sites found in the promoter region of the gene as shown previously in Figure 2.1b (Du *et al.*, 2003).

MART1 is frequently expressed in melanoma tumour cells and melanoma cell lines, but is not expressed in non-melanoma malignancies or in the blood of humans without cancer (Wascher *et al.*, 2003). Several studies have investigated the relationship between the presence of MART1 in patient sera and the stage of melanoma progression. The frequency of detection was found to increase significantly with advancing stage of melanoma (Keilholz *et al.*, 2004).

2.5.4. *SFRP* expression in melanoma

The ability of SFRP to facilitate or impede WNT signaling allows it to control important signaling pathways. At high levels, SFRP acts as an antagonist to WNT/Frizzled interaction, blocking production of β-catenin; at low levels, it up-regulates β-catenin (Polakis, 2000). As well as binding with LEF1 to allow the transcription of *MITF,* β-catenin also plays a crucial role in the regulation of the growth of melanoma cells, with its down-regulation associated with increased tumour thickness and disease progression (Kageshita *et al.*, 2001). Thus, by regulating β-catenin, SFRP functions to regulate melanoma progression making it an important marker in the detection of melanoma cells in peripheral blood and possibly a key indicator of disease progression.

Low levels of expression of SFRP have been observed in breast, kidney and ovarian cancers, revealing a relationship between these proteins and cancer. The most likely SFRP family member to have an effect on the metastasis of malignant melanoma is SFRP5 which is expressed in epithelial cells, such as retinal pigment epithelium (Jones and Jomary, 2002).

2.5.5. *TGFβ* expression in melanoma

Pathways that activate cell proliferation and cell cycling are important features of both development and tumourgenesis. During development, Transforming Growth Factor Beta (TGF-β) is involved in the process of neural tube closure due to its control of cell proliferation (Sanford *et al.*, 1997).

The TGF-β signaling pathway is shown below in Figure 2.7. This pathway implicates TGF-β as a regulator of many genes and transcription factors responsible for cell apoptosis and neogenesis (Mulder, 2000). In recent *in vivo* studies, *TGFβ2* was expressed in malignant melanoma cells but not found to be associated with normal melanocytes; it was therefore determine to be a critical factor in the development of deep invasion and metastasis in malignant

melanoma (Reed *et al.*, 1994). These findings indicate its potential as a valuable marker for use in the detection of melanoma cells in the blood.

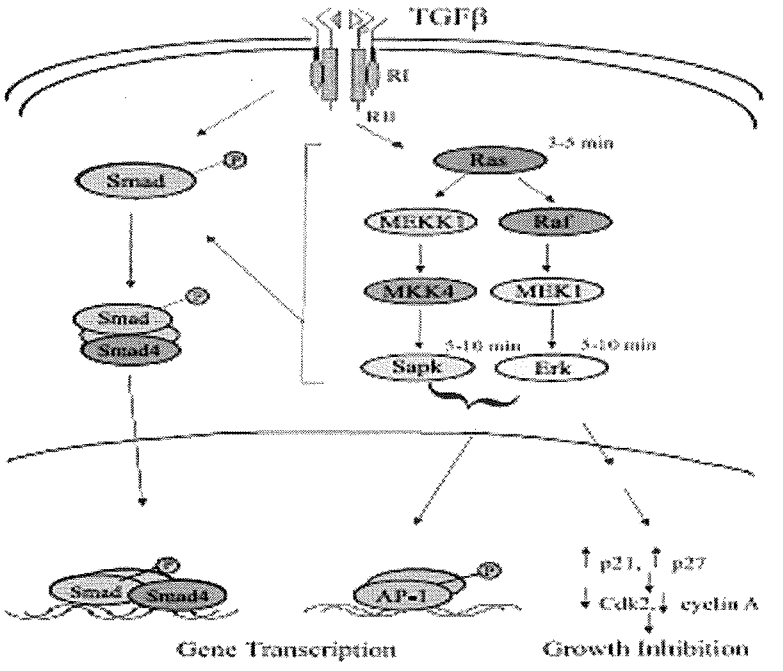


Figure 2.7. TGF-β pathway controlling gene transcription and growth inhibition. TGF-β attaches to a cell receptor and activates Ras and Smad proteins. These proteins then follow a signaling pathway leading to the activation of *Smad4* and *Erk*, genes responsible for gene transcription, growth inhibition and apoptosis (Adapted from Mulder, 2000).

3. SUMMARY AND THEORETICAL FRAMEWORK

The metastasis of CMM is responsible for a dramatic increase in mortality of patients with the disease. Consequently, studies focussing on the early detection of metastasis are essential. Since the advent of the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), many studies have analysed the use of molecular markers for the detection of melanoma metastasis (Lewis *et al.*, 2005; Smith *et al.*, 1991).

Several molecular markers have been identified as reliable indicators of melanoma progression (Medic *et al.*, 2007). These markers have been studied individually and in groups using multi-marker RT-PCR. However the ideal combination that accurately predicts disease outcome has yet to be identified. Accordingly, further investigation to find an optimal array of markers is warranted.

In this study, several markers have been trialled with RT-PCR. Multi-marker RT-PCR allows the comparison of the expression of these markers in peripheral blood from patients with CMM of different primary tumour depths. Results were compared with peripheral blood of normal healthy volunteers to determine which of these markers would be suitable for use in a predictive and/or prognostic blood test of Cutaneous Malignant Melanoma.

4. HYPOTHESIS AND AIMS

4.1. Hypothesis

PAX3d, MITF, SFRP5, MART1 and TGF-β2 are involved in formation and development of CMM and can be used as markers in the diagnosis and prognosis of CMM.

4.2. Aim

OVERALL AIM:

To develop an assay that can be used for CMM diagnosis and prognosis.

AIM:

To detect *PAX3d, MITF, SFRP5, MART1* and *TGF-β2* expression in peripheral blood of patients diagnosed with malignant melanoma and to compare this expression with that found in blood from healthy volunteers.

5. MATERIALS AND METHODS

5.1. Experimental Design and Procedures

5.1.1. Collection of human tissue and blood samples

All procedures performed as part of this study were ratified by the Faculty of Computing, Health and Science Human Research Ethics Committee (Appendix A) and conformed to NHMRC guidelines as stated on the website (http://www.nhmrc.gov.au/funding/policy/researchprac.htm). All tissues and blood samples were obtained from patients identified by clinicians, and from whom informed consent was obtained. Blood samples were also obtained from healthy volunteers who were classified as melanoma free by clinicians or were undiagnosed but regarded as disease free based on self assessment. Healthy volunteers also signed consent forms allowing the use of their blood in the study (Information sheet and consent form are Appendix B and C, respectively).

Lymph node tissues were obtained from melanoma patients by surgical removal for diagnostic and treatment purposes. Only tissues that were extraneous to the diagnostic procedure were used. Tissue was immediately cryopreserved in liquid nitrogen following excision and stored at -80°C prior to use.

Blood samples from patients or healthy volunteers were obtained by venous puncture and blood was collected in an EDTA tube (to eliminate melanocyte contamination from the site of injection), and then in a specially formulated RNA blood collection tube was (5-10ml). Each sample was subjected to RNA isolation within a week of being collected, and the RNA was stored at -80°C until used. For each patient blood sample, the depth of tumour and the time since surgical removal of the primary tumour, as indicated by the pathological reports, was recorded in an excel spreadsheet coded by number so that no private details were recorded.

5.1.2. Total RNA isolation from freshly isolated cryogenically preserved tissue

Total RNA was extracted from frozen tissue samples using Aurum Total RNA Mini Kit (Bio-Rad, 732-6820) according to the manufacturer's instructions.

Approximately 20-30mg of tissue was cut from the frozen lymph node sample and crushed under liquid nitrogen using a mortar and pestle. The tissue powder was then placed into a sterile RNase-free eppendorf tube and allowed to thaw before 700µl of Lysis Solution was added. The lysate was mixed well, centrifuged for three minutes at 14,000rpm and the resulting supernatant was transferred into a fresh eppendorf tube.

Following that, 700µl of 60% ethanol was added to the tissue lysate, mixed well and 700µl of the whole lysate was transferred to the RNA extraction column and centrifuged for 60 seconds at 14,000rpm, after which the filtrate was discarded. This procedure was repeated for the remainder of the lysate. Low Stringency Solution (700µl) was added to the column, centrifuged for 30 seconds at 14,000rpm and the filtrate was discarded. DNase Mix (80µl; 5µl DNase I and 75µl DNase Dilution Solution) was added to the membrane of the column and incubated for one hour at room temperature and then centrifuged for 30 seconds at 14,000rpm. The sample was washed with 700µl of High Stringency Solution, centrifuged for 30 seconds at 14,000rpm. A second wash of 700µl of Low Stringency Solution was added and centrifuged for 30 seconds. Additional centrifugation for one minute was performed to eliminate the remaining ethanol. The column was transferred into a fresh eppendorf tube and 25µl of Elution Solution, previously warmed to 70^0C, was added to the membrane and allowed to saturate for one minute after which time it was centrifuged for 2 minutes at 14,000rpm. The eluted total RNA was aliquoted and stored at -80^0C until used for RT-PCR.

5.1.3. RNA extraction from peripheral-blood samples

Blood was collected into PAXgene blood collection tubes (PreAnalytiX, 762165) and the RNA was extracted using a PAXgene Blood RNA Kit (Qiagen, 762132) according to the manufacturer's instructions described below. Additional DNase treatment with DNA-*free* (Ambion, AM-1906) was required after RNA isolation as genomic DNA was still present in the majority of the samples.

Blood samples collected in PAXgene tubes were incubated at room temperature for at least 2 hours to allow optimal lysis of cells. The blood was then centrifuged at 2,800rpm for 10 minutes and the supernatant was decanted. RNase-free water (4ml) was added to the tube and the pellet was resuspended by thorough mixing, then the tube was re-centrifuged at 2,800rpm for 10 minutes. The supernatant was then discarded and 350µl of Resuspension Buffer BR1 was added to the pellet. The sample was vortexed until the pellet dissolved, then transferred into a fresh microcentrifuge tube. Binding Buffer BR2 (300µl containing guanidine thiocyanate) and Proteinase K (40µl) were added to the lysate, mixed and incubated for 10 minutes in a shaker-incubator at 55°C. Following incubation, the lysate was transferred into a shredder spin column and centrifuged at 14,000rpm for 3 minutes. The supernatant was transferred into a fresh tube and 350µl of 100% ethanol was added and mixed. The lysate was transferred into a spin column and centrifuged for 1 minute at 14,000rpm then transferred to a fresh processing tube and the flowthrough was discarded. Wash Buffer BR3 (350µl containing guanidine thiocyanate and ethanol) was added to the column, centrifuged at 14,000rpm for 1 minute and the flowthrough was discarded. DNase I (10µl, RNase-free DNase Set, Qiagen, 79254) and 70µl Buffer RDD (RNase-free DNase Set, Qiagen, 79254) were gently mixed and added to the column membrane. The column was then incubated for 15 minutes at room temperature and Buffer BR3 (350µl) was again added to the column and centrifuged for 1 minute at 14,000rpm, after which the flowthrough was discarded. The column was transferred into a fresh 2ml processing tube and 2x 500µl of Buffer wash BR4 containing 100% ethanol were added, then the tube was centrifuged for 1 minute once and a second time for 3 minutes at 14,000rpm. The flowthrough was discarded and the column centrifuged for 1

minute at 14,000rpm to ensure complete removal of ethanol. The column was then placed into a fresh 1.5ml eppendorf tube and 40µl of elution Buffer BR5 was added directly to the column membrane and the column was centrifuged at 14,000rpm for 1 minute. The eluted RNA was incubated at 65°C for 5 minutes and immediately chilled on ice.

RNA from the blood samples was then retreated with DNase I (DNA*free*, Ambion) to completely eliminate genomic DNA, as follows: RNA samples at concentrations less than 200ng/µl were treated with 1µl of rDNase I and those at higher concentrations, with 2-3µl of rDNase I. Reaction mixture containing rDNase I and 0.1 volume of 10x DNase I Buffer and water to a total volume of 30-50µl, was incubated for 30 minutes at 37^0C. 1x volume of resuspended DNase Inactivation Reagent was added and the reaction mixture incubated for 2 minutes at room temperature with occasional mixing. Samples were then centrifuged for 1.5 minutes at 14,000rpm. The supernatant, containing RNA, was aliquoted into RNase-free tubes and stored at -80°C until used in RT-PCR reactions.

5.1.4. Assessment of RNA quality and quantity

The integrity of RNA was assessed on a 1% agarose gel by electrophoresis at 100mV for 30 minutes in TAE Buffer. The gel was stained with ethidium bromide and visualised under UV light and results were recorded using the GelDoc system (Bio-Rad). RNA samples of poor quality, defined by a lack of two clear ribosomal RNA bands, were excluded from further analysis. RNA concentration was determined by UV spectrophotometry and optical density recorded at 260nm and 280nm so that a ratio of 260:280 was obtained. Samples with an optical density ratio of 260:280 <1.8 were discounted. RNA concentration was calculated using the following formula:

$$\text{Concentration} = Abs_{260} \times 40\text{ng/µl} \times \text{Dilution Factor}$$

5.1.5. RT-PCR analysis of gene expression

Two-step Reverse Transcriptase PCR (RT-PCR) was used to assess the expression of *PAX3d, MITF, MART1, SFRP5* and *TGFβ2* in blood and tissue samples obtained from CMM patients and healthy volunteers. The total numbers of samples used are detailed in table 5.1 below.

Table 5.1. Blood and tissue samples used for RT-PCR analysis

Sample type	**Number**
Healthy volunteer peripheral blood	33
CMM patient lymph node metastases	4
CMM patient peripheral blood	68

Healthy volunteer peripheral blood was used to critique all markers. The presence of a marker in healthy volunteer blood could indicate a false positive result, or a non-informative marker. With this information, the rate of false positives or the number of informative markers can be estimated from the general population and thus compared with results from CMM patients.

CMM patient lymph nodes were used as positive controls as these tissue samples are known to contain melanoma cells. For each set of PCR reactions, cDNA obtained from any one of these lymph nodes was used as a positive reaction control. Negative control samples containing no template cDNA were used in each set of reactions to ensure there was no contamination of stock solutions.

CMM patient peripheral blood was analysed to determine marker presence in the blood and to assess the relationship between marker expression, depth of tumour (disease progression) and time since removal of the primary tumour.

5.1.6. Two-step RT-PCR

Two-step Reverse Transcriptase PCR consists of two phases: total cDNA synthesis from total RNA followed by PCR amplification of specific genes. This method of RT-PCR was preferred over one-step RT-PCR, which completes both phases in a single reaction, as the two-step method allows each sample to be tested for multiple genes, including *GAPDH* (as a measure of RNA integrity). Thus each sample was first reverse transcribed and then a PCR reaction was performed to assess *GAPDH* expression.

5.1.6.1. Step one: Reverse transcription (RT)

cDNA synthesis was performed using Omniscript Reverse Transcription kit (Qiagen, 205111), according to the manufacturers' instructions.

Total RNA (250ng) was reverse transcribed in a 20µl reaction mixture containing: 1x Buffer RT, 0.5mM each dNTP (Fisher Biotec, DN-25-100), 1µM Oligo-dT primer (Qiagen, 79237), 10 units of RNase OUT Recombinant Ribonuclease Inhibitor (Invitrogen, 10777-019), 4 units of Reverse Transcriptase and RNase-free water. The reaction was then incubated for 1 hour at 37^0C, followed by 5 minutes incubation at 95^0C followed by cooling at 4^0C. Following this procedure, samples were either used immediately in a PCR reaction, or stored at -20^0C until used.

5.1.6.2. cDNA analysis using *GAPDH* as a housekeeping gene

To ensure that the RT process had been completed successfully, PCR amplification of *GAPDH* was completed for each sample using the reaction mixture described below (section *5.1.6.3.*), and the following cycling conditions: an initial PCR activation for 3 minutes at 94°C, followed by 3-step cycling process: Denaturation at 94°C for 45 seconds; annealing at 50 °C for 45 seconds; extension at 72°C for 45 seconds repeated 40 times, before a final

extension at 72°C for 10 minutes. The reaction used Taq DNA Polymerase (Qiagen, 201205) and the following primers:
Fwd: 5' GGGTGTGAACCATGAGAA 3'
Rev: 5' GACTGTGGTCATGAGTCCT 3' (Takeuchi *et al.*, 2003)

The *GAPDH* forward primer is located in exon 6 at position 474-493 of human *GAPDH* mRNA, and the reverse primer is at position 607-591 spanning exons 7 and 8 (GenBank accession number NM_002046). These primers amplify a PCR product of 134bp.

PCR reaction products were assessed by agarose gel electrophoresis, and only following identification of the PCR product was the sample accepted for further analysis involving PCR amplification of selected genes.

5.1.6.3. Step two: PCR amplification to detect presence of specific markers

PCR was performed using Taq DNA Polymerase (Qiagen, 201205) and gene specific primers. Primers for gene amplification were designed and analysed using PrimerSelect software (DNASTAR, Lasergene). All primers were subjected to BLAST analysis (NCBI) to ensure they did not bind elsewhere in the human genome.

Primers:

PAX3d (fwd)	5'-AGACTGATTACGCGCTCTCC-3'
PAX3d (rev)	5'-GGCTGCGAAGACCAGAAAC-3'
MITF (fwd)	5'-GCTTAACTCCAACTGTGAAAAAGAG-3'
MITF (rev)	5'-GGGAAAAATACACGCTGTGAGC-3'
MART1 (fwd)	5'-ATGCCAAGAGAAGATGCT-3'
MART1 (rev)	5'-TCAAGGCTCTGTATCCAT-3'
SFRP5 (fwd)	5'-CAGTGTGAGATGGAGCACAG-3'
SFRP5 (rev)	5'-CTCCTTATTCTTCTTGTCCCAGC-3'
TGF-β2 (fwd)	5'-AAAACAAGAGCAGAAGGCGAATG-3'
TGF-β2 (rev)	5'-ATGTGGAGGTGCCATCAATACC-3'

The PCR reaction was performed in a total volume of 20µl containing: 1x PCR Buffer (or CoralLoad PCR Buffer), 1x Q Solution, 0.2mM of each dNTP, 2.5 units of Taq, 0.4µM of both the forward and reverse primers, PCR grade water and 2µl of template cDNA (obtained from 250ng of Total RNA).

The following cycling conditions were used: an initial PCR activation for 3 minutes at 94°C, followed by 3-step cycling process: Denaturation at 94°C for 30 seconds; annealing at 56°C of *PAX3d*, 57°C for *MITF*, 46°C for *MART1*, 60°C for *SFRP5* and 64°C for *TGF-β2* for 30 seconds; extension at 72°C for 30 seconds repeated 45 times, before a final extension at 72°C for 10 minutes.

Although there is a very small risk of genomic DNA contamination of samples due to rigorous DNase treatments, the risk should not be ignored. Accordingly, the forward and reverse primers of each marker were located on different exons in order to allow the distinction between PCR amplicons from cDNA and genomic DNA due to the length of the PCR product. cDNA contains only exons; which are included in the mRNA sequence, whereas genomic DNA would contain introns between these exons and thus the resulting PCR product would larger.

The forward primer for *PAX3d* is located in exon 8, position 1586-1605bp and the reverse primer is in exon 9, position 1873-1855bp, of human *PAX3* mRNA, (GenBank accession number NM_181458.1), and are designed to give a PCR product of 288bp. Exon 8 is contained in *PAX3c*, *PAX3d* and *PAX3e* transcripts, however the reverse primer in exon 9 recognises *PAX3d* and *PAX3g* transcripts only, thus together these primers amplify *PAX3d* exclusively.

The *MITF* forward primer is located in exon 7, position 358-382bp and the reverse primer at position 694-673bp, spaning exons 11 and 12 of human *MITF* mRNA, (GenBank accession number NM_000248.2). These primers are designed to amplify a PCR product of 336bp. Although exon 7 is contained in *MITF* transcript variants 1, 2, 3, 4, 5 and 6, the reverse primer spanning exons 11 and 12 recognises the *MITF* transcript variant 4 exclusively, which is also known as *MITF-M*.

The *MART1* forward primer is located in exon 2, position 54-71bp and the reverse primer is in exon 3, position 225-208bp of human *MLANA* mRNA, (GenBank accession number NM_005511.1), and these primers are designed to give a PCR product of 171bp.

The forward primer for *SFRP5* is located in exon 1, at position 728-747bp and the reverse primer is in exon 3, position 1051-1029bp of human *SFRP5* mRNA, (GenBank accession number NM_003015.2), and these primers are designed to give a PCR product of 323bp.

The forward primer for *TGFβ2* is located in exon 3, at position 752-774bp and the reverse primer is in exon 5, position 956-935bp of human *TGFβ2* mRNA, (GenBank accession number NM_003238.1), and these primers are designed to give a PCR product of 204bp.

PCR products were analysed by 2% agarose-gel electrophoresis and ethidium bromide staining. Gels were run at 100 mV for 30 minutes in TAE buffer and PCR-product size was compared to pUC19 DNA ladder. PCR products were visualised and recorded using the Gel-Doc system (Bio-Rad).

5.1.7. Sequencing of RT-PCR products

Sequencing reactions were performed to confirm the identity of all RT-PCR products, checking that the PCR reactions amplified *PAX3d, MITF, MART1, SFRP5* and *TGFβ2* respectively.

Prior to sequencing, cDNA from the PCR reaction was cleaned using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Briefly, 5 volumes of Buffer PB was added to 1 volume of PCR sample and the mixture was placed into the column and centrifuged for 30-60 seconds at 13,000rpm. Buffer PE (750µl) was added to the column and centrifuged for 30-60 seconds at 13,000rpm. The column was placed into a fresh tube and DNA

was eluted with 30µl ddH_2O by centrifugation for 1 minute at 13,000rpm. This purification method was used to remove all PCR reagents and DNA fragments under 70bp in length, including primers.

The BigDye Terminator v3.1 Cycle Sequencing Kit (AB Applied Biosystem) was used to sequence the amplicons. Sequencing reactions contained: 5ng of PCR product (approx. 300bp in length), 3.2 pmol of either the forward or the reverse primer, 2.5x Ready Reaction Premix and 5x BigDye Sequencing Buffer, diluted in ddH_2O to a total reaction volume of 15µl. Sequencing cycling conditions were: 96°C for 3 minutes, then 30 cycles of 96°C for 10 seconds, rapid thermal ramp 10°C per second to 50°C, 50°C for 5 seconds and 60°C for 4 minutes.

For the removal of excess dye terminators prior to sequencing, ethanol/sodium acetate precipitation of DNA was performed by addition of 50µl of precipitation mix (containing 100µl of 96% ethanol, 2µl 3M Sodium-Acetate (pH 4.6), 4µl 0.1mM EDTA) and 5µl H_2O to each completed sequencing reaction. After mixing and centrifugation for 20-25 minutes at 4,000rpm at 4°C, supernatant was discarded. Chilled 70% ethanol (100µl) was added to each pellet prior to centrifugation at 4,000rpm at 4°C for 4 minutes. Supernatant was discarded and the resultant pellet was vacuum dried. Lyophilized DNA was wrapped in foil and kept at -20°C until analysed by the DNA sequencing service at Royal Perth Hospital (Perth) using an ABI Prism 3730 48 capillary sequencer. Resultant sequences were aligned with known sequences in GenBank using the multialign tool in the Angis computer program, available in GenBank.

5.2. Statistical analyses

All results were analysed using the Statistical Packages for the Social Science (SPSS) and its statistical analysis functions.

5.2.1. Presence of individual markers

The efficacy of each marker was assessed separately by comparing the expression of each marker in CMM patients relative to its expression in healthy controls, with results statistically analysed using Chi-square or Fisher's Exact Tests.

Following that, patients were allocated into groups dependent on the depth in millimetres of their primary tumour. Individual marker expression was assessed in relation to patient tumour depth, with results again statistically analysed using Chi-square or Fisher's Exact Tests.

5.2.2. Presence of marker expression

In order to assess the multi-marker assay as a whole, the expression of one or more markers was assessed in the CMM patients relative to healthy controls. This analysis allows the rate of positive identification of melanoma cells to be calculated in each group, assuming that the presence of one marker would indicate a positive result. Results were statistically analysed using Chi-square or Fisher's Exact Tests.

5.2.3. Number of markers expressed

Further statistical analysis of the multi-marker assay was performed to assess the number of markers expressed in the healthy control group compared with that of the CMM patient group. This analysis may lead to an insight into the number of markers necessary to conclude a true positive result (compared with a false positive in a healthy person). These results were statistically analysed using Chi-square or Fisher's Exact Tests.

Additionally, the number of markers expressed in relation to CMM patient tumour depth was examined to determine whether primary tumour depth

influences marker expression. Results were statistically analysed using Chi-square or Fisher's Exact Tests.

5.2.4. Marker expression relative to time since diagnosis

Individual marker expression and the number of markers expressed were analysed in relation to the time since diagnosis of CMM patients of in situ, ≤2.00mm and >2.00mm tumour depths. This analysis helps to determine whether markers would still present in the peripheral blood of a patient more than a year after diagnosis. Results were statistically analysed using Chi-square or Fisher's Exact Tests.

6. RESULTS

6.1. Introduction

This study used two-step Reverse Transcriptase PCR (RT-PCR) to identify the expression of *PAX3d, MITF, MART1, SFRP5* and *TGFβ2* genes in the peripheral blood of healthy volunteers, and CMM patients with differing primary tumour depths. Melanoma tissue was used as a positive control. Positive expression is then assessed for association with CMM and regarded as arising from migrating melanoma cells.

6.2. Samples

154 patients from the private practice of Mr Robert Pearce at Hollywood Specialist Centre volunteered to donate one or more blood samples for use in this study. Of these, 150 were diagnosed with Cutaneous Malignant Melanoma, and 4 were non-melanoma patients. Overall, a total of 271 patient blood samples have been obtained. In instances where there were several blood samples available from a single patient, only one was used in this study. Five lymph nodes containing metastatic tumours were obtained from patients with metastatic melanoma, of which 4 were utilised in this study. A total of 33 healthy volunteer blood samples, for use as negative controls, were analysed in this study.

6.2.1. CMM patient cohort

68 blood samples from patients with CMM were analysed in this study. This cohort consisted of 45 (66%) males and 23 (34%) females, ranging in age from 31 to 93 years; the median age was 77 years. The time between initial diagnosis and blood sample collection ranged from zero (collected at the time of diagnosis) to 4 years and 10 months.

Of the 68 patients, 20 (30%) were diagnosed with *in-situ* melanoma and of these, 11 (55%) were diagnosed within one year prior to blood collection and 9 (45%) were diagnosed more than one year prior to blood collection. Twenty-four of the 68 patients (35%) had a primary tumour depth of ≤2.00mm, of which 16 (67%) were diagnosed within one year prior to blood collection and 8 (33%) were diagnosed more than one year prior to blood collection. The remaining 24 (35%) of the 68 test patients had a primary tumour depth of >2.00mm and of these, 15 (62%) were diagnosed within one year prior to blood collection and 9 (38%) were diagnosed more than one year prior to blood collection. The structure of the CMM patient cohort is analysed below in Table 6.1.

For each *in situ* patient, the pathology report specified that their primary tumour was either *in situ* or Clarks level 1. Importantly, all *in situ* patients had a tumour depth of 0.00mm and for the purposes of this study they were analysed separately. The ≤2.00mm group refers to patients with a primary tumour depth of 0.01mm-2.00mm. In this study, the >2.00mm group refers to patients with a tumour depth of 2.01-16.00mm.

Table 6.1. Analysis of CMM patient cohort.

		Time since diagnosis	
Depth of primary tumour	Total number of patients	≤1 year. Number of patients	>1 year. Number of patients
In-situ	20	11	9
≤2.00mm	24	16	8
>2.00mm	24	15	9

6.3. Quality assurance of cDNA product

RNA was isolated from blood and tissue; its quality was assessed by agarose gel and its amount by optical density. Moreover, for use in the study, RNA was required to be of sufficient quality to successfully amplify the housekeeping gene *GAPDH* in an RT-PCR reaction. A PCR product, with an expected size of 134bp indicating *GAPDH* expression, was obtained for all samples utilised. Any samples unable to amplify *GAPDH* were not used in the study; since the same

amount of cDNA was utilised in each reaction, the amount of PCR product for GAPDH expression was the same. Figure 6.1 shows an example of the agarose gel analysis of several samples amplified to assess *GAPDH* expression. As a result of the positive identification of *GAPDH*, all RNA and subsequent cDNA was suitable for marker expression analysis by RT-PCR.

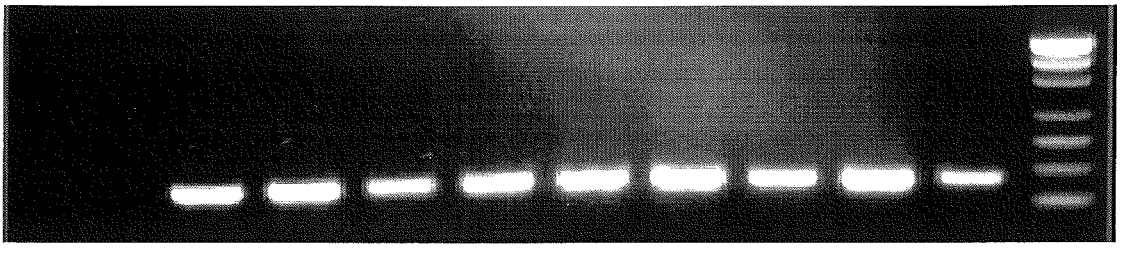


Figure 6.1. 2% agarose gel showing PCR products amplified for analysis of *GAPDH* expression in CMM patient peripheral blood samples. p=CMM patient sample number, pUC19=pUC19 DNA ladder. - denotes negative control with no cDNA, + denotes positive control.

6.4. Gel analysis of RT-PCR products

Following the analysis of RNA quality, samples showing *GAPDH* expression were then analysed by RT-PCR for the presence of each of the genetic markers: *PAX3d, MITF, MART1, SFRP5* and *TGFβ2.* The agarose gel displaying the PCR products from some healthy volunteer samples analysed for *MART1* is shown in Figure 6.2 and the agarose gel displaying the PCR products from some patient blood samples analysed for *PAX3d* is shown in Figure 6.3 below. Since only presence or absence was recorded in this project, the level of expression of individual genes was not assessed relative to GAPDH.

Figure 6.2. 2% agarose gel showing PCR products amplified to analyse *MART1* expression in a representative set of control samples. h=healthy control samples, pUC19=pUC19 DNA ladder. - denotes negative control in which there was no cDNA, + denotes positive control (lymph node cDNA).

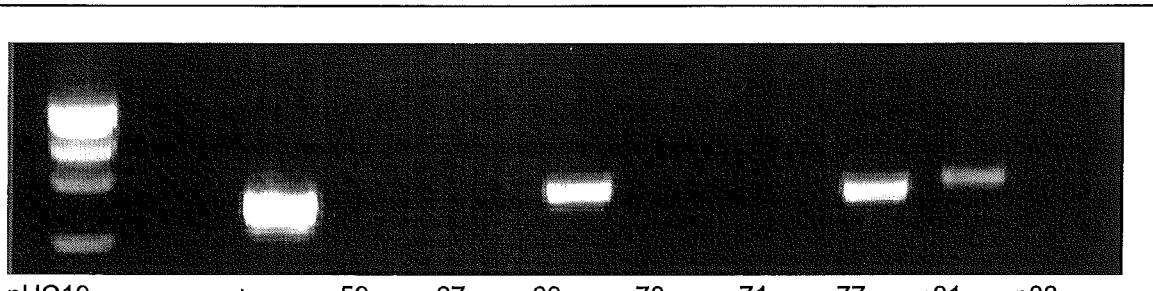


Figure 6.3. 2% agarose gel showing PCR products amplified for the analysis of *PAX3d* expression in a representative set of patient samples. p=CMM patient sample number, pUC19=pUC19 DNA ladder. - denotes negative control (no cDNA), + denotes positive control (lymph node cDNA).

6.5. Marker sequencing reactions

RT-PCR products from each marker were sequenced to confirm that the product was in fact the mRNA product produced from gene of interest. Sequenced PCR products were tested for *GAPDH, PAX3, MITF, MART1, SFRP5* and *TGFβ2* and results were aligned with the corresponding NCBI mRNA sequences for each gene (detailed in section 5.1.6. above).

The results were positive for each marker; that is, all PCR products aligned with their expected mRNA sequence as shown below in Figure 6.4. The number of base pairs aligned varies due to the different product size of each marker.

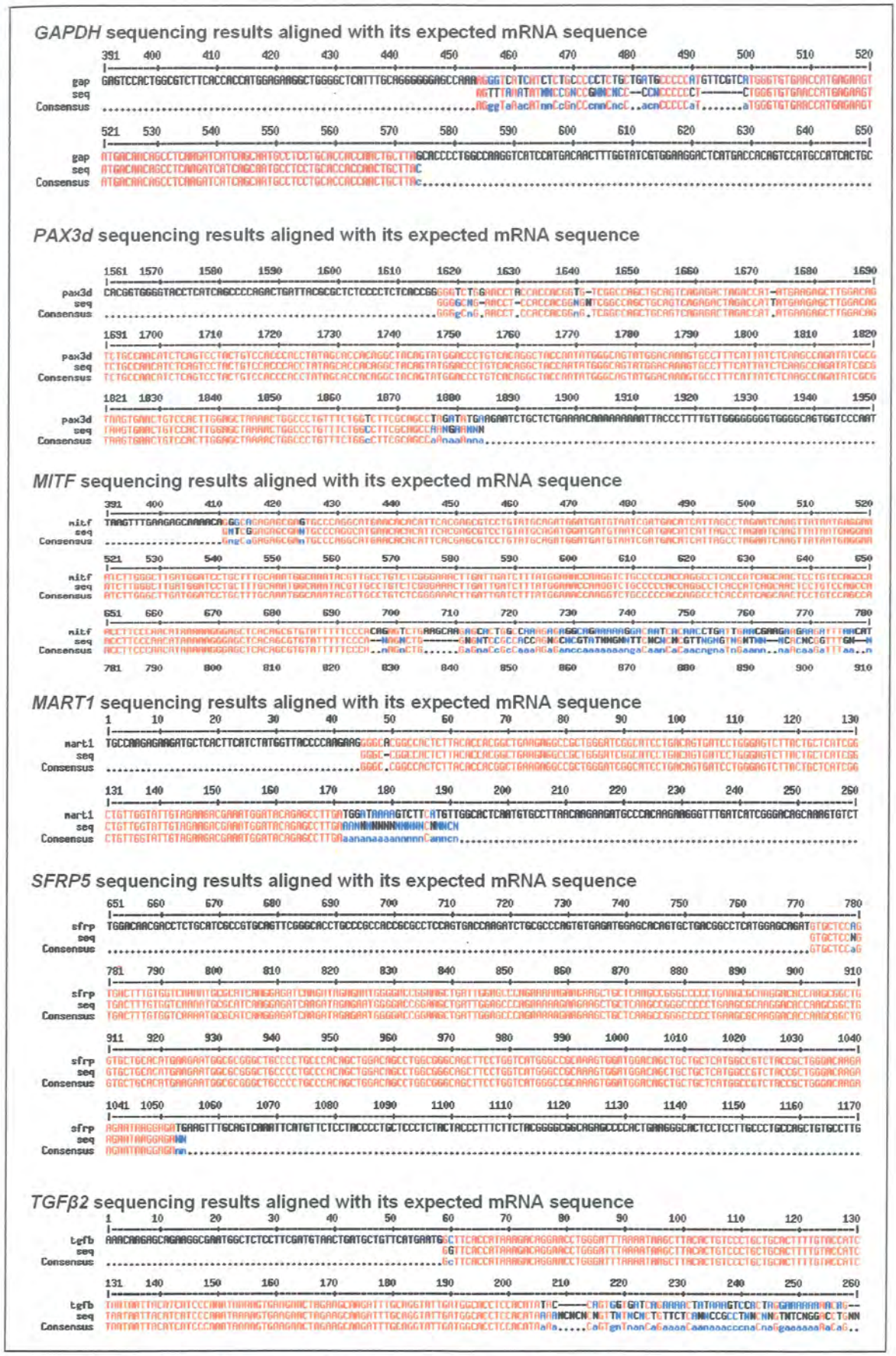
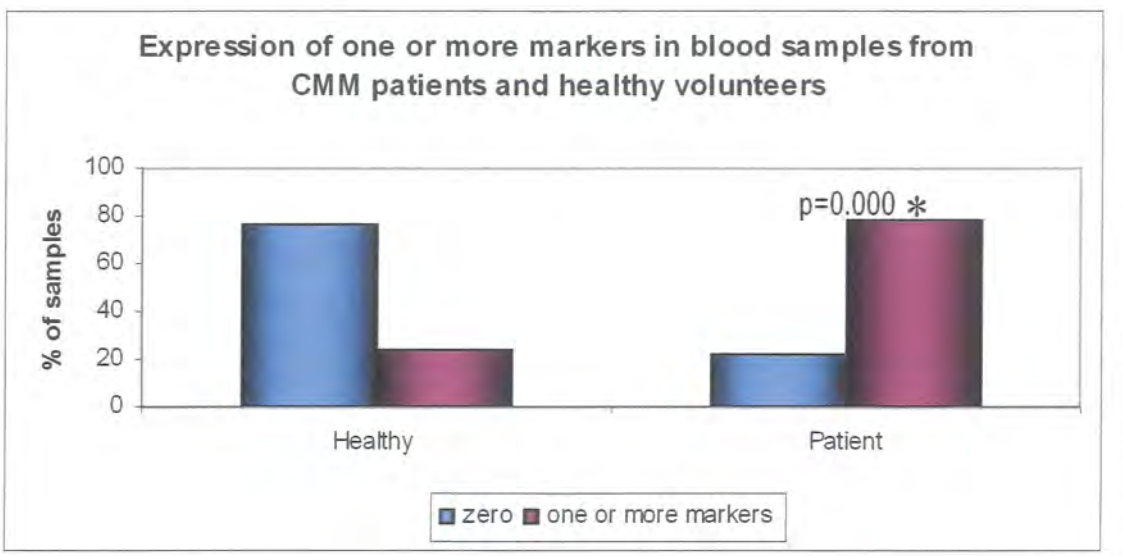


Figure 6.4. *GAPDH, PAX3d, MITF, MART1, SFRP5* and *TGFβ2* RT-PCR product sequences aligned with their expected mRNA sequence. Red letters indicate base pairs that align between the RT-PCR product sequence and the mRNA sequence.

6.6. Presence of marker expression in CMM patient and healthy blood

Due to the heterogeneity of CMM, it is understood that marker expression will vary between samples. The purpose of a multi-marker assay is to identify expression of at least one marker in all patient samples. Figure 6.5 shows the expression of one or more markers in the peripheral blood of CMM patients and healthy volunteers. Chi square analysis of expression of one or more markers as a percentage of the total number of samples, shows that the CMM patients express one or more markers significantly more frequently than healthy volunteers (Fisher's exact test: p=0.000,df=1; table 6.2).



Number of CMM patients and healthy volunteers showing expression of zero markers and one or more markers

Sample type	Zero	One or more markers
Healthy	25	8
Patient	15	53

Figure 6.5. A graphical representation of the analysis of expression of one or more markers in blood samples from CMM patients, relative to healthy volunteers. * denotes a statistically significant difference between CMM patients and healthy volunteers. Table shows the total number of CMM patients and healthy volunteers expressing zero and one or more markers.

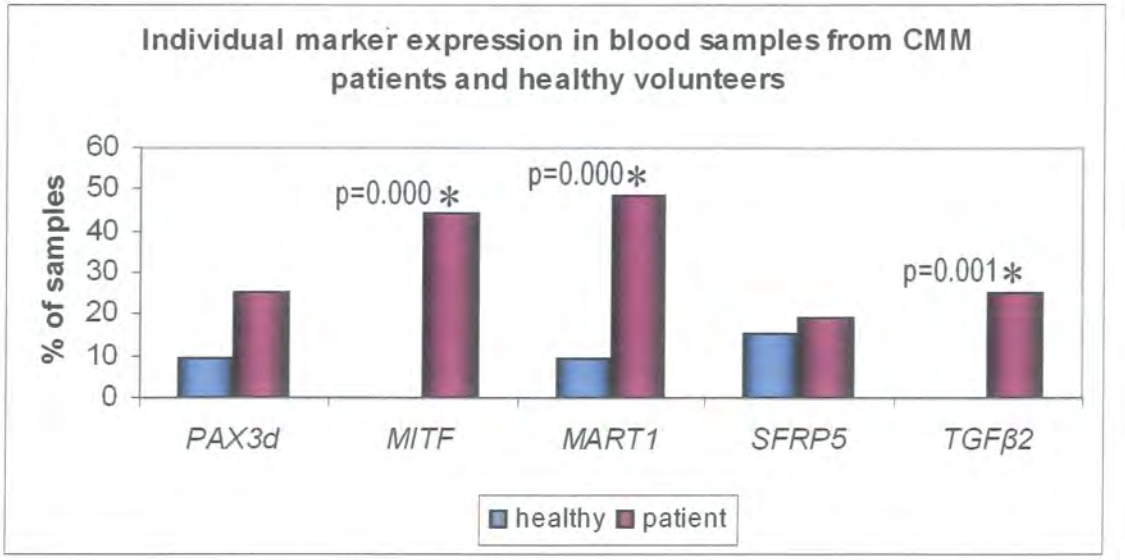
Table 6.2. Statistical analysis of expression of one or more markers in the peripheral blood of CMM patients relative to healthy volunteers (Chi-Square Tests).

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	26.784(b)	1	.000		
Continuity Correction(a)	24.586	1	.000		
Likelihood Ratio	27.302	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	26.519	1	.000		
N of Valid Cases	101				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 13.07.

6.7. Expression of individual markers in CMM patient and healthy blood

The number of CMM patient and healthy volunteer samples that were positive for each individual marker was analysed by SPSS. The percentage and number of samples positive for each marker is shown in Figure 6.6 below. It is important to note that in this graphical representation, the sum of the percentages of marker expression may exceed 100% for CMM patient and healthy volunteer groups as each sample may express more than one marker. Chi-square analysis, of marker expression as a percentage of the total number of samples, indicates that *PAX3d* and *SFRP5* are not significantly differentially expressed in CMM patients relative to healthy volunteers. However *MITF, MART1* and *TGFβ2* showed significantly higher expression in CMM patient samples than in healthy volunteer samples (Fisher's exact test: p=0.000,df=1 and p=0.000,df=1 and p=0.001,df=1 respectively; table 6.3).



Number of CMM patients and healthy volunteers showing expression of each marker

Marker	CMM patients	Healthy volunteers
PAX3d	17	3
MITF	30	0
MART1	33	3
SFRP5	13	5
TGFβ2	17	0

Figure 6.6. A graphical representation of the analysis of individual marker expression in the peripheral blood of CMM patients relative to healthy volunteers. * denotes a statistically significant difference between CMM patients and healthy volunteers. Table shows the total number of samples expressing each gene in CMM patients and healthy volunteers.

Table 6.3. Statistical analysis of *MITF, MART1* and *TGFβ2* expression in blood samples from CMM patients relative to healthy volunteers.

Statistical analysis of *MITF* expression in CMM patients relative to healthy volunteers (Chi-Square Tests)

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	20.710(b)	1	.000		
Continuity Correction(a)	18.651	1	.000		
Likelihood Ratio	29.557	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	20.505	1	.000		
N of Valid Cases	101				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 9.80.

Statistical analysis of *MART1* expression in CMM patients relative to healthy volunteers (Chi-Square Tests)

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	15.065(b)	1	.000		
Continuity Correction(a)	13.395	1	.000		
Likelihood Ratio	17.255	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	14.916	1	.000		
N of Valid Cases	101				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.76.

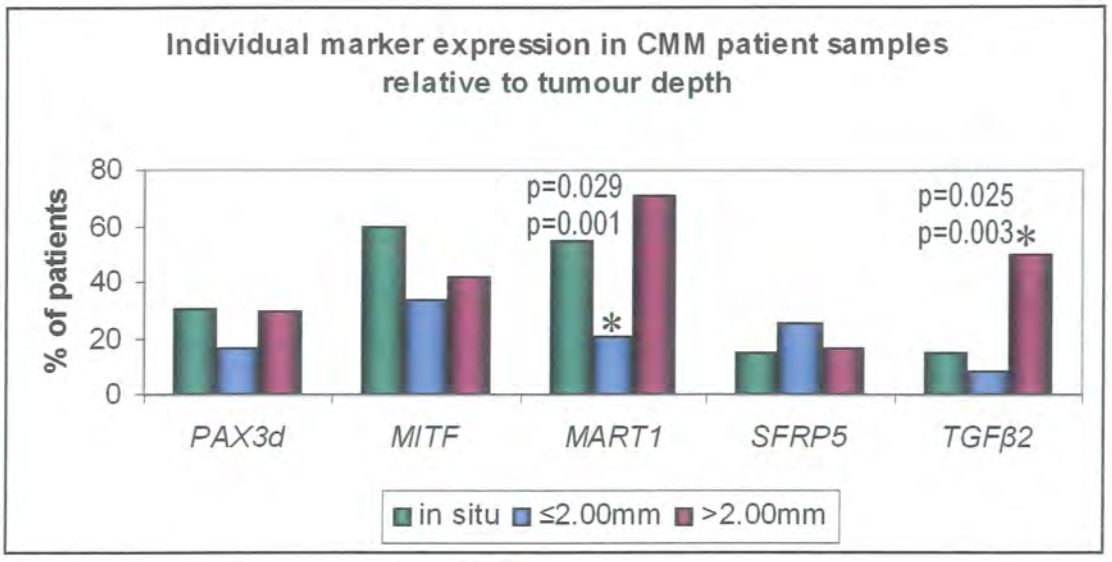
Statistical analysis of *TGFβ2* expression in CMM patients relative to healthy volunteers (Chi-Square Tests)

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	9.920(b)	1	.002		
Continuity Correction(a)	8.214	1	.004		
Likelihood Ratio	15.070	1	.000		
Fisher's Exact Test				.001	.001
Linear-by-Linear Association	9.821	1	.002		
N of Valid Cases	101				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.55.

6.8. Markers expressed in relation to patient tumour depth

To assess the relationship between tumour progression and gene expression, patients were divided into groups dependent on the level of invasion and the depth in millimetres of their primary tumour, as indicated on their pathology report. Tumours were regarded as either *in situ* with a depth of 0.00mm, ≤2.00mm (spanning 0.01mm-2.00mm in depth) or >2.00mm in depth (spanning 2.01-16.00mm). Figure 6.7 indicates the expression of individual genetic markers in relation to the depth of the primary tumour of CMM patients. Again, the sum of the percentages of marker expression may exceed 100% as each patient may express more than one marker. Chi-square analysis of marker expression as a percentage of the total number of patient samples shows that CMM patients with a tumour depth ≤2.00mm are significantly less likely to express *MART1* than patients with either *in situ* melanoma or >2.00mm tumour depths (Fisher's exact test: p=0.029,df=1 and p=0.001,df=1 respectively; table 6.4). Additionally, CMM patients with a tumour depth >2.00mm were significantly more likely to express *TGFβ2* than patients with *in situ* melanoma, or ≤2.00mm tumour depth (Fisher's exact test: p=0.025,df=1 and p=0.003,df=1 respectively; table 6.5).



Number of patients with *in situ*, ≤2.00mm and >2.00mm tumour depths expressing each marker

	in situ	≤2.00mm	>2.00mm
PAX3d	6	4	7
MITF	12	8	10
MART1	11	5	17
SFRP5	3	6	4
TGFβ2	3	2	12

Figure 6.7. A graphical representation of the percentage of CMM patients expressing each marker in relation to the depth of their primary tumour. * denotes a statistically significant difference between patients of differing primary tumour depth. Table shows the number of CMM patients with *in situ*, ≤2.00mm and >2.00mm tumour depths expressing each marker.

Table 6.4. Statistical analysis of *MART1* expression in the peripheral blood of patients with ≤2.00mm tumour depth in relation to patients with *in situ* or >2.00mm tumour depth.

Statistical analysis of *MART1* expression in the peripheral blood of patients with ≤2.00mm tumour depth in relation to patients with an *in situ* primary tumour (Chi square Tests)

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.503(b)	1	.019		
Continuity Correction(a)	4.126	1	.042		
Likelihood Ratio	5.593	1	.018		
Fisher's Exact Test				.029	.021
Linear-by-Linear Association	5.378	1	.020		
N of Valid Cases	44				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.27.

Statistical analysis of *MART1* expression in the peripheral blood of patients with ≤2.00mm tumour depth in relation to those with >2.00mm tumour depth (Chi-Square Tests)

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	12.084(b)	1	.001		
Continuity Correction(a)	10.154	1	.001		
Likelihood Ratio	12.670	1	.000		
Fisher's Exact Test				.001	.001
Linear-by-Linear Association	11.832	1	.001		
N of Valid Cases	48				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.00.

Table 6.5. Statistical analysis of *TGFβ2* expression in the peripheral blood of patients with ≤2.00mm tumour depth in relation to patients with *in situ* or >2.00mm tumour depth.

Statistical analysis of *TGFβ2* expression in the peripheral blood of patients with >2.00mm tumour depth in relation to patients with an *in situ* primary tumour (Chi square Tests)

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.948(b)	1	.015		
Continuity Correction(a)	4.492	1	.034		
Likelihood Ratio	6.285	1	.012		
Fisher's Exact Test				.025	.016
Linear-by-Linear Association	5.812	1	.016		
N of Valid Cases	44				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 6.82.

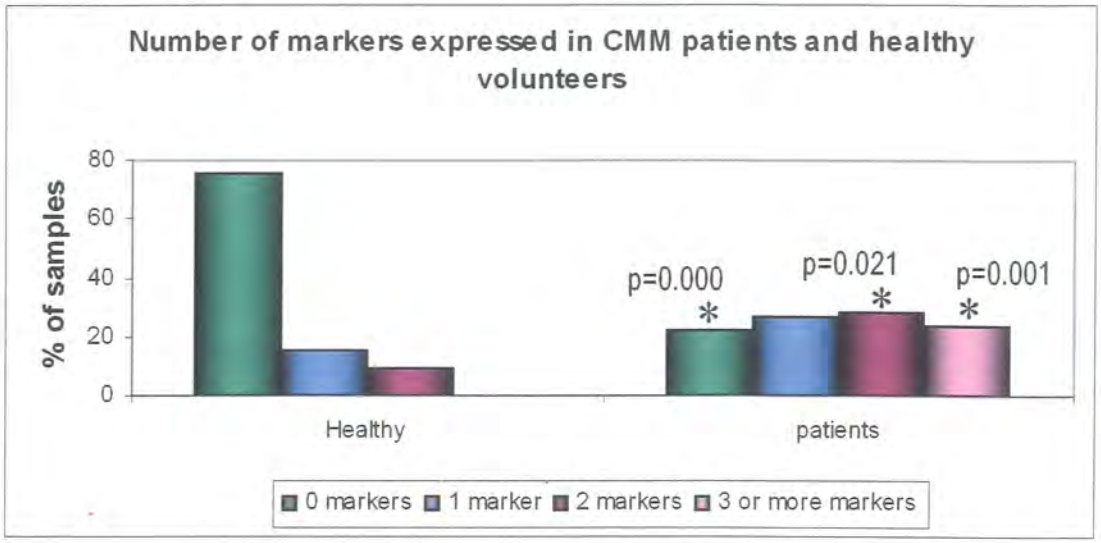
Statistical analysis of *TGFβ2* expression in the peripheral blood of patients with >2.00mm tumour depth in relation to those with ≤2.00mm tumour depth (Chi square Tests)

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	10.084(b)	1	.001		
Continuity Correction(a)	8.168	1	.004		
Likelihood Ratio	10.910	1	.001		
Fisher's Exact Test				.003	.002
Linear-by-Linear Association	9.874	1	.002		
N of Valid Cases	48				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.00.

6.9. Number of makers expressed in CMM patient and healthy blood

In order to evaluate the number of markers that are diagnostically relevant in the multi-marker assay, the number of markers expressed per sample was examined in CMM patients relative to healthy volunteers. Figure 6.8 below shows the number of markers expressed in patients relative to those in healthy volunteers. Analysis shows that 75.8% of healthy volunteers shows no expression of markers, whereas only 22.1% of CMM patients expressed no markers, a significant difference (Fisher's exact test: p=0.000,df=1 - table 6.6). Moreover, patients were significantly more likely to express two and three or more markers than healthy volunteers (Fisher's exact test: p=0.021,df=1 and p=0.001,df=1 respectively; table 6.7).



Number of CMM patients and healthy volunteers expressing zero, one, two or three or more markers

	0 markers	1 marker	2 markers	3 or more markers
Healthy	25	5	3	0
Patients	15	18	19	16

Figure 6.8. A graphical representation of the analysis of the numbers of markers expressed in blood samples from CMM patients and healthy volunteers. * denotes a statistically significant difference between CMM patients and healthy volunteers. Table shows the number of CMM patients and healthy volunteers that expressed zero, one, two or three or more markers.

Table 6.6. Statistical analysis of the difference in expression of zero markers in blood samples from CMM patients and healthy volunteers (Chi-Square Tests).

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	26.784(b)	1	.000		
Continuity Correction(a)	24.586	1	.000		
Likelihood Ratio	27.302	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	26.519	1	.000		
N of Valid Cases	101				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 13.07.

Table 6.7. Statistical analysis of the difference in expression of two, and three or more markers in the blood of CMM patients and healthy volunteers.

Statistical analysis of expression of two markers in CMM patients relative to healthy volunteers. (Chi-Square Tests)

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.623(b)	1	.018		
Continuity Correction(a)	4.477	1	.034		
Likelihood Ratio	6.305	1	.012		
Fisher's Exact Test				.021	.014
Linear-by-Linear Association	5.564	1	.018		
N of Valid Cases	95				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.64.

Statistical analysis of expression of three or more markers in CMM patients relative to healthy volunteers. (Chi-Square Tests)

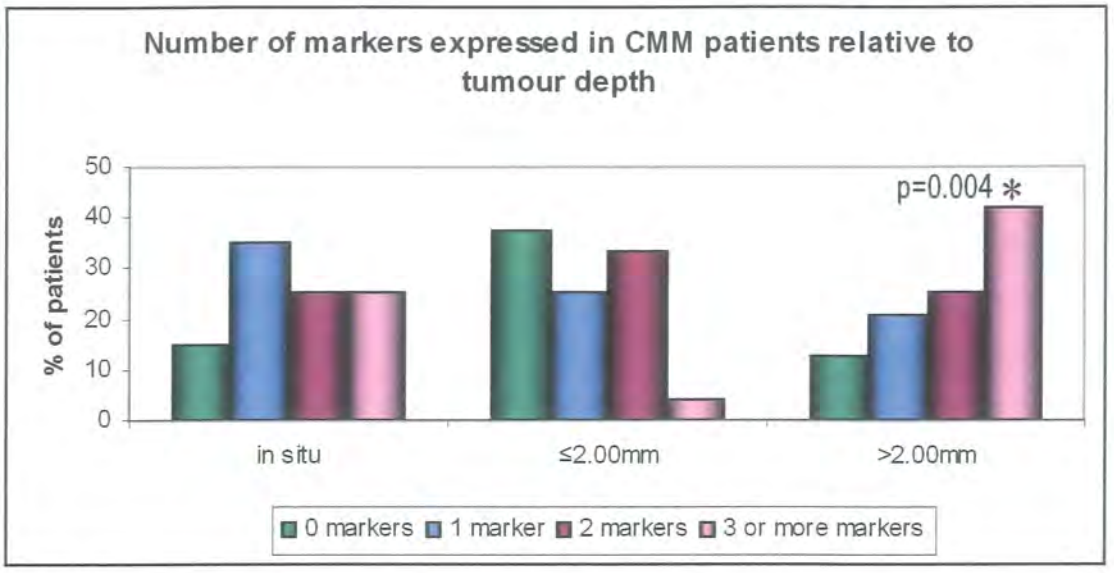
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	9.226(b)	1	.002		
Continuity Correction(a)	7.546	1	.006		
Likelihood Ratio	14.080	1	.000		
Fisher's Exact Test				.001	.001
Linear-by-Linear Association	9.135	1	.003		
N of Valid Cases	101				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.23.

6.10. Number of makers expressed in relation to tumour depth

To facilitate analysis of whether primary tumour depth influences marker expression, the number of markers expressed in each of the CMM patient depth groups were evaluated separately as shown below in Figure 6.9. Analysis shows that while CMM patients with a tumour depth ≤2.00mm expressed no markers more frequently than those with *in situ* and >2.00mm tumour depths,

these differences were not significant. In contrast, patients with a tumour depth >2.00mm were significantly more likely to express three or more markers than those with ≤2.00mm tumour depth (Fisher's exact test: p=0.004,df=1; table 6.8).



Number of patients with *in situ* tumours and tumours ≤2.00mm and >2.00mm in depth expressing zero, one, two or three or more markers

	0 markers	1 marker	2 markers	3 or more markers
in situ	3	7	5	5
≤2.00mm	9	6	8	1
>2.00mm	3	5	6	10

Figure 6.9. Graphical representation of the analysis of the number of markers expressed in the peripheral blood of CMM patients with tumours >2.00mm in depth relative to those with tumours ≤2.00mm in depth. * denotes a significant difference between CMM patients with a tumour depth >2.00mm and those with a tumour depth ≤2.00mm. Table shows the number of patients in each depth category expressing zero, one, two and three or more markers

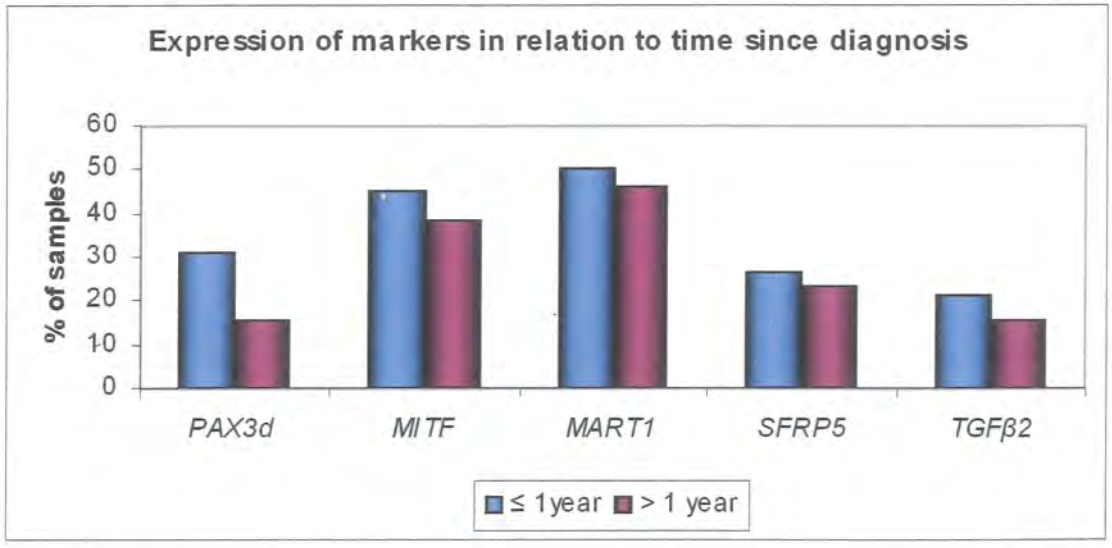
Table 6.8. Statistical analysis of the expression of three or markers in the blood of CMM patients with a tumour depth >2.00mm in relation to those with a tumour depth ≤2.00mm (Chi square tests).

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	9.553(b)	1	.002		
Continuity Correction(a)	7.548	1	.006		
Likelihood Ratio	10.759	1	.001		
Fisher's Exact Test				.004	.002
Linear-by-Linear Association	9.354	1	.002		
N of Valid Cases	48				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.50.

6.11. Marker expression in relation to time since diagnosis

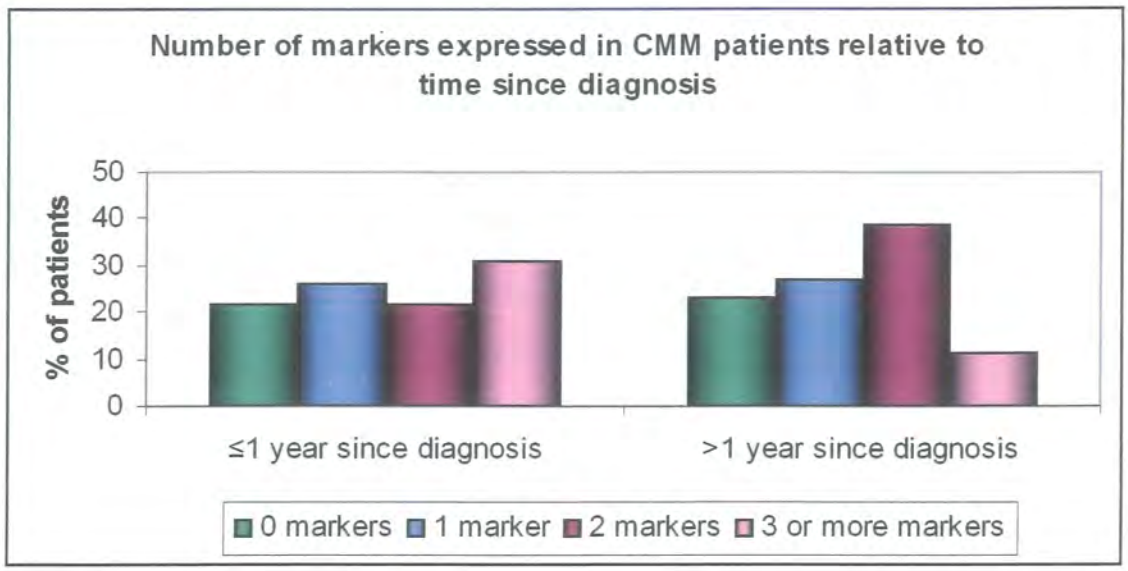
In order to assess whether the time since diagnosis of CMM and removal of the tumour has an effect on marker expression in patients, gene expression was analysed relative to the time since the patient's diagnosis and tumour removal (ie. ≤1 year or >1 year since tumour removal). Figure 6.10 depicts expression of each marker in CMM patient blood relative to time since diagnosis. Analysis reveals that although marker expression is generally less common in the >1 year group and *PAX3d* shows the biggest difference relative to time since diagnosis, there is no significant difference in marker expression relative to time since diagnosis. Figure 6.11 shows the number of markers present in CMM patient blood relative to time since diagnosis. Similarly, analysis shows that although three or more markers show the biggest difference relative to time since diagnosis, there is no significant difference between the numbers of markers expressed in relation to time since diagnosis.



Number of CMM patients expressing each gene relative to time since diagnosis

Marker	≤ 1year since diagnosis	> 1 year since diagnosis
PAX3d	13	4
MITF	19	10
MART1	21	12
SFRP5	11	6
TGFβ2	9	4

Figure 6.10. Graphical representation of the analysis of CMM patient marker expression in relation to time since diagnosis. Table shows the number of patients showing expression of each marker relative to time since diagnosis.



Number of patients showing zero, one, two or three or more markers relative to time since diagnosis

	0 markers	1 marker	2 markers	3 or more markers
≤1 year since diagnosis	9	11	9	13
>1 year since diagnosis	6	7	10	3

Figure 6.11. Graphical representation of the analysis of the number of markers expressed in CMM patient blood in relation to time since diagnosis. Table shows the number of patients expressing zero, one, two or three or more marker relative to their time since diagnosis.

7. DISCUSSION

7.1. Overall marker expression

Smith and colleagues (1991) determined that molecular markers specific to melanocytes could be used to detect migrating melanoma cells in peripheral blood. Following this discovery, many studies have investigated the use of these and other markers of melanoma metastasis, including genes responsible for the proliferation, invasiveness and survival of melanoma cells in peripheral blood assays. Assays to determine this expression included RT-PCR and ELISA methodologies. (Carreira *et al.*, 2005; Lang *et al.*, 2005; Wagner and Fisher, 2005). Since melanomas are highly heterogeneous with respect to tumour-related gene expression, multi-marker RT-PCR was developed to improve assay sensitivity (Medic *et al.*, 2007). This procedure uses the simultaneous detection of several markers of melanoma cells to allow for the variation in expression of genes (Hoon *et al.*, 2000; Reynolds *et al.*, 2003). In this study, markers of melanocyte development were used together with markers of melanoma progression, to determine the efficacy of this combination of markers in a multi-marker assay. Markers were assessed in combination and individually for efficacy by statistical analysis.

Of the markers used here, *PAX3d, MITF* and *MART1* have previously been identified individually as significant markers of melanoma metastasis in tissue and peripheral blood samples (Blake and Ziman, 2003; Koyanagi *et al.*, 2006; Medic, 2006; Wascher *et al.*, 2003). To date the efficacy of *SFRP5* and *TGFβ2* have not been investigated as markers of melanoma cells in peripheral blood (Jones and Jomary, 2002; Reed *et al.*, 1994). Although *TGFβ2* has been implicated as a marker of melanoma progression in tissue (Reed *et al.*, 1994), it remains to be conclusively tested in both tissue and peripheral blood.

In this study, the choice of markers and their use in combination proved successful; overall, 77.9% of patients examined showed expression of at least one marker. This is a considerable improvement on the results of some multi-marker assays from several other recent studies, which showed 60% (Schmidt

et al., 2006), 55% (Koyanagi *et al.*, 2005), 51.5% (Samija *et al.*, 2004) and 47% (Reynolds *et al.*, 2003) overall expression of at least one marker. The markers selected for multi-marker assay in the current study differed from the markers trialed in these previous studies. This increased frequency of marker expression suggests that this combination was better suited to the detection of heterogeneous melanoma cells. Alternatively, this difference in results may be due to the smaller number of patients (68) used in the current study, relative to previous studies of 118 (Reynolds *et al.*, 2003) and 94 (Koyanagi *et al.*, 2005) patients, respectively. The lower sample size increases the significance of detection in a single patient. However the study undertaken by Samija and colleagues (2004) used a smaller patient cohort, dispelling this reasoning. This increase in detection of mRNA markers is more likely to be due to the careful optimization of blood collection and RT-PCR protocols performed in this study (see section 5: methods).

Multi marker trials that have resulted in higher frequency of marker detection such as 82% (Palmieri *et al.*, 2003) and 93% (Hoon *et al.*, 2000) overall expression of at least one marker, used very different combinations of markers, but similar techniques. Ultimately, a set of markers that optimally detects melanoma cells in peripheral blood is likely to include markers used in this and other successful studies.

7.2. Analysis of individual marker suitability for inclusion in a blood test for CMM metastasis

This study showed that *MITF* and *TGFβ2* are reliable markers of melanoma cells in peripheral blood, in line with previous studies that have described the expression of these genes in CMM tissue (*MITF* and *TGFβ2*) (Reed *et al.*, 1994; Wascher *et al.*, 2003) and peripheral blood (*MITF*) (Koyanagi *et al.*, 2006). The fact that neither gene was expressed in the blood of healthy volunteers strengthens the assumption that their observed expression in peripheral blood of CMM patients arises from melanoma cells.

MITF expression was detected in 44% of CMM patients, which is notable when compared to other recent studies which showed expression of this gene in 39% (Koyanagi *et al.*, 2006) and 30% (Samija *et al.*, 2004) of patients tested. Since the number of patients used here was midway between those used in the previous studies, the higher frequency of detection is more likely due to careful optimization of methodologies of blood collection and RT-PCR procedures.

TGFβ2 was expressed in 25% of melanoma patients and with no previous studies of this type utilizing this gene, no comparisons are possible. However relative to other markers in this study expressing 44% (*MITF*), 48.5% (*MART1*), 25% (*PAX3d*) and 19% (*SFRP5*), this can be deemed a considerable proportion. Accordingly, both *MITF* and *TGFβ2* are considered to be valuable markers of migrating melanoma cells, and are suitable for inclusion in a blood test for CMM metastasis.

In this study, *MART1* was found to be expressed in the peripheral blood of both CMM patients (48.5%) and healthy volunteers (9%). Chi-square analysis of these results show that *MART1* is significantly more likely (p=0.000) to be expressed in CMM patients than in healthy volunteers. Therefore the gene is considered a valuable marker of melanoma cells in peripheral blood and suitable for inclusion in a blood test for CMM metastasis.

MART1 expression in 48.5% of patients is considerably higher than in previous studies which showed 30% (Koyanagi *et al.*, 2005), 32% (Palmieri *et al.*, 2003), 17% (Reynolds *et al.*, 2003) and 10% (Wascher *et al.*, 2003) expression in CMM patients respectively. Interestingly, Keilholz and colleagues (2004) also found expression of *MART1* in the blood of healthy volunteers (14.3%). To offset the effect of the false positives in healthy volunteer samples, Keilholz and colleagues (2004) used quantitative real-time RT-PCR to set a cut-off level of expression of the gene to distinguish between healthy and patient samples as expression levels of *MART1* were lower in controls. This option was not possible in the current study, as the RT-PCR assay used here was not quantitative. Future studies will employ Real-Time RT-PCR to further evaluate

the efficacy of the markers identified as suitable in this study. This proposition will be explained in more detail later.

In this study, *PAX3d* and *SFRP5* were found to be poor markers of melanoma cells in the blood due to the expression of these genes in healthy volunteers. When analyzed statistically, expression of both genes was found to be similar in CMM patients and healthy volunteers, with no significant difference in expression noted between these groups.

The *PAX3d* result contrasts significantly with that of Medic (2006) in which *PAX3d* expression was found in 52.4% of CMM patients but not in healthy volunteers. *PAX3d* was therefore considered to be a useful marker of CMM metastasis (Medic, 2006). The variation in results may indicate that some of the healthy volunteers recruited in the current study may have CMM but be clinically undiagnosed. Alternatively, a much smaller number of healthy volunteers was used in the previous study and this may explain the lack of expression of the marker in healthy volunteers (Medic, 2006).

This is the first study to date to investigate the use of *SFRP5* as a marker of melanoma cells in the blood. Results suggest that this is not a suitable marker of migrating melanoma cells in peripheral blood of CMM patients. Analysis of expression of this and other *SFRP* genes will be tested in tissue sampled prior to further blood tests.

Overall this study found *MITF, TGFβ2* and *MART1* to be useful markers of melanoma cells circulating in peripheral blood and suggest that they would be suitable for inclusion in a multi marker assay designed to identify migrating melanoma cells in peripheral blood of CMM patients.

7.3. Analysis of efficacy of individual markers as indicators of disease progression

Breslow (1975) showed a significant correlation between patient tumour depth and prognosis. This correlation remains the gold standard in CMM prognostic techniques. This study utilized this correlation to investigate marker efficacy, by analyzing marker expression relative to primary tumour depth. The only marker that shows increased expression with increased tumour depth is *TGFβ2*; that is *TGFβ2* is significantly more likely to be expressed in CMM patients with a primary tumour depth greater than 2.00mm than in patients with in-situ melanoma or tumours ≤2.00mm in depth. The observation that *TGFβ2* is expressed at a higher rate in patients with thicker tumours supports the association between this gene and the deep invasion and metastasis of CMM (Reed *et al.*, 1994). Accordingly, this gene may be useful as a prognostic marker if included in a blood test for CMM progression.

Another marker that showed differing expression in relation to primary tumour depth was *MART1*. However, unlike *TGFβ2* this marker did not align with advancing clinical stage of the disease. Rather, patients with in situ melanoma or those with >2.00mm tumour depth were significantly more likely to express *MART1* than patients with a primary tumour depth ≤2.00mm. This suggests that *MART1* may be down-regulated at certain stages of the disease, before being up-regulated again during deeper invasion. Alternatively, and a more likely explanation, is that these results are inaccurate due to small group numbers.

7.4. Number of markers relative to disease progression

Several studies have shown that the number of markers detected in blood samples from CMM patients is directly related to the clinical stage of the disease, with more advanced tumours showing an increased number of markers (Hoon *et al.*, 2000; Koyanagi *et al.*, 2005; Wascher *et al.*, 2003). By contrast, this study found that there was no significant correlation between the tumour depth and the number of markers.

This difference in results may be due to the small number of patients utilized here, or to the difference in classification of disease progression between studies. In the current study, disease progression was attributed to a deeper primary tumour according to the Breslow system of tumour grading (Breslow, 1975), which is still the only system used by pathologists in Western Australia. Previous studies have used the more accurate AJCC classification system of disease progression (Balch *et al.*, 2001). While Breslow's system of tumour grading shows a relationship between tumour depth and patient survival, it does not take into account the presence of ulceration or the presence of disease spread measured by lymph node involvement and/or distant metastases.

Alternately, the markers used in this study were not necessarily associated with tumour progression. While *TGFβ2* is thought to be associated with tumour progression (Reed *et al.*, 1994), confirmed by frequent expression in patients with thicker tumours (this study), its role in tumour metastasis remains to be determined.

It was expected that patients with in situ tumours would express the fewest markers of CMM patients. At this early stage of disease, a tumour is not considered to be invasive (Pantel *et al.*, 1999) and therefore not expected to have as many tumour cells circulating in the blood stream (Koyanagi *et al.*, 2005). Also, at an earlier stage of CMM there is thought to be less genetic instability and therefore less likelihood of expression of multiple melanoma mRNA markers (Hoon *et al.*, 2000). In this study, the number of markers detected in samples from CMM patients with in situ melanoma was similar to the number of markers detected in samples from patients with a >2.00mm tumour depth. This suggests that even at this early stage of the disease, there is some invasion of melanoma cells into the bloodstream, although their metastatic potential has yet to be assessed. This is not necessarily a limitation, and may in fact be a positive finding. Firstly, the markers utilized here could be used in a blood test for melanoma detection rather than progression. Secondly, the finding that patients with early clinical staged tumours express markers of migrating melanoma cells in peripheral blood is a novel finding and may lead to more careful monitoring of these patients (discussed further later).

7.5. Number of markers expressed in patients relative to healthy volunteers

This study shows that CMM patients are significantly more likely to express two or more markers than healthy volunteers. This suggests that a blood test incorporating these markers would require expression of at least two markers to give an accurate result for melanoma markers circulating in peripheral blood.

In this study, 24.2% of healthy volunteers showed marker expression, of which 9.1% expressed two markers. Due to its poor result as a marker of melanoma cells in the blood, *SFRP5* would not be incorporated in future blood tests for CMM. Had this marker not been included in this study, fewer than 20% (18.2%) of healthy volunteers would show positive marker expression, and none would show expression of more than one marker. However, the removal of *SFRP5* from the multi-marker assay does not lead to a change in statistical significance of the number of markers expressed in CMM patients relative to healthy volunteers; that is, there is no significant difference in the expression of one marker between patients and healthy controls, and CMM patients would still be significantly more likely to express two or more markers than healthy volunteers.

The expression of markers in the peripheral blood of healthy volunteers may be due to contamination during the processes of RNA extraction and purification or to the unspecific nature of the markers to CMM. That is, the markers detected in healthy volunteers may be expressed in cells other than melanoma cells. As described previously, the identification of markers in the blood of healthy volunteers may also be caused by melanoma as yet undetected by the individual.

7.6. Marker expression relative to time since diagnosis

Interestingly, this study showed no significant difference between the individual markers, or the number of markers expressed, in relation to the time since

patient diagnosis and surgical excision of the tumour. It was expected that fewer markers would be detected or fewer patients would express molecular markers in their blood of patients when analyzed at a greater length of time since tumour removal. It was expected that melanoma cells in the blood would either form metastases or expire (Pantel *et al.*, 1999).

The ability of tumour cells to evade the immune system is well documented (reviewed in Gorter and Merib, 1999), The up-regulation of anti-apoptotic genes such as *BCL2* is regarded as one of the ways in which these cells avoid destruction (Campana *et al.*, 1993). As described previously, aberrant expression of *MITF* in melanoma may cause over-expression of *BCL2* (McGill *et al.*, 2002) generating immortal melanoma cells that may continue to circulate in the blood for several years after diagnosis and surgical excision of the primary tumour. Furthermore, *TGFβ2* is known to act as an immunosuppressive molecule in several other cancers and is thought to contribute to decreased immune surveillance, allowing tumour development, by inhibiting interleukin-1 stimulated proliferation of T cells (Bodmer *et al.*, 1989; Ellingsworth *et al.*, 1988).

7.7. Novel findings: marker expression in the blood of in situ melanoma patients

Surprisingly, this study showed that in situ melanoma patients were as likely to express markers of melanoma cells in peripheral blood as patients with a tumour depth of ≤2.00mm and even >2.00mm. This was unexpected as in situ melanomas are considered to be non-invasive and therefore not expected to have invasive cells, or cells that invade the reticular dermis and blood (Meier *et al.*, 1998; Miller and Mihm Jr, 2006; Pantel *et al.*, 1999).

This is a disturbing discovery as it suggests that there may be some potential for melanoma cells to circulate within the blood of in situ melanoma patients, formerly thought to be safe from CMM metastasis. Furthermore, as previously stated there is no significant difference in the expression of markers in patients

with in situ tumours relative to their time since diagnosis. This implies that possibly metastatic melanoma cells may be circulating in the blood of in situ patients for several years after the surgical removal of their tumour. This is an area not previously studied as in situ tumours are not included in studies of markers of melanoma cells in peripheral blood. These results suggest that patients with these tumours be included in future marker expression studies of migrating melanoma cells.

7.8. Limitations of the study

The principal limitation of this study was sample size. The low number of CMM patient samples analyzed affected the results since many of the analyses failed to reach statistical significance due to small group sizes, limiting the conclusive observations that can be made from the data.

Time was a second limitation. With only four months in which to process, purify and test RNA, as well as performing RT-PCR analysis for six markers (*GAPDH, PAX3d, MITF, MART1, SFRP5* and *TGFβ2*) the number of samples that could be tested was restricted.

7.9. Conclusion

Following the isolation, purification and quality assurance of total RNA and mRNA, CMM patient and healthy control samples were analyzed using RT-PCR for the expression of genetic markers *PAX3d, MITF, MART1, SFRP5* and *TGFβ2*. Overall, the multi-marker assay trialed in this study proved successful with 77.9% of blood samples from CMM patients expressing one or more markers, while only 24.2% of blood samples from healthy volunteers expressed one or more markers. Of the genes tested, *MITF, TGFβ2* and *MART1* were found to be reliable markers of melanoma cells circulating in peripheral blood. Furthermore, *TGFβ2* expression was found to correlate with the presence of thicker primary tumours from CMM patients. Although no association was found

between the number of markers expressed and increased tumour depth, CMM patients were found to be significantly more likely to express two or more markers than healthy volunteers. Surprisingly however, there was no correlation between marker expression and time since CMM patient diagnosis and tumour removal, a fact that may be attributed to the gene regulation of tumour cell survival.

Based on these results, further investigation is required to assess the levels of expression of these markers in peripheral blood relative to tumour progression.

7.10. Further studies

In order to further investigate the level of expression of these markers in CMM patient and healthy volunteer peripheral blood, it is necessary to utilize quantitative Real Time RT-PCR. It is thought that levels of expression are likely to be related to primary tumour depth and/or tumour progression as shown in previous studies (Hoon *et al.*, 2000; Koyanagi *et al.*, 2005; Wascher *et al.*, 2003). Preliminary attempts were made during the study to utilize this methodology; however limitations hampered the development of this technique.

In future studies it is also important to clarify that marker expression is definitively associated with CMM tumour presence. That is, healthy volunteers must be clinically tested by a dermatologist and declared melanoma free prior to their inclusion in the study. Furthermore, peripheral blood from patients diagnosed with skin cancers other than CMM should be tested with these markers to clarify that marker expression is associated with melanoma cells in peripheral blood.

Finally, the inclusion of more samples would allow significant statistical values to be obtained for all study groups. A higher number of samples would ideally incorporate patients with differing clinical stages of CMM as well as different times since diagnosis and tumour removal.

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9. APPENDICIES

Appendix A

EDITH COWAN UNIVERSITY MEMO
FACULTY OF COMPUTING, HEALTH AND SCIENCE

Human Ethics Subcommittee

TO: **Tamara Harold, Admin. Officer, Higher Degrees**
FROM: **Angus Stewart, Chair, Faculty Human Ethics Subcommittee**
SUBJECT: **Human Ethics Clearance Application/s**
DATE: **11th July, 2007**

Dear Tammie,

The following ethics application

Rebecca Slattery	Development of a blood test for the detection of Cutaneous Malignant Melanoma.

is approved (category 1).

Best wishes,

Angus.

Appendix B

RNA (GENE EXPRESSION) TESTING

The purpose of this document is to provide information relating to procedures for RNA (Gene Expression) testing

This document explains the ethical issues and provides a code of practice for gene testing as laid down by the National Health and Medical Research Committee of Australia, and is provided in addition to the specific project information sheet.

WHAT IS RNA?

Your genes are composed of a chemical molecule called DNA. RNA, a similar chemical compound, is copied from DNA by enzymes in each cell of your body. RNA provides the blueprint for the synthesis of proteins. These compounds are often referred to as the BUILDING BLOCKS OF LIFE.

Each RNA molecule is expressed from a specific gene present on DNA. RNA provides the code and signal for manufacture of proteins. Therefore once the specific signal RNA is received in the cell the corresponding protein is made. Each gene in your DNA is only expressed when needed by the cell; in fact the expression of most genes into RNA is carefully controlled occurring only at specific times and in specific cells. Many genes are specifically expressed during embryonic development and their expression specifies the carefully controlled specification, migration, proliferation and differentiation of particular types of cells required for the development of organs and tissues. Aberrant unregulated expression of these genes into RNA and then into protein in adult cells could result in abnormal proliferation and/or migration of these adult cells and formation of a tumour.

RNA testing can provide information on what may be happening in your cells right now (specific RNA testing) or what may happen to your general health, or perhaps that of your family, either now or in the future (general RNA testing).

In this project, an RNA test is performed to assist the researchers to assess abnormal gene expression as a marker of cells in peripheral blood of patients which will assist with identification of abnormal gene expression in the process of skin cancer.

WHY IS RNA TESTED?

RNA testing can assist us to understand how your cells function or it can assist us to understand a disease (specific test), or it can be used for general medical research (general test). Medical research helps us find out about our cells and tissues and diseases and what causes them. This document gives you information about providing samples for research for a set of RNA tests to be used in this research.

INFORMED CONSENT

RNA testing is carried out with your full consent.

Should you agree to RNA testing for **research purposes**, you are given an information sheet and consent form to read and sign.

We recommend that you give careful consideration to the important supplementary information set out in this document before providing your consent.

HOW IS RNA OBTAINED?

RNA is usually obtained from a sample of tissue or blood. The amount of tissue required may vary but usually, $2mm^2$ is required. For blood, only 5ml of blood is required and from the blood, RNA is obtained by chemical separation and column centrifugation to remove blood cells. For this study, only a small sample of blood is taken as this is sufficient for the RNA tests that we are to perform. For this study you will be required to provide samples of blood once as a control or more than once as a patient (to a maximum of four times per person – please see schedule on consent form).

Isolation of the RNA results in the destruction of the cancer cells. The extracted RNA appears as a clear fluid (like water) and is kept in a small plastic tube. The sample is stored in a freezer until the end of the research. The sample will be labelled with an identification number given by your doctor and by the principal researcher. The number is not in any way connected to you and does not include your personal identification details. Only routine clinical data in your medical records may be given to the researcher, such as your age and sex as well as your clinical diagnosis.

RNA is long lasting and may be stored for an indefinite period of time and for the purposes of this research your RNA will be stored while the researchers conduct their research and then it will be discarded at the end of the research.

-That is, the sample is to be discarded after it has been used for melanoma research only and will not be used for any general RNA testing at a later stage.

You will be required to acknowledge on your Consent Form that you are prepared to donate your RNA sample on the understanding that you will not receive, or be entitled to, any financial reward or remuneration for providing this sample.

The value of the research is not known at this time. You will be notified of the results of the RNA research in general terms at your request – i.e. your specific details are not recorded but the outcomes of the research as a whole is provided to you upon completion of the project.

TISSUE SAMPLE HANDLING

If you decide to provide your tissue sample for research purposes, you can be assured that in this case, your sample is only used for melanoma research and there is no possibility that future RNA testing may result in new information about other diseases that you may carry.

THE CHIEF INVESTIGATOR'S COMMITMENT TO YOU

RNA testing is a complex and changing area. The Chief Investigator will endeavour to protect your interest at all times. Your RNA sample is treated exactly as indicated above and is monitored by the Chief Investigator at all times and is dealt with as indicated in the signed consent form.

Your sample is stored carefully, but a guarantee cannot be given against inadvertent loss or damage which is beyond the Chief Investigator's control. We guarantee that your sample is NOT to be used for other tests, and is discarded upon completion of the research.

All research to be conducted has the approval of Edith Cowan University Human Research Ethics Committee and the Human Research Ethics Committee at Sir Charles Gairdner Hospital.

All experiments are conducted under the strict guidelines of the Australian National Health and Medical Research Council.

Should you advise your family members?

1. You may advise your family members of the existence of your RNA sample; perhaps even provide them with a copy of this Information Brochure and your signed Consent Form.
2. Advise your family members of the purpose for which you have provided your RNA sample and bring them with when you donate your sample if you wish.
3. However in this project we are **not** testing for association with genetically determined diseases. The information will not be used to alter your treatment in any way. It may however at some future stage provide information about the relationship between gene expression and tumour progression. Your sample will assist with this research but we cannot provide conclusive details about this until the conclusion of the research. We are **not specifically** testing your future health or that of your family in this research project.

WHAT IF I CHANGE MY MIND?

You maintain the right to withdraw your consent and sample at any time.
If you wish to have your sample withdrawn, notify the Chief Investigator in writing. It will then be discarded in an appropriate and timely manner.

DO YOU HAVE FURTHER QUESTIONS?

If you have any questions about RNA testing, please contact your doctor or the Chief Investigator of the research study.
Any concerns you may have regarding the manner in which the research project is conducted, may be directed to:-

Kim Gifkins
Research Ethics Officer
Edith Cowan University
100 Joondalup Drive
JOONDALUP WA 6027
Phone: (08) 6304 2170
Fax: (08) 6304 2661
Email: research.ethics@ecu.edu.au

Appendix C

JOONDALUP CAMPUS
100 Joondalup Drive, Joondalup Western Australia 6027 Telephone (08) 6304 5555

EDITH COWAN UNIVERSITY

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Dr. Mel Ziman
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Consent for Blood to be utilized for Gene Expression Testing Research

I, ...of .....
.....
.....

have read the Information Document entitled "RNA (Gene expression) Testing" and the Information Sheet for the study.

I hereby consent to a blood sample being taken and donate the sample for research as indicated in the information document.

As my sample is a control sample and I have not been diagnosed with skin cancer and I do not wish my personal details to be recorded then I have every right not to provide these details. This means that the

researchers cannot contact me at a future date and the risks and benefits of this have been explained to me in detail.
If the research produces information relevant to my health and wellbeing, this information will be provided to me (if I wish to be notified) and my clinician will be consulted to allow me to obtain appropriate medical attention should this be necessary.

It is important for you to understand that the information gathered from your sample forms part of a research project. If any abnormal result is obtained from your sample then this needs to be verified by clinical testing.

If you do not provide contact details now so that we can contact you at a later date then this information is not available to you and your samples will be completely de-identified unless you specifically request that you remain contactable, in which case details that allow us to contact you, are required. Your sample will then be provided with a code for use by the researchers and all personal information will be held in locked filing cabinets.

In making my donation of blood, I understand and agree that:

(a) the sample (which in this consent form includes its constituents and any genetic material (RNA) will be used only in relation to melanoma research; and
- will be stored in a tissue bank for the duration of the melanoma research conducted by researchers at Edith Cowan University.

(b) Taking and storage of samples will be conducted in accordance with the National Health & Medical Research Council's* *Guidelines for Genetic Material*.

(c) Storage of RNA will be conducted under a coded system, to ensure that donor confidentiality is maintained;

(d) samples of RNA will be discarded upon completion of the research or upon my written request;

(e) the results of these studies may be of interest to me and my family and I may decide whether or not the information may be disclosed to my family; in accordance with **Options for disclosure to family members**. However, the research will not at this stage provide me with any detailed information about any genetic diseases. However donation of my sample may assist researchers to provide a more detailed and specific diagnosis of melanoma in the future.

*The National Health & Medical Research Council advises the Australian community and Commonwealth and the State Governments on standards of individual and public health, and supports research to improve these standards as follows:

(f) if a research worker wishes to obtain additional information or samples from me, my name will not be divulged to that researcher without my written permission;

(g) the fact that I have had a test, and the results of my test, will not be revealed or made available to any other person or organisation except in accordance with my written consent,

(h) the Chief Investigator of this project and his or her associates involved with this project as well as Edith Cowan University will not be liable for any loss or damage to the sample taken or used in accordance with this form.

- I consent to the storage of my RNA for melanoma research as explained in the related Information Sheet

I acknowledge the public interest in the research and donate the sample absolutely on the understanding that I will not receive, or be entitled to, any reward or remuneration for providing my RNA.

I understand the potential benefits and adverse consequences (if any) involved in testing of this sample. These have been explained to me and I accept the risks involved. I have had the opportunity to ask questions and am satisfied with the explanation and the answers to my questions.

I understand that I may withdraw my consent at any stage without reason and without prejudice.

.....	.....	.....
Name of Participant	**Signature**	**Date**
.....	.....	.....
Name of Witness	**Signature**	**Date**
.....	.....	.....
Name of Chief Investigator	**Signature**	**Date**

The Human Research Ethics Committee at Edith Cowan University requires that all participants are informed that, if they have any complaint regarding the manner in which a research project is conducted, it may be given to the researcher or, alternatively, to

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