

**IDENTIFICATION OF NOVEL TUMOUR SUPPRESSOR GENES
INVOLVED IN THE DEVELOPMENT OF CUTANEOUS
MALIGNANT MELANOMA**

A thesis submitted for the degree of Doctor of Philosophy by

Lylia Ouboussad

School of Health Sciences and Social Care
Brunel University

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Abstract

Skin cancer is one of the most common forms of adult solid tumour. The incidence is increasing rapidly making skin cancer a major health problem in several countries. Cutaneous Malignant Melanoma (CMM) is the least common but the most life threatening type of skin cancers and is responsible for 90% of all skin malignancy associated deaths.

The precise cellular and molecular etiology of malignant melanoma is quite complex and the molecular events directly related to melanoma progression are yet to be elucidated. However, recent advances in molecular biology have resulted in a clearer understanding of the cellular and molecular events of skin cancer development. The best-characterized locus associated with CMM development is the CDKN2A that maps to chromosome 9p21 and encodes for the cell cycle regulator p16 tumour suppressor gene (TSG), and is frequently inactivated in melanoma tumours. In addition to p16, other loci located in 9p21 appear to be important in CMM development and functional evidence for the presence of TSG(s) has been provided (Parris *et al.*, 1999). The aim of our study is to contribute to the understanding of CMM development by isolating and characterising novel TSG(s) at this location.

In order to pursue identifying potential TSG(s), we have developed several monochromosome hybrids using microcell mediated chromosome transfer, and evaluated the tumorigenicity of the constructed hybrids by anchorage independent growth in soft agar. For the molecular biology aspects, expression analysis of the genes in the 9p21 region was carried out by reverse transcription PCR. Potential candidate tumour suppressor genes were then carefully evaluated by generating expression profiles via conducting real time PCR. Experimental evidence is provided which supports the candidacy of interferon alpha 1 (IFNA1) as a tumour suppressor gene for melanoma development.

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For my Parents

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Abbreviations

- AMS:** Atypical Mole Syndrome.
- ARF:** Alternative Reading Frame.
- BCC:** Basal Cell Carcinoma.
- BHK:** Baby Hamster Kidney.
- BLAST:** Basic Local Alignment Search Tool.
- Bp:** base pair.
- CDK:** Cyclin-Dependent Kinase.
- CFE:** Colony Forming Efficiency.
- CMM:** Cutaneous Malignant Melanoma.
- Ct:** Threshold Cycle.
- DEPC:** DiEthyl PyroCarbonate.
- DMEM:** Dulbecco's Modified Eagle's Medium.
- DMSO:** DimethylSulfoxide.
- Dnase I:** Deoxyribonuclease I.
- DNS:** Dysplastic Nevus syndrome.
- dNTP:** DeoxyNucleoside TriPhosphate.
- DTIC:** Dacarbazine.
- EDTA:** Ethylenediaminetetraacetic Acid.
- EMBL-EBI:** European Molecular Biology Laboratory- European Bioinformatics Institute.
- ES:** Embryonic Stem cells.
- EtBr:** Ethyidium Bromide.
- FCS:** Fetal Calf Serum.
- HyTK:** Hygromycin phosphotransferase-Thymidine Kinase fusion gene.
- IFN:** Interferon.
- IL:** Interleukin.
- Kb:** Kilobase.
- LAK:** Lymphokine-Activated Killer.
- LOH:** Loss of Heterozygosity.
- MAA:** Melanoma-Associated Antigens.
- Mb:** Megabase.
- MgCl₂:** Magnesium Chloride.
- MMCT:** Microcell-Mediated Chromosome Transfer.

MMS: Mismatch Repair.

MSI: Microsatellite Instability.

NCBI: National Center for Biotechnology Information.

NCS: Newborn Calf Serum.

NRK: Normal Rat Kidney.

NSCLC: Non-Small Cell Lung Cancer.

PBS: Phosphate Buffered Saline.

PCR: Polymerase Chain reaction.

PEG: Poly Ethylene Glycol.

PHA-P: Phytohaematogglutinin-P.

PTEN/MMAC1: Phosphatase and Tensin homologue deleted on chromosome Ten/ Mutated in Multiple Advanced Cancers 1.

Rb: Retinoblastoma gene.

RGP: Radial Growth Phase.

Rn: Normalised Reporter Signal.

Rpm: revolutions per minute.

RQ: Relative Quantification.

RT-PCR: Reverse Transcription Polymerase Chain Reaction.

SCC: Squamous Cell Carcinoma.

SCID: Severe Combined Immunodeficiency.

SCLC: Small Cell Lung Cancer.

SFM: Serum Free Medium.

STS: Short Tagged Sequences.

TBE: Tris/Borate/EDTA.

TGF: Transforming Growth Factor.

TIL: Tumour Infiltrating Lymphocytes.

TSG: Tumour Suppressor Gene.

UV: Ultraviolet.

VGP: Vertical Growth Phase.

CHAPTER 1

INTRODUCTION

1.1. Skin cancer

1.1.1. Incidence

Skin cancer is one of the most common forms of cancer. In fact, one in three cancers are skin-related (WHO, 2005). The incidence is increasing rapidly making it a major health problem in several countries. Official figures show that the occurrence of skin cancer has doubled in the past decade in the UK which makes it the seventh most common form of cancer. There were 70,038 cases registered in the UK in 2001 including 7,300 cases of melanoma (Cancer Research UK, 2005). The same source indicates that not only the number of skin cancer patients is increasing but also the mortality. In fact, in 2003, 2,280 people died from skin cancer of which 1,770 were attributable to malignant melanoma.

In the United States, skin cancer accounts for about half of all cancers and affects approximately one million Americans every year (American Cancer Society, 1999). Moreover, it is estimated that approximately 60,000 new cases of

melanoma have been reported and 8,110 melanoma related deaths in 2007 (Jemal *et al.*, 2007). However, Australia has the highest skin cancer incidence in the world where over 382,000 people are treated for skin cancer and over 1400 die each year (The Cancer Council Australia, 2006).

1.1.2. Skin biology and structure

The skin is the largest organ of the body. It has several roles, it is responsible for shielding the rest of the body from excessive light and extreme temperatures (Poole and Guerry, 2005), protecting the body from infection and injury. The skin is kept clean from excess of deleterious substances by the action of sweat glands. The human skin is composed of two layers: epidermis (outer layer) and dermis (inner layer), and below them is the subcutaneous layer also called subcutis (Figure 1.1).

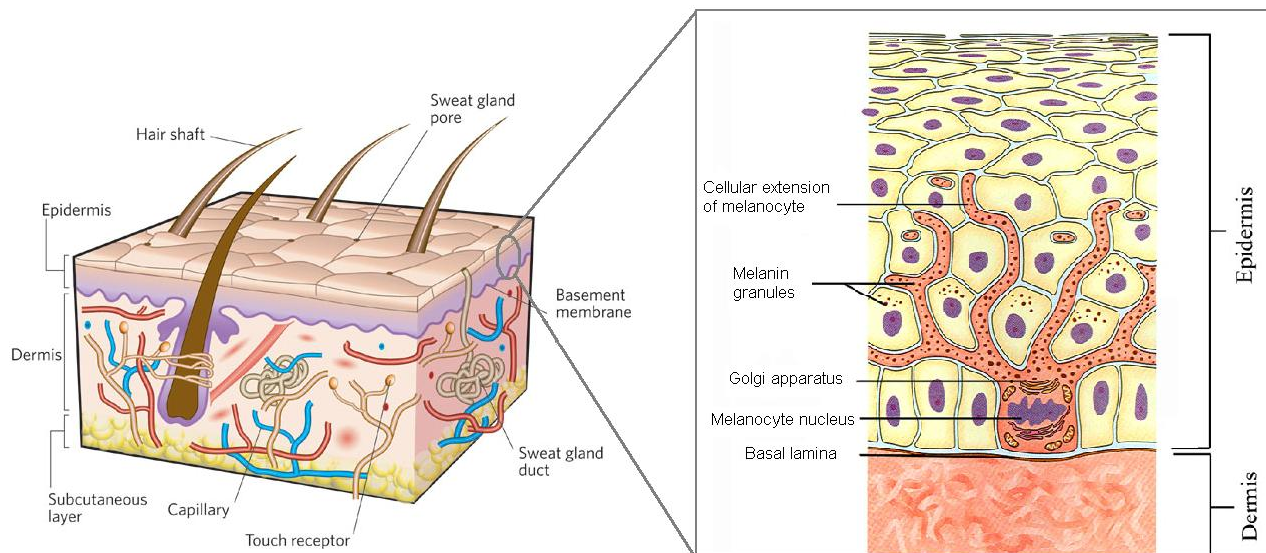


Figure 1.1: A diagram showing the structure of the skin. The outer surface is the epidermis and the lower layer is the dermis much thicker and composed of connective tissue, blood vessels, nerve endings, hair follicles, sweat and oil glands. However, most skin cancers develop from the epidermis (image from MacNeil, 2007).

Most skin tumours arise from the epidermis which contains three different cell types that correspond to the 3 types of skin cancer. In other words, the names Basal Cell Carcinoma, Squamous Cell Carcinoma and Cutaneous Malignant Melanoma originate from the types of cells that they derive from. For instance, melanoma is the uncontrolled growth of melanocytes located in the lower part of the epidermis (Figure 1.2). Melanocytes function is the synthesis of the pigment

called melanin that gives the skin its colour (Montagna and Prakkal, 1974; Slominski *et al.*, 2001).

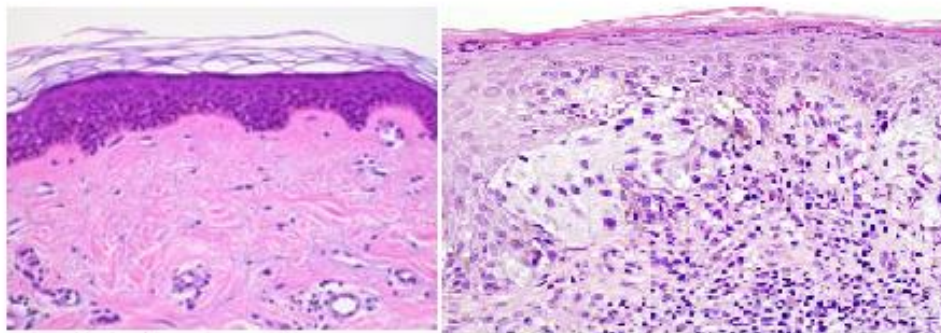


Figure 1.2: Histology of a normal skin (left): the dermis and epidermis are clearly delineated with a non proliferative layer of melanocytes between them. Histology of melanoma skin (right): Highly proliferative melanocytes represented by dark staining cells (stained with hematoxylin and eosin, and viewed on a microscope slide, from www.Pathologypics.com)

1.1.3. Types of skin cancer

There are three common types of skin tumour:

1.1.3.1. Basal Cell Carcinoma (BCC):

First described in 1827, BCC is the most common malignancy, also called basal cell epithelioma but the term carcinoma is widely used to describe this tumour. This type of tumour arises from the basal cells of the epidermis and it can develop into a very large size lesion if neglected by the patient leading to destruction of

the skin or disfigurement at the site of the lesion. It usually occurs on chronic sun exposed sites such as face and neck.

BCC of higher risk for metastasizing are those of long duration, larger than 2cm, located in the mid-face or ear region and metastatic sites have been described most commonly in lung, bone and lymph nodes (Ionescu *et al.*, 2006). However, metastasis appears very rarely in basal cell carcinoma.

Like most skin cancers, sun exposure plays a role in the development and transformation of BCC, in addition there are other risk factors such as exposure to arsenic, coal tar derivative and irradiation (Crowson, 2006). In white populations in USA, the incidence of basal cell carcinoma has increased at more than 10% a year (Miller *et al.*, 1994). Also, 53,000 new cases of BCC are estimated every year in the UK and figures are continuing to rise on a yearly basis (Bath-Hextall *et al.*, 2007).

1.1.3.2. Squamous Cell Carcinoma (SCC):

Squamous cell cancer is a malignant skin lesion that affects the epidermis and is derived from the epidermal keratinocytes. It is usually painless initially, but may become painful with the development of larger and invasive lesions that do not heal if neglected. SCC is common on white and Caucasian skin and it appears to be due to excessive exposure to UV light but it can also arise from industrial exposure such as x-ray, tar or coal (MacKie, 1996).

Cutaneous SCC is divided into categories of low, intermediate and high risk based on the malignant potential and an additional category reserved for those rare subtypes whose origin cannot be accurately assigned (Cassarino *et al.*, 2006). Studies have revealed that in more than 90% of SCCs, as in most BCCs, inactivation of p53 by induced mutagenesis plays a key role in the pathogenesis of both non- melanoma skin cancers (Campbell *et al.*, 1993; Brasch *et al.*, 1997). In addition, SCCs tend to arise from premalignant lesions such as actinic keratoses (AK) also known as solar keratoses. They are extremely common skin neoplasm and develop as a consequence of prolonged exposure to UV radiation. There are strong similarities between AK and SCC both at the histological and molecular levels and it can sometimes be difficult to distinguish between them clinically (Chia *et al.*, 2007). A proportion of 5 to 20% of AKs will progress in 10 to 25 years to superficial squamous cell carcinoma. However, the progression towards SCC might be increased in immuno-compromised patients such as organ transplant recipients who are more likely to develop AKs (Salasche, 2000; Euvrard *et al.*, 1995).

1.2. Cutaneous malignant melanoma

Malignant melanoma represents the fifth and sixth most common cancers in men and women, respectively, in the US. Also, the average lifetime risk is estimated at 1 in 75 that shows increasing rates during the last decades (Pho *et al.*, 2006).

1.2.1. History of melanoma

Melanoma was referred to in ancient literature. Melanotic masses were found approximately 2400 years ago on the extremities of nine Colombian Inca mummies of Peru, also metastases were detected on their bones particularly the skull (Urteaga and Pack, 1966). The first description of melanoma was by Hippocrates in the 5th century BC, who referred to it as black tumour (*melas*: black / *oma*: tumour) (Urteaga and Pack, 1966).

However, the first description of melanoma as a disease was by René Laennec (Faculté de Médecine in Paris) in 1812 (Chin *et al.*, 1998). Furthermore, Norris identified in 1820 that melanoma is a hereditary disease in the case of two family members having tumours originated from moles and other relatives having “many moles of various parts of their bodies” (Palamaras and Stavrianeas, 2004).

1.2.2. Types of cutaneous malignant melanoma

CMM is classified into four principal types that are distinct from each other regarding to etiology, biological properties and prognosis (Weyers *et al.*, 1999). Pictures of the different types of melanoma are shown in Figures 1.3, 1.4, 1.5, 1.6.

1.2.2.1. Superficial spreading melanoma

It is the most common type accounting for 65-70% of diagnosed melanomas and is most frequently localised in the legs for women and the trunk for men especially the back. The growth consists in two phases (biphasic), starting by an initial growth phase and followed by a vertical growth when melanocytes invade deeply the dermis (Figure 1.3).



Figure 1.3: A melanoma of the superficial spreading type showing a flat lesion with irregular borders. The pigmentation varies from light to dark shades (Poole and Guerry, 2005).

1.2.2.2. Nodular melanoma

It is most commonly found in middle-aged people and occurs in the back and chest. It accounts for 12% of all cutaneous melanomas and spreads almost exclusively in a vertical phase only. These nodular lesions are very aggressive and

develop quickly and they tend to be more common in women than men (Figure 1.4).



Figure 1.4: Nodular melanoma that has only vertical growth phase. It appears as a black dome that can however have a dark brown, red or blue colour (Poole and Guerry, 2005).

1.2.2.3. Lentigo malignant melanoma

It is most commonly found on the face and neck, particularly in older people. It grows slowly and may take several years to develop and can remain in a non-invasive form for years but will penetrate into the deeper layers of the epidermis, increasing the potential of metastases (Cancerbackup 2003). It is associated with intense sun exposure and occurs usually in areas of the body that have been chronically exposed to solar radiation (Figure 1.5).



Figure 1.5: A lentigo malignant melanoma on the cheek of an old person showing a bumpy surface which is a marker of vertical growth phase and may be invasion (Poole and Guerry 2005).

1.2.2.4. Acral lentiginous melanoma

It is a form of malignant lentigo melanoma that occurs in palms, soles, and subungual. It is the most common melanoma in dark skinned populations and the least common among caucasians (Figure 1.6).



Figure 1.6: A form of acral lentiginous melanoma that grows under the foot. This type of melanoma does not appear to be related to UV exposure (Poole and Guerry, 2005).

Apart from the skin, melanoma can occur in other locations of the body such as the eye called uveal melanoma which appears as a small freckle beneath the retina and can eventually metastasise.

Other uncommon melanomas are amelanotic (no melanin pigment), desmoplastic which are often amelanotic and the head and neck are the most common location for occurrence (reviewed in Foote *et al.*, 2008). Desmoplastic melanomas are characterised by the presence of atypical spindle cells separated by collagen fibres, occurring usually in individuals with chronic sun damage (Scolyer and Thompson, 2005). Also, mucosal melanomas that are also rare but usually diagnosed in an advance stage because of the location (inside nose, mouth, genital areas).

1.2.3. Etiology and risk factors of melanoma

Environmental, phenotypic and genetic factors interact in the development of melanoma. However, the exact etiology of malignant melanoma is quite complex and the molecular events directly related to melanoma progression are yet to be fully elucidated.

1.2.3.1. Ultraviolet radiation

Although it is a commonly accepted fact that solar radiation (natural or artificial) is a major environmental factor leading to melanoma, the evidence is not straight

forward. It is supported by the fact that the incidence and mortality among Caucasians is increasing proportionally with the intensity of the sun. In other words, the evident correlation between sun exposure and ethnic background is demonstrated by studies showing that people with fair skin living at low latitudes are more likely to develop a skin tumour than dark skinned-populations (Crombie, 1979).

Ultraviolet radiation is composed of UVA with a wavelength of (320-400nm), UVB (280-320nm) and UVC (100-280nm) that is completely blocked by the ozone layers and are therefore not involved in solar-induced carcinogenesis. Although UVB is partially filtered and only a minor amount of radiation reaches the earth, it has been demonstrated that it induces skin cancer. At a molecular level, UVB exposure increases DNA damage, inhibits antioxidants, and suppresses the immune system (Sauter and Herlyn 1998).

The most frequent DNA photoproducts made by UV radiation are the results of joining adjacent cytosines or thymines resulting in the formation of pyrimidine dimers that can be cyclobutane-pyrimidine dimer or pyrimidine pyrimidone (4-6) photoproducts (Brash, 1997). Both photoproducts cause mutations and change the structure of DNA by insertion of adenine opposite the damaged cytosine during DNA replication, in about 70% of cases C is then replaced by T making CC to TT mutations that are distinctive feature of UV-induced mutations (Hutchinson, 1994) (Figure 1.7). Such mutations are commonly associated with p53 inactivation in BCC and SCC. However UV induced signature mutations as

described above are not observed in any known melanoma-associated tumour suppressor gene.

Studies based on animal models have proven that UVB radiation suppresses immune reaction and induces tolerance to antigens. UVB is also known to induce DNA damage and upregulate gene expression through intracellular signal transduction pathways (Ichihashi *et al.*, 2003).

Although UVA is generally less carcinogenic than UVB, it is the major UV radiation that reaches the earth and can damage DNA directly and indirectly by generating oxidative stress. In addition, UVA can induce DNA strand breaks and chromosomes translocations by generating free oxygen radicals in the cell (de Gruijl, 1999). Also, intermittent repeated exposures to sunlight from childhood are epidemiologically shown to be major cause of melanoma.

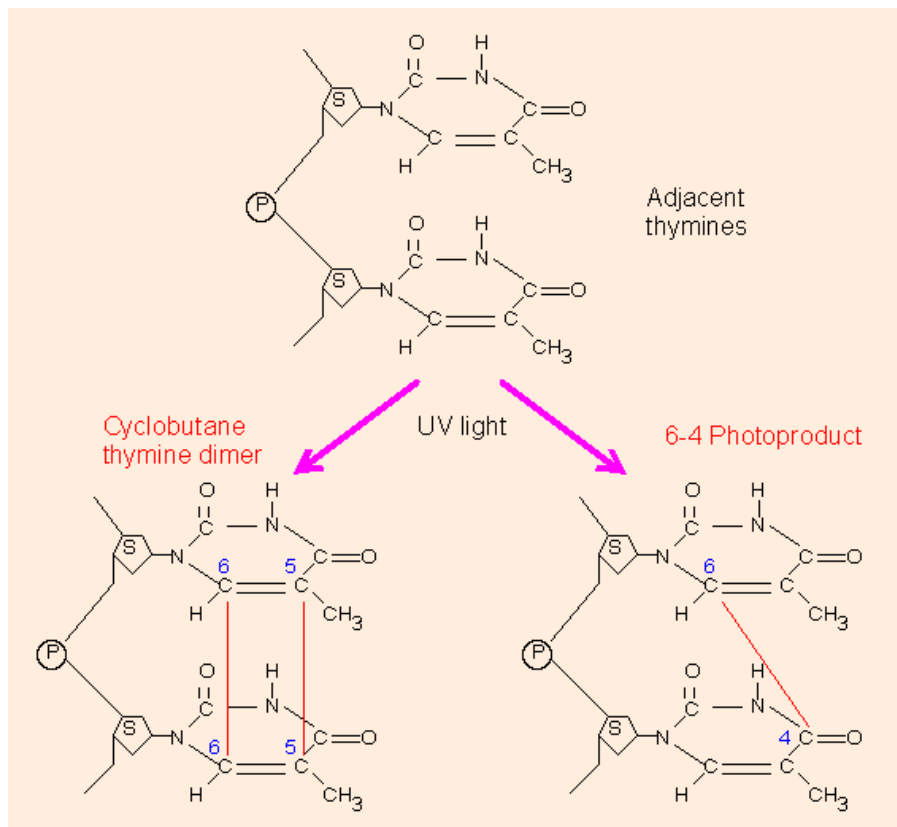


Figure 1.7: UV radiation induces formation of cyclobutane pyrimidine dimers (linking carbons 5 and 6 of each pyrimidine) and 6-4 photoproducts (adjacent thymine or cytosine bases are photoactivated forming a 4-6 link) (Web Book Publishing, 2007).

1.2.3.2. Oxidative damage

Mutations caused by oxygen free radicals can alter the function of oncogenes, tumour suppressor genes and important house keeping genes and potentially leading to loss of cell cycle control and transformation of melanocytes (Hussein, 2005).

Cellular stresses induced for instance by DNA damage may activate the tumour suppressor gene P53 and cause accumulation of p53 in the nucleus which can lead to G1 cell cycle arrest.

The transition from G1 to S phase is controlled by p53, therefore cells with DNA damage will undergo a repair before progression to DNA replication in S-phase of the cell cycle. If the damage is not repaired, the cells may progress towards apoptosis or programmed cell death (Levine *et al.*, 1994). In non-melanoma skin cancer such as BCC and SCC, alterations in the P53 tumour suppressor gene are early events involved in the progression and are often point mutations that are UV-related such as CC-TT tandem base substitutions (Ziegler *et al.*, 1994).

In melanomas, P53 gene mutations are rare and late events that occur during progression to a higher step of malignancy (Hussein *et al.*, 2003). Despite the strong evidence of the implication of UV radiation in the initiation of BCC and SCC, the role of UV concerning the development of CMM is still unclear.

1.2.3.3. Skin Type

Besides environmental factors, endogenous factors play an important role in skin cancer development such as skin type (fair skin populations are more prone to develop skin cancer), genetic predisposition (familial melanoma) and immune deficient status.

1.2.3.4. Familial predisposition

The other significant risk factor for melanoma mentioned above is familial predisposition, which is described mostly by the inheritance of mutations in

highly-penetrant genes (Thompson *et al.*, 2005). In the majority of these families, the propensity for melanoma is associated with the presence of abnormal melanocytic naevi, the so-called atypical mole syndrome (AMS) phenotype (Newton, 1994). Familial predisposition for melanoma accounts for approximately 10% of the cases of melanoma (Greene and Fraumeni, 1979) and is believed to be associated with familial aggregation of dysplastic nevi (Dysplastic Nevus Syndrome DNS). However, studies based on inherited melanoma have been instrumental in leading to a better understanding of the genes involved in melanoma progression, by identifying the genetic alterations and cytogenetic losses involving many chromosomal regions.

1.2.4. Development and pathogenesis of cutaneous malignant melanoma

It is important to detect a melanoma at its early stages in order to maximise treatment options. Most melanoma cases can be diagnosed clinically by the recognition of the symptoms of the so-called ABCDE system which corresponds to: Asymmetry, Border irregularity, Color variation, Diameter and Elevation (Poole and Guerry, 2005).

However, the prognosis of a patient diagnosed with melanoma depends on two factors: the thickness of the primary tumour and the presence or absence of metastasis to regional lymph nodes (Thompson *et al.*, 2005). Therefore, the survival rate of the patient is closely linked to the stage of tumour development. In 2002, a staging system was proposed by the American Joint Committee on Cancer

(AJCC 2002) estimating melanoma-survival rates at different stages as shown in

Table 1.1:

Stages	Phase	Survival Rates	
		5 years	10 years
I	RGP	93%	85%
II	RGP	68%	55%
III	VGP	45%	36%
IV	VGP+metastasis	11%	6%

Table 1.1: Melanoma-specific survival estimates, according to the 2002 AJCC system (Thompson *et al.*,2005).

As shown in the table above, there are 4 melanoma stages corresponding to the tumour thickness and ulceration. Stages I and II represent the radial growth phase and the stages III and IV characterise the vertical growth phase. Also, the stage IV of a melanoma tumour is characterised by the metastatic phase which is the invasion or spread of the tumour to other sites of the body, mostly in visceral sites such as lung, liver, brain, bone and small intestine (Chin *et al.*, 1998).

Melanoma develops as a result of an accumulation of changes in the genetic pathway that controls the division and development of the melanocytes, these changes are the result of genetic alterations occurring in oncogenes, tumour suppressor genes or DNA repair genes.

There are a number of factors influencing the changes from a benign melanocyte to the proliferation into a melanoma. This initiation occurs at both molecular and chromosomal levels and can be applied to most cancer types. The diagram below (Figure 1.8) shows these various factors, the events can happen in an overlapping manner (like the growth and resistance to apoptosis) or can be extended in time (tumour initiation to invasion and metastasis).

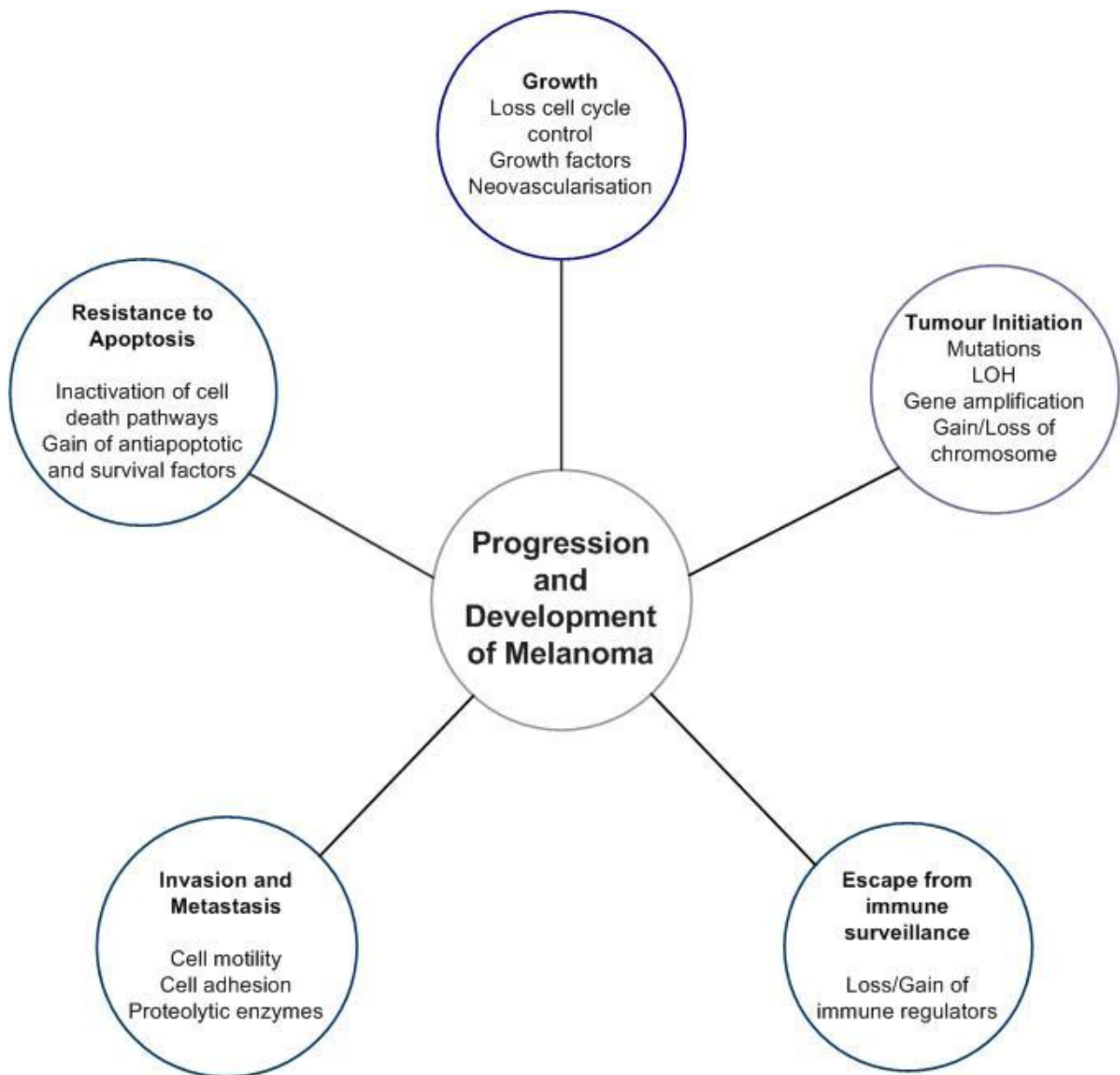


Figure 1.8: Molecular pathogenesis of melanoma (adapted from Carlson *et al.*, 2005). Various factors leading to the progression and development of cancer in general.

1.3. Molecular Genetics of Cutaneous Malignant Melanoma

Recent advances in molecular biology have led us to a much better understanding of the biological events of skin cancer formation. As it is the case for most cancers, RB, p53, and RAS pathways appear to play prominent roles in the pathogenesis of several skin cancer types (Tsai and Tsao, 2004). However, unlike most cancers, mutations affecting p53, the retinoblastoma gene product RB, PTEN are rare or relatively late events in melanoma progression (Carlson *et al.*, 2005).

Although more than 90% of melanomas are sporadic rather than familial, several studies have been conducted on individuals with familial predisposition to melanoma in order to identify the genetic basis for melanoma susceptibility (Pho *et al.*, 2006). In addition, the analysis of susceptibility genes in patients with dysplastic nevus syndrome (DNS) or multiple atypical naevi are strong markers for melanoma risk irrespective to family history (Thompson *et al.*, 2005).

Karyotype analysis and cytogenetics studies of melanoma have revealed genetic changes in different chromosomes which include:

1p, 6q, 9p, 10q and 11q.

Consistent deletions and loss of heterozygosity (LOH) in 1p, 6q and 9p suggest the involvement of tumour suppressor genes in these regions (Smedley *et al.*, 2000). The genetic changes at these locations are discussed below and summarised in Table 1.2.

1.3.1. Chromosomal aberrations in melanoma

- Chromosome 1 abnormalities in CMM

Chromosome 1 aberrations are found in a number of various cancers including cutaneous melanoma. A study conducted by Goldstein and colleagues in 1993 on a set of 13 families with CMM and DN (dysplastic naevus) revealed a melanoma susceptibility locus on chromosome 1p36. Loss of heterozygosity has been located in the short arm of chromosome 1, in the region flanked by the Pronatrodilatin (PDN) and the anonymous marker (D1S47) using multipoint linkage analysis on 99 relatives of 6 families having cutaneous melanoma susceptibility (Bale *et al.*, 1989).

In addition, it was demonstrated that LOH at loci in the distal region of 1p appears to accumulate during tumour progression (Dracopoli *et al.*, 1989). In other words, alterations in chromosome 1 arise late in tumour progression. The transfer of chromosome 1 into highly metastatic human malignant melanoma cell line suppressed metastasis without altering tumourigenicity (Miele *et al.*, 1996).

- Chromosome 6 abnormalities in CMM

Trent *et al.*, in 1989 presented in a report some of the various alterations of a recurring translocation site involving the long arm of chromosome 6 (6q) in

malignant melanoma and especially a translocation (t) between chromosomes 1 and 6.

Different studies have also shown that loss of genetic material on chromosome 6 is associated with the progression of human melanomas, and that the introduction of chromosome 6 into metastatic human melanoma cell lines suppresses metastasis without affecting the ability of the hybrids to form progressively growing tumours. This suggests the evidence of the presence of a tumour suppressor gene(s) on 6q (Goldberg *et al.*, 2003).

It has also been demonstrated that the deletions on 6q are related with the losses on 9p and 10q which suggests that 6q losses occur after initiation of tumourigenesis (Walker *et al.*, 1995).

- Chromosome 10 abnormalities in CMM

A region on the long arm of chromosome 10 (10q24-26) has attracted substantial attention due to its involvement in a wide spectrum of human cancers (Chin *et al.*, 1998). Another study has suggested the fact that although segmental deletions are relatively rare, they have been useful in defining a common region of loss of heterozygosity and chromosomal rearrangements spanning 10q24-27 (Robertson *et al.*, 1998).

The region 10q23 is also deleted or mutated in a wide range of advanced tumours including melanoma. It has been identified that alterations in the tumour suppressor gene PTEN/MMAC1 (Phosphatase and Tensin/Mutated in Multiple

Advanced Cancers 1) occur in some metastatic melanomas, and that mutations of this gene plays a role in the progression of some forms of melanoma (Celebi *et al.*, 2000).

- Chromosome 11 abnormalities in CMM

In 1996, a report published by Tomlinson and colleagues suggested that multiple melanoma tumour suppressor genes may exist on chromosome 11, and also play a role in other types of cancer.

Cytogenetic analysis of a number of patients with regional or disseminated melanoma provides the evidence of chromosomal breakpoints at 11q23-ter (Thompson *et al.*, 1995).

The aim of a study conducted by Utikal and associates in 2005 was to evaluate cytogenetic aberrations of the gene cyclin D 1(CD1) locus at chromosome 11q13 in cutaneous malignant melanoma. Cyclin D1 is associated with progression through G1 and entry into S phase of the cell cycle, and acts as a partner of cyclin-dependent kinases (CDKs). The results obtained have suggested that cyclin D1 could well be involved in the pathogenesis of a subset of malignant melanoma. Also, results suggested that CD1 may be an oncogene in melanoma and that targeting the expression of this gene can have beneficial effect in therapeutic treatment for melanoma (Sauter *et al.*, 2002). Additional studies will have to elucidate the role of further genes located at chromosome 11q13.

Table 1.2 is a summary of some of the chromosomal rearrangements that occur at different locations. The chromosomal changes in 9p and 10q are generally early events in the melanoma progression. The alterations detected in 1p and 6q occur after the initiation of melanoma tumourigenesis and are considered as later events.

Chr	Mutations	Regions	LOH	References
1	Deletions, Breakpoints, LOH	1p36; 1p22-q21	22%	Bale <i>et al.</i> , 1989 Dracopoli <i>et al.</i> , 1989 Walker <i>et al.</i> , 1995
6	Deletions, translocations, 6p isoforms, LOH	6q22-27	30%	Trent <i>et al.</i> , 1989 Cowan <i>et al.</i> , 1986 Walker <i>et al.</i> , 1995
9	LOH, deletions, recombinations	9p21	50-60%	Hussein 2004 Walker <i>et al.</i> , 1995
10	LOH, chromosomes rearrangements	10q24-26	32%	Robertson <i>et al.</i> , 1998 Walker <i>et al.</i> , 1995
11	Deletions, loss of chromosome, LOH	11q23-ter	25%	Thompson <i>et al.</i> , 1995 Robertson <i>et al.</i> , 1996b Walker <i>et al.</i> , 1995

Table 1.2: Chromosomal aberrations in cutaneous malignant melanoma.

1.3.2. Melanoma and the 9p21 region

Carcinogenesis is the combined effect of the inactivation of tumour suppressor genes and activation of oncogenes. The inactivation of TSGs happens either by acquiring somatic mutations or by the presence of inherited mutations. Based on the two hit hypothesis, the inactivation of TSGs requires two genetic events to inactivate both alleles of the gene. Evidence of the presence of TSGs can be obtained from tumour loss of heterozygosity (LOH) studies, which can be the result of chromosomal deletions, mitotic recombination, non-disjunction, or unbalanced translocation (Hussein, 2004).

Loss of heterozygosity studies have revealed the existence of tumour suppressor gene(s) in chromosome 9. Several studies of cytogenetics, linkage, and molecular analyses of familial and sporadic melanomas provided strong evidence that loss of a tumour suppressor residing at 9p21 represents a critical step in the development of almost all melanomas (Chin *et al.*, 1998). The INK4A locus on 9p21 encodes for the best-characterised high-penetrance gene p16. In addition, various studies have demonstrated that some of the genes implicated in cutaneous malignant melanoma map to 9p21 which include p14^{ARF}, p16 (CDKN2A/MTS1/INK4A) and p15 (CDKN2B, MTS2/INK4B) (Haber, 1997) (Figure 1.9). The INK4 locus encodes two distinct tumour suppressor proteins: p16 composed of exon 1 α , 2 and 3, and p14^{ARF} which is a product of an alternatively spliced transcript with coding regions composed of exons 1 β and 2 (Pho *et al.*, 2006). Also, The p15 (INK4b)

gene is located adjacent to p16 (INK4a) on 9p21 and is codeleted in a high proportion of established human cancer cell lines (Kamb *et al.*, 1994).

The p16 gene is deleted or mutated in more than half of cell lines derived from sporadic melanoma tumours (Kamb *et al.*, 1994; Kumar *et al.*, 1998). Germline mutations for the p16^{INK4a} gene are found in about 25% of families with inheritable melanoma (Carlson *et al.*, 2005). In addition to mutations and LOH, promoter methylation is a common mechanism of silencing p16 gene activity in sporadic melanoma (Carlson *et al.*, 2005).

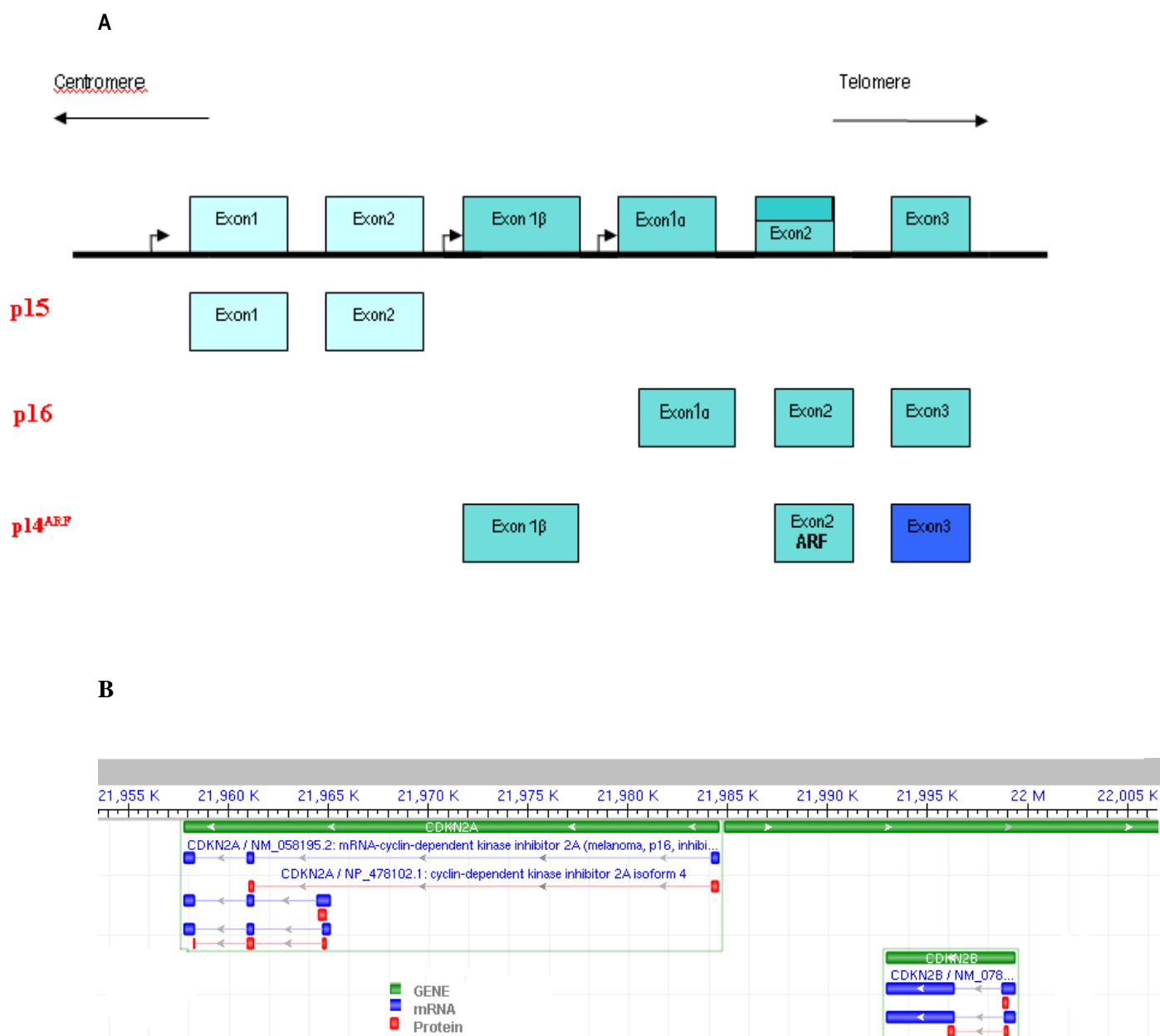


Figure 1.9: **A:** The INK4 locus in chromosomal region 9p21. The INK4 contains two genes: CDKN2A encodes for two transcripts p16 (Exons 1 α , 2 and 3) and p14^{ARF} (exon 1 β , 2 containing an alternative reading frame, and exon 3 untranslated coloured in blue). The other gene is CDKN2B that encodes for p15 composed from exon1 and 2 represented here in light blue boxes (Adapted from Haber, 1997). **B:** schematic view of the CDKN2A and CDKN2B genes (in green) showing exons (in blue) sizes and intronic distances using the NCBI sequence view.

Cell cycle control is dependent on complex interaction between cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors.

The cyclin-dependent kinases (CDKs) are enzymes that regulate a number of target molecules by phosphorylation, which will lead to DNA replication and mitosis. The CDKs are activated by a group of positive regulators called cyclins and inhibited by the proteins: p15, p16, p21 and p27 (Kamb *et al.*, 1995).

The complex action of CDKs and cyclins phosphorylates the Retinoblastoma RB gene (13q14). Phosphorylation of RB occurs through the action of cyclin D1/CDK4-6 in early G1 phase of the cell cycle, by cyclin E/CDK2 in late G1 and early S, and by cyclin A/CDK2 in S phase. At its unphosphorylated state, Rb does not act directly to control transcription but it interacts with the transcription factor E2F, inhibiting transcription and suppressing cell growth. However, phosphorylated RB (pRB) by the complex cyclin/CDK action does not bind to E2F allowing the initiation of the transcription (Figure 1.10). When p16 is defective, it is unable to inactivate CDK4 and CDK6, which phosphorylates Rb, releasing the transcription factor E2F and leading to the cell cycle progression (Serrano, 1997).

The alternative product of the gene CDKN2A p14^{ARF} has been implicated in melanoma however strong evidence is lacking. One of the identified functions of p14^{ARF} is stabilising p53 through inhibition of MDM2 activity, although p53-independent activities of p14^{ARF} and can be upregulated by oncogenes such as RAS and MYC (Lowe and Sherr, 2003). In addition, Robertson and Jones (1998) have proposed the existence of an autoregulatory feedback loop which allows

tight control over the levels of p53 in normal cells. In other words, ARF is considered as a critical element of the p53 pathway, and it increases the level of functional p53 through interaction with the proto-oncogene MDM2 leading to apoptosis and cell cycle arrest Robertson and Jones (1998).

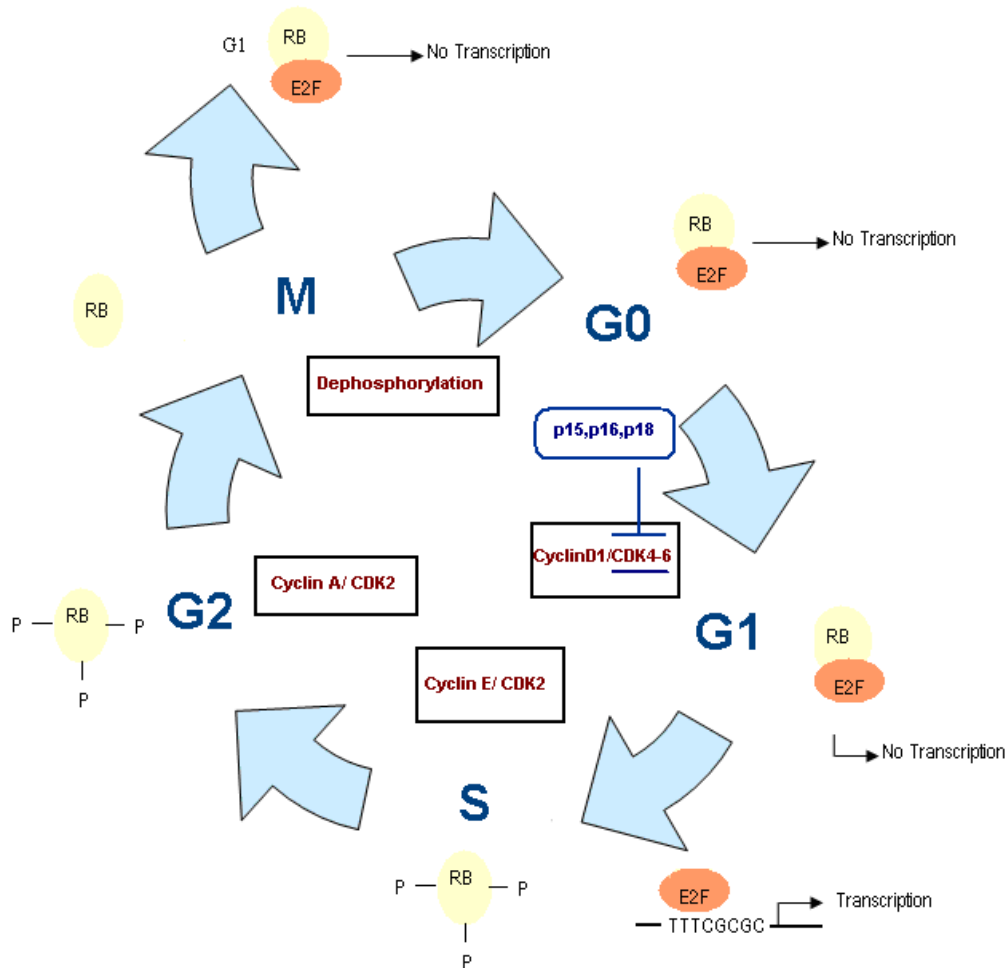


Figure 1.10: Cell cycle. The association of the cyclins and the cyclin-dependent kinases phosphorylates the protein Rb. Phosphorylated pRB doesn't interact with E2F, therefore it will induce the transcription resulting into the progression from G1 to S phase. The INK4 family of tumour suppressor genes p15, p16, and p18 inhibits the interaction of cyclins/CDK, RB remains unphosphorylated and interacts with E2F, and consequently the cell cycle is arrested at G1/S. (Adapted from MacDonald and Ford, 1997).

1.3.3. Melanoma susceptibility genes

The literature suggests that a number of genes are involved in the predisposition to melanoma. Studies conducted on a proportion of familial melanoma cases have demonstrated the existence of high-penetrance genes (conferring a high-risk of developing CMM) and low-penetrance genes (conferring a low-risk of developing CMM).

1.3.3.1. High-penetrance susceptibility genes:

CDKN2A

CDKN2A (cyclin-dependent kinase inhibitor 2A) was the first melanoma susceptibility gene to be identified (Kamb *et al.*, 1994) and it codes for two unrelated proteins known as p16 and p14ARF (alternative reading frame). P16 acts as a tumour suppressor by arresting cells at the G1 phase, ultimately leading to the negative regulation of cell growth. More specifically, p16 inhibits the activity of the cyclin D1 complex by preventing its interaction with cyclin-dependent kinase 4 (CDK4) or CDK6. Allowing cyclin D1 to interact with CDK4 or 6 results in the phosphorylation of the retinoblastoma protein (Rb) (leading to cell cycle progression beyond the G1 phase). However, since p16 blocks these interactions, it is able to act as a tumour suppressor. P14ARF on the other hand behaves as a tumour suppressor by enhancing apoptosis and blocking oncogenic

transformation. Both of these proteins act on the Rb and p53 pathways; two critically important pathways underlying cell cycle control.

Although CDKN2A is an important melanoma susceptibility gene, the incidence of germline CDKN2A mutations in the general population is remarkably low (Hayward, 2003). Furthermore, the occurrence of CDKN2A mutations in familial cases is only 20-40% (Hayward, 2000), which suggests the possible involvement of other candidate genes that may be implicated in melanoma.

CDK4

The cyclin dependent kinase 4 gene is an oncogene located in 12q13 and encodes for the CDK 4 which is one of the targets of p16. Although few cases of CDK4 mutations have been reported, familial melanoma studies have demonstrated mutations in codon 24: R24C (Arginine to Cysteine) and R24H (Arginine to Histidine) changes (Zuo *et al.*, 1996; Soufir *et al.*, 1998), these changes occur exclusively at the binding domain; the arginine normally present at this location is important for the binding of p16. Therefore, these mutations induce the loss of cell-cycle regulation downstream of p16.

1.3.3.2. Low-penetrance susceptibility genes:

MC1R

The Melanocortin-1 receptor gene, located on 16q24, is the most common low-penetrance melanoma-susceptibility gene so far identified. MC1R codes for the receptor of α -melanocyte stimulating hormone (α -MSH), the binding of α -MSH and MC1R results in the stimulation of production of eumelanin (brown/black pigments over pheomelanin (red/yellow pigments) (Suzuki *et al.*, 1999).

MC1R is involved in the pigmentation pathway, and therefore appears to play a key role in hair colour, skin type and response of the skin to tanning.

In 1995, Valverde and colleagues have shown that MC1R is highly polymorphic, and reported the presence of variants for this gene in humans. It appears that there are more than 20 allelic variants and some are associated with increased melanoma susceptibility because they change skin pigmentation and tanning ability. Three particular variants of MC1R are associated with red hair and fair skin [R151C (Arginine to Cysteine), R160W (Arginine to Tryptophan), D294H (Aspartic acid to Histidine)] (Box *et al.*, 1997).

Other melanoma susceptibility loci have been mapped, although the putative causal genes at these loci have yet to be identified (Fargnoli *et al.*, 2006). Some of these loci are: CDKN2A polymorphisms, EGF (epidermal growth factor gene), GSTM1 (glutathione S-transferase gene), CYP2D6 (cytochrome P450

debrisoquine hydrolase locus), VDR (vitamin D receptor gene) (reviewed in Hayward, 2003).

1.3.4. Pathways involved in melanoma

The transformation of a normal melanocyte to a malignancy results from an abnormal proliferation of the cells that have lost cycle regulation mechanisms. Oncogenes and tumour suppressor genes control the regulation of the cell cycle via a number of interactions between the proteins in the form of activation/inhibition pathways. There are two major cell cycle regulatory pathways that are altered in melanoma, the RAF-MAPK pathway and Pi3K-Akt pathway which involve the retinoblastoma (Rb) and p53 tumour suppressor gene.

An extensive knowledge of cell cycle regulatory mechanisms will increase the understanding of the process by which a normal cell develops into a malignancy (Li *et al.*, 2006). The mammalian RAS gene family consists of three members which are located on different chromosomes: H-RAS and K-RAS genes are the cellular counterparts of the viral Harvey and Kirsten genes, respectively, and the N-RAS gene is derived from a human neuroblastoma cell line (Bar-Sagi, 2001).

The RAS genes are signal transducers frequently mutated in human cancers. Recent studies have estimated that 10-20% of the RAS gene family, and more specifically NRAS, are mutated in human melanoma (Gorden *et al.*, 2003). Alterations of oncogenic RAS activate both the PI3K and RAF cascades which have been strongly associated with melanoma development. The frequent loss of

the tumour suppressor gene PTEN (Phosphatase and TENsin homolog) in melanocytic lesions is evidence of the involvement of PI3K-Akt pathway in the progression of the malignancy. LOH in chromosome 10q has been extensively studied and found to account for 30-50% of melanoma (Herbst *et al.*, 1994). PTEN has both lipid phosphatase and protein phosphatase activities that inhibit Akt activation by PI3K. The Akt protein, when phosphorylated, acts mainly as an antagonist of apoptosis (Haluska *et al.*, 2006).

In other words, PTEN reduces PIP3 level (Phosphatidylinositol tri-phosphate) and acts downstream on serine/threonine Akt by decreasing its activity, therefore PTEN is a negative regulator of the PI3K-AKT pathway. Inactivation of PTEN results in the hyperphosphorylation of Akt which increases cell proliferation and enhances resistance to apoptosis (Wu *et al.*, 2003).

However, it is not yet clear whether PTEN loss of activity occurs at an early or late stage in melanoma progression.

The role of RAF-MEK-ERK pathway in melanoma progression has been the main focus of several studies in the past years, especially after the identification of BRAF gene mutations in a number of melanomas. In fact, mutations of BRAF gene have been estimated at 67% by Davies and colleagues in 2002. The most frequent mutation in the BRAF gene is V599E, and occurs at the BRAF kinase domain which leads to elevated protein activity and results in increasing activation of ERK. Activation of the RAF-MEK-ERK pathway is a critical component of tumour cell proliferation and survival in general (Gordon *et al.*, 2003). In addition to the high rate of BRAF mutations (approximately 70%),

expression of Brn-2 POU domain transcription factor has been shown to be high in melanoma cell lines but not in melanocytes resulting in increased proliferation (Eisen *et al.*, 1995). It has been found that high levels of Brn-2 expression might be triggered by activation of the MAP kinase cascade arising from mutations in the BRAF gene resulting in increased proliferation (Goodall *et al.*, 2004).

Gene type	Gene	Frequency	Type of modification
Oncogenes	BRAF	70%	Substitution (V599E)
	NRAS	10-20%	Mutation (codon61)
Tumour suppressor genes	CDKN2A	30-70%	Deletion, silencing, mutation
	PTEN	20-40%	Deletion, mutation
	P53	10%	Loss, mutation

Table 1.3: Summary of main genetic alterations in cutaneous malignant melanoma.

1.3.5. Epigenetic events in melanoma

Modifications in DNA methylation are regarded as epigenetic events rather than genetic changes because they do not alter the genetic code. Recent studies have demonstrated the existence of a strong correlation between methylation alterations and transcriptional inactivation. In addition, genetic alterations as well as epigenetic events can contribute to the carcinogenic process (Strathdee, 2002).

DNA methylation occurs at the position 5 of cytosine residues in the pyrimidine ring of CpG dinucleotides (Cytosine-phosphate-Guanine sites) by the covalent addition of a methyl group (-CH₃) by DNA methyltransferases (Figure 1.11). CpG

islands are regions of DNA that contain high percentage of CG nucleotides and are commonly found near the promoter region of the genes. DNA methylation has been reported to regulate gene expression by transcriptional silencing when occurring at CpG islands (Razin and Riggs, 1980).

Methylation of CpG rich sequences of the promoter region of the genes and the role that epigenetic events play in the development of various cancers have recently become an important area of investigation in assessing the mechanisms of tumour suppressor and regulatory gene inactivation (Hoon *et al.*, 2003) and are at least as important as the inactivation of tumour suppressor genes and activation of oncogenes in the progression of cancer (Jones and Baylin, 2002).

In normal somatic cells, CpG islands are usually unmethylated in the promoter regions of tumour suppressor genes. However during cancer progression there is an aberrant hypermethylation of these regions which lead to effective transcription silencing of the selected tumour suppressor genes (Robertson, 2005).

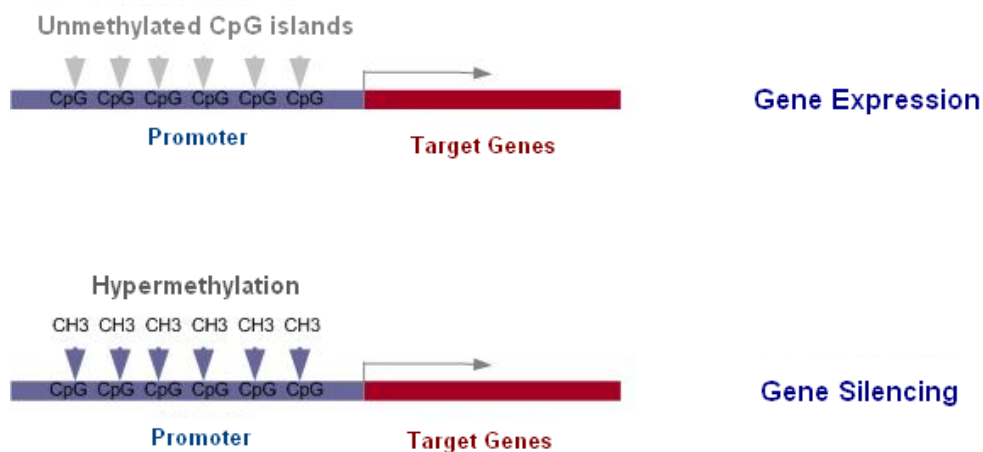


Figure 1.11: A promoter region showing methylated CpG islands which results in transcriptional silencing of several target genes such as p16, p14, MTAP, Apaf-1 for melanoma.

Studies of malignant melanoma have revealed that at least 50 genes have been identified to be silenced by promoter hypermethylation during disease development and progression (Gallagher *et al.*, 2005; van der Velden *et al.*, 2003). The most well known gene involved in the progression of melanoma is CDKN2A tumour suppressor which has been shown to be regulated via chromosomal deletions of the 9p21 region, inactivating mutations and also by promoter hypermethylation (van der Velden *et al.*, 2001).

Van der Velden and colleagues showed in 2001, that the p16 promoter was hypermethylated in 50% of uveal melanoma cell lines and 32% of primary uveal melanomas. Moreover, another study reported the loss of p16 protein expression by hypermethylation of the CDKN2A gene promoter in 19% of primary cutaneous melanomas and 33% of metastases derived from melanoma (Straume *et al.*, 2002).

Another tumour suppressor gene MTAP (Methylthioadenosine phosphorylase) has been demonstrated to carry a homozygous deletion in only one out of nine melanoma cell lines and all others showed a reduction of MTAP expression due to promoter hypermethylation of MTAP gene (Behrmann *et al.*, 2003). In addition, the same study demonstrated the existence of an inverse correlation between MTAP protein levels and tumour progression: MTAP expression decreased from benign melanocytic nevi to primary melanoma and to metastatic melanoma *in vivo*. In a same manner, another important tumour suppressor whose expression is negatively correlated with the development of malignant melanoma is Apaf-1

which is a cell death effector that mediates p53-dependent apoptosis (Soengas *et al.*, 2001).

The use of methylation inhibitors or demethylating agents such as 5-azacytidine and its derivative 5-aza-2'-deoxycytidine (5Aza-dC) has been a useful tool to demonstrate the gain of expression of target genes that have been silenced by DNA methylation in the promoter regions. Azacytidine is incorporated in the DNA during replication and inhibits methyltransferase activity because the enzyme becomes irreversibly bound to the azacytidine residues into the DNA (Christman, 2002).

Following treatment with (5Aza-dC), mRNA transcripts could be re-expressed in melanoma cell lines having hypermethylated genes (Hoon *et al.*, 2004). These agents have been used as potential chemotherapeutics agents for cancer because of their ability to restore gene function to treated cells in culture and patients with malignant diseases (Cihak, 1974). In a study conducted by Muthusamy *et al.* (2006), 17 genes have been screened in a microarray-based assay showing a re-expression following treatment with 5AzadC. Similar results for the gene MTAP re-expression have been demonstrated after exposure to 5-azacytidine in combination with interferon- γ (Rothhammer and Bosserhoff, 2007).

1.4. Treatment of cutaneous malignant melanoma

1.4.1. Surgical excision

The current treatment options for melanoma are surgical excision, which is the first step of treatment. This surgery depends on the tumour thickness and stage of progression of the disease, melanoma *in situ* or stage I and II are usually treated with surgical removal of the lesion and surrounding tissues (margins), and can also cure stage III melanoma if the lymph nodes are removed. However, once the tumour has spread from the skin to distant organs, it is unlikely to be curable with surgical excision.

Regional recurrence has been associated with the excision margin, as studies have demonstrated that a 1-cm margin of excision of a tumour of 2 mm thickness is associated with a significant greater risk of regional recurrence than an excision of 3-cm margin; however the survival rate is similar (Thomas *et al.*, 2004).

In most of cases, further treatment is not needed after surgical excision especially for the early melanomas, since the recovery of the patients is assumed. However, in some cases recurrence of melanoma can occur by appearance of a lesion beside the site of the original one or it may appear in the lymph node draining that area, also the local recurrence rate is significantly affected by the thickness of the primary lesion and the presence or not of ulceration (Karakousis *et al.*, 1996). Many agents have been investigated for treatment of melanoma, but few have

shown promising results. In addition, attempts to improve survival after treatment require considerable effort since some therapies have failed to be successful.

1.4.2. Adjuvant therapy

However, early detection and appropriate treatment such as adjuvant therapy have improved outcomes (Hamm *et al.*, 2007). Adjuvant therapy is the treatment given after surgery and is either chemotherapy or radiotherapy; the purpose of this therapy is to destroy remaining cancer cells after the surgery.

Systemic chemotherapy applied for melanoma cases consists on use of anticancer drugs that can be single agent or multi-drug combinations. The main drug used for treatment of melanoma is Dacarbazine (DTIC) that demonstrated an overall response rate of 6.8-12.1% (Middleton *et al.*, 2000). This drug can be combined to other agents such as carmustine, cisplatin and tamoxifen (Dartmouth regimen) but no proof has been provided yet that multi agent chemotherapy is superior to single-agent DTIC chemotherapy (Lee *et al.*, 1995). Temozolomide is another agent that has an equivalent efficacy to that of DTIC and is for patients with advanced metastatic melanoma (Middleton *et al.*, 2000).

1.4.3. Immunotherapy

In addition, chemotherapy drugs can be combined to immunotherapies including interferon alpha (IFN) and interleukin-2 (IL-2). Cancer immunotherapy stimulates

the patient's immune system to recognize and destroy cancer cells more efficiently. IL-2 is a cytokine that plays an important role in T cell activation and its anti-tumour activity has been demonstrated in a number of murine tumour models (Rosenberg *et al.*, 1985). Data from various trials have shown that single agent IL-2 given in high doses had response rates of 10-20% (Atkins *et al.*, 1999). The latter techniques used for immunotherapy of melanoma use two types of immune cells in combination with IL-2: lymphokine-activated killer (LAK) and tumour-infiltrating lymphocytes (TIL). However, randomised studies have shown that there is no superiority for IL-2 with LAK cells therapy compared to single-agent IL-2 therapy (Tarhini and Agarwala, 2006).

Interferon alpha 2b (IFN- α) is commonly used as a treatment for patients who are at a high risk of recurrence of melanoma, also for adjuvant therapy after surgical removal of the tumour. This immunotherapeutic agent is a cytokine exhibiting a potential anti-tumour effect and is used mainly in phase II and III melanoma patients. Trials using IFN- α as an adjuvant therapy have shown an improvement of overall disease-free survival (from 2.8 to 3.8 years) (Kirkwood *et al.*, 1996).

However, trials of single-agent IFN have demonstrated disappointing results and the same concerning combination of IFN and DTIC (Hamm *et al.*, 2007). In addition, IFN displays a significant toxicity as 25% of patients show moderate to severe flu-like symptoms after completion of IFN therapy (Kim *et al.*, 2002).

Studies have shown that UV radiation is immune suppressive. In other words, the immune suppressive effects acquired from exposure to UV radiation contribute to skin cancer development by depressing cell-mediated immune reactions that

normally serve to destroy the developing skin tumours (Kripke, 1974; Kripke and Fisher, 1976). Highly antigenic properties of UV-induced tumours were identified by the growth of these tumours after transplantation in immune suppressed recipient mice while they were recognised and rejected in normal immune system (Kripke, 1974).

Findings of several studies have supported the hypothesis that UV radiation was having two effects: immune suppression and induction of skin cancer, for instance immune suppressed transplant patients have a high risk of developing skin cancer (Penn, 2000). Therefore, it is clear that there is an association between UV-induced immune suppression and carcinogenesis, and it is critical to identify the mechanisms that suppress immunologic reactions after solar stimulation (Ullrich, 2005). Furthermore, a novel treatment has been developed targeting melanoma-associated antigens (MAA) which are highly immunogenic human antigen that are recognised by cytotoxic T lymphocytes. Downregulation of MAA is one of the mechanisms that aids in evasion of immune surveillance, (van Dinten *et al.*, 2005).

1.4.4. Vaccine therapy

Development of vaccines to treat cancer has attracted a lot of attention especially with the success of vaccines against infectious diseases. But the difference is that cancer vaccines are therapeutic rather than prophylactic and are administered after the occurrence of the disease (Morton and Barth, 1996). In addition in the case of

cancer vaccines, the immune response requires between 12 to 14 weeks and induction of regression requires 6 to months compared to chemotherapeutic agents that induce a regression after 2 to 4 weeks because of their high cytotoxicity (Morton *et al.*, 1992).

Various vaccine strategies are being tested in patients with stage III or stage IV melanoma but have not yet been able to demonstrate a reliable clinical tumour regression and a proven efficacy (Terando *et al.*, 2007).

A number of clinical trials are currently being carried out using new agents that can possibly provide a more effective therapy for cutaneous malignant melanoma such as developing molecular therapy targeting the Ras-MAPK and PI3K/AKT signal transduction pathways, the proteasome, histone deacetylases, methyltransferases, and melanoma-induced angiogenesis (Becker *et al.*, 2006).

A recent clinical trial of Hodi et al 2008 of the drug imatinib mesylate (Gleevec) which targets Kit gene, showed major response of the drug in KIT-mutated melanoma. The same group reported that treatment using Gleevec has also forced metastatic melanoma into remission for the first time.

1.5. Multistep melanoma tumourigenesis model

The clinical, histopathological and biological aspects of cutaneous malignant melanoma and its progression are well recognized. However, the molecular aspects and the exact genetic events involved in the proliferation of melanoma are yet to be identified and understood.

The genetic analysis of malignant melanoma has shown that the progression into a metastatic tumour starts from the transformation of the melanocytes. This progression is controlled by the accumulation of genetic alterations as shown in the figure below.

A model published by Hussein (2004), has proposed to divide the process of melanoma tumourigenesis into a multi-step genetic model. The progression consists in lesional steps from benign naevi to metastatic malignancy.

Multistep tumourigenesis involves the inactivation of TSGs activation of oncogenes and defect in mismatch repair (MMR) genes. These events are poorly understood in melanoma proliferation. However, strong evidence has been provided about the implication of allelic alterations at several chromosomes in the development of cutaneous malignant melanoma and the transformation of melanocytes to a malignant phenotype (Fountain *et al.*, 1990). Recent findings have revealed that melanoma tumourigenesis is a very complex process that involves allelic loss, microsatellite instability, and alterations of TSGs, mismatch repair proteins, oncogenes, and some growth factors (Hussein, 2004) (Figure 1.12). Furthermore, tumour progression markers have been identified such as integrins are transmembrane glycoproteins cell surface receptors that mediate adhesion between the cell membrane and the extracellular matrix (Buck and Horwitz, 1987). Certain classes of integrin expression have been shown to be upregulated during transition from radial to vertical growth phase in melanoma. In particular, integrin $\alpha V\beta 3$ has been identified as a tumour progression marker and

the increase in its expression correlates with the conversion of melanoma growth from radial to vertical (Seftor, 1998).

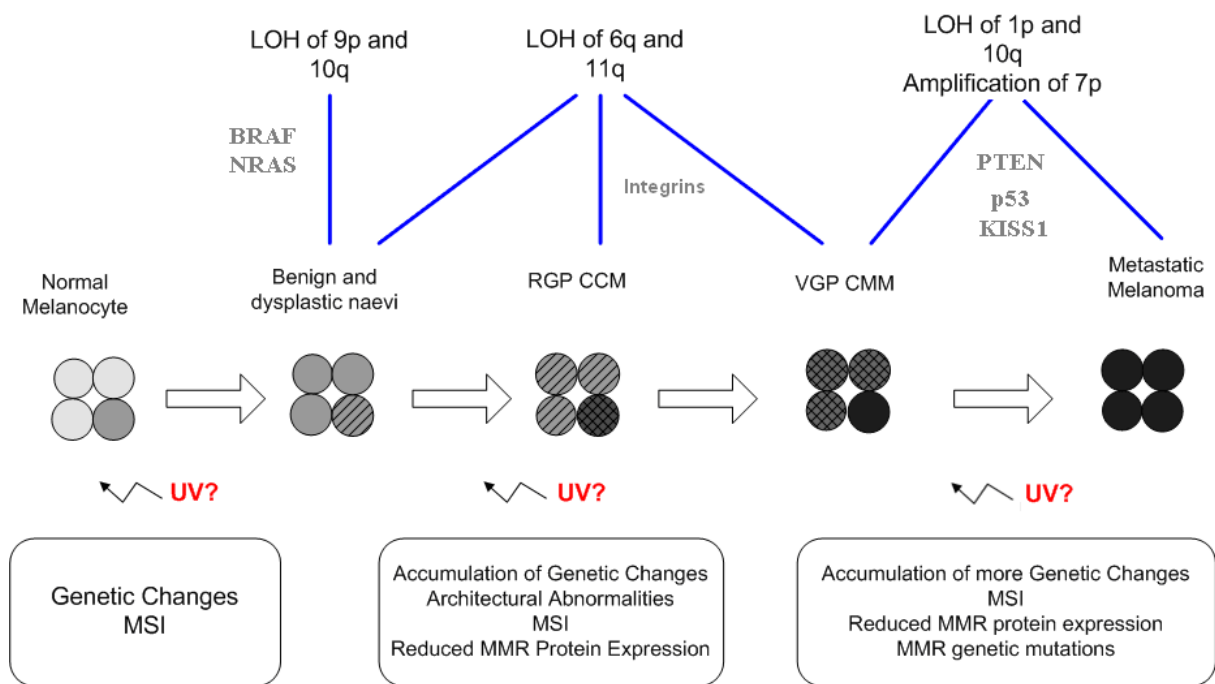


Figure 1.12: Multistep melanoma tumorigenesis model. Accumulation of LOH and genetic instability from benign nevi to metastatic melanoma and inactivation of TSGs and activation of oncogenes, also effect from UV radiation.

RGP: Radial Growth Phase. **VGP:** Vertical Growth Phase. **MSI:** Microsatellite Instability.

MMR: Mismatch repair.

1.6. Research aim and objectives

The efforts undertaken by various research groups in order to identify the molecular events leading to cutaneous malignant melanoma have resulted in the understanding of some of the mechanisms such as abrogation of TSGs, activation of oncogenes and accumulation of genetic changes, however, a clear picture is yet to be elucidated. The implication of the 9p21 region in CMM has been well documented and the CDKN2A locus has been demonstrated to carry homozygous deletions in various types of cancers. In addition to harbouring p16, other loci important to CMM progression appear to be located in the 9p21 region.

Functional evidence of the presence of tumour suppressor gene(s) that functions independently of CDKN2A locus has been provided by Parris and colleagues in 1999. These findings were later on supported by Pollock *et al* in 2001 who demonstrated LOH on the region 9p21 leaving CDKN2A intact.

Hence, the aim of this project is to identify and characterise potential novel tumour suppressor gene(s) involved in the pathogenesis of cutaneous malignant melanoma. In doing so, we have set a number of objectives that were achieved during this work. First of all, constructing several monochromosome hybrids using microcell mediated chromosome transfer, and evaluate their tumourigenicity by soft agar assays. Then, carry out an expression analysis of the genes in the 9p21 region by RT-PCR. Potential candidate TSGs were then carefully evaluated by generating expression profiles via conducting quantitative real time PCR.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Cell lines

UACC-903: Highly tumourigenic human malignant melanoma cell line carrying deletions in the exons 1 α and 2, therefore generating inactive p16 and p14 proteins (Trent *et al.*, 1990).

A9HyTK9: Mouse fibroblasts containing human chromosome 9 used as donors for MMCT (Cuthert *et al.*, 1995).

1BR3: Normal human fibroblasts (ECACC European Collection of Cell Culture, Arlett *et al.*, 1988). Although there is no published record of p14 and p16 mutations in this cell line, the status of this locus is presumed normal.

PC-3: Human prostate, adenocarcinoma (ECACC European Collection of Cell Culture, Ohnuki *et al.*, 1980)

PB-1: Provided by Prof Robert Newbold (Institute of Cancer Genetics and Pharmacogenomics, Brunel University UK) Human breast cancer cells. 21NTs transfected with hTERT. Other melanoma cell lines have been used for routine tissue culture work: SK-MEL5, MEL-154, MRI-H-221 and HS695T.

There was no published evidence of mutations in p16 and p14 genes in all other cell lines used in this work.

2.2. Cell Culture

The cell lines were routinely cultured in a Holten LaminAir Class II Haeraus Instruments hood. The human malignant melanoma cell line UACC-903 (a kind gift from Dr J.M.Trent, NIH, Bethesda, MD, USA (Trent *et al.*, 1990) was used as a recipient during microcell mediated chromosome transfer (MMCT). This cell line is highly tumourigenic and has been chosen in several studies in order to identify tumour suppressor genes in malignant melanoma (Trent *et al.*, 1990; Robertson *et al.*, 1996).

The UACC-903 cells were normally cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Newborn Calf Serum (NCS) both from Gibco, Invitrogen (Paisley, Scotland), 2 mM of L-Glutamine and 100 µg/ml of Penicillin and 100 µg/ml of Streptomycin (Gibco) in a humidified incubator at 37°C and 10% CO₂ (Heraeus 6000, Heraeus Instruments, Hanau, Germany). The cells were grown as a monolayer and were confluent after 48 to 72 hours. Sub-culture of UACC-903 cells consisted in splitting the cells 1:4 or 1:5 into 15 ml of complete DMEM for each Petri dish. After washing the monolayer with 10 ml versene (0.2mg/ml EDTA in PBS (Sigma, UK)), the cells were detached by incubation in 1 ml of Trypsin (Gibco, Scotland) for few minutes and by tapping the dish gently or retropipetting few times.

The cell suspension was put in a 15 ml tube after adding 9 ml of medium to neutralise the trypsin, and was centrifuged for 4 minutes at 1200 x g using a megafuge (Heraeus 1.0, Heraeus Instruments).

2.3. Cell lines storage and recovery from cryopreservation

After checking the cultures using an inverted microscope (Olympus Europa GmbH, Hamburg, Germany) to assess the degree of cell density and confirm the absence of bacterial and fungal contaminants, the cells were stored in nitrogen liquid, after washing the monolayer with versene, and trypsinise with 1ml of Trypsin/EDTA. After centrifugation, the cells were resuspended in an appropriate volume of 70% medium, 20% NCS (Newborn Calf Serum, Gibco, Scotland), and 10% of the cryoprotectant DMSO (Dimethylsulfoxide) (Sigma, UK).

2.4. Testing cells for Mycoplasma contamination

Cells in culture were screened for mycoplasma contamination using two different methods. One adapted from Hoechst method, 2×10^5 cells were plated onto a 40 mm x 24mm coverslip in a 10 cm Petri dish and incubated at 37°C and 10% CO₂. After 48 hours, the cells were washed in PBS (Phosphate Buffered Saline, Sigma, UK), then fixed onto the coverslips with freshly made 3:1 100% methanol to glacial acetic acid (Carnoy's fixative) for 5 minutes. Cells were washed three times in PBS and then briefly air dried. The coverslips were treated with Hoechst 33258 stain (Sigma, UK) at 20 ng/ml in PBS and incubated at room temperature for 5 minutes in the dark, then rinsed again 3 times with PBS. After that the coverslips were placed inverted onto microscope slides for viewing by fluorescence microscopy.

The second method utilises PCR technology (see section 2.7) for the detection of mycoplasma in cell cultures. It consists in two PCR rounds, for the first PCR a reaction mix of 20 μ l was prepared using 1 μ l of 100-150 ng of DNA, 1 μ l of forward primer GPO1 (5' ACTCCTACGGGAGGCAGCAGTA 3') at a concentration of 4 μ M, 1 μ l of reverse primer MGSO (5' TGCACCATCTGTCACTCTGTTAACCTC 3') at a concentration of 4 μ M and 17 μ l of ReddyMix mastermix (Thermo Scientific Abgene, UK). The reaction conditions were 95°C for 30 seconds then 35 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 minute. For the second PCR round, the same conditions were used except the used of the forward primer GPO2 (5' CTAAAGCAATTGACGGGAACCCG 3'). The PCR products of both rounds were visualised by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide. A strong positive sample gives two bands, one at 720 bp from the first round and the other at 145 bp from the second round and a weak positive sample gives a single band at 145 bp showing the absence of mycoplasma contamination. All cells were found to be free of mycoplasma using both methodologies.

2.5. Isolation of Genomic DNA

Genomic DNA was isolated from tissue culture cells using Wizard[®] Genomic DNA Purification Kit (Promega, UK). Initially, a confluent cell monolayer adherent in a 10 cm plate was trypsinised and harvested in a 15 ml centrifuge tube (Nunclon, Fisher scientific, UK). The cell pellet was resuspended in 1 ml of PBS

and transferred to 1.5 ml microfuge tube and centrifuged at 13,000-16,000 x g in a benchtop microfuge (Heraeus Instruments). Remove the supernatant, leaving behind 10 to 15 μ l of residual liquid. After resuspending the cells by vortexing vigorously, 600 μ l of Nuclei Lysis Solution was added to lyse the cells and pipetting until no visible cell clumps remain. A volume of 3 μ l of RNase was added to the nuclear lysate and the sample mixed by inverting the tube 2 to 5 times, and incubated at 37°C for 15 to 30 minutes. After allowing the samples to cool down for 5 minutes at room temperature, 200 μ l of Protein Precipitation Solution was added and the mixed using the vortex for 20 seconds and put on ice for 5 minutes. After centrifugation for 4 minutes at 13,000-16,000 x g, the precipitated protein was visible as a tight white pellet; the supernatant containing the DNA was removed and transferred to a clean 1.5 ml tube containing 600 μ l of room temperature isopropanol. By gentle inversion, the solution was mixed until white thread-like strands of DNA was observable, and centrifuged using the microfuge for 1 minute, the DNA was then visible as a small white pellet and the supernatant carefully removed. At room temperature, the pellet was washed by adding 600 μ l of 70% Ethanol. After centrifugation for 1 minute, the Ethanol was aspirated very carefully using either a Pasteur pipette or a pipette tip. The pellet was air-dried for 10 to 15 minutes, and the DNA rehydrated by adding 100 μ l of DNA Rehydration Solution followed by an overnight incubation at room temperature or at 4°C.

The concentration of genomic DNA was quantified using Ultra-Violet spectrophotometer at a wavelength of 260 nm (A₂₆₀). In addition, the purity of

the DNA extracted was calculated by using the ratio between the absorbance values at 260 and 280 nm.

2.6. Extraction of RNA using TRIzol reagent

TRIzol reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi in 1987. TRIzol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components.

After aspirating the media from the cells, the monolayer at 90 to 95 % confluent was washed twice with 10 ml PBS. The amount of TRIzol added is based on the area of culture dish, in our case 1ml of TRIzol reagent was added (usually 2 ml per $\sim 2 \times 10^6$ cells). The lysate was transferred to a 15 ml conical tube after retropipetting and stored at -80°C . The above homogenisation step is followed by a phase separation, after incubation of the homogenised samples for 5 min at $15-30^{\circ}\text{C}$ to allow the complete dissociation of nucleoprotein complex, 200 μl of chloroform per ml of TRIzol was added and the tubes mixed vigorously by hand for 15 seconds and incubated at $15-30^{\circ}\text{C}$ for 2-3 minutes. Then the samples were centrifuged at 13000 rpm for 30 minutes at 4°C . Following centrifugation, the mixture is separated into a lower red phenol/chloroform phase, an interphase and a colourless upper aqueous phase. The RNA remains exclusively in the upper

aqueous phase which is about 60 % of the volume of TRIzol reagent used for homogenisation.

Once the aqueous phase was transferred to a fresh tube, the RNA was precipitated by gently mixing with 500 μ l of isopropyl alcohol per ml of TRIzol reagent used for the initial step. The samples were incubated at 15-30°C for 10 minutes and then centrifuged at 13000 rpm (revolutions per minute) during 20 minutes at 4°C. After centrifugation, the RNA precipitate forms a gel-like pellet on the side and bottom of the tube.

The supernatant is removed and the RNA pellet washed with 1 ml of 75 % Ethanol (1 ml of ethanol for 1 ml of TRIzol), the sample was mixed by vortexing followed by a centrifugation at 8000 rpm for 5 minutes at 4°C. The supernatant was decanted and the pellet air-dried at room temperature for 5-10 minutes, at the end of the procedure the RNA was dissolved by the addition of 20 μ l of Diethyl PyroCarbonate (DEPC) treated water (Sigma-Aldrich, UK). The samples were incubated on ice for an hour or at 55-60°C for 10 minutes then stored at - 80°C.

The concentration and quality of RNA were determined using a spectrophotometer by measuring the ratio between the absorbance values at 260 and 280 nm. Pure preparations of RNA have A_{260}/A_{280} ratio values in the range 1.8 to 2.1. Preparation of 1 μ g was visualised by electrophoresis in 1 % agarose gel stained with Ethidium Bromide and resulted in 2 bands that correspond to 18S and 28S ribosomal RNA sub units. The total RNAs were then stored at - 80°C in 1.5 ml microcentrifuge tubes for the next experiments.

2.7. Standard polymerase chain reaction (PCR)

PCR is a reaction that allows the researchers to generate a large number of amplicons for a specific DNA sequence. A basic PCR set up requires several components and reagents usually in a final volume of 25 μ l: a target DNA template to be amplified, two primers of approximately 20 bases which are complementary to the DNA regions at the 5' end (forward primer) and 3' end (reverse primer), and a DNA polymerase enzyme like Taq polymerase. Also, dNTPs (Deoxynucleoside triphosphates) mix is necessary to synthesize the new DNA strand. All this reagents should be in an optimum reaction for the stability of the DNA polymerase which is a buffer solution containing magnesium and potassium.

PCR consists of a series of 30 to 40 cycles of temperature changes, the temperatures used and the time applied in each cycle depend on a variety of parameters such as the melting temperature of the primers, concentration of dNTPs (Deoxynucleoside triphosphates). There are usually three different steps which can sometimes be preceded by a hot start step ($>90^{\circ}\text{C}$) to activate the DNA polymerase if it is thermoactivated, between 1 to 10 minutes.

The first PCR stage is the denaturation which consists of melting the double stranded DNA by disrupting the hydrogen bonds between the complementary bases and the temperature is usually between $94-98^{\circ}\text{C}$ for 20-30 seconds.

The next step is annealing, the temperature is lowered to $50-65^{\circ}\text{C}$ for 20-40 seconds in order to allow the primers to anneal to the DNA template and the

temperature is about 3-5 degrees Celsius below the T_m (melting temperature) of the primers used. Followed by elongation/extension step that is commonly at 72°C for around 30 seconds, the synthesis of the new DNA takes place during this step by adding complementary dNTPs. The last step of PCR is the final extension for 5 to 10 minutes at 72°C to ensure that any remaining single-stranded DNA is fully extended.

2.8. Agarose gel electrophoresis

Agarose gel electrophoresis is a method to separate negatively charged DNA or RNA molecules according to size by applying an electric field through the gel, smaller molecules travel further on the agarose matrix which is a highly purified polysaccharide derived from agar.

The preparation of 1% agarose gel consists of dissolving 1 g of agarose (Fisher Scientific, UK) in 100 ml of 1X TBE (Tris/Borate/ EDTA) (Fisher Scientific, UK) in a microwave for approximately 3 minutes until the homogenous mixture is transparent. After cooling the solution, 5 μ l of ethidium bromide (10 mg/ml) (Fisher Scientific, UK) was added. Ethidium bromide (EtBr) is the most common dye used to detect DNA or RNA bands by fluorescence during exposure to UV light.

After swirling the bottle gently, the gel was slowly poured into a casting tray containing sample combs to form the wells, and allowed to solidify at room temperature. After solidification, the combs were removed and the gel was

covered by few millimetres by TBE buffer. About 10-15 μ l of each sample was loaded slowly into each well and about 5-8 μ l of 1Kb plus ladder (Invitrogen, UK) was loaded into one well too in order to determine the size of the amplification products. Electrodes were connected to the apparatus and a constant current of about 60 to 70 volts was applied for half to one hour until the dye reached approximately $\frac{3}{4}$ across the gel. Finally, the gel was visualised under a UV illuminator and a picture taken by Alpha Imager 2200 (AlphaInnotech Corporation, USA).

CHAPTER 3

MICROCELL MEDIATED CHROMOSOME TRANSFER TO IDENTIFY NOVEL MELANOMA TUMOUR SUPPRESSOR GENE ON CHROMOSOME 9p21

3.1. Introduction

Microcell Mediated chromosome Transfer (MMCT) is an approach originally developed in the 1970s as a consequence of somatic cell fusion studies between normal and malignant tumour cells (Harris *et al.*, 1969). During that period, various techniques were developed for gene transfer such as chromosome-mediated gene transfer (McBride and Ozer, 1973), microcell-mediated gene transfer (Fournier and Ruddle, 1977) and DNA-mediated gene transfer (Wigler *et al.*, 1979). However, MMCT provides a practical way for the introduction of one single chromosome into recipient cells in order to identify loci associated with a variety of phenotypes including cancers, DNA repair genes, senescence-inducing genes and telomerase suppression genes (Meaburn *et al.*, 2005) (Table 3.1). In fact, MMCT is a method of functional complementation whereby any measurable phenotype can be investigated.

Phenotype	Gene Identified	Chromosomal Location	References
Tumour suppression	p53	17p13.1	Stanbridge 1992
	pRB	13q14.2	
	KAI1	11p11.2	Dong <i>et al.</i> , 1995
	CDKN2A	9p21	England <i>et al.</i> , 1996
	THY1	11q23-24	Abeysinghe <i>et al.</i> , 2003
	ADAMTS9	3p14.2	Lo <i>et al.</i> , 2007
Senescence	To be identified	2q37	Uejima <i>et al.</i> , 1998
	To be identified	4q33-q34	Bryce <i>et al.</i> , 1999
	SEN16	16q24.3	Reddy <i>et al.</i> , 1999
DNA repair	XRCC4	5q15-q12	Athwal and Kaur, 1996
	XRCC5	2q35	Hafezparast <i>et al.</i> , 1993
Telomerase suppression	To be identified	3p14.2-p21.1	Tanaka <i>et al.</i> , 1998
	To be identified	10p15.1	Nishimoto <i>et al.</i> , 2001
	To be identified	3p12-21.1	Cuthbert <i>et al.</i> , 1999
Mismatch repair	hMLH1	3p21	Koi <i>et al.</i> , 1994

Table 3.1: Examples of genes identified by MMCT. This technology played a key role in the identification of genes, loci or genomic regions that carry genes associated in different important cellular mechanisms.

This approach has been a useful tool for the identification of chromosomes that carry tumour suppressor genes by the introduction of single human chromosomes into human cancer cell lines. The first successful transfer of single chromosome was reported by Fournier and Ruddle in 1977. Somatic cell hybrids were constructed by transferring murine chromosomes to mouse, Chinese hamster and human cells using MMCT and maintaining these chromosomes as functional elements within the host cell background. This methodology has been used by a number of research groups for various purposes such as the identification of genes

involved in diseases especially cancer. Chromosome transfer is a useful method for hunting tumour suppressor genes on chromosomes with high levels of loss of heterozygosity, for instance it has been successfully used for the identification of the functions of p53 and pRB as the most important tumour suppressor genes (Stanbridge, 1992).

MMCT technique has been mainly used for tumour suppression studies. However, it has also been utilised to prove the presence or absence of specific genes that alter or reverse the phenotype of a cell in a detectable manner. As an example of this, transfer of chromosome 3 into a human colon tumour cell line HCT116 mutated in the mismatch repair gene hMLH, restored normal levels of the mismatch repair activity (Koi *et al.*, 1994).

In addition to the discovery of tumour suppressors and mismatch repair genes, chromosome transfer was used to identify DNA repair genes, senescence-inducing genes and telomerase repressor genes (reviewed in Meaburn *et al.*, 2005). Also, it has been shown that heterospecific human:rodent microcell hybrids offer a better advantage compared to homospecific combinations (*eg*: human:human or rodent:rodent), because it is more straight forward to define the foreign chromosome using PCR analysis of sequence-tagged-sites (STS), genetic markers and chromosome painting (Newbold and Cuthbert, 1998).

MMCT has also been valuable in creating animal models to analyse the functions of human genes *in vivo*, as shown by the study of Tomizuka and colleagues in 1997, where human chromosomes or fragments of chromosomes were introduced into mouse embryonic stem cells (ES) generating transgenic mice.

Chimeric mice were created from the ES hybrids after injection into mice blastocysts and human genes were expressed in specific tissues such as immunoglobulin (Ig) that was detected in the sera of adult chimeric mice (Tomizuka *et al.*, 1997).

Construction of a highly stable panel of 23 hybrids representing all 22 human autosomes and the X chromosome was realised by Cuthbert and colleagues in 1995 (at Brunel University, West London, UK). The library of hybrids was used in several genetic studies as a resource for the determination of gene functions, mapping and cloning. This panel consists of human:rodent monochromosomal hybrids, each hybrid contains a single intact human chromosome maintained in the rodent background by drug selection (Cuthbert *et al.*, 1995). In order to construct these hybrids, the normal adult male dermal fibroblast cell strain 1BR.2 was used as the source for the human chromosomes to be transferred and mouse A9 fibroblasts were used as recipient cells. Prior to this transfer, the introduced individual human chromosomes had to be tagged with a selectable marker. In doing so, the retroviral vectors tgLS(+)*HyTK* were used for transfection of the human cell line 1.BR2 by adding supernatants obtained from a packaging cell line (Lupton *et al.*, 1991). The *HyTk* marker is a fusion gene that contains both enzymatic activities of hygromycin transferase (*hph*) gene and herpes simplex thymidine kinase (*TK*), this fusion gene permits positive selection (*in*) for the presence of the transferred chromosome by supplementing the culture media with hygromycin B antibiotic, and negative selection (*out*) by adding the toxic nucleoside analogue Ganciclovir to the media; in other words the marker provides

a facility to select for the absence as well as the presence of the human chromosome (Lupton *et al.*, 1991; Newbold *et al.*, 1995) (Figure 3.1).

Exposure of the donor cells to colcemid during MMCT causes microdeletions of different sizes to transferred chromosomes, as a result of this, a subset of hybrids arising from monochromosome transfer experiment will fail to exhibit the studied phenotype such as tumour suppression. Thus, the segregant hybrids are very useful and provide a resource for mapping of novel genes using deletion analysis (England *et al.*, 1996; Parris *et al.*, 1999).

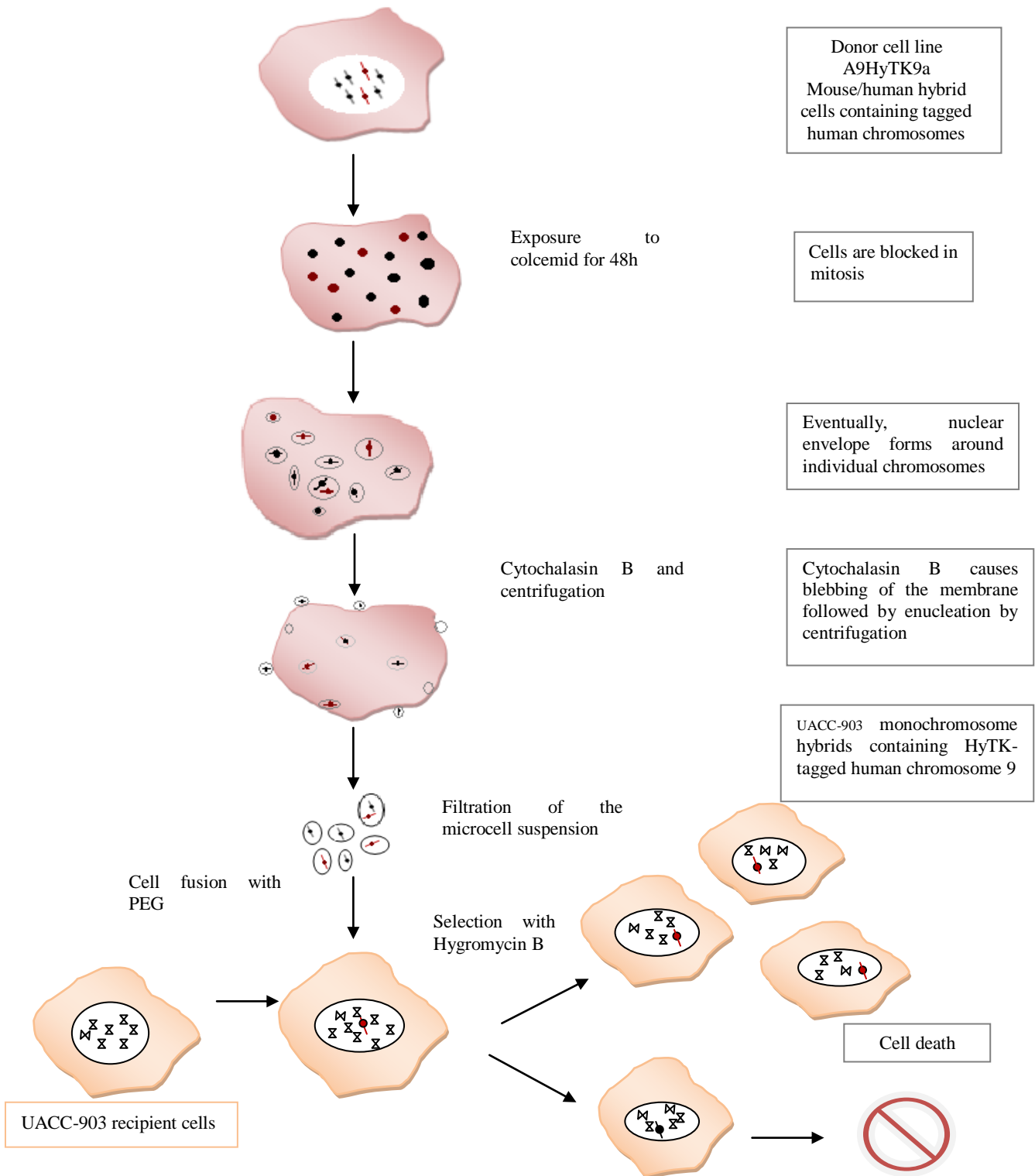


Figure 3.1: A simplified diagram of the transfer of chromosome 9 into UACC-903 recipient melanoma cell line using MMCT.

Previous study from Parris and colleagues in 1999, used two variants of chromosome 9 carrying microdeletions. Chromosome 9a contains small deletions in the locus INK4 for the CDKN2A and CDKN2B genes, and chromosome 9b harbours a larger deletion of approximately 3.07 Mbp between IFNA1 (telomeric) and the marker D9S171 (centromeric) (Figure 3.2). Suppression of tumourigenicity was observed in nude mice and no metastasis was seen after injection of suppressing hybrids constructed with the 9a transferred chromosome. In contrast, mice injected with hybrids constructed by the transfer of the 9b chromosome variant that harbours a larger deletion, displayed more rapid tumour growth than hybrids of 9a chromosomes. The difference between the two variants 9a and 9b indicates the presence of TSG(s) at 9p21.

In the present work, microcell mediated chromosome transfer was used where a variant 9a chromosome derived from a normal human fibroblast primary cell line (1BR.2) and maintained into a murine background (A9) was transferred into the highly tumourigenic malignant melanoma cell line (UACC-903).

The transferred chromosome 9a harbours exon-specific microdeletions at the INK4 locus which contains the two genes CDKN2A and CDKN2B.

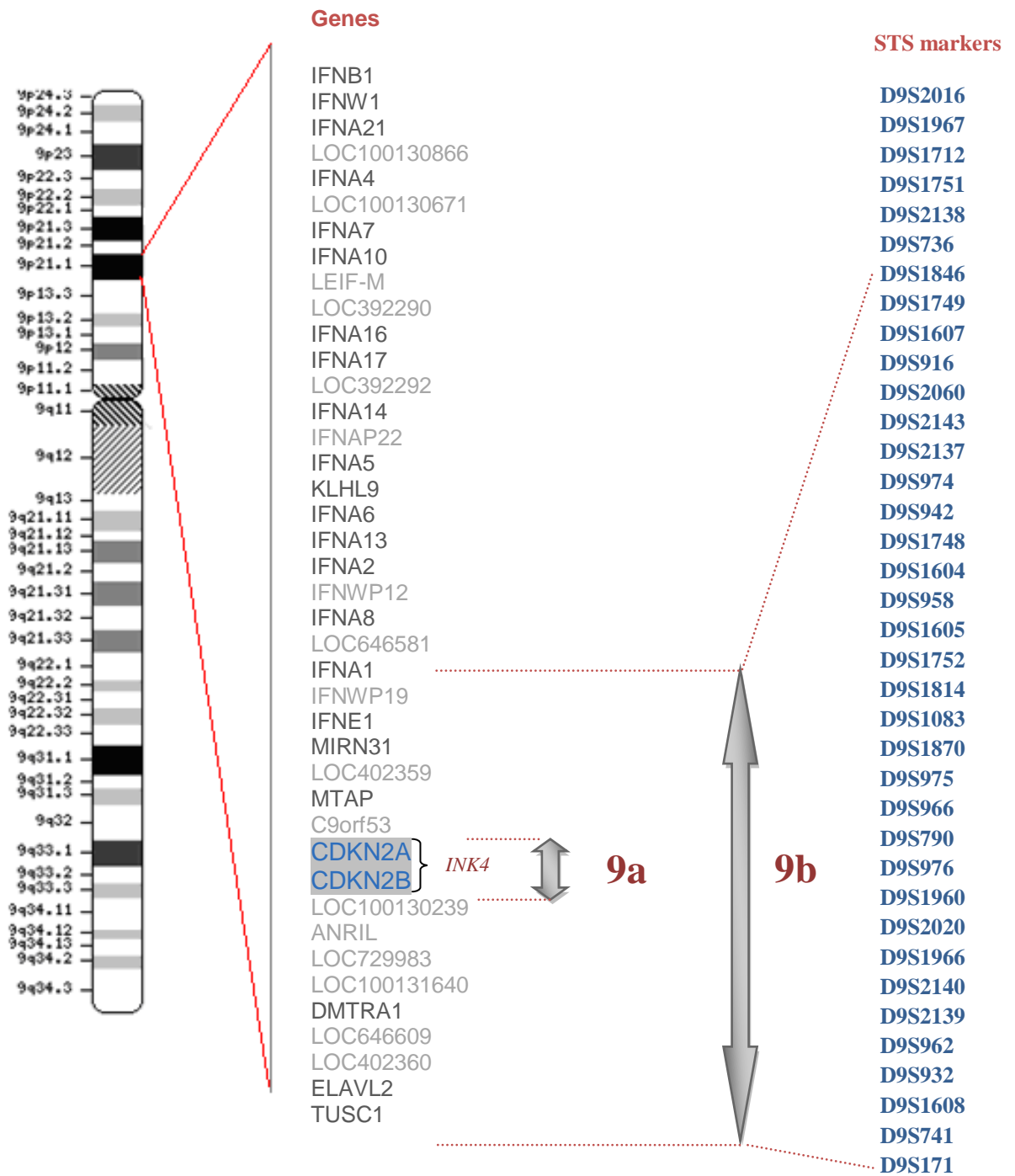


Figure 3.2: Chromosomal region 9p21 and location of the deletions in 9a and 9b chromosomes transferred into the UACC-903 melanoma cell line. Differences between the two variants 9a and 9b indicates the presence of TSG between the markers D9S1846 and D9S171.

3.2. Method

3.2.1. Microcell-Mediated Chromosome Transfer

The donor cells used for MMCT were monochromosome hybrids containing the human chromosome 9 carrying a deletion at the INK4 locus, maintained in the murine background cells A9 by the selective antibiotic hygromycin B. The donor cell line will be referred to in the present work as A9HyTK9 cells. A9 mouse cells containing the human monochromosome were cultured in DMEM with 10% FCS, 2 mM of L-Glutamine and 100 µg/ml of Penicillin and 100 µg/ml of Streptomycin. In addition 800 units of hygromycin B were added in the medium to maintain the human chromosome in the donor cells.

MMCT method is performed as follows. On the first day, 1.5×10^6 donor cells A9HyTK9 were seeded in 24 x 25cm² flasks containing 20% FCS (10% already in the complete medium + 10% of the volume of FCS) in a volume of 5 ml per flask, without hygromycin B. The recipient cells UACC-903 were seeded at 1×10^6 in 10 cm tissue culture dish in a final volume of 15 ml complete medium. On the following day, a concentration of 0.075 µg/ml of colcemid was added to the donor cells and left to incubate for 48 hours. Exposure of cells to colcemid for 48 hours results in the formation of micronuclei containing the genetic material of fragments of chromosome or sometimes a single chromosome (Newbold and Cuthbert, 1998) (Figure 3.3).

After 48 hours of incubation, the medium in the 24 flasks was aspirated and replaced by 30 ml of pre-warmed (37°C) serum free medium SFM supplemented with 10 µg/ml cytochalasin B (Sigma, UK) and incubated for 20 minutes at 37°C. Cytochalasin B makes the cell membrane more permeable which allows the microcells to be extruded to the cell surface which is confirmed visually by light microscopy. The flasks containing cytochalasin B with extruded microcells are weighed and paired by two (± 0.02 g) to be centrifuged. To cushion the flasks during the centrifugation, 24 bottles of 75 ml of water are prepared the previous day and are stored at 37°C. The flasks were placed in the pots with the cell monolayer facing inwards and the lids pointing towards the rotor axis. The flasks were centrifuged for 1 hour and 15 minutes at 9500 x g in a Sigma manufacturer 6k10 centrifuge. After centrifugation, the supernatant containing cytochalasin B was aspirated leaving about 2ml, was transferred into a 1 litre bottle, filtered and stored at 4°C for use in the next MMCT experiment.

The flasks were flicked and the microcells resuspended by thorough pipetting. The microcells were then collected in 4 tubes of 15 ml and centrifuged for 5 minutes at 4500 rpm in Heraeus megafuge 1.0. The medium was aspirated and the pellet resuspended in 1.5 ml of SFM for approximately 2 to 3 minutes to create a suspension of microcell. Serum free medium was added in the tubes up to 12 ml, each tube was then filtered and purified once through 8 µm sterile 25 mm nucleopore polycarbonate filters and twice through 5 µm filters (LabScale Company, Over Industrial Park, Cambridge, UK), held in swinlock filter holders (LabScale) and followed by a centrifugation at 4500 rpm for 5 min. The

supernatants were aspirated off, and the pellets resuspended in 3 ml of Phytohaematogglutinin PHA-P (150 μ l of 100 μ g/ml of PHA-P + 3 ml of SFM).

Meanwhile, the UACC-903 recipient cells were washed three times with 10 ml SFM for each wash. The 3 ml of PHA-P containing the microcells were transferred to recipient cells and were incubated at 37°C and 10% CO₂ during 25 minutes (PHA-P agglutinates the microcells to the recipient cells). Another 3 ml of PHA-P only is added to a UACC-903 plate for control.

After the incubation of 25 to 30 minutes, the microcells were aspirated off and 3ml of Polyethylene Glycol (PEG) solution containing 42.5% of PEG and 8.5% DMSO in SFM was slowly added to the edge of the plate, then covering all the monolayer. The timer was set at 1 minute but the PEG was aspirated after 50 seconds, this is important in order to avoid fusion of whole cells. The same was done for the control plate.

The plates were washed with 10ml of prewarmed SFM three times, the plates were gently swirled and the medium aspirated after 40, 50 and 60 seconds respectively. Then 15 ml of complete medium (DMEM + 10% FCS) was added and the plates were incubated at 37°C and 10% CO₂. The same steps were followed for the control plate.

The next day, each plate was subcultured in ten dishes in 15 ml of complete medium. After 48 hours, the medium was aspirated from the 10 dishes and 15 ml of medium was added containing 300 units/ml of hygromycin B and was replaced every week by fresh medium containing the same concentration of hygromycin B.

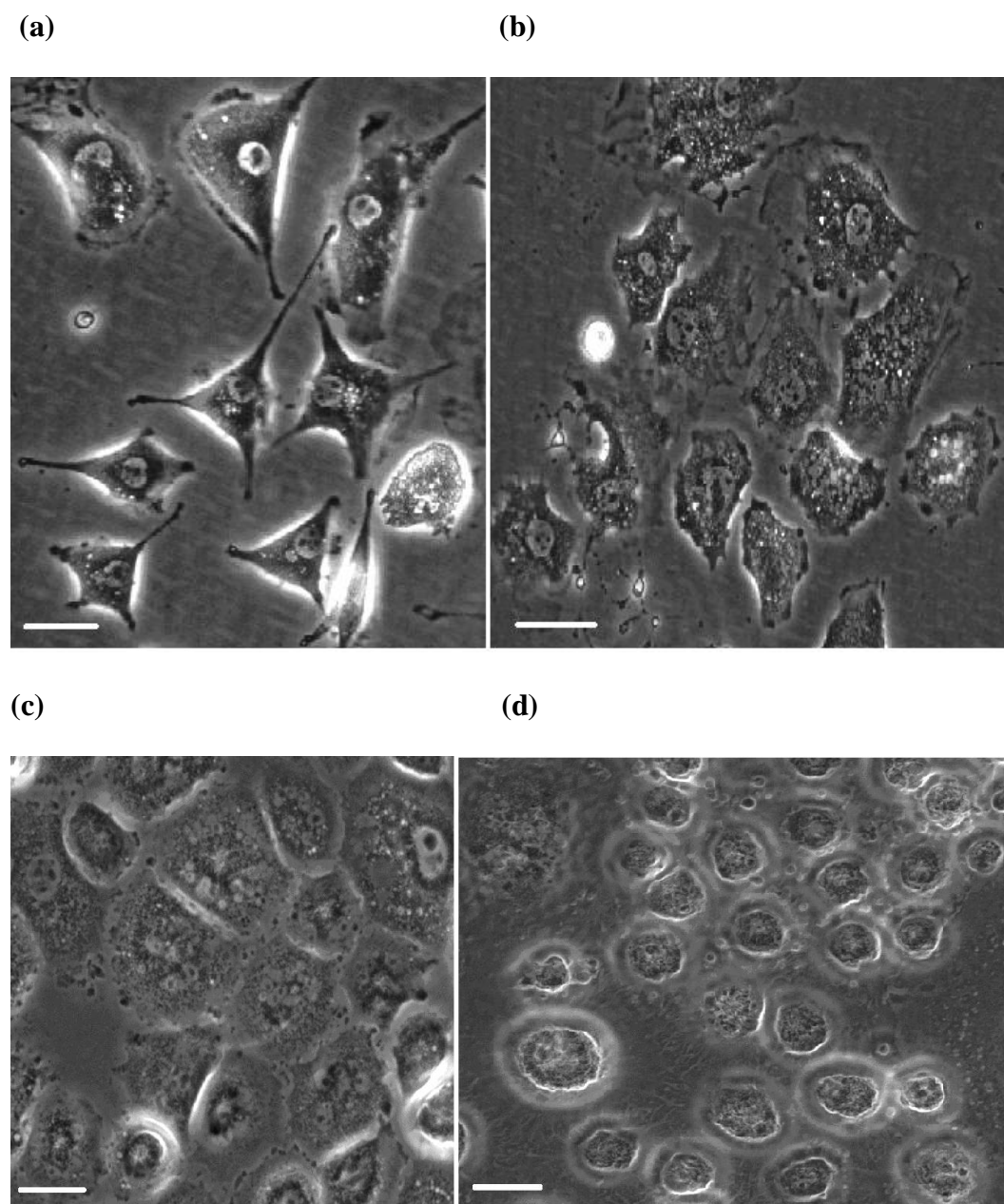


Figure 3.3: Images showing the different steps of MMCT.

(a) The human: rodent A9 somatic donor cells containing a single human chromosome 9a tagged with the hygromycin gene. (b) Donor cells A9a treated for 48 hours with colcemid causing micronucleation. (c) Exposure of micronucleated cells to cytochalasin B. (d) Micronucleated cells after 25 minutes exposure to cytochalasin B showing blebbing of the membrane. **Scale Bar:** 40 μ M.

3.2.2. Isolating UACC-903 hybrids containing a transferred chromosome 9a

After 3 to 4 weeks, hygromycin B resistant colonies were detected by visualisation using phase contrast microscopy. The colonies were chosen and marked by drawing a circle under the dish. The medium was aspirated and cells washed with versene. The colonies were harvested using cloning rings placed on top of the marked colony with grease (Vaseline) on the edge allowing the cloning ring to make a tight seal. The cylinder was pressed slowly with forceps and trypsin was added inside with a fine bore pipette then was aspirated when the cells were detached; and transferred to a T25 flask containing 150 units/ml of hygromycin B. The cells were then maintained in culture and expanded.

3.3. Results

Hybrid cell lines have been generated by the transfer of single chromosome 9a present in murine:human hybrid cell lines. The tagged chromosomes have been maintained into the A9 recipient cells by adding 400 units/ml of hygromycin B in the culture media. After harvesting the clones, the different hybrids were expanded by subculturing them and stored in liquid nitrogen.

In the present study, hybrids were constructed after isolation of the clones (Table 3.2). The number of clones was different from one chromosome transfer to the other.

Transfer of chromosome 9			
MMCT	Donor cells	Recipient cells	Number of hybrids
1	A9HyTK9a p 3	UACC-903 p 85	0
2	A9HyTK9a p 3	UACC-903 p 78	5
3	A9HyTK9a p 10	UACC-903 p 79	0
4	A9HyTK9a p 9	UACC-903 p 84	9
5	A9HyTK9a p 9	UACC-903 p 84	2
6	A9HyTK9a p 9	UACC-903 p 85	12

Table 3.2: Microcell-mediated chromosome transfer of chromosome 9a into UACC-903 melanoma cell line.

3.4. Discussion

A number of hybrids have been constructed via microcell mediated chromosome transfer technique by the introduction into UACC-903 melanoma cell lines of a derivative of chromosome 9 carrying a deletion at the INK4 locus tagged with HyTK dominant marker which is a fusion gene that contains both enzymatic activities of hygromycin transferase (*hph*) gene and herpes simplex thymidine kinase (*TK*) thus conferring resistant to the antibiotic hygromycin B.

The transfer of a single intact copy of any chromosome into the UACC-903 cell line used in this study did not affect their growth in culture (Parris *et al.*, 1999).

However, 24 hours after the transfer, the UACC-903 confluent monolayer was split into 10 Petri dishes in media with hygromycin B and most of the cells were dead after 24-48 hours of selection. After approximately one week to ten days, clusters of cells were observed indicating the formation of colonies.

However, MMCT is a technique that has variability in occurrence. Table 3.1 shows this variability, the efficiency for the production of hybrids depends upon the sensitivity of the chromosome transfer. In fact, one of the important factors to a successful chromosome transfer is the recipient cells. The recipient UACC-903 cells had to be at 95% confluency and there should be no space during the cells in order to increase the chances of the microcells to fuse with UACC-903 cells.

Several methods have been developed for testing tumorigenicity. One of the main procedures used to assess the potential of cell lines to form tumours, is the development of tumour models by injection of the cells subcutaneously in nude athymic (nu/nu) mice (T-cell deficient) or SCID (Severe Combined Immunodeficiency) mice. The SCID mouse show an immunodeficiency that affect both T and B lymphocytes (Bosma *et al.*, 1983) which makes it an ideal animal model for tumorigenicity studies because it allows growth of human tumours over a short period of time. Also, human xenografts tumours method (cross-species transplantations) allowed efficient proliferation of human tumour tissues in strain nude or SCID mice without rejection of the transplants (Morton and Houghton, 2007). However, these *in vivo* methods for assessing tumorigenicity have some disadvantages such as the costs for breeding and maintaining that could not be afforded during the PhD.

Clonogenic assays are a good indicator for tumorigenicity and have been used as a major application for testing tumours and xenografts. The method is commonly used in oncological research to evaluate the proliferative capacity of cancer cells

(Hamburger and Salmon, 1977). Fiebig and his colleagues (2004) have compared the response of human tumours established as xenografts in nude mice and tumour clonogenic assay in a study for drug sensitivity and resistance. Their findings show that the results obtained by the *in vitro* assay and the clinical response to the drug were about 90%. Therefore, tumour clonogenic assays have an important role in oncological research such as testing anticancer drugs.

The ability to proliferate in semi-solid medium, a property described as anchorage-independent growth, is demonstrated by the soft agar assay which was the *in vitro* technique used in the present study in order to evaluate tumourigenicity which is also widespread for mapping for novel genes. The constructed hybrids were tested for their ability to form colonies of soft agar as explained in chapter 4.

CHAPTER 4

TUMOURIGENICITY OF UACC-903/9a HYBRIDS MEASURED BY ANCHORAGE INDEPENDENT GROWTH IN A SOFT AGAR ASSAY

4.1. Introduction

Most of the cells of the body have to be physically attached to the extracellular matrix in order to grow and differentiate normally. Anchorage dependent growth is a characteristic of most type of cells with the exception of blood cells. The attachment of the cell to a solid surface is required for the control of cell division (Assoian, 1997). Many transformed cells are characterised by the loss of anchorage dependent growth, and the close relationship between anchorage independent growth and tumourigenicity has been demonstrated by several studies.

Many tumour cells are characterised by phenotypic and molecular changes, these features correlate with oncogenic transformation of the cells. A summary of some of the properties acquired by transformed cells is shown in Table 4.1 (Hall, 2002).

Phenotypic and molecular features of tumour cells	Description
Loss of contact inhibition	Normal cells stop dividing when they touch each other as a consequence of signals between neighbour cells. Tumour cells continue dividing because of interruption of the signals to stop division.
Anchorage independent growth	Normal cells (eg. Fibroblasts) grow only if they are attached to solid substrate. In contrast, tumour cells lose this feature.
Immortality	Unlimited number of cell division.
Molecular alterations in key regulatory genes	Mutations that can activate oncogenes and inactivate tumour suppressor genes.
Altered surface properties	Changes in surface carbohydrates and antigens and charge.
Increased motility	Develop invasive properties
Reduced requirements for serum (<i>in vitro</i>)	Tumour cells can grow and developed even without additional growth factors in the medium.

Table 4.1: Some of the properties characterising tumour cells (Hall, 2002).

Anchorage independent growth is a hallmark of cell's transformation, the cells become able to divide and grow with the absence of adhesion to the extracellular matrix. This feature can be introduced by the modification of the signalling pathways, changes in growth factors, changes in the cytoskeleton but the exact mechanisms underlying loss of anchorage dependent growth are being investigated by various research groups.

It is known that normal cells require signalling functions between growth factors and growth factor receptors and also interactions between cell surface molecules and the extra cellular matrix for growth and survival (Assoian, 1997). In response to anchorage deprivation, normal cells undergo programmed death by triggering

apoptosis mechanisms also called anoikis (Assoian, 1997; Frisch and Screaton, 2001). There has been evidence suggesting that the ability of transformed and cancer cells to have reduced requirements for adhesion to the substratum was due to the inhibition of apoptotic-related pathways (Grossmann, 2002). Furthermore, integrins are cell surface receptors that interact with the ECM. They play a major role in the attachment between the cells and the surface; also integrins mediate several intracellular signalling pathways (Buck and Horwitz, 1987). It has been demonstrated that disruptions of some signalling pathways was due to the loss of cell adhesion mediated by integrins. Over-expression of the integrin-linked kinase promotes anchorage independent cell cycle progression, resulting in the stimulation of G1/S cyclin-CDK activities (Radeva *et al.*, 1997).

The transforming growth factor β (TGF β) induces anchorage independent growth. This phenotypic transformation induced by TGF β has been observed in few cell lines only such as NRK fibroblasts (Guadagno *et al.*, 1993). However, this induction is rarely observed because TGF β is considered as a proliferation inhibitor but a study conducted by Zhu *et al.*, in 2000, showed that induction of anchorage independent growth needs other factor to activate this transformation through TGF β such as the expression of cyclin D1.

Cytoskeletal alterations are frequently observed in tumour cells, and changes of the cytoskeleton are very important for the anchorage independent growth of transformed cells (Pawlak and Helfman, 2002). Actin microfilament organisation is disrupted because of the reduction of the actin binding protein tropomyosin I,

this observation is believed to be due to the down-regulation of the topomyosin I synthesis (Boyd *et al.*, 1995).

Benecke and colleagues showed in 1978 that the incubation of cells in the absence of solid surface or anchorage, results in a reduction of 5 fold in mRNA production within a few hours, and consequently a reduction in protein synthesis. However, this type of responses to the loss of anchorage dependence by putting the cells in suspension are reduced or modified as the cells become progressively transformed in behaviour (Wittelsberger *et al.*, 1981).

A study conducted in 1975 by Shin and co-workers, showed that rodent cells displaying anchorage-independent growth were able to form tumours in nude mice. Mouse 3T3 cells and primary rat embryo cells infected by simian virus (SV40) have been tested for tumourigenicity into the deficient nude mice. As a result, the injected cells displayed an ability to proliferate *in vitro* in the absence of anchorage.

One convenient assay of the anchorage-independent growth is the colony forming ability on a semisolid agar medium. In addition, the assessment of oncogenic capability of cells in soft agar has been proven to be a reliable technique for the study of the properties of tumour cells. The anchorage independent growth was first developed in human cells by Hamburger and Salmon in 1977. The authors developed an *in vitro* assay that allowed the formation of colonies of human myeloma stem cells in soft agar. They took adherent spleen cells from BALB/c mice previously injected with 0.2 ml mineral oil 4 weeks, to prepare conditioned medium for the assay. Then, 5×10^5 cells derived from bone marrow of myeloma

patients were suspended in 0.3% agar in growth medium and plated. Final counts of the colonies were made 2 to 3 weeks after plating and the counted colonies consisted of 40 to hundreds cells with a diameter $>2 \mu\text{m}$. The number of colonies counted from bone marrow cells from the patients were 80 to 150 colonies. The bone marrow cells from normal volunteers developed 1 to 4 colonies per 5×10^5 . This assay indicated a difference between tumourigenic and non tumourigenic cell populations and demonstrated that tumourigenic cells display anchorage-independent growth.

Soft agar assay is a reliable technique for tumourigenicity by measuring the ability of the cells to proliferate in a semi-solid medium. It has been used for the screening of hybrids resulting from the fusion of normal human fibroblasts and carcinogen-transformed baby hamster kidney (BHK) immortalised cell line, the hamster cell line displayed an anchorage independent growth (Stoler and Bouck, 1984). However, the anchorage independent growth phenotype was suppressed by only hybrids that retain chromosome 1, thus providing an evidence of the presence of growth suppressing genes located in chromosome 1.

Also, *in vivo* studies have confirmed the correlation between anchorage independent growth and tumourigenicity. Lugo and Witte (1988) demonstrated that the expression of the oncogene tyrosine kinase P210, the product of the BCL-ABL fusion gene in Rat-1 cells resulted in lost of the cell contact inhibition and a transformed phenotype and morphological change. Furthermore, subcutaneous inoculation of P210 expressing Rat-1 cells into nude mice resulted in tumour

formation after 12 weeks. On the other hand, inoculation of parental Rat-1 cells developed slow growing tumours.

It has been demonstrated that there are phenotypic differences between cells derived from different stages of melanoma, when grown in tissue culture or injected in animals (Herlyn *et al.*, 1985). Only metastatic and VGP melanoma cells formed tumours in nude mice, and show non random chromosomal abnormalities involving chromosomes 1, 6 and 7.

The colony-forming efficiency in soft agar is an *in vitro* indicator of tumourigenesis making it an ideal assay to use in the present study where the constructed hybrids were assessed for colony formation ability.

The tumourigenic human melanoma cell line UACC-903 has been used in several studies for the understanding of the mechanisms underlying cutaneous malignant melanoma (Robertson *et al.*, 1996a; Su *et al.*, 1996). Therefore, the use of UACC-903 as the recipient cell line during chromosome transfer and the soft agar assays in this study was an ideal choice for this study.

4.2. Method

These assays were conducted in order to demonstrate the anchorage-independent growth which is described as the ability to proliferate in a semi solid medium, a characteristic of tumour cells. In the present study, soft agar assays have been used to identify the hybrid cells UACC-903 containing the human chromosome 9, and to display suppression of anchorage-independent growth.

The method consists in the preparation of the stock solutions for both the top and the base layers. The bottom layer containing 6% was prepared by making up 6 gm of Agar Noble with 10 gm Bacto Peptone and bringing the volume with ddH₂O to a total of 100 ml. For the base layer, in a total volume of 100 ml ddH₂O, 3.3 gm of Agar Noble was mixed with 10 gm of Bacto Peptone and a 3.3% agar solution was obtained. The agar solutions were sterilised by autoclave or pressure cooker and aliquots of 10-20 ml were prepared before the agar sets hard.

On the day of the experiment, the appropriate amount of both 6% and 3.3% soft agar was solubilised by boiling. The agar was then placed in the water bath set at 55-60°C to keep the agar molten. The base layers were set by pouring 4 ml of soft agar solution in the appropriate number of 6 well plates (Nunclon, UK) and was left to set. For instance, the dilution of the 6% soft agar mixture can consist of mixing 90 ml of complete growth medium with 10 ml of molten agar 6%. The cells were trypsinised and counted to derive a final appropriate concentration required for each plate. Then, 1.6 ml of molten 3.3% agar was mixed with 13.4 ml of medium and 1 ml of cells (1×10^4 cells) was added and also the appropriate volume of 300 units/ml of hygromycin B (about 11-12 μ l), giving a final volume of 16 ml of top layer that was distributed by pouring 4 ml in each well, each hybrid was screened in triplicate. The plates were placed in the incubator at 37°C and 10% CO₂. After 3 to 4 weeks, the colonies were counted.

4.3. Results

Cells possessing a tumourigenic phenotype and anchorage independent growth can be assessed by the ability to proliferate on a semi-solid medium. The hygromycin resistant hybrids constructed using MMCT have been taken through soft agar assay to analyse whether the transfer of single chromosome 9 deleted for the INK4 locus can suppress tumourigenesis. Colonies of different sizes were visualised using an inverted light microscope (Olympus, UK), small colonies were formed by less than 50 cells and were not counted. However, colonies formed by more than 50 cells were counted and the colony forming efficiency (CFE) was calculated by dividing the number of colonies by the number of cells seeded and expressed as a percentage. The monochromosome hybrids that display colony formation efficiencies of less than 1% were regarded as suppressed. In this hybrid set, it was observed that the majority of colonies were small in size and comprised less than 50 cells. This is in contrast to parental UACC-903 and non-suppressed hybrids where colonies were microscopically visible and composed of many thousands of cells. The results obtained for this study are summarised in Figure 4.1, and the values are the means based on two soft agar assays. The parental melanoma cell line was used as a control in soft agar and produced colony forming efficiency of 21 %.

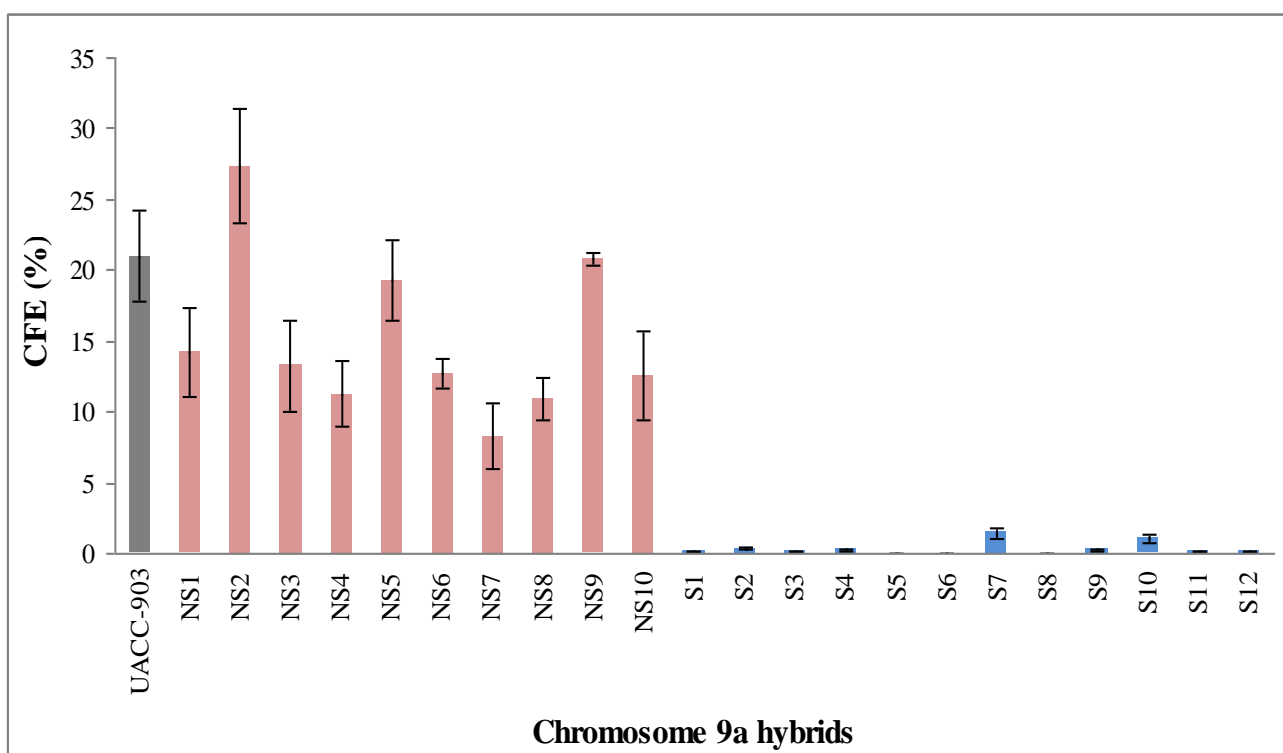


Figure 4.2: Average colony forming efficiency (CFE) of the constructed hybrids UACC-903/A9HyTK9a. 22 hybrids with hybrids in red showing segregation (NS = non-suppressor) and hybrids in blue demonstrating growth suppression with CFE < 1% (S=suppressor). Errors bars represent standard error of the mean which were no greater than 4.03% (NS2 standard error).

4.4. Discussion

Soft agar assay of the hybrids generated by the transfer of chromosome 9 harbouring a small deletion in the INK4 locus (deletion of p16 and p15) into the highly tumourigenic human melanoma cell line UACC-903 resulted in the characterisation of two categories of hybrids. Hybrids demonstrating growth suppression in soft agar (suppressors) and others did not exhibit growth suppression (non suppressor hybrids).

Chromosome 9 has been implicated in the etiology of cutaneous melanoma and p16 has been demonstrated by various studies to be the most well characterised gene involved in development of melanoma. However, a study conducted by England and colleagues in 1996 showed that the transfer of an intact chromosome 9 into human malignant melanoma cell lines deficient for p16 induces growth arrest in the cultures. Hence, growth suppression conferred by the transfer of chromosome 9 is very interesting as it implies that another growth suppressor locus is present on 9p21 that functions independently of p16 gene. This observation was further confirmed by Parris and colleagues in 1999 where tumour suppression was observed after the introduction of chromosome 9 derivatives (9a and 9b variants) into the UACC-903 melanoma cell line, the results observed were significant as the introduced chromosomes conferred growth suppression in soft agar in the absence of p16 which was inactivated or deleted in the chromosome 9 variants as well as in the parental melanoma cell line, further implying that another growth suppressor locus is present on 9p21 that functions

independently of p16. Pollock and colleagues also confirmed the presence of at least three tumour suppressor loci in the chromosomal region 9p21 involved in the development and progression of cutaneous malignant melanoma (Pollock *et al.*, 2001).

In addition, the construction of segregant hybrids that demonstrate the reverse phenotype of anchorage independent growth because of micro deletions in regions that may carry a tumour suppressor gene locus in the transferred chromosomes, are an invaluable resource for identifying regions that contain the genes which confer growth suppression in soft agar.

CHAPTER 5

GENE EXPRESSION ANALYSIS OF GENES IN CHROMOSOMAL REGION 9p21

5.1. Introduction

The discovery of all tumour suppressor genes implicated in human cancer is a central goal of the study of cancer genetics. The genomic location and function of these genes will likely provide novel therapeutic targets for the treatment of cancer in the future. Tumour suppressor genes act as negative regulators of cell division. This control is lost when tumour suppressor genes are inactivated which can cause the development of a malignancy.

This study aims to characterise potential tumour suppressor genes located in the chromosomal region 9p21 which may be involved in the development of CMM. There is a strong evidence for the presence of a tumour suppressor gene(s) functioning independently of p16 residing between IFNA and MTAP genes and the availability of two variants of chromosome 9 (9a and 9b) as described in the previous chapter harbouring different microdeletions provides an opportunity to identify the tumour suppressor gene(s). In addition, mice injected with hybrids constructed by the transfer of the 9b chromosome variant which harbours a larger

deletion compared to chromosome 9a, displayed more rapid tumour growth than hybrids of 9a chromosomes which exhibited prolonged tumour suppression (Parris *et al.*, 1999).

The introduction of a variant of chromosome 9 deleted for the INK4 locus only (A9HyTK9a) into the highly tumourigenic melanoma cell line UACC-903 by microcell mediated chromosome transfer resulted in the construction of thirty hybrids. They were then tested for tumourigenicity by evaluating the ability to form colonies using soft agar assays (anchorage independent growth). A proportion of the constructed hybrids exhibited segregation (did not exhibit suppression of anchorage independent growth) whilst other hybrids demonstrated suppression of tumourigenicity. The generation of suppressor and segregant hybrids, due to microdeletions occurring during the transfer, is a valuable resource for the characterisation of the regions that are deleted in the segregant hybrids. The association of a deletion and the lack of tumour suppression potentially identifies the location of novel tumour suppressor loci.

The following chapter describes and analyses the approach undertaken in order to conduct a gene expression analysis of all the genes present in the region of chromosome 9p21 between markers IFNA and MTAP. Differences in gene expression patterns between the suppressor and segregant hybrids might be associated with the presence or absence of tumour suppressor genes. In doing so, several methods have been taken into consideration for gene expression analysis. Microsatellite analysis can be used to define regions of loss of heterozygosity, for instance the tumour suppressor gene TUSC1 located in the region 9p21 has been

identified using STS (sequence tagged site) mapping and homozygously deleted regions were detected by reverse transcription PCR (Shan *et al.*, 2004). However, this method could not be applied for this study because it is usually used for genotyping for identification of deleted loci. In addition, the aim of the gene expression analysis was to specifically check for expression for each gene located at chromosome 9p21 rather than detecting regions of homozygous deletion.

Another approach used for gene expression profiling is microarray technology which is considered as a valuable strategy for identifying novel disease-related genes and to understand the initiation and progression of cancer and other diseases.

DeRisi and colleagues in 1996 were one of the first groups to use high throughput DNA microarray technology to examine gene expression in melanoma. They used a chip of around 1100 genes associated with tumour progression in the human melanoma cell line UACC-903. This approach had led to the identification of several candidate genes and melanoma-antigens that play a role in melanoma progression.

Recent advances in bioinformatics and high-throughput technologies such as microarrays have been widely used in identifying molecular mechanisms underlying normal and dysfunctional functions. Microarray is a technology consisting of an array of samples representing thousands of DNA nucleotides that can be analysed for expression in a highly efficient manner. Gene expression profiling using microarray technology has been useful to assess gene expression levels of RNA.

Hence, complementary DNA microarray technology has been used to study gene expression patterns in cutaneous malignant melanoma (Carr *et al.*, 2003).

It has been shown that most studies utilising high-throughput gene expression analysis have had a major impact in melanoma research and the development of gene expression platforms provided important data for the future development of melanoma prognostic markers and therapies (Ren *et al.*, 2008). Despite the fact that microarray technology had helped in the identification of several genes involved in melanoma, this approach was not chosen for the current study because the gene expression analysis was carried out for 23 genes only. Also, the cost required for the microarray chip and reagents was relatively high for the analysis of the expression of 23 genes.

Northern blot is a standard method for detection and quantification of RNA levels but has some limitations such as the sensitivity compared to reverse transcription PCR.

Reverse transcriptase polymerase chain reaction (RT-PCR) is a very powerful and sensitive technique to measure and compare gene expression at the mRNA level among samples by reverse transcribing RNA into cDNA (complementary DNA).

RT-PCR method was used for the present study in order to investigate the expression of the genes located in the chromosomal region 9p21. More precisely, the investigation was based on the expression of the genes only in suppressed cell lines but not in non suppressed constructed hybrids.

5.2. Materials and Methods

5.2.1. Candidate genes in the region of chromosome 9p21

It was possible to obtain an updated map of the position of the genes in the region 9p21 using the NCBI database (National Center for Biotechnology Information), more specifically by accessing Map Viewer which is available on the database (<http://www.ncbi.nlm.nih.gov/mapview/>).

Map Viewer provides a wide variety of genome mapping and sequencing data from different organisms. The map was constructed by accessing the current *Homo sapiens* (Human) genome build 36.3 that offers an up-to-date gene position. However, it was important to check the location of the genes as the database is often updated and genes location is modified. New entries are added and even some genes discontinued.

Based on the NCBI search, some of the genes in the 9p21 region were pseudogenes. Pseudogenes are non-functional copies of genes that lost their protein-coding ability (Vanin, 1985). Most of the pseudogenes can not be expressed because they lack the ability to encode RNA or proteins because of various genetic disruptions such as stop codons, frameshifts, or lack of transcription (Harrison *et al.*, 2003). However, pseudogenes are important resource for comparative genomics because of their evolutionary conservation of gene sequences.

In the present study, 17 pseudogenes were present in the region 9p21 (Figure 3.2) and were not included in the gene expression analysis because they do not code for a protein and they can complicate molecular genetic studies because PCR amplification of these genes can result in amplification of genes that have similar sequences.

Below is the list of the 23 functional genes present in the region of 3.5 Mb at the chromosomal region 9p21 that have been analysed for expression using RT-PCR (Table 5.1).

Genes on chromosome 9p21		
Gene	Name	Description/ Function
IFNB1	Interferon Beta 1	Induces transcription of the p53 gene and increase p53 protein levels (Takaoka <i>et al.</i> , 2003)
IFNW1	Interferon Omega 1	Member of type I interferons family
IFNA21	Interferon Alpha 21	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
IFNA4	Interferon Alpha 4	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
IFNA7	Interferon Alpha 7	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
IFNA10	Interferon Alpha 10	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
IFNA16	Interferon Alpha 16	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
IFNA17	Interferon Alpha 17	ile184arg (I184R): role in risk and prognosis of cervical cancer (Kim <i>et al.</i> , 2003)
IFNA14	Interferon Alpha 14	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
IFNA5	Interferon Alpha 5	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
KLHL9	Kelch-like 9 (Drosophila)	Interacts with 370KDa protein complex in HeLa cell lysates (Sumara <i>et al.</i> , 2007)
IFNA6	Interferon Alpha 6	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
IFNA13	Interferon Alpha 13	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
IFNA2	Interferon Alpha 2	Used for adjuvant therapy to treat high-risk cutaneous melanomas (Dithmar <i>et al.</i> , 2000)
IFNA8	Interferon Alpha 8	Positioned in the cluster of IFN- α genes by deletion mapping (Olopade <i>et al.</i> , 1992)
IFNA1	Interferon Alpha 1	Induces transcription of the p53 gene and increase p53 protein levels (Takaoka <i>et al.</i> , 2003). Used in adjuvant therapy of melanoma.
IFNE1	Interferon Epsilon 1	Unknown function but the structure and mRNA expression pattern suggest that it may have a function distinct from those other members of type I INF (Hardy <i>et al.</i> , 2004)
MTAP	methylthioadenosine phosphorylase	Major role in polyamine metabolism, and is important for the salvage of both adenine and methionine. Complete or partial deletion in some malignant cell lines (Nobori <i>et al.</i> , 1996)
CDKN2A	cyclin-dependent kinase inhibitor 2A	Encodes for p16 an inhibitor of CDK4 kinase and an important tumour suppressor gene frequently mutated or deleted in a wide variety of tumours. Encodes for p14 ARF functions as a stabilizer of the tumour suppressor protein p53 as it can interact with, and sequester, MDM1, a protein responsible for the degradation of p53
CDKN2B	cyclin-dependent kinase inhibitor 2B	Encodes for p15 a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6
DMRTA1	DMRT-like family A1	Unknown function.
ELAVL2	embryonic lethal (Drosophila)	Homolog to human Hel-N1, expressed in neurons. Absence causes multiple structural defects in the central nervous system (King <i>et al.</i> , 1994).
TUSC1	tumor suppressor candidate 1	Deleted in nonsmall cell lung carcinomas (NSCLC) and SCLC cell lines, potential lung cancer tumour suppressor genes in a region of LOH on 9p (Shan <i>et al.</i> , 2004).

Table 5.1: List of the functional genes present in the chromosomal region 9p21 with description and protein function.

5.2.2. Primer design

Primer sequences were designed prior to RT-PCR. A primer is a short segment of DNA that is complementary to the desired target genomic DNA or cDNA sequence to be amplified. Also, primers are selected to specifically match a cDNA and no other sequence in the genome. A set of primer sequences that matches different DNA targets results in non-specific amplification.

Several rules are followed when designing primers because the length and the sequence are critical parameters of a successful amplification. The primers consist in a set of two short sequences: forward primer (5' to 3' sense) and reverse primer (reversed and complemented 3' to 5' anti-sense). In general, primer's length should be between 20 and 25 bp (base pair) with a GC percentage of 50 to 60.

Another important parameter is the melting temperature (T_m) which is the temperature at which 50% of the double stranded DNA will dissociate to become single stranded. The annealing temperature (T_a) is usually around 5°C below the T_m . Also, the 3' ends of the primers should not be complementary in order to avoid the synthesis of primer dimers during amplification.

Different methods have been used in order to design primers for the target genes to be amplified. The primers for the interferon β 1 (IFNB1) gene have been selected from a published paper by Zhang *et al.*, 1996 as well as the PCR conditions and the product size.

Some tools that design primers are available online such as Primer 3 (v. 0.4.0) (Rozen and Skaletsky, 2000). Primer 3 has been used to design a set of primers specifically for the interferon α 1 gene (IFNA1) only. The IFNA1 and IFNA13 genes have 73.7% similarity when the transcript sequences were compared using Pairwise Alignment Algorithms from EMBL-EBI database (Labarga *et al.*, 2007). Thus, the forward and reverse primers have been designed from the 5' end of the IFNA1 where there are no similarities to any other gene.

Other genes have been analysed for expression using UniSTS polymorphic markers from the NCBI website.

A tool has been used for designing the primers of the other 22 genes in the region 9p21 called Primer Express Software v2.0 from Applied Biosystems (California, USA). Primer Express is a software that designs primers and probes for PCR, the sequence of the gene is entered in the query box and a list of available primer sets is provided with primer sets specifications such as CG%, Tm (Figure 5.1).

In addition, it is possible to blast both forward and reverse primers at the same time using the new tool NCBI primer-BLAST in order to retrieve the primer pairs that can cause amplification of targets other than the input template or the single target sequence to be amplified. Below is an example of the use of primer-BLAST showing that the primers set for the gene IFNB1 matches only IFNB (Figure 5.2).

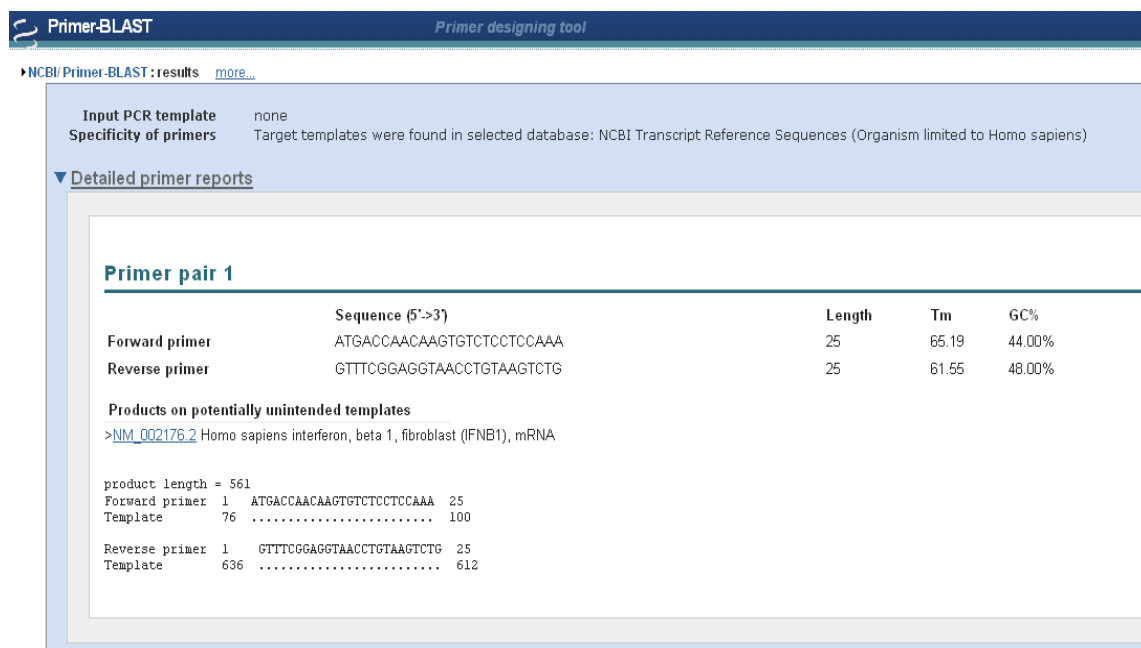


Figure 5.2: Primer-BLAST results for IFNB1 primers. The primers pair matched one single product which is the IFNB1 transcript showing also the primers position in the target sequence and the amplicon size which is 561 bp.

However, the presence of the human type I interferon (IFN) genes clustered on human chromosome 9p21 (Hardy *et al.*, 2004) caused some difficulties in the design of specific set of primers for each gene. The 13 subtypes of interferon α found on chromosome 9 are: IFNA1, IFNA2, IFNA4, IFNA5, IFNA6, IFNA7, IFNA8, IFNA10, IFNA13, IFNA14, IFNA16, IFNA17, IFNA21. Figure 5.3

shows the alignment of the genes at the IFN locus which are individually separated and do not overlap.

Therefore, the primers matched more than one target causing difficulties for analysis of the expression and the interpretation of the results obtained after amplification. Table 5.3 displays the percentage of similarity of the transcriptional sequences between the IFNA genes by using NCBI-BLAST.

All the selected primers have been ordered from Sigma-Aldrich (UK) and each oligonucleotide was solubilised with DEPC water to a concentration of 100 μ M, and was aliquoted in 0.2 ml tubes and stored at -20°C.

Genes	Primers (5' to 3')	Tm (°C)	GC (%)	Primer design
IFNB1	F: ATGACCAACAAGTGTCTCTCCAAA	65	44	(Zhang <i>et al.</i> , 2004)
	R: GTTTCGGAGGTAACCTGTAAGTCTG	61	48	
IFNW1	F: GCCCATGTCATGTCTGTCTCT	61	55	Primer Express
	R: GCAAGCAGGTCTCCAGGTGT	63	60	
IFNA21	F: GATCTGCCTCAGACCCACAG	61	60	Primer Express
	R: GAGCCTTCTGGAAGTGGTTG	60	55	
IFNA4	F: GCTGTGATCTGCCTCAGACC	61	60	Primer Express
	R: GCTGTGATCTGCCTCAGACC	61	60	
IFNA7	F: CTGGGCTGTGATCTGCCTC	62	63	Primer Express
	R: GGAAGTGGTGGCCATCAAAC	63	55	
IFNA10	F: GATCTGCCTCAGACCCACAG	61	60	Primer Express
	R: GAGCCTTCTGGAAGTGGTTG	60	55	
IFNA16	F: ATCCCAATGGCCCTGTCC	63	61	Primer Express
	R: TCAGGCAGGAGAAATGAGAGAT	60	45	
IFNA17	F: GTGATCTGCCTCAGACCCAC	61	60	Primer Express
	R: GTCTTCTGGAAGTGGTTGCC	60	55	
IFNA14	F: CAGCAGCATCTTCGGGATT	61	52	Primer Express
	R: TGTGCCATGAGCATCAAAGT	60	45	

IFNA5	F:	GATCTGCCTCAGACCCACAG	61	60	Primer Express
	R:	GAGCCTTCTGGAAGTGGTTG	60	55	
KLHL9	F:	AGTGAAGCCATCTCTGTCTC	60	45	Primer Express
	R:	GCCGTTACCAAGGGACACTT	61	55	
IFNA6	F:	GCTGAAGCCATCTCTGTCTC	61	57	Primer Express
	R:	CTCACAGCCAGGATGGAGTCC	64	62	
IFNA13	F:	GGACCTTGATGCTCCTGGCAC	66	62	Primer Express
	R:	CACAGCCAAGATGGAGTCCGC	67	62	
IFNA2	F:	CTGGCACAGATGAGGAGAATC	60	52	Primer Express
	R:	GCTTCCAGGTCATTGAGCTGC	64	57	
IFNA8	F:	TGCCAAAACCATGCTATGAA	60	40	D9S1712
	R:	CATCTGTAAGGGACTAGTGCCC	60	55	
IFNA1	F:	CATACACCAGGTCACGCTTT	59	50	Primer 3
	R:	CACTAACCACAGTGTAAGG	51	45	
IFNE1	F:	GGCCATTCTCCATGAGATGC	63	55	Primer Express
	R:	GGTAATCATGGATCCTTCGG	59	50	
MTAP	F:	GCCCACTGCAGATTCCTTTC	62	55	Behrmann <i>et al.</i> , 2003
	R:	CTGGGCAGCCATGCTACTTT	63	55	
CDKN2A	F:	CATCAGTCACCGAAGGTCCT	60	55	D9S2060
	R:	AATGGACATTTACGGTAGTGGG	60	45	
CDKN2B	F:	GCCTTTGCTCTTACTACA	48	44	D9S958
	R:	GTTCTTCCTCACATTTTA	45	33	
DMRTA1	F:	GGTCTAATGGTGTCATTGGG	58	50	Primer Express
	R:	CAATGGCTTGGACCACATCC	64	55	
ELAVL2	F:	CTCAGAACATGACACAGGAGG	58	52	Primer Express
	R:	CTGAACTTGGGCGAGCATAGG	64	57	
TUSC1	F:	AGCCAAGACAGGCATGAGTT	60	50	Primer Express
	R:	CAGCTGTTCTTTCATATGTGGC	60	45	

Table 5.2: List of the selected primers for the genes in the chromosomal region 9p21 with melting temperature (T_m) of each primer and percentage of GC content.

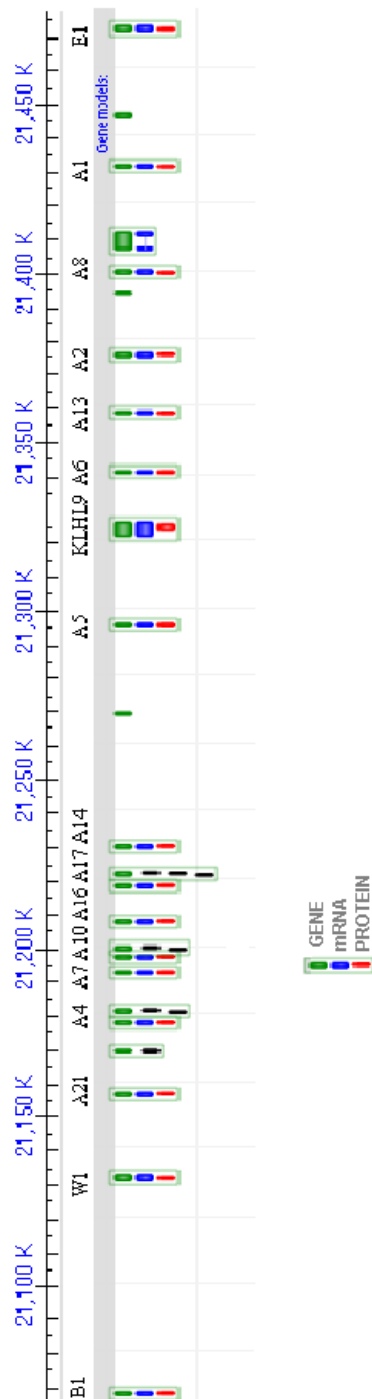


Figure 5.3: IFN genes alignment. Sequences alignment of the interferon genes at 9p21, the other genes which do not produce a protein (red) are the pseudogenes. All the interferon genes have a single exon which is shown in the vertical lines.

IFN genes (%)	IFNA1	IFNA2	IFNA4	IFNA5	IFNA6	IFNA7	IFNA8	IFNA10	IFNA13	IFNA14	IFNA16	IFNA17	IFNA21
IFNA1	100	81	-	64	65	-	-	78	74	80	74	80	74
IFNA2	62	100	-	49	49	-	90	-	56	84	79	-	86
IFNA4	-	-	100	58	57	75	82	87	-	86	82	90	86
IFNA5	99	100	100	100	99	100	-	100	99	100	100	100	100
IFNA6	100	99	99	99	100	97	99	99	100	99	99	99	99
IFNA7	-	-	99	77	75	100	88	99	-	95	95	100	95
IFNA8	-	99	78	54	54	62	100	78	-	92	90	91	92
IFNA10	71	-	89	59	58	76	84	100	60	95	95	100	95
IFNA13	99	87	-	80	80	-	-	89	100	87	89	96	90
IFNA14	83	94	94	73	72	90	96	94	72	100	94	100	94
IFNA16	69	96	85	60	60	74	-	100	60	99	100	100	99
IFNA17	71	-	90	58	57	75	95	99	64	-	95	100	95
IFNA21	67	99	87	55	55	72	93	94	57	90	90	95	100

Table 5.3: Summary of the similarities between the genes transcriptional sequences. This table summarises the similarities between the 13 genes of the IFN locus after using NCBI-Blast for each transcript. The homology is displayed as a percentage of coverage of the sequences, showing that some genes have up to 100% similarities generating difficulties for designing the primers. For example the IFNA5 gene has a DNA sequence of 570 bp and displays a homology of 100% with most of the IFN subtype genes (A2, A4, A7, A10, A14, A16, A17 and A21). However, the IFNA1 mRNA sequence is not homologue at 100% to any of the IFN subtypes.

5.2.3. Reverse Transcription PCR (RT-PCR)

Reverse transcription polymerase chain reaction is a sensitive method for the detection of mRNA transcription levels. The process traditionally involves two steps: the first step consists of the generation of the complementary DNA (cDNA) or first strand reaction, and the second step is the amplification of the cDNA by polymerase chain reaction (PCR). This method is widely used in molecular biology and is usually referred to as RT-PCR.

Before commencing it is important to remove DNA contamination by treating the RNA with DNase that digests single and double-stranded DNA to oligodeoxyribonucleotides containing a 5' phosphate. After dilution of RNA to a concentration of 4 µg using DEPC treated water, 2 µl of 10X buffer (Sigma-Aldrich, UK) and 0.5 µl of RNase Out which acts as an RNase inhibitor and 2 µl of Deoxyribonuclease I (DNase I from Sigma Aldrich, UK). The final volume was 20 µl and was incubated at room temperature for 60 minutes. This was followed by adding stop solution by adding 2 µl of 25 mM EDTA that blocks DNase activity. The tubes were incubated for 15 minutes at 65°C, then put on ice. The first step of reverse transcription PCR is the generation of cDNA from RNA. The method found the most efficient for our study was the use of random primers (random hexamers) for first strand complementary DNA synthesis in order to obtain various random sequences along the mRNA. The other commonly used methods for cDNA synthesis are: gene specific primer pairs which amplify

exclusively the gene of interest and is mainly used for amplification of low abundance transcripts; and oligo (dt)-based priming.

In PCR tubes, to 5 μ l of DNase treated RNA were added the following, 1 μ l of random primers at a concentration of 250 ng and 1 μ l of 10 mM dNTP mix that was prepared by diluting 1 in 10 of the 100 mM stock of all four dATP, dCTP, dGTP, dTTP and mixing equal volumes of each. On each tube, a volume of 4 μ l of DEPC water was added for a final volume of 20 μ l. Once the tubes were spun briefly, they were incubated for 10 minutes at 70°C in a thermal cycler and then placed on ice.

The following mix was added to the samples: 4 μ l of 5X First-Strand Buffer [250 mM Tris-HCL (pH 8.3 at room temperature), 375 mM KCL, 15 mM $MgCl_2$], 2 μ l of 0.1 M DTT, 0.5 μ l of RNase Out and 1 μ l of SuperScriptIII (Invitrogen, UK), the total volume was made up to 20 μ l by adding 0.5 μ l of DEPC water. The tubes were incubated at 42°C for 50 minutes then at 70°C for 15 minutes in a thermal cycler. Finally, the synthesised cDNA was placed on ice then stored at - 20°C, or processed directly for reverse transcription steps.

The next step of the reverse transcription was PCR amplification, however prior to the amplification of specific genes it was important to check the synthesis and the quality of the cDNA, the gene GAPDH has been used for this purpose. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the best known housekeeping genes expressed at very high levels. Hence, their amplification profile reaches significant level compared to the relatively low abundant RNAs studied.

The RT-PCR conditions vary from one gene to another according to the primer sets specifications, it was important to optimise the PCR conditions for each target gene by modifying cDNA concentration, cycle numbers, annealing temperature, MgCl₂ concentration.

However, the preparation was similar to all the genes with some changes depending on the cDNA concentration used, 17 µl of ReddyMix PCR Master Mix (1.5 mM MgCl₂) (ABgene, UK) was added in PCR tubes. Then 1 µl of diluted forward primer (1/10 dilution) and 1 µl of diluted reverse primer (1/10 dilution) were added to the master mix. Finally, 1 or 2 µl of cDNA was added in the PCR tube specifically corresponding to the cell lines.

For the present study, 3 control cell lines have been used for the gene expression analysis, a normal human fibroblast cell line (1BR.3) and two cancer cell lines: PC-3 (prostate cancer) and PB-1 (breast cancer).

The expression analysis of the 23 genes of the 9p21 region was carried out in 12 suppressor (S1 to S12) and 10 non suppressor hybrids (NS1 to NS10), UACC-903 parental cell lines and three control cell lines (1BR.3, PC-3, PB-1) and a negative control (blank) where 1 µl of DEPC water was added instead of cDNA.

The PCR cycle conditions used for most of the genes were as followed:

The first step corresponded to 'hot start' with a temperature at 94°C during 2 minutes followed by 35 or 40 cycles of denaturation (94°C for 45 seconds), annealing step with a temperature that varies between 55 to 65°C depending on the gene (Table 5.3) for 45 seconds, and extension step at 72°C for 25 seconds. The final step consisted on a final extension for 5 minutes at a temperature of

72°C followed by an additional step at an indefinite time for short-term storage of the reaction.

The PCR products generated by amplification were visualised by electrophoresis on a 1% agarose gel stained with ethidium bromide. The size of the amplicon was determined by using 1Kb plus ladder (Invitrogen, UK) (Figure 5.4).

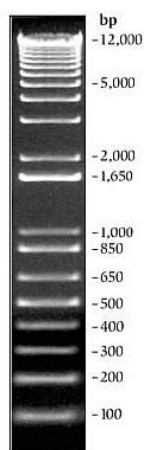


Figure 5.4: 1 KB plus ladder used as a marker to determine PCR products size.

5.3. Results

Reverse transcription PCR was carried out for 23 genes located at chromosome 9p21. After extraction of the RNA using the TRIzol method and generating complementary DNA, expression of these genes has been assessed by PCR amplification. Each gene has been tested for expression in suppressor and non suppressor hybrids, the original parental tumourigenic UACC-903 melanoma cell line and three control cell lines: the normal fibroblast 1BR.3 cell line, the PC-3 prostate cancer cell line and the PB-1 breast cancer cell line.

The house keeping gene GAPDH has been used as a control for checking the quality and quantity of synthesised cDNA by using the following primers:

Forward 5' GAAGGTGAAGGTCGGAGT 3' and reverse 5' GAAGATGGTGATGGGATTTTC 3'. The PCR conditions to amplify GAPDH were as followed: The first step of GAPDH amplification was a hot start at 95°C for 5 minutes, then 30 cycles of 95°C for 45 seconds, 55°C for 45 seconds and 72°C for 45 seconds. The final extension was at 72°C for 10 minutes followed by a final hold step for unlimited time at 4°C. The expected product size is 213 bp shown as a single band in Figure 5.5.

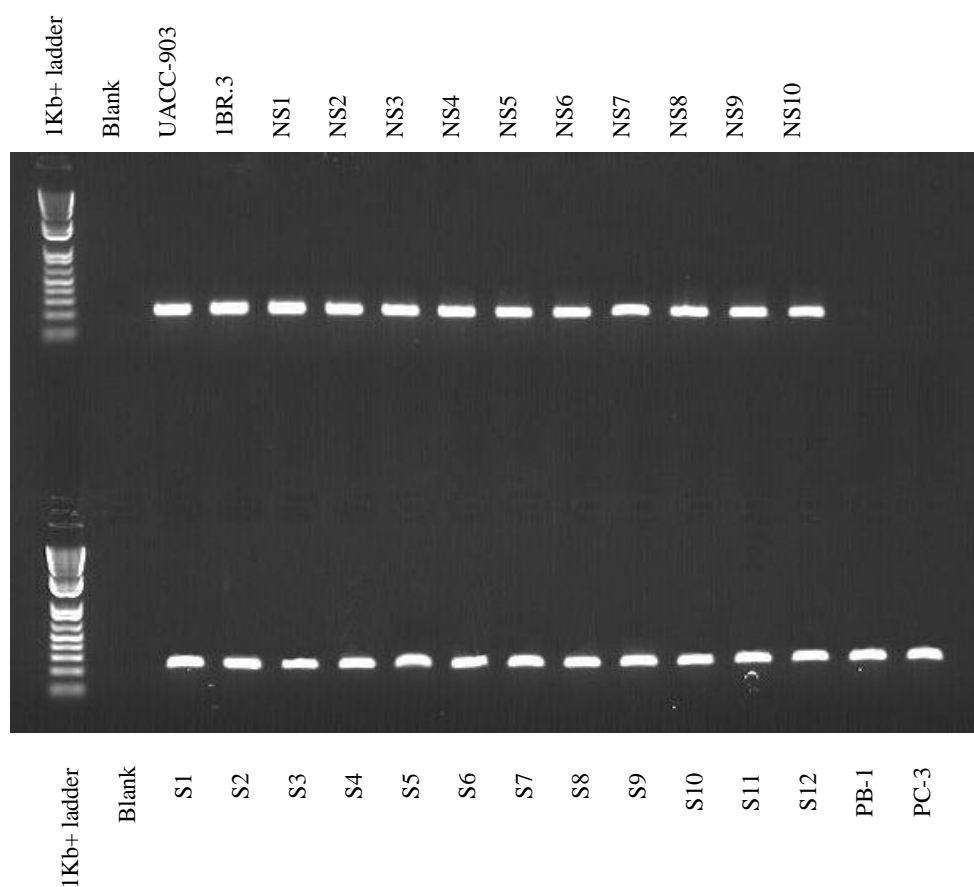


Figure 5.5: RT-PCR gel demonstrating the quality of the cDNA from all the samples using GAPDH primers set that generates a 213bp product. Amplified products were run on a 1% agarose gel stained with ethidium bromide.

RT-PCR was performed for all the genes of the 9p21 region and each gene was tested for expression in 23 melanoma cell lines (parental, NS and S hybrids), 1BR-3 normal fibroblast cell line and 2 cancer cell lines (PB-1 and PC-3).

The optimisation of the PCR conditions was carried out for each gene, and the final annealing temperature specific to each gene is showed on Table 5.4.

Genes	Annealing temperature (°C)	Expected product size (bp)
IFNB1	60	561
IFNW1	60	151
IFNA21	59	151
IFNA4	59	151
IFNA7	59	151
IFNA10	60	151
IFNA16	57	166
IFNA17	57	158
IFNA14	58	151
IFNA5	59	151
KLHL9	57	151
IFNA6	59	212
IFNA13	60	323
IFNA2	51	239
IFNA8	58	101
IFNA1	60	176
IFNE1	58	230
MTAP	60	899
CDKN2A	60	150
CDKN2B	60	295
DMRTA1	57	325
ELAVL2	57	219
TUSC1	60	139

Table 5.4: Optimal annealing temperature (T_a) and expected amplicon size for the genes present in the chromosomal region 9p21.

The results of the amplification of a representative sample of genes in the suppressor and non-suppressor hybrid cell lines are shown below (Figure 5.6 to 5.13). The RT-PCR products were analysed on 1 % agarose gel electrophoresis stained with ethidium bromide and visualised under UV transillumination.

The non suppressor hybrids have been loaded on the top wells, and the suppressor in the bottom wells. Both top and bottom lanes had a negative control sample (blank) and positive sample (1BR-3, PB-1 and PC-3).

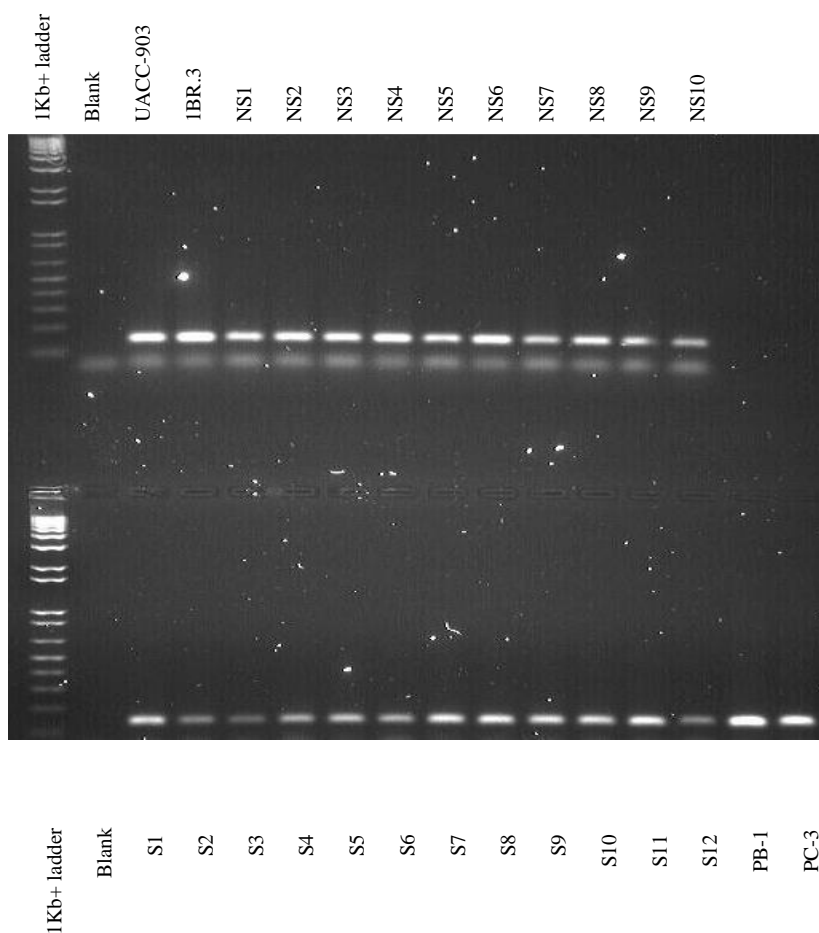


Figure 5.6: The figure shows products of RT-PCR of the KLHL9 gene. The size of the products is 151 bp. KLHL9 was expressed in the UACC-903 parental cell line and all the NS and S hybrids as well as the 1BR-3 normal fibroblast, the PB-1 breast cancer cell line and PC-3 prostate cancer cell line.

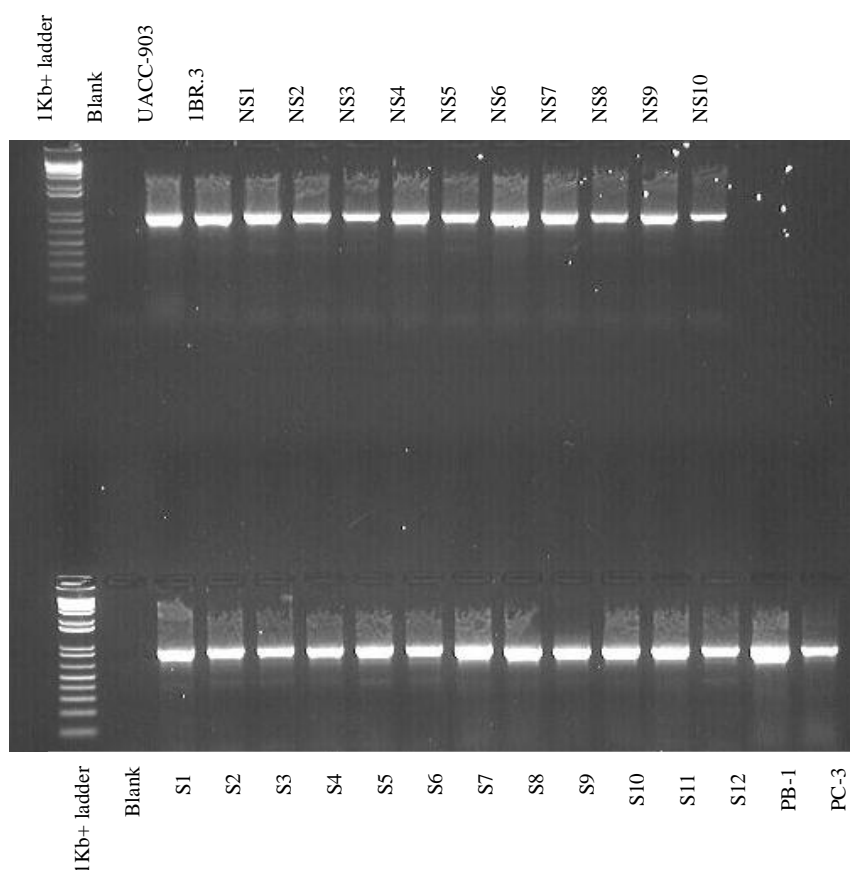


Figure 5.7: Amplification products of MTAP gene with bands at 899bp. Expression has been observed in all the hybrids, the parental cell line UACC-903 and the controls cell lines. The intensity of the band was quite strong which can be an indication of the quantity of the amplified product.

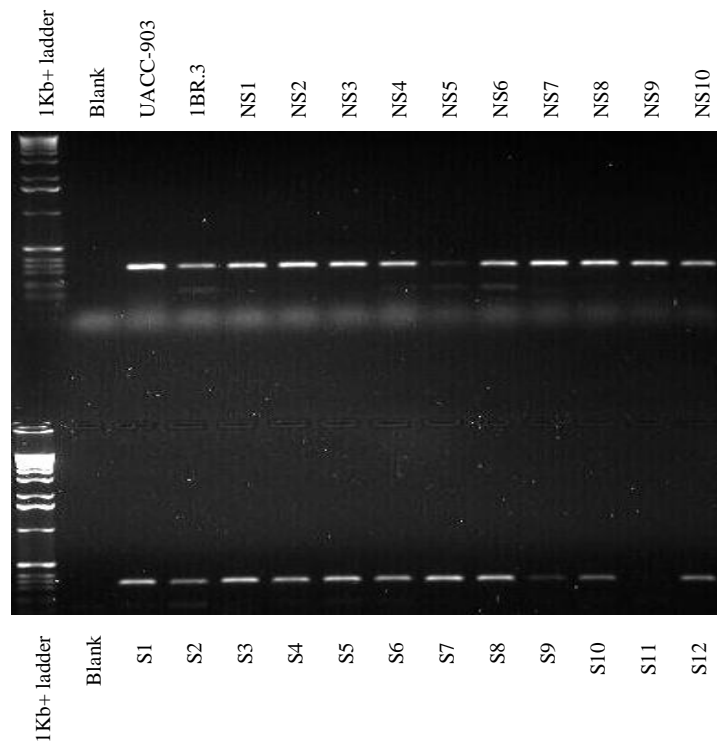


Figure 5.8: RT-PCR of IFNA13 gene generating 323 bp products and is expressed in almost all the hybrids

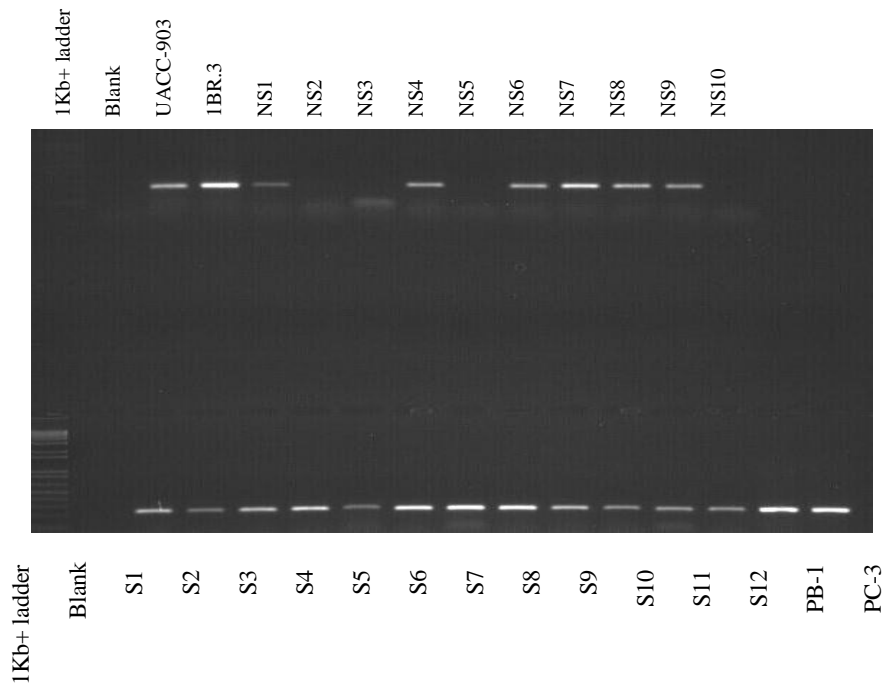


Figure 5.9: RT-PCR products of the IFNE1 gene with product size of 230bp. The IFNE1 is expressed in all suppressor hybrids, and was not expressed in 4 of NS hybrids (NS2, NS3, NS5 and NS10).

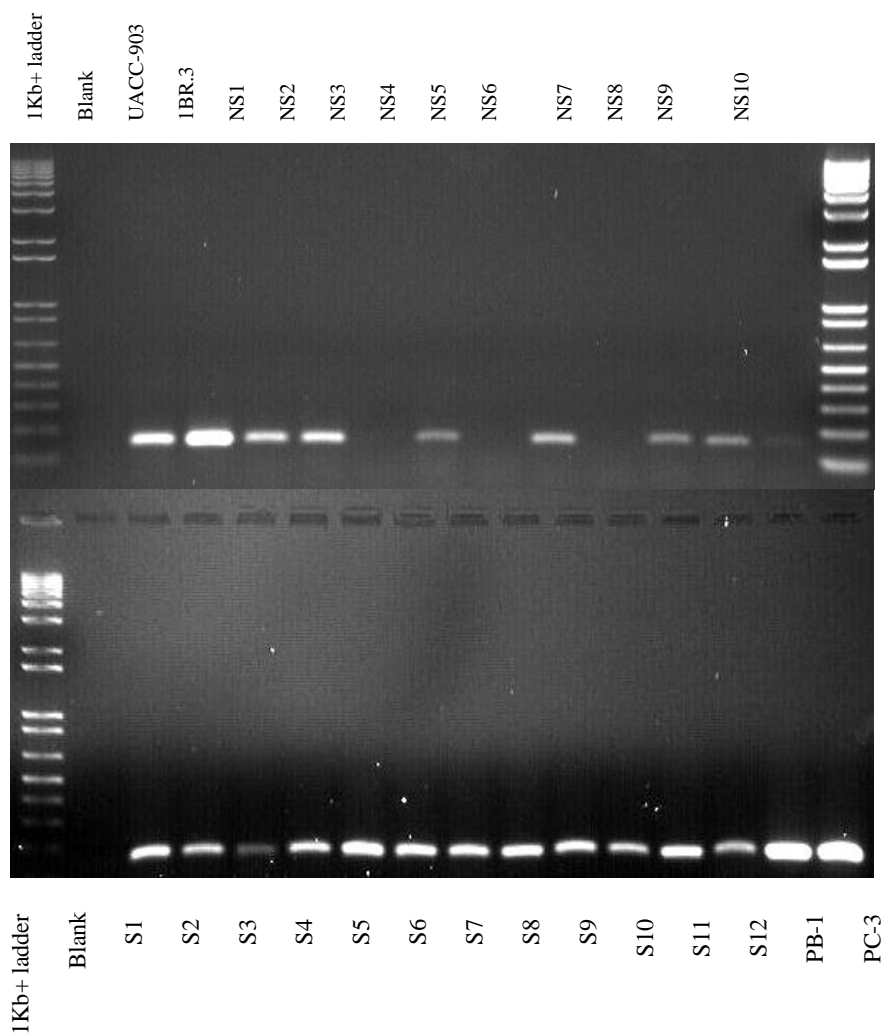


Figure 5.10: Agarose gel electrophoresis after RT-PCR of the CDKN2A gene with product size of 150bp. CDKN2A was expressed in all suppressor hybrids at high levels but not in 4 of the non suppressor hybrids (NS3, NS5, NS7 and NS10). Expression the CDKN2A gene was very high in the control cell lines 1BR-3, PB-1 and PC-3.

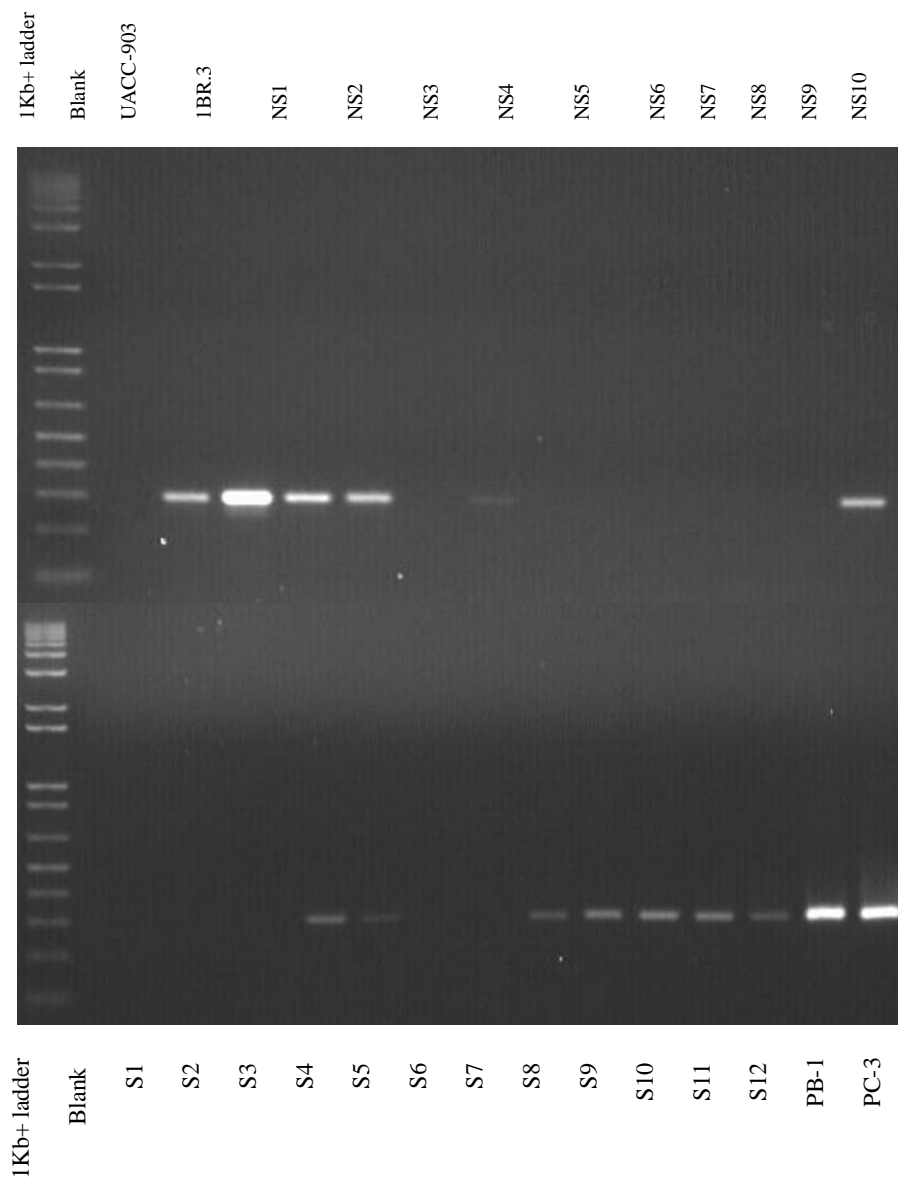


Figure 5.11: RT-PCR products of the CDKN2B gene with product size of 295bp. The CDKN2B is not expressed in 6 of the non suppressor hybrids and 5 of the suppressor hybrids. However, it is expressed in the parental cell line UACC-903.

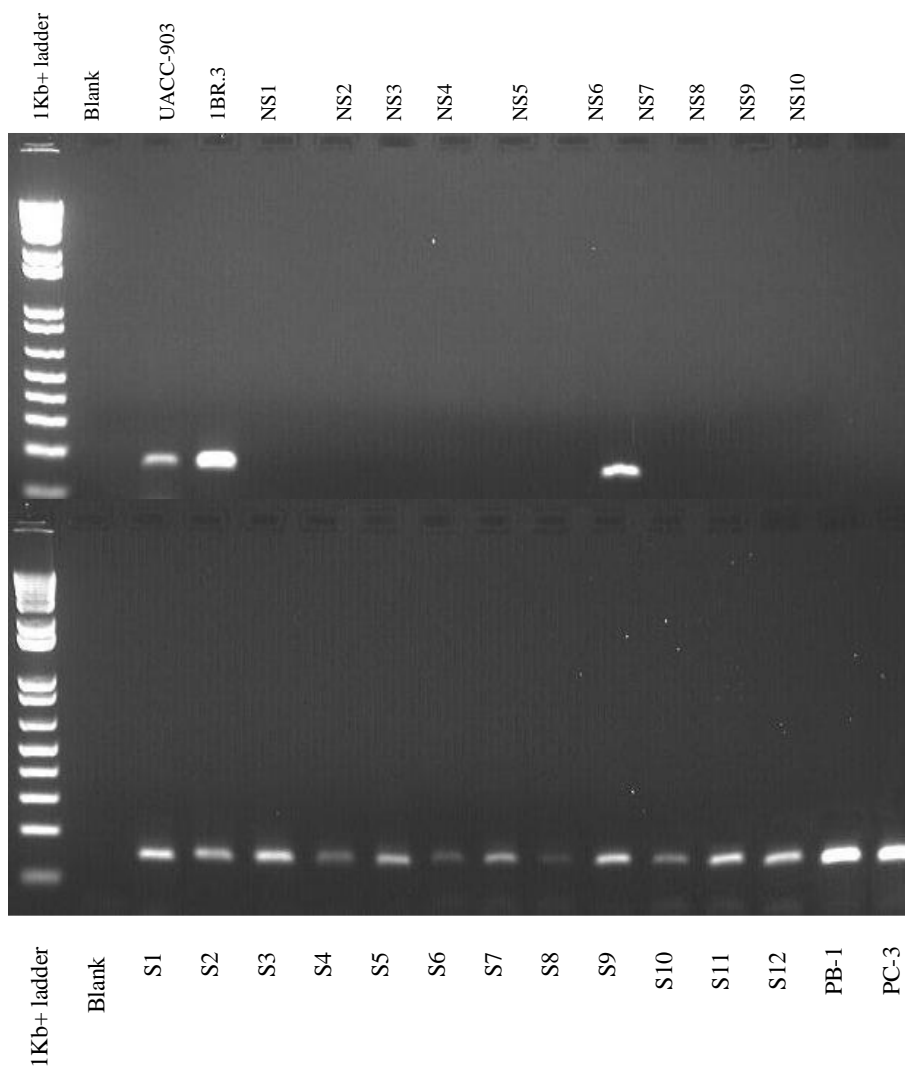


Figure 5.12: RT-PCR of the gene IFNA1 with products of 176 bp. The IFNA1 was expressed in NS7 only and not expressed in all other non suppressor hybrids. Amplification generated products in the 12 suppressor hybrids as well as the parental melanoma cell line UACC-903, and at high levels in the control cell lines 1BR.3, PB-1 and PC-3.

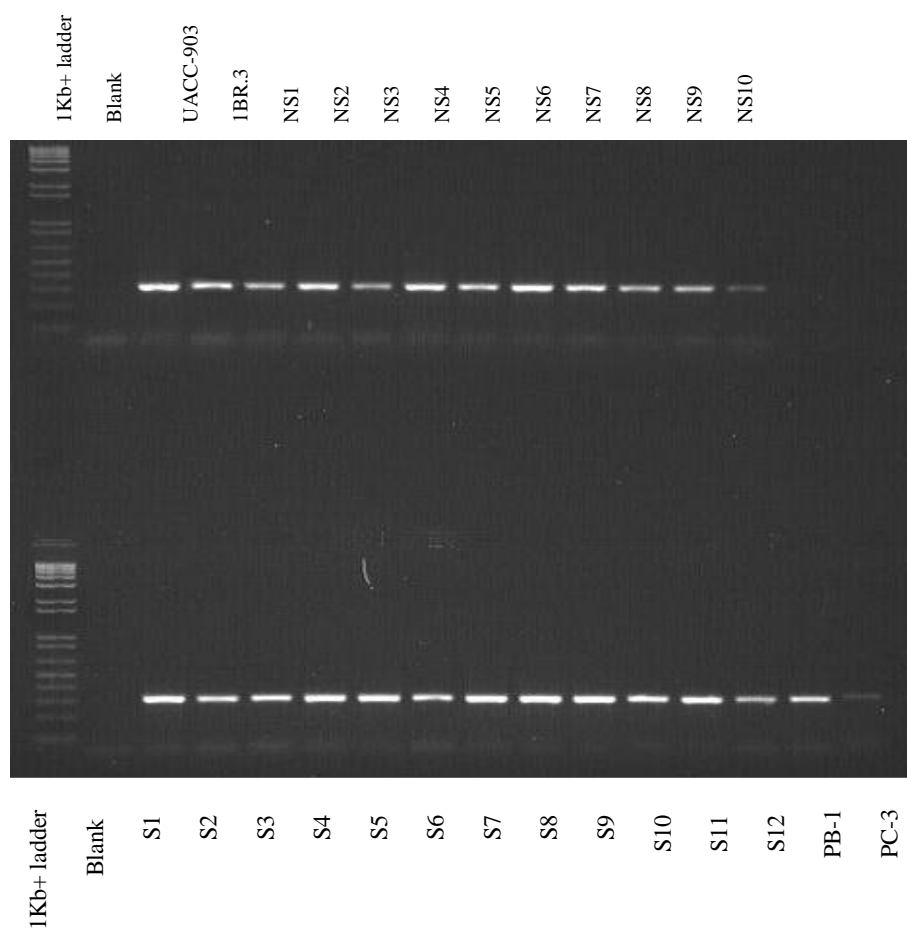


Figure 5.13: Amplification products of the DMRTA1 gene with size of 325 bp expressed in both suppressor and non suppressors hybrids. The function of this gene is still unknown.

Samples Genes	UACC-903		Non Suppressor Hybrids										Suppressor Hybrids										PB-1		PC-3	
	UACC-903	1-BR3	NS1	NS2	NS3	NS4	NS5	NS6	NS7	NS8	NS9	NS10	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	PB-1	PC-3
IFNB1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNW1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA21	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA4	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA7	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA10	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA16	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA17	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA14	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA5	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
KLHL9	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA6	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA13	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	/	/
IFNA2	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA8	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNA1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
IFNE1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
MTAP	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
CDKN2A	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
CDKN2B	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
DMRTA1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
ELAVL2	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
TUSC1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

Table 5.5: Summary of the results of gene expression analysis carried out for the 23 genes present in the 9p21 region in 10 non suppressor hybrids, 12 suppressor hybrids, the parental melanoma cell line UACC-903 and three control cell lines 1BR-3, PB-1 and PC-3. The results were visualised on 1% agarose gel electrophoresis after RT-PCR.

- = High level of expression
- = Lower level of expression
- = No expression / = not available

5.4. Discussion

The gene expression analysis of the genes in the chromosomal region 9p21 was carried out in order to characterise potential tumour suppressor gene(s) involved in the development of cutaneous malignant melanoma. During the gene expression analysis, the pseudogenes have not been studied or analysed as they code for non functional proteins. Consequently, the study focused on the functional genes only.

The 23 genes in the region 9p21 and several studies have showed the evidence of the presence of one or more tumour suppressor genes functioning independently of p16 which play a role in the development of CMM. Reverse transcription PCR was carried out individually for each gene after designing primer sets that match the target gene only. The synthesis of complementary DNA was performed using random primers after extraction of the RNA with TRIzol.

IFN α genes

The design of primer pairs was made difficult due to the presence of the IFN I cluster in the 9p21 region. The type I interferons are a family of cytokines that inhibit viral replication and cell proliferation and also activation of the immune system (Stark *et al.*, 1998). The IFN cluster is located in the short arm of chromosome 9 and consists to date of 26 genes (13 IFNA, 1 IFNB, 1 IFNW and 11 IFN pseudogenes) (Diaz *et al.*, 1994). The IFN α locus consists of 13 subtypes

that are called IFNA1, IFNA2, IFNA4, IFNA5, IFNA6, IFNA7, IFNA8, IFNA10, IFNA13, IFNA14, IFNA16, IFNA17, IFNA21. These genes have high homology and similarity (Table 5.4) and most of the primers designed matched different genes of the IFN locus. Therefore, amplification of a specific target gene could result in the amplification of other IFN α genes. However, it was possible to design specific primers for the genes IFNA1, IFNA8, IFNA13 and IFNA14 by using STS markers selected in the genes. In addition, the genes which primers sets matched only a single target after using NCBI blast are: IFNB1, IFNW1, IFNA14, KLHL9, IFNA8, IFNA1, IFNE1, MTAP, CDKN2A, CDKN2B, DMRTA1, ELAVL2 and TUSC1.

The RT-PCR results of the IFNA genes are displayed in the Table 5.5, the genes IFNA2, IFNA4, IFNA5, IFNA6, IFNA7, IFNA10, IFNA16, IFNA17 and IFNA21 are expressed in all the suppressor and non suppressor hybrids, as well as the parental melanoma cell line UACC-903. These genes are also expressed at a higher level in the 1BR.3 fibroblast cell line, PC-3 prostate cancer cell line and PB-1 breast cancer cell line. Therefore, these genes are not considered as candidate TSG due to the fact that they expressed in all the cell lines. However, the availability of specific primer sets for each gene would have given more accurate results.

IFNA8 and IFNA14

From the results of the amplification IFNA8 and IFNA14 are not candidate TSG genes because expression is observed in the 12 suppressor and 10 non suppressor hybrids with higher levels of expression in the control cell lines.

IFNB1

IFNB1 gene is expressed in all the cell lines and hybrids which can lead to the conclusion that interferon beta is not the candidate TSG. However, recent work from Kubo *et al.*, 2008 demonstrated that IFNB1 has an anti-tumour effect on malignant melanoma; it inhibits proliferation and induces apoptosis on melanoma cells *in vitro* depending on the injected dose into the melanoma lesions. These observations depend among the cell lines so it is possible to conclude that gene expression analysis of IFNB1 gene should be tested in other melanoma cell lines.

IFNW1

IFNW1 is not considered as the candidate TSG because the expression was observed in almost all the non suppressor hybrids except for the NS4 sample, and was expressed in all the suppressor hybrids but not in the S12 sample.

KLHL9

KLHL9 is not a potential TSG because it is expressed in all the control cell lines, the UACC-903 melanoma parental cell line and all the suppressor and non suppressor hybrids.

IFNA13

The gene IFNA13 is expressed in high levels in most of the hybrids and is not expressed in one suppressor hybrid. This gene was not tested in the PB-3 and PC-1 cell lines but was expressed in UACC-903 parental cell line and 1BR.3 normal fibroblast. It can be concluded that the IFNA13 is not a potential TSG involved in the development of CMM.

IFNA1

The interferon IFNA1 is the potential candidate gene in this study. This gene was expressed in all of the hybrids which demonstrate tumour suppression in the soft agar assay. In addition it was expressed in only one of the non-suppressed hybrids. The expression of IFNA1 gene in only 1 of the non suppressor hybrids (NS7) is an indication that the IFNA1 may play a role in the suppression of tumourigenicity. Studies have demonstrated that interferon α is used in immunotherapy treatment of melanoma and it possibly induces mechanisms to inhibit the progression of the disease which remain unclear. The fact that IFNA1

is expressed in suppressor and not in non suppressor hybrids is a strong evidence that it plays a role in reducing tumourigenicity.

IFNE1

Similarly to IFNA1 gene, IFNE1 was not expressed in 40% of the non suppressor hybrids (NS2, NS3, NS5 and NS10) and expressed in all the suppressor cell lines suggesting that it can also be a candidate TSG. However, the role of IFNE1 gene remains unclear.

MTAP

Methylthioadenosine phosphorylase plays a major role in polyamine metabolism and for the salvage of both adenine and methionine. MTAP catalyses the phosphorylation of methylthioadenosine (MTA) which acts as an inhibitor of polyamine aminopropyltransferases and methyltransferases (Behrmann *et al.*, 2003).

MTAP gene has attracted a lot of attention because of its location next to INK4 locus. Findings indicated that MTAP and the p16 TSG are codeleted in many cancers (Nobori *et al.*, 1996) and that a significant down-regulation of MTAP expression was observed in many melanoma cell lines (Behrmann *et al.*, 2003).

The MTAP gene is expressed at high levels in both suppressor and non suppressor hybrids and the control cell lines, and also UACC-903 melanoma cell line.

CDKN2A and CDKN2B

The INK4 locus genes coding for p16 and p15 were expressed in some of the suppressor and non suppressor hybrids. However, the p16 gene is mutated and therefore non functional in the UACC-903 cell line as mentioned in Trent *et al.*, 1990. Loss of p16 can consequently lead to the loss of p15 gene because both genes can be simultaneously deleted or mutated. It has been demonstrated that these two genes are codeleted in a high proportion of established human cancer cell lines (Kamb *et al.*, 1994).

DMRTA1

RT-PCR of this gene showed high levels of expression of this gene in all the suppressor and non suppressor hybrids. The DMRTA1 function remains unknown.

ELAVL2

Despite testing different PCR conditions such as the annealing temperature, cycle number, MgCl₂ concentration, but no RT-PCR products were detected for this gene. This gene is also referred to as Hel-N1 (human *elav*-like neuronal protein 1), an RNA-binding protein expressed in neurons (King *et al.*, 1994) suggesting

that this gene might be cell specific and unlikely to be a primary target for tumour suppressor.

The results provide evidence that IFNA1 and IFNE1 are likely to be potential tumour suppressor genes involved in the development of melanoma. Expression of IFNA1 and IFNE1 has been detected in all the suppressor hybrids and some of the non suppressor hybrids only. Reverse transcription PCR is a qualitative method to detect genes expression but does not give an indication of the precise levels of expression. Real time quantitative PCR approach has been utilised in order to measure the level of expression of the IFNA1 and IFNE1 genes in the different hybrids.

CHAPTER 6

DETERMINATION OF EXPRESSION LEVELS OF CANDIDATE MELANOMA TUMOUR SUPPRESSOR GENES USING QUANTITATIVE PCR

6.1. Introduction

PCR is considered as one of the most important techniques for the detection of any nucleic acid sequence (RNA or DNA). Standard PCR allows the generation of a large number of identical copies of a target sequence for different purposes such as the detection of expression of a gene. The products are detected at the final phase of the amplification using agarose gel and viewed using a transilluminator. However, the standard PCR method has some limitations. For example, the results obtained are based on the size of the amplicon which may not be very precise as it does not measure the amount of amplified products. Therefore, quantification of the amplified nucleic acid can be difficult because the PCR generates the same amount of product independently of the initial amount of template molecules. Therefore, the results are size-based only and quantitative data cannot be obtained.

This limitation was resolved in 1992 by Higuchi and colleagues with the development of real-time PCR. Real-time polymerase chain reaction also called quantitative PCR (qPCR) has become a very useful method for the quantification of mRNA (Bustin, 2000).

Typical uses of real-time PCR include pathogen detection, gene expression analysis, single nucleotide polymorphism (SNP) analysis, analysis of chromosome aberrations and recently protein detection by real-time immuno PCR (Kubista *et al.*, 2006). Also, measurements of DNA copy number which is known as absolute quantification real-time PCR is possible (Bustin, 2000).

In real-time PCR, the amount of amplified DNA is proportional to the cycle number and the product formed is detected, quantified and monitored in real time.

The DNA is measured using fluorescent reporter dyes (Higuchi *et al.*, 1993) that bind to the double stranded DNA formed, the amount of product accumulates as the fluorescent signal increases exponentially. A number of probes and dyes are available for real-time PCR; in the initial quantitative assay the common nucleic acid stain EtBr was used and the fluorescence was detected upon intercalating into the double stranded DNA (Higuchi *et al.*, 1993). However, classical intercalators may interfere with the polymerase reaction, therefore asymmetric cyanine dyes such as SYBR Green and BOXTO have become more popular (Zipper *et al.*, 2004). These agents bind to the minor groove of the DNA double helix and have no fluorescence when free in solution but they are brightly fluorescent when they bind to double stranded DNA, and the fluorescence increases with the amount of double stranded product formed during the PCR reaction. These dyes are

considered as non-specific reporters and they give fluorescent signal when they bind to any double stranded DNA, including primer dimers which may interfere with the formation of target products, leading to the generation of errors in the results. It is therefore important to check for the formation of primer dimers by adding a melting curve analysis after the amplification (Kubista *et al.*, 2006). The melting or dissociation curve consists of increasing the temperature gradually until the fluorescent signal drops when the temperature is reached at which the double stranded DNA separates, therefore the dye is detached and the fluorescent signal stops suddenly (Ririe *et al.*, 1997). The temperature at which the two strands of the target DNA separate is known as the melting temperature (T_m), and the primer dimers are usually shorter than the amplicon, they melt at a lower temperature than the T_m and they can be easily detected during a dissociation curve analysis.

In addition to non-specific detection using DNA binding probes like SYBR Green I, there are specific probes which detect specific targets. The detection is done with oligonucleotide probes labelled with two dyes: a reporter fluorescent and a quencher dye. Examples of probes with two dyes are the Taqman probes and Molecular Beacons (Holland *et al.*, 1991; Tyagi and Kramer, 1996).

As mentioned previously, the fluorescent signal increases gradually as the product accumulates, until it reaches a point where it saturates. Saturation of the signal is due to the fact that some reagents or reaction components may run out, such as primers, the reporter or dNTPs (Kubista *et al.*, 2001). It is important to know that during real-time PCR a particular threshold fluorescent signal level is reached at a

certain number of amplifications which is shown in the samples' response curve. It is the point where the fluorescent signal first rises above a defined background fluorescence (Nolan *et al.*, 2006). The number of cycles required to reach this threshold is represented as the Ct value (cycle threshold) and this value is inversely proportional to the amount of amplified DNA in the sample. In other words, the lower the Ct value is, the greater is the amount of target DNA in the sample. This threshold is usually selected automatically by the thermal cycler software but it can be set manually by the user.

Prior to gene expression measurements it is worth noting that the mRNA should be copied to cDNA by reverse transcription (RT-PCR), this step is critical for the accuracy and the sensitivity of the quantification. Also, in order to increase the accuracy, the real-time PCR is performed using duplicates or triplicates for each analysed sample.

Real-time PCR is very useful quantification method and has been proven to be successful in various molecular biology applications and this quantification of DNA can be either relative or absolute. Absolute quantification allows the precise determination of copy number per cell and total RNA concentration. In contrast, relative quantification compares the expression of a gene to that of a calibrator gene. In other words, the target Ct value is compared directly with the calibrator Ct and is monitored and evaluated as containing either more or less mRNA (Bustin, 2000).

In the present study, relative quantification has been used where cDNA samples prepared from suppressor and non suppressor hybrids for the genes IFN epsilon 1

(IFNE1) and IFN alpha 1 (IFNA1) have been monitored for expression levels. These two genes were selected because our basic expression analysis described and discussed in chapter 5 demonstrated differential expression levels in UACC-903 melanoma cell hybrids with an additional chromosome 9a. Thus in this study they are regarded as potential tumour suppressor genes. For each gene, two separate 96 wells plates were prepared, one for the analysis of expression levels in the non suppressor hybrids along with the GAPDH gene, and the second 96 wells plate for the suppressor hybrids cDNA samples as well as GAPDH gene.

6.2. Materials and methods

6.2.1. Real-time PCR 96 wells plate set-up and samples preparation

The procedure for real-time PCR follows the principles of standard PCR. The samples were prepared by pipetting the PCR mastermix (see below) into a 96 wells plate MicroAmp™ Fast Optical 96-Wells Reaction Plate with Barcode 0.1 ml (Applied Biosystems, UK) kept on ice. Each reaction was performed in triplicate and the mix consisted of 1µl of forward primer diluted 1/10 in DEPC water and 1 µl of reverse primer diluted as well, both primers were added in 11 µl of SYBR green (Applied Biosystems, UK) and 11 µl of DEPC water. Finally 1 µl of specific cDNA was added in each sample, except for the non template control (NTC) sample where 1 µl of DEPC water was added instead of the cDNA and was considered as a negative control for the experiment in order to check for non

specific signal arising from primer dimers or contamination. After putting 25 µl of each sample in the wells, the plate was covered with a MicroAmp™ optical adhesive film (Applied Biosystems, UK) and sealed tightly to avoid evaporation. The plate was then centrifuged for 1 minute at 3000rpm, and loaded into the real-time PCR system.

6.2.2. Real time PCR program set-up

The real time PCR system used in the present study is the ABI Prism® 7900 HT real-time PCR instrument (Applied Biosystems) which is a powerful platform for quantitative PCR analysis. The machine is connected to a PC and uses the SDS 2.1 software from Applied Biosystems. After opening the program, the selected assay is relative quantification which determines the changes in mRNA levels of a gene across multiple samples and evaluates the expression by comparing to the levels of an internal control RNA.

The probe used in the current study was SYBR Green I (Applied Biosystems, UK) and two detectors were added in the program: GAPDH detector was assigned as endogenous control which is usually used as a reference for normalization during real-time PCR, and IFN detector which was assigned as target. It is important noting that the same name of samples should be assigned for the triplicates (Figure 6.1).

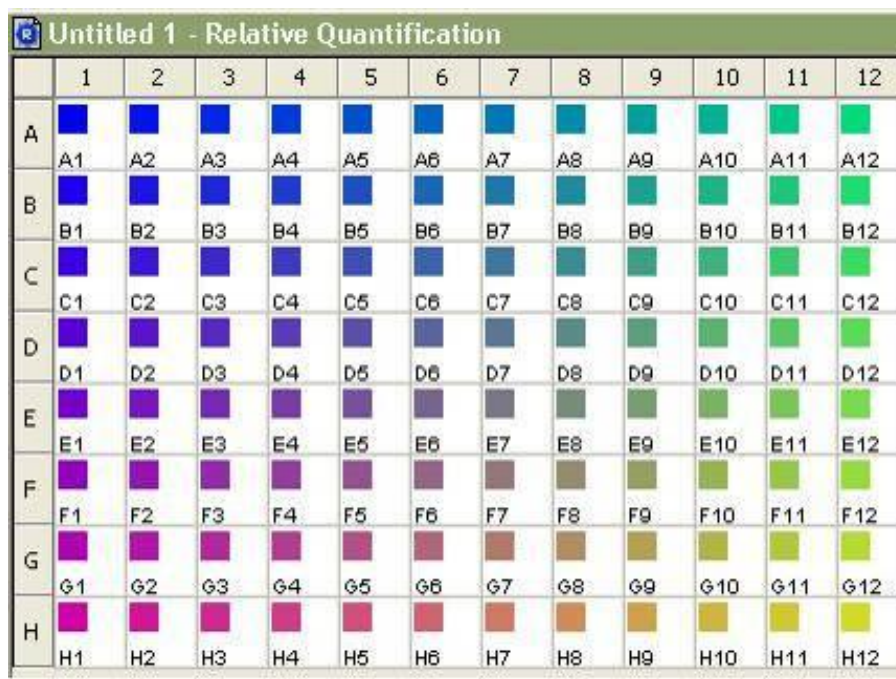


Figure 6.1: Real-time PCR 96 wells plate set-up.

Two different plates have been prepared for the gene expression analysis of the IFNE1 and IFNA1 genes because of the high number of samples and control cell lines, the non suppressor and suppressor hybrids cell lines of UACC-903 were run on separate 96 wells plates as illustrated in Figures 6.2 and 6.3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	IFN 1BR3	IFN 1BR3	IFN 1BR3	IFN NS8	IFN NS8	IFN NS8	GAPDH 1BR3	GAPDH 1BR3	GAPDH 1BR3	GAPDH NS8	GAPDH NS8	GAPDH NS8
B	IFN NS1	IFN NS1	IFN NS1	IFN NS9	IFN NS9	IFN NS9	GAPDH NS1	GAPDH NS1	GAPDH NS1	GAPDH NS9	GAPDH NS9	GAPDH NS9
C	IFN NS2	IFN NS2	IFN NS2	IFN NS10	IFN NS10	IFN NS10	GAPDH NS2	GAPDH NS2	GAPDH NS2	GAPDH NS10	GAPDH NS10	GAPDH NS10
D	IFN NS3	IFN NS3	IFN NS3	IFN PC3	IFN PC3	IFN PC3	GAPDH NS3	GAPDH NS3	GAPDH NS3	GAPDH PC3	GAPDH PC3	GAPDH PC3
E	IFN NS4	IFN NS4	IFN NS4	IFN PB1	IFN PB1	IFN PB1	GAPDH NS4	GAPDH NS4	GAPDH NS4	GAPDH PB1	GAPDH PB1	GAPDH PB1
F	IFN NS5	IFN NS5	IFN NS5	IFN UACC-903	IFN UACC-903	IFN UACC-903	GAPDH NS5	GAPDH NS5	GAPDH NS5	GAPDH UACC-903	GAPDH UACC-903	GAPDH UACC-903
G	IFN NS6	IFN NS6	IFN NS6	IFN NTC	IFN NTC	IFN NTC	GAPDH NS6	GAPDH NS6	GAPDH NS6	GAPDH NTC	GAPDH NTC	GAPDH NTC
H	IFN NS7	IFN NS7	IFN NS7				GAPDH NS7	GAPDH NS7	GAPDH NS7			

Figure 6.2: Set up of the 96 wells plate for 10 non suppressor hybrids as well as the parental melanoma cell line UACC-903 and the three control cell lines 1BR.3, PC-3 and PB1. A negative control sample noted NTC (non template control) containing water instead of cDNA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	T IFN 1BR3	T IFN 1BR3	T IFN 1BR3	T IFN S8	T IFN S8	T IFN S8	E GAPDH 1BR3	E GAPDH 1BR3	E GAPDH 1BR3	E GAPDH S8	E GAPDH S8	E GAPDH S8
B	T IFN S1	T IFN S1	T IFN S1	T IFN S9	T IFN S9	T IFN S9	E GAPDH S1	E GAPDH S1	E GAPDH S1	E GAPDH S9	E GAPDH S9	E GAPDH S9
C	T IFN S2	T IFN S2	T IFN S2	T IFN S10	T IFN S10	T IFN S10	E GAPDH S2	E GAPDH S2	E GAPDH S2	E GAPDH S10	E GAPDH S10	E GAPDH S10
D	T IFN S3	T IFN S3	T IFN S3	T IFN S11	T IFN S11	T IFN S11	E GAPDH S3	E GAPDH S3	E GAPDH S3	E GAPDH S11	E GAPDH S11	E GAPDH S11
E	T IFN S4	T IFN S4	T IFN S4	T IFN S12	T IFN S12	T IFN S12	E GAPDH S4	E GAPDH S4	E GAPDH S4	E GAPDH S12	E GAPDH S12	E GAPDH S12
F	T IFN S5	T IFN S5	T IFN S5	T IFN UACC-903	T IFN UACC-903	T IFN UACC-903	E GAPDH S5	E GAPDH S5	E GAPDH S5	E GAPDH UACC-903	E GAPDH UACC-903	E GAPDH UACC-903
G	T IFN S6	T IFN S6	T IFN S6	T IFN PC3	T IFN PC3	T IFN PC3	E GAPDH S6	E GAPDH S6	E GAPDH S6	E GAPDH PC3	E GAPDH PC3	E GAPDH PC3
H	T IFN S7	T IFN S7	T IFN S7	T IFN NTC	T IFN NTC	T IFN NTC	E GAPDH S7	E GAPDH S7	E GAPDH S7	E GAPDH NTC	E GAPDH NTC	E GAPDH NTC

Figure 6.3: Set up of the 96 wells plate for 12 suppressor hybrids. The control cell line PB1 was not added.

The amplification programs for the IFNE1 and IFNA1 were the same as the one used during the RT-PCR for gene expression analysis as seen in the previous chapter. However, the cycle number was increased to 50 cycles in order to determine more clearly the exponential phase as it is the optimal point for analysing the data (Figure 6.4). After loading the 96 wells plate in to the real-time thermal cycler, the machine was connected to the computer and the program run for 1 hour and 30 minutes. An additional step was added after the real-time amplification run was completed, which was the dissociation curvestage (Figure 6.5), this step is important because SYBR Green binds to any double stranded

DNA molecule including primer dimers which can interfere with the target molecules.

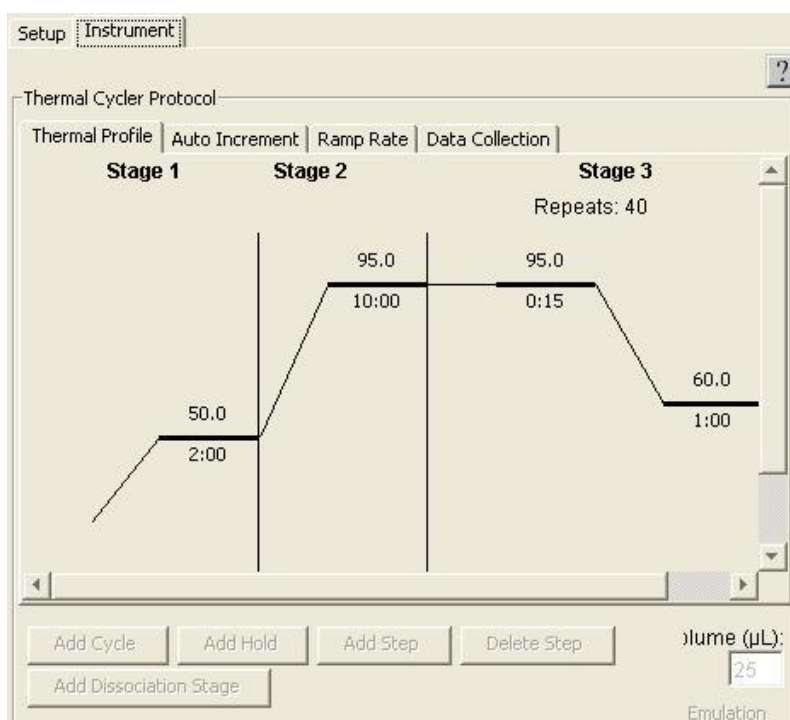


Figure 6.4: Real-time PCR cycle conditions set up. The different stages of amplification can be set with temperature and time as well as the number of cycles.

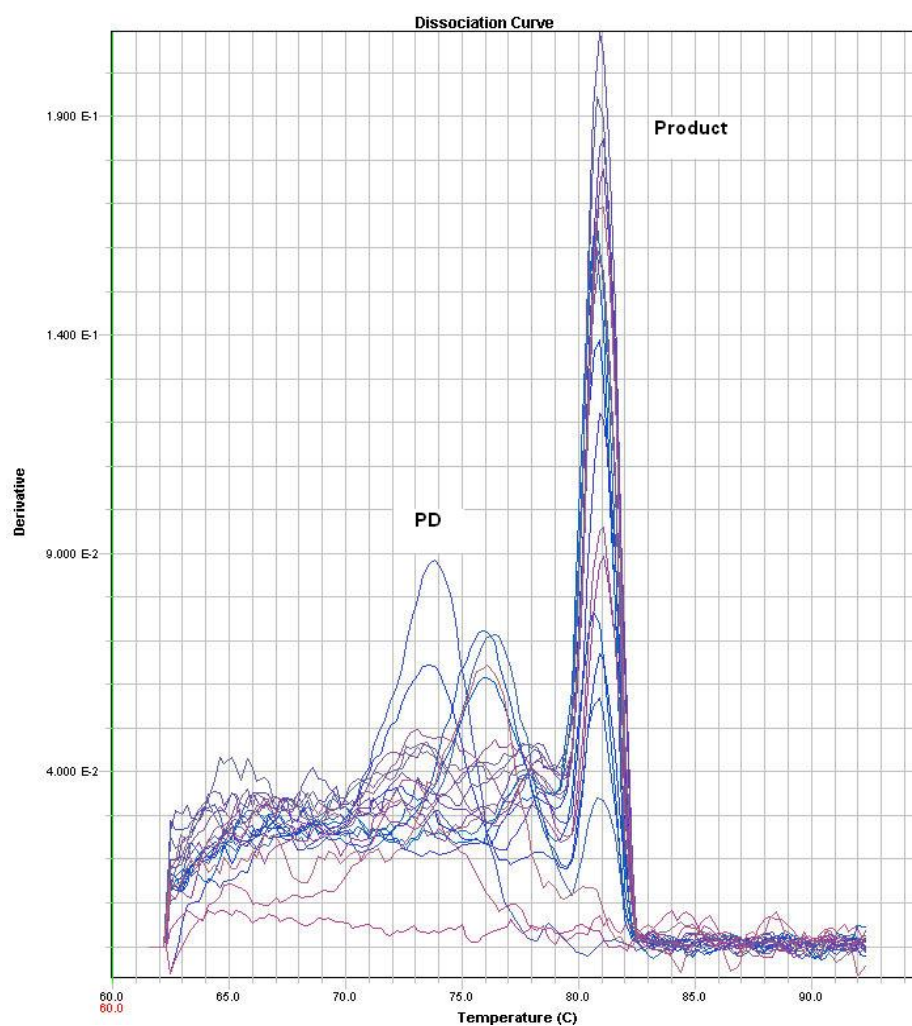


Figure 6.5: Dissociation curve stage conditions. The temperature is increased progressively from 60 to 95°C, the double stranded DNA strands will melt when the temperature reached the T_m . The amplicon produced from the targeted product is longer and will melt at a higher temperature than primer dimers (PD). The x axis represents the temperature (°C) and on the y axis is the negative derivative of the fluorescence emitted by each sample during the melting curve analysis.

6.3. Results

6.3.1. Interferon epsilon 1 (IFNE1)

Although the role of IFNE1 gene remains unknown, the structure and mRNA expression pattern of IFNE1 suggest that it may have a function distinct from those other members of type I IFN which are cytokines that play a role in assisting the immune response. Moreover, from the standard RT-PCR results displayed in chapter 5, the gene IFNE1 is believed to be one of the candidate genes that might be involved in the development of cutaneous melanoma. The IFNE1 gene was expressed in all the suppressor hybrids but not in some of the non suppressor hybrids (not expressed in NS2, NS3, NS5 and NS10).

Analysis of gene expression by real-time PCR using relative quantification results in monitoring the levels of mRNA of the target IFNE1 gene in the suppressors and the non suppressor hybrids as well as for the GAPDH gene which was considered as an endogenous control used in order to normalise the qPCR.

6.3.1.1. qPCR of non suppressor hybrids for IFNE1 gene

A relative quantification was carried out for the IFNE1 target gene and it consisted of analysing the changes in fluorescence signal cycle by cycle. Each sample was tested in triplicate for IFNE1 target gene and also for GAPDH endogenous control gene.

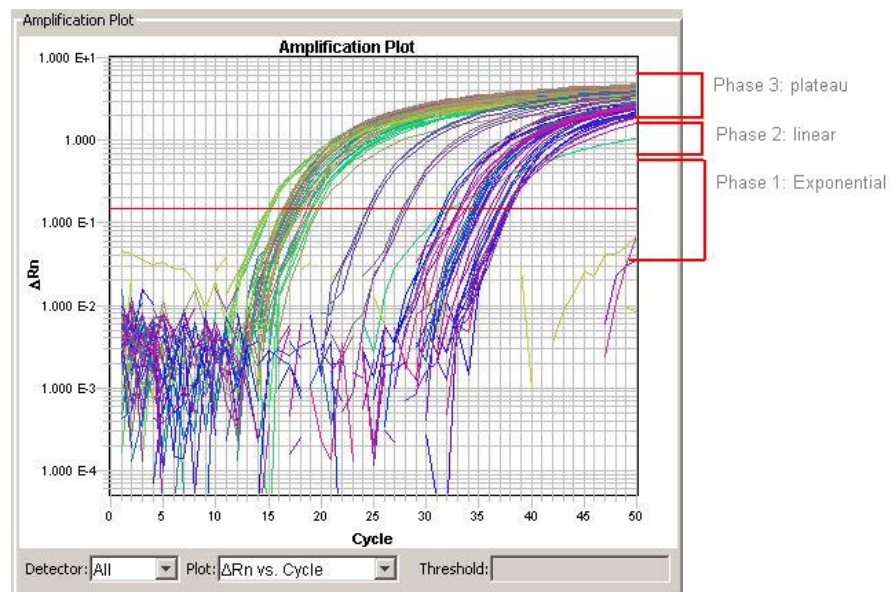
The samples were prepared and loaded in the 96 wells plate as shown previously in Figure 6.2. After approximately two hours of amplification, the data were collected for analysis under the program name $\Delta\Delta\text{Ct}$ study. The results are displayed as an amplification plot representing the measurement of the fluorescent signal for each sample in each well versus the cycle number as displayed in the Figure 6.6. The graphs showed in (a) are the amplification curves on a linear scale where R_n (normalised reporter signal) represents the fluorescence emission intensity at each cycle number. This amplification curve is characterised by three distinct regions corresponding to the progression of the PCR that are viewed more clearly in a logarithmic scale as shown in Figure 6.6 (b). An exponential phase which is a cycle range of high precision characterised by constant amplification efficiency and the first detectable rise in fluorescence occurs during this phase.

A linear phase characterised by a decrease of the amplification efficiency due to a low concentration of one of the samples components. The third and final phase is the plateau where the amplification stops and the R_n signal remain relatively constant (Theory of operation, Applied Biosystems 7900HT manual).

The crossing points of the threshold line in the exponential phase of the curve are the C_t values as shown in Figure 6. 6, it is possible to see that the average C_t values for the GAPDH gene samples is around 17 cycles, and the average C_t value for the IFNE1 gene is approximately 35 cycles. This observation shows that the levels of mRNA of the gene IFNE1 is less abundant than the ones of GAPDH genes. This is not surprising as it is well known that GAPDH is a housekeeping gene expressed in abundance. The NTC negative controls containing no input

cDNA are shown as background under the threshold red line. For these samples, the Rn curves appear as a flat line below the fluorescent signal detection limit. However, we can observe on the Figure 6.6 that the lines corresponding to the NTC samples tend to be less flat towards the end of the amplification which can be due to the accumulation of primer dimers because real-time PCR is extremely sensitive technique and amplification signals can be detected even if they are very low.

(a)



(b)

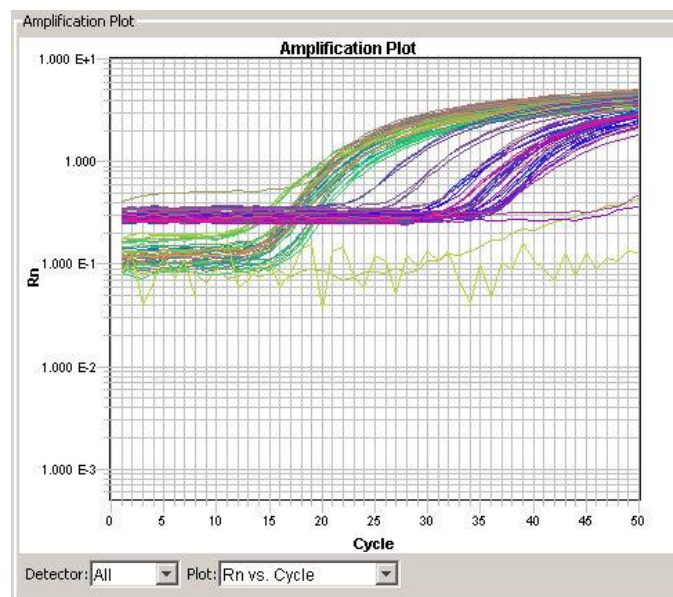


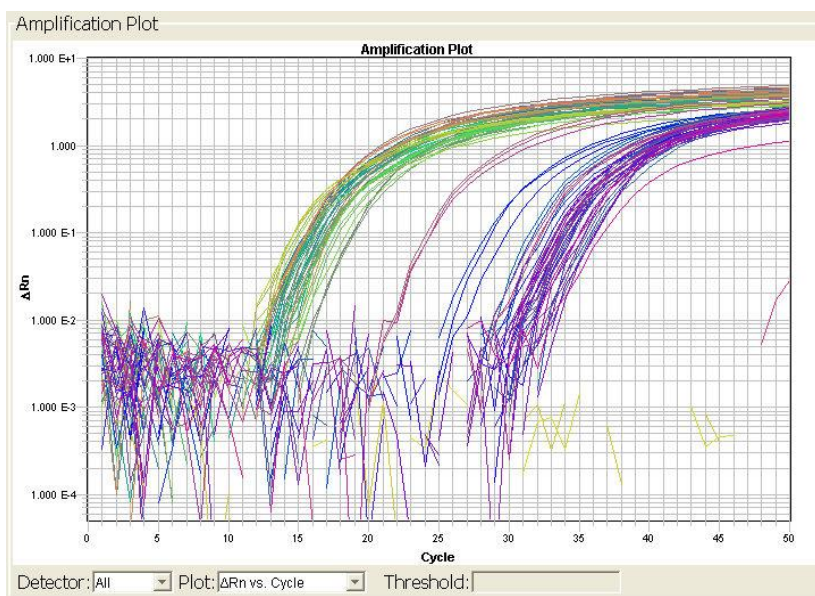
Figure 6.6: Amplification curves for non suppressor hybrids expression levels analysis for both IFNE1 gene (blue and purple) and GAPDH gene (green colour). (a) Linear scale of the amplification plot for non suppressor hybrids, on the y axis is ΔRn (normalised Reporter signal) which is the fluorescence intensity and the cycle numbers are on the x axis. (b) The logarithmic view of the amplification plot shows three phases as well, the exponential phases for IFNE1 (purple) occurs later than the one of GAPDH (green) indicating a difference of mRNA concentration between both genes.

The relative quantification of the expression of the genes was given by measuring each individual relative quantification (RQ) value which allows the comparison of the levels of cDNA of the target gene and therefore gene expression level in each hybrid, to a calibrator which is a single reference sample used as the basis for the expression level. In the present study, the calibrator was the control normal dermal fibroblast cell line 1BR.3 and the aim of this analysis was to determine whether the levels of expression of the IFNE1 gene in each one of the suppressor as well as the non suppressor hybrids was more or less than the amount of mRNA of the IFNE1 gene in the control cell line 1BR.3. Levels of expression of all the non suppressor hybrids and the UACC-903 parental melanoma cell line were lower than the calibrator 1BR.3 which is set here at one. However, the expression of the IFNE1 gene in the two other cancer cell line was higher than in normal fibroblasts cell line 1BR.3 (Figure 6.8).

6.3.1.2. qPCR of suppressor hybrids for IFNE1 gene

Real-time PCR was also carried out for the suppressor hybrids and the samples were placed into the 96 wells plate as explained in Figure 6.4. Twelve suppressor hybrids were tested in order to assess the expression levels of the IFNE1 gene also in the UACC-903 parental melanoma cell line and one of the PC-3 prostate cancer cell line. The amplification plots in both linear and logarithmic views are shown in Figure 6.7.

(a)



(b)

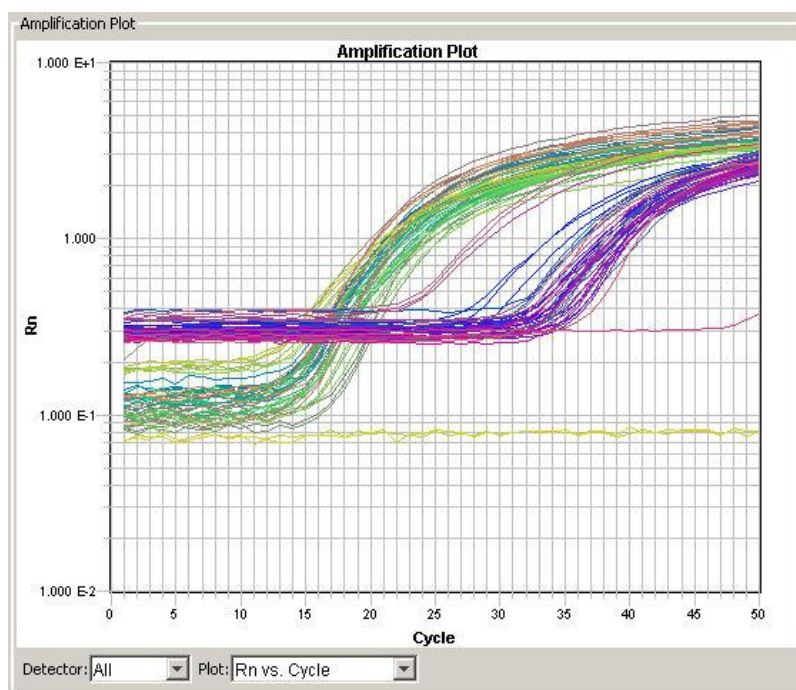


Figure 6.7: Amplification plot for IFNE1 suppressor hybrids. (a) the linear view of PCR curve and (b) is the logarithmic view of amplification plot. The 3 lines which are between the two major groups of curves (green and yellow curves-GAPDH on left and purple curves of right-IFNE1) represent the PC-3 sample triplicate and the flat lines are the NTC samples.

The gene expression plot of the suppressor hybrids for the IFNE1 gene is displayed in Figure 6.8. By taking 1BR.3 sample as the calibrator, we can see that all the levels of gene expression were below the calibrator except for the prostate cancer cell line where the IFNE1 is highly expressed.

By taking the individual RQ values for each sample from suppressor and non suppressor hybrids it was possible to draw a graph for the expression of the IFNE1 gene. The RQ value is the calculated relative quantity of the sample expressed as a multiple of the calibrator. In other words, it represents the fold change of the level of expression compared to the calibrator. In the present work, the calibrator 1BR.3 level of expression was set to the value of 1 (Figure 6.8). An impaired t-test was carried out for the expression levels of the IFNE1 gene, the results of the test suggests that the difference of RQ values between the two types of hybrids is non-significant as the calculated p value was 0.135.

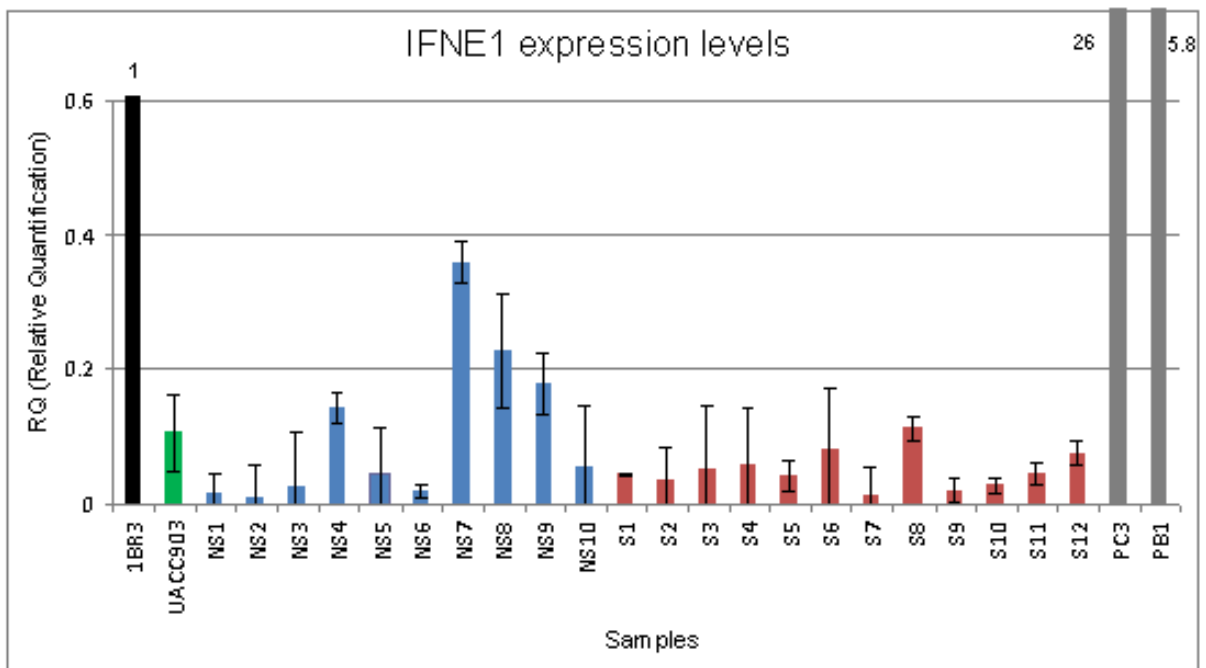


Figure 6.8: Gene expression levels of the non suppressor and suppressor hybrids which are below the 1.BR3 calibrator level except for PC3 and PB1 cell lines. By comparing the relative quantification values between the two types of hybrids, it appears that IFNE1 is expressed as higher amount in the non suppressor than the suppressor hybrids.

6.3.2. Interferon alpha 1 (IFNA1)

Similarly to the IFNE1 gene, from the standard reverse transcription PCR carried out for the constructed suppressor and non suppressor hybrids, the IFNA1 might be a candidate gene which can play a role in the development of cutaneous malignant melanoma. In fact, expression of this gene has been detected in all the suppressor hybrids and in only one the non suppressor hybrids (NS7) (figure 6.10). Real-time PCR has been chosen as a method to measure the levels of expression of the interferon alpha 1 gene in the different constructed hybrids, as it a highly sensitive method able to detect very low levels of double stranded DNA.

6.3.2.1. qPCR of non suppressor hybrids for IFNA1 gene

The non suppressor hybrids have been tested in order to quantify the expression of the IFNA1 gene. The 96 wells plate was prepared in the same manner as for the IFNE1. The amplification plot at a logarithmic scale is shown below (Figure 6.9).

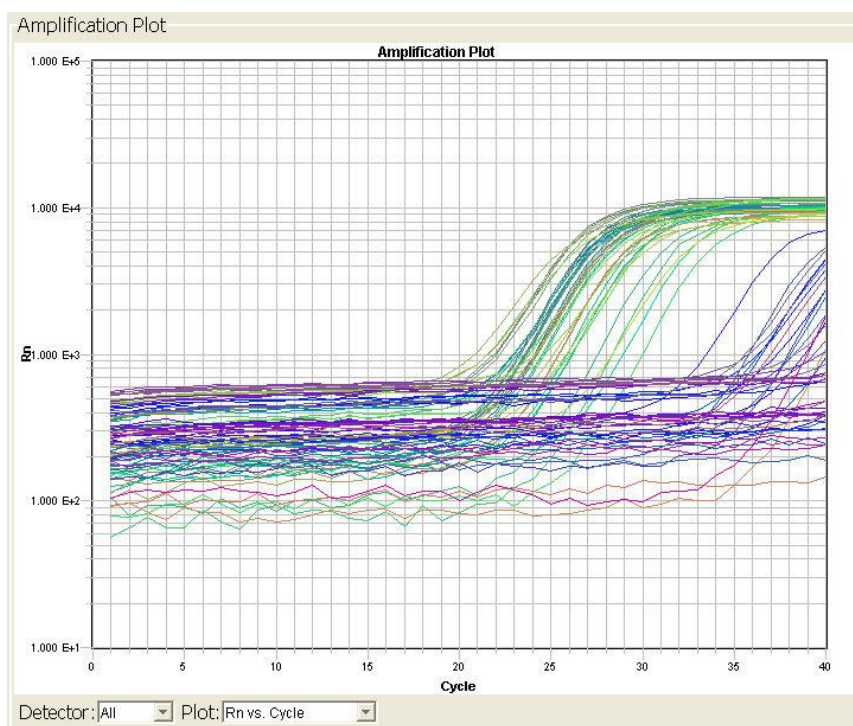


Figure 6.9: Logarithmic view of the amplification plot of the non suppressor hybrids for the GAPDH (green curves) and IFNA1 genes (blue/purple curves). Most of the IFNA1 curves are flat which indicates that no fluorescent signal was detected.

Expression levels plot was also available. As for the analysis of expression levels for the gene IFNE1, the calibrator chosen was 1BR.3 normal fibroblast cell line. Therefore, the levels of expression of the non suppressor hybrids for the IFNA1 gene were compared to the one of 1BR.3 by measuring individual RQ values (Figure 6.12).

6.3.2.2. qPCR of suppressor hybrids for IFNA1 gene

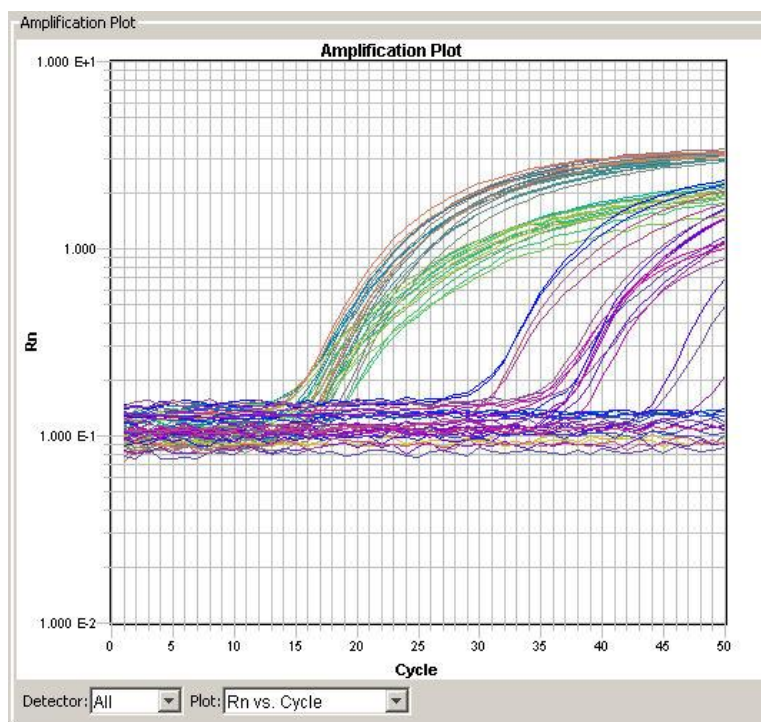


Figure 6.10: Logarithmic view of the amplification plot of the suppressor hybrids for the GAPDH (green) and IFNA1 genes (blue/purple).

As shown in the above figures, the level of expression of the IFNA1 between the suppressor and the non suppressor hybrids show significant differences. Therefore, additional real-time PCR have been carried out in order to compare the relative quantification of both suppressor and non suppressor hybrids. In doing so, a 96 wells plate was prepared with duplicate of non suppressor and suppressor hybrid cell lines, the UACC-903 parental melanoma cell line, the 1BR.3 normal fibroblast cell line and the PC-3 prostate cancer cell line (Figure 6.10).

	1	2	3	4	5	6	7	8	9	10	11	12
A	IFN 1BR3	IFN 1BR3	IFN S8	IFN S8	IFN NS6	IFN NS6	GAPDH 1BR3	GAPDH 1BR3	GAPDH S8	GAPDH S8	GAPDH NS6	GAPDH NS6
B	IFN S1	IFN S1	IFN S9	IFN S9	IFN NS7	IFN NS7	GAPDH S1	GAPDH S1	GAPDH S9	GAPDH S9	GAPDH NS7	GAPDH NS7
C	IFN S2	IFN S2	IFN S10	IFN S10	IFN NS8	IFN NS8	GAPDH S2	GAPDH S2	GAPDH S10	GAPDH S10	GAPDH NS8	GAPDH NS8
D	IFN S3	IFN S3	IFN NS1	IFN NS1	IFN NS9	IFN NS9	GAPDH S3	GAPDH S3	GAPDH NS1	GAPDH NS1	GAPDH NS9	GAPDH NS9
E	IFN S4	IFN S4	IFN NS2	IFN NS2	IFN NS10	IFN NS10	GAPDH S4	GAPDH S4	GAPDH NS2	GAPDH NS2	GAPDH NS10	GAPDH NS10
F	IFN S5	IFN S5	IFN NS3	IFN NS3	IFN UACC-903	IFN UACC-903	GAPDH S5	GAPDH S5	GAPDH NS3	GAPDH NS3	GAPDH UACC-903	GAPDH UACC-903
G	IFN S6	IFN S6	IFN NS4	IFN NS4	IFN PC3	IFN PC3	GAPDH S6	GAPDH S6	GAPDH NS4	GAPDH NS4	GAPDH PC3	GAPDH PC3
H	IFN S7	IFN S7	IFN NS5	IFN NS5	IFN NTC	IFN NTC	GAPDH S7	GAPDH S7	GAPDH NS5	GAPDH NS5	GAPDH NTC	GAPDH NTC

Figure 6.11: Set-up of suppressor and non suppressor hybrids for IFNA1 gene.

Individual relative quantification (RQ) values for a set of suppressor and a set of non suppressor hybrids have been measured for each sample by taking 1BR.3 as the calibrator which RQ value was set at 1 as displayed in Figure 6.11. By taking the individual RQ values for each sample from suppressor and non suppressor hybrids it was possible to draw a graph for the expression of the IFNE1 gene. The RQ value is the calculated relative quantity of the sample expressed as a multiple of the calibrator. In other words, it represents the fold change of the level of expression compared to the calibrator. In the present work, the calibrator 1BR.3 level of expression was set to the value of 1.

Statistical analysis of IFNA1 levels of expression between the S and NS hybrids resulted on a p value of 0.321 which suggests that there is no significant difference between the two sets of RQ values. Although, there is not a significant difference, some of the suppressor hybrids show to highly express INFA1.

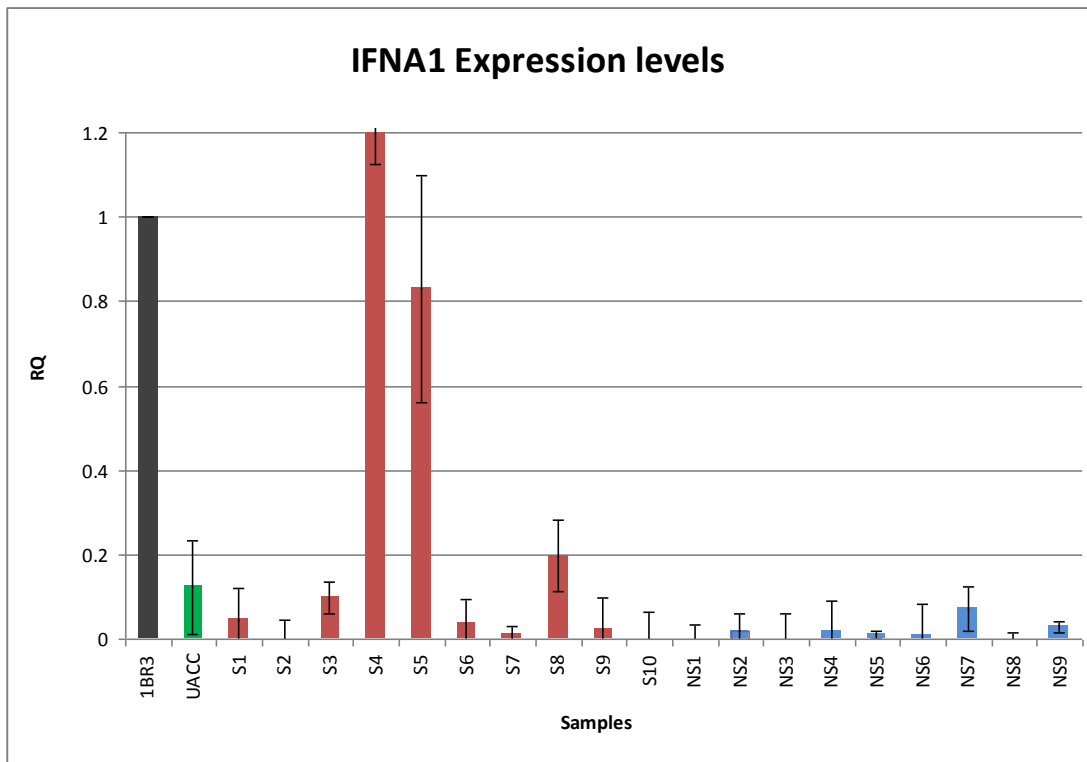


Figure 6.12: Gene expression levels of the non suppressor and suppressor hybrids which are below the 1.BR3 calibrator level except for PC3 prostate cancer cell line and for one of the suppressor hybrid (S4). Most of the RQ values of the suppressor hybrids are higher than the ones of the non suppressor hybrids.

A qualitative comparison of non-quantitative RT-PCR (Figure 5.12) with results from quantitative real-time PCR (Figure 6.12) for the expression analysis of IFNA1 has been carried out and showed that the NS7 sample, which is the only expressed hybrids among the non suppressor hybrids, has the highest RQ values when compared with the other non suppressor hybrids. The RQ values of the other hybrids were much lower than the RQ of NS7 and were therefore not detectable using standard PCR. However, the suppressor hybrids did not show clear

correlation as some intense bands of some samples such as S1, S3, S9 and S11 did not have display high levels of expression of the IFNA1 gene.

6.4. Discussion

The aim of performing real-time PCR in this study was to measure the levels of expression of the genes IFNE1 and IFNA1 in the suppressor and non suppressor constructed hybrids. Gene expression analysis using standard RT-PCR revealed that the IFNE1 and IFNA1 genes might be the candidate tumour suppressor genes involved in the development of cutaneous malignant melanoma, this suggestion resulted from the expression of these genes in all of the suppressor hybrids and only some of the non suppressor hybrids.

Real-time PCR is a highly sensitive method for quantitative reverse transcription PCR of mRNA levels from genes. Applying this method for the expression of the genes IFNE1 and IFNA1 showed different profiles of level of expression between the suppressor and non suppressor hybrids. Firstly, the cDNA amounts were measured between non suppressors using GAPDH as an endogenous control. On a second 96 wells plate, suppressor hybrid samples were assessed for level of cDNA as well which resulted in amplification plots showing that cDNA levels of the genes IFNE1 and IFNA1 were low compared to the cDNA levels of the GAPDH housekeeping gene (Figures 6.6, 6.7, 6.9, and 6.10). However, this observation was not surprising as it is known that housekeeping genes transcripts are abundant and their expression remains constant in the cells and tissues under investigation (Barber *et al.*, 2005). The fluorescent signal for the GAPDH gene

was detected in first and the average Ct value was around 17, which means that the exponential phase of amplification started from the cycle number 17. On the other hand, the average Ct values for IFNE1 and IFNA1 were approximately 30 and 35 respectively.

Relative quantification (RQ) of the levels of mRNA was given by setting the 1BR.3 fibroblast cell line as a calibrator where its RQ value was 1. This reference sample is used to measure the fold change of levels of expression, which means that if the RQ value is superior to 1 the mRNA level of the target gene is higher than the one of 1BR.3 and vice versa.

The results obtained for the IFNE1 gene expression levels show that there was no major difference in the amount of mRNA between the suppressor and non suppressor hybrids, and that all the RQ values were below the one of the calibrator 1BR.3. From the results presented in figure 6.8 it is possible to say that no significant difference in the amount of cDNA for the IFNE1 gene between the suppressor and the non suppressor hybrids. Despite the fact that standard RT-PCR resulted in the absence of expression of this gene in 4 out of 10 of the constructed non suppressor hybrids. Real-time PCR data for the analysis of levels of IFNE1 transcripts illustrate that: (a) IFNE1 is expressed at high levels in the human prostate cell line (PC-3) and breast cancer cell line (PB-1) compared to its expression in the 1BR.3 fibroblast cell line, (b) IFNE1 gene is expressed at low levels in both the suppressor and non suppressor hybrids, (c) there is no significant differences in the expression of the IFNE1 gene in the hybrids.

Consequently, it is suggested that the IFNE1 gene might not be a candidate tumour suppressor gene involved in the development of CMM. The interferon epsilon 1 gene has been recently identified (Hardy *et al.*, 2004) and its function remains unclear but it is believed to have a different role than the other type I interferons which are cytokines with pleiotropic activities such as inhibition of viral replication, cell proliferation and activation of the immune system (Hardy *et al.*, 2004). This difference of function results from the fact that IFNE1 gene has limited homology to other type I IFN genes. Also, their ability to bind to the type I IFN receptor is unknown. Hence, it is difficult to establish how IFNE1 can suppress tumorigenicity and if its deletion affects the development of CMM.

In the case of the interferon alpha 1 (IFNA1) gene, real time PCR was performed in order to estimate the levels of expression in the two types of constructed hybrids as well as in other control cell lines. Similarly to the analysis carried out for the IFNE1 gene, levels of cDNA of the IFNA1 gene were compared to the GAPDH endogenous control and the relative quantification values were monitored. The amplification plots for some of the samples were almost flat as shown in Figures 6.9 and 6.10. The fluorescent signal was weak which can be interpreted as an indication of the presence of extremely low levels of dsDNA of the target gene or absence of amplification of the IFNA1 target gene.

As illustrated in Figure 6.12, the relative quantification values for the IFNA1 gene in the suppressor hybrids were high when compared to the RQ values of the non suppressor hybrids. As a result, these data provide evidence that there is a

measurable difference of expression between the two types of hybrids. Expression of IFNA1 gene is higher in the suppressor than its expression in the non suppressor hybrids. This observation provides tantalising evidence that the IFNA1 gene might be a candidate tumour suppressor gene involved in the pathogenesis of cutaneous malignant melanoma. The interferon alpha 1 gene is widely used in the treatment of specific types of tumour including melanoma. This immunotherapeutic agent is a cytokine exhibiting a potential anti-tumour effect and is used mainly in phase II and III melanoma patients. In addition, it has been suggested that IFNA1 might induce other tumour suppressor genes. For instance, IFNA1 anti tumour action has been proven by the fact that it induces the transcription of the p53 gene (Takaoka *et al.*, 2003). Furthermore, the same researchers show that induction of the p53 gene by IFNA1 and IFNB1 contribute to tumour suppression but the mechanism(s) by which IFNA1 induces p53 is (are) still unclear. In order to confirm the involvement of IFNA1 in the development of melanoma, it would be possible to use siRNA knock down against IFNA1 and analyse its effects on tumourigenicity. Plasmid vector expressing appropriate small interfering RNAs of IFNA1 could be readily transfected into the parental tumourigenic UACC-903 cells. Published evidence suggests the knockdown of a targeted gene occurs within few hours and persists within less than one week (Bartlett and Davies, 2006). While this approach is possible, it would be difficult to assay for tumourigenicity by soft agar since these assays monitor colony growth over 2-3 weeks; by which time the transient effect of siRNA knockdown would have passed. Another informative approach would be to analyse IFNA1

protein expression using western blotting in cells taken at different stages of melanoma progression in tumours from patients would provide an indication of changes of expression occurring in CMM as no data has been provided yet relating the expression of IFNA1 during tumour progression.

CHAPTER 7

SUB-CLONING AND CHARACTERISATION OF IFNA1 GENE

7.1. Introduction

Although the anti-proliferative and pro-apoptotic mechanisms of interferon alpha (IFNA1) which have been recognised, its antitumour effects are not entirely known. Interferon alpha is a cytokine that displays anti-viral and immunomodulatory activities, and it is the most used type of cytokine being used as a therapy for the treatment of different types of cancer including some haematological malignancies and solid tumours such as melanoma (Ferrantini and Belardelli, 2000). Despite the wide clinical use, it is still unclear whether IFNA therapy is an effective strategy in melanoma therapy and more importantly the involvement of the IFNA1 gene in the pathogenesis of cutaneous melanoma remains to be fully established. After the identification of the IFNA1 gene as the potential candidate tumour suppressor gene in melanoma in the present study, the next step undertaken was the expression of the IFNA1 cDNA in the UACC-903 using an expression vector to evaluate suppression of tumourigenicity by anchorage independent growth and real-time PCR. These series of experiments aim to further determine the role of IFNA1 as a potential suppressor gene in melanoma.

7.2. Materials and methods

The cell line used for the sub-cloning experiment was the normal dermal fibroblast 1BR.3 described previously in Chapter 2. After isolation of IFNA1 RNA using TRIzol method (see section 2.6) and using reverse transcription method to obtain complementary DNA, and finally amplification IFNA1 cDNA (described in section 5.2.3), the amplification product was cloned into a vector as described in the sections below:

7.2.1. Preparation of LB Agar plates

The first step for the IFNA1 cDNA cloning consisted of the transformation step of the plasmid cDNA. In doing so, agar plates have been prepared by weighing out the following: 10 g of bacto tryptone, 5 g of bacto yeast extract, 10 g of sodium chloride and 15 g of bacto agar and dissolving them in 950 ml of distilled water. After autoclaving the bottle at 121°C for 15 minutes, the hot agar bottle was placed in a water bath set at 55°C and 100µg of ampicillin per ml of agar was added from 10mg/ml of stock solution. The bottle was swirled in order to mix the ampicillin and poured quickly into the plates (P-110 Nunclon, Fisher Scientific). The lids of the plates were flamed using a Bunsen burner and the plates were covered partially with their lid for the agar to set normally after 30 minutes. The plates were wrapped into cling film and stored at 4°C for no longer than one month.

7.2.2. Cloning of PCR products

The TOPO TA cloning[®] Kit (Invitrogen, UK) was used for the direct insertion of the IFNA1 PCR product into a plasmid vector for sequencing. The plasmid vector (pCR[®]4-TOPO[®]) was supplied linearised with single 3' thymidine (T) overhangs for TA cloning and topoisomerase covalently bound to the vector. It also uses T7 and T3 primers to allow for sequencing of + and – strands (Figure 7.1).

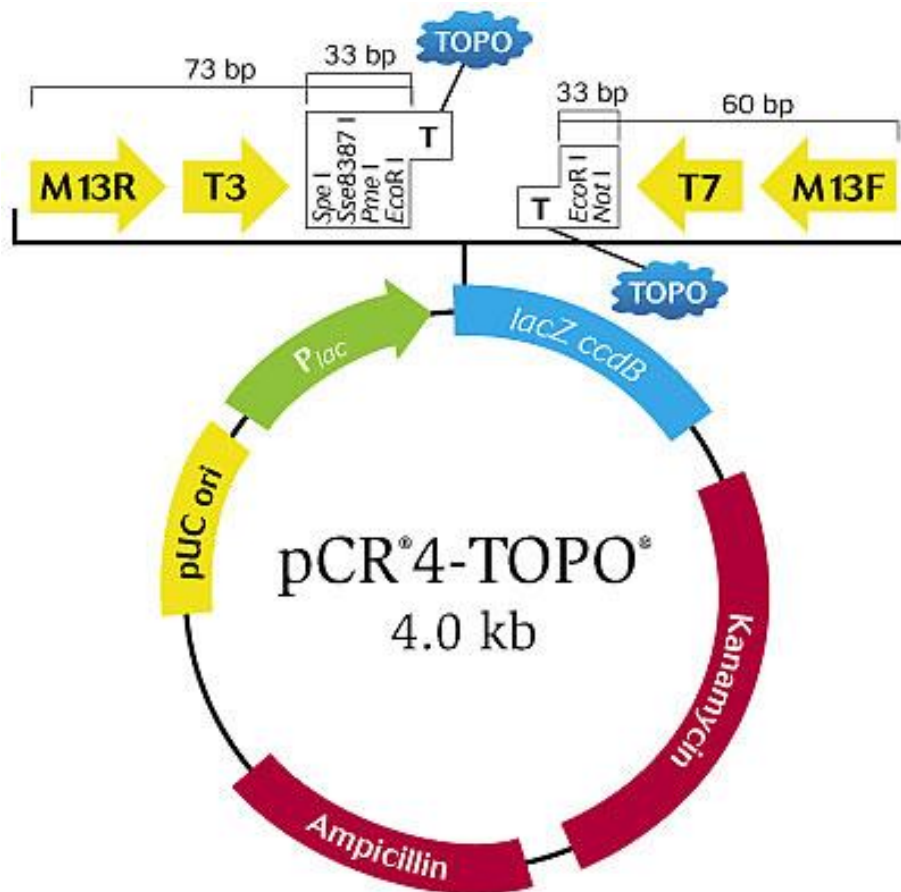


Figure 7.1: The pCR[®]4-TOPO[®] vector displaying 3' T overhangs and the T7 and T3 primers as well as the EcoRI sites flanking the cloning site that simplify excision of cloned PCR products (Invitrogen, UK)

(<http://products.invitrogen.com/ivgn/en/US/adirect/invitrogen?cmd=catProductDetail&entryPoint=adirect&productID=K457502&messageType=catProductDetail>).

The total ligation mixture was prepared by adding 1 μ l of salt solution and 1 μ l of vector solution provided in the kit, 2 μ l of DEPC water was added as well as 2 μ l of PCR product resulting from the amplification of IFNA1 cDNA. The ligation mixture was then incubated at room temperature for 5 minutes and 2 μ l were taken and added into the competent cells that were thawed on ice. The tubes were mixed gently without retro-pipetting as it might shear the DNA TA ligation), and incubated on ice for 15 minutes. The cells were put for 30 seconds in a water bath at 42°C to create a heat shock, and transferred immediately on ice. After that, 250 μ l of warm SOC TOP 10 medium was added into the tubes that were then placed into a shaker set at 37°C for an hour at 200rpm. After the shaking step, 50 μ l and 100 μ l of the plasmid were spread onto the pre-warmed LB agar plates containing 100 μ g/ml of ampicillin. The plates were left to dry for approximately 10 minutes and placed into the 37°C incubator. The colonies were observed from the next day, and approximately 8 colonies were picked and spread onto an agar plate that was divided into 8 parts by drawing lines on the plate.

On the following day, 5 ml of agar solution containing ampicillin was added into 15 ml tubes. With a tip, the colonies were picked by touching them carefully and placing the tip in the 5 ml agar solution. Around 10 colonies were picked in addition to a control tube that does not contain cells to confirm the absence of infection. The tubes were then incubated overnight in a shaker set at 37°C.

7.2.3. Extraction and purification of plasmid DNA using Miniprep Kit

After shaking the tubes overnight, they all appear to be full of cells except the control tube which should be clear. An aliquot of each clone should be frozen down by taking 250 µl of warm glycerol into 1.5 ml Eppendorf tubes and adding 750 µl of culture medium for each tube and storing them at -80°C.

The miniprep kit used for the extraction of plasmid DNA was the PureLink™ Quick Plasmid Miniprep (Invitrogen, UK). From the E-coli overnight culture, 2 ml were taken and put into a 2 ml Eppendorf tube and centrifuged at 13,000 rpm for 1 minute. After removing the supernatant medium, the pellet was resuspended in 250 µl Resuspension Buffer (R3) with RNase A, 250 µl of Lysis Buffer (L7) were added and the tubes were mixed gently by inverting the capped tubes 5 times. The tubes were then incubated for no more than 5 minutes at room temperature and 350 µl of Precipitation Buffer (N4) were added to the cells and the tubes mixed immediately by inverting the tubes until the solution is homogenous. The mixture was centrifuged at 12,000 x g for 10 minutes at room temperature using a microcentrifuge to clarify the lysate from the lysis debris. The supernatant was loaded onto a Spin Column which was then placed into a 2 ml wash Tube and centrifuged at 12,000 x g for 1 minute. After discarding the flow-through, the column was put back into the Wash Tube and 700 µl of Wash Buffer (W9) containing ethanol were added, the column was centrifuged at 12,000 x g for 1 minute. After discarding the flow-through, the column was placed back into the Wash Tube and centrifuged again at 12,000 x g for 1 minute to remove any residual Wash Buffer (W9). The Wash Tube was discarded with the

flow-through and the column placed in a clean 1.5 ml Recovery Tube. The next step consisted of adding 75 µl of preheated TE Buffer (TE) to the centre of the column which was left to incubate for 1 minute at room temperature. Another centrifugation was carried out for 2 minutes at 12,000 x g and the purified plasmid DNA was retrieved in the Recovery Tube and can be stored at -20°C.

7.2.4. EcoR1 restriction enzyme digestion of DNA plasmid

In order to check the presence of the target gene (in this case IFNA1) into the DNA plasmid, 5 µl of the DNA solution was transferred in Eppendorf tubes with 1 µl of EcoR1 (Invitrogen, UK) enzyme, 2 µl of 10X buffer from EcoR1 kit and 12 µl of DEPC water. The tubes were then incubated at 37°C for 60 minutes. The products were run on a 1% agarose gel electrophoresis to confirm the EcoR1 treatment; control tubes have been prepared by adding 4 µl of uncut DNA plasmid with 4 µl of water and 2 µl of 6X loading buffer. For the digested samples, 5 µl of 6X loading buffer was added in each tube. All the samples were loaded on the gel by alternatively putting uncut and cut products. Visualisation of the gel using the alpha imager confirmed the presence the IFNA1 cDNA into the plasmid vector (Figure 7.1). Different clones were selected and sent for sequencing to a company called Cogenics which provides a full range of genomics services for both research and regulated projects (Essex, UK).

7.3. Results

The digestion of the plasmid DNA with EcoR1 enzyme was verified by running cut and uncut DNA on a 1% agarose gel electrophoresis. Figure 7.2 shows the digestion of eight DNA plasmid products with the products of the digested DNA showing two bands where the bottom one corresponds to the IFNA1 cDNA with a size of 691bp and the upper band corresponds to the plasmid cDNA.. For sample number 2, the results of the digestion are negative which may be caused by the absence of cDNA.

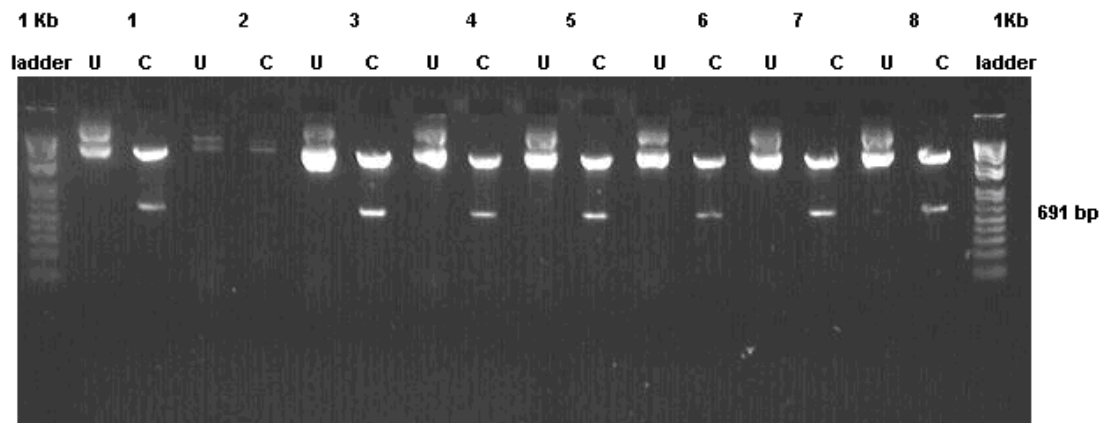


Figure 7.2: Agarose gel image of plasmid cDNA treated with ECOR1 enzyme. U (Uncut) and the control samples where no enzyme was added and C (Cut) are the cDNA samples digested with EcoR1 resulting on 2 bands: the upper one corresponds to plasmid cDNA and the bottom one to IFNA1 cDNA with a size of 691 bp.

The results from the sequencing obtained after three weeks from Cogenics are summarised in Table 7.1. The different sequences have been analysed using Chromas software (version 2.33) available from the internet

(<http://www.technelysium.com.au/chromas.html>). After analysing the sequences using Chromas, they were compared to the cDNA sequence of the gene IFNA1. The percentage of similarity between the sequences and the IFNA1 gene are shown in the Table 7.1.

Sample number	Sample name	Type	Template / Conc (ng/ μ l)	Similarity (%)
1	CPA86245_A12_T3P_L03.ab1	Plasmid DNA	100	99
2	CPA86245_B01_T3P_N03.ab1	Plasmid DNA	100	99
3	CPA86245_B02_T3P_P03.ab1	Plasmid DNA	100	100
4	CPA86245_B03_T3P_B05.ab1	Plasmid DNA	100	99
5	CPA86245_B04_T3P_D05.ab1	Plasmid DNA	100	99
6	CPA86245_C04_T7P_L07.ab1	Plasmid DNA	100	99
7	CPA86245_C05_T7P_N07.ab1	Plasmid DNA	100	99
8	CPA86245_C06_T7P_P07.ab1	Plasmid DNA	100	99
9	CPA86245_C07_T7P_B09.ab1	Plasmid DNA	100	99
10	CPA86245_C08_T7P_D09.ab1	Plasmid DNA	100	99

Table 7.1: Results from sequencing of the clones DNA obtained after transfecting IFNA1 cDNA into plasmid vector. The clone number 3 displays 100% similarity to IFNA1 gene which means that it has no mutations.

7.4. Discussion

The cDNA sequence obtained from the clone number 3 displays 100% similarity with the full cDNA sequence of the interferon alpha 1 gene. In other words, no PCR errors have occurred in that clone which will then be transferred into mammalian cell expression vector (pcDNA3) and transfected in to the UACC-903 cell line as well as other melanoma cell lines in order to assess the suppression of tumourigenicity.

CHAPTER 8

CONCLUSIONS AND FURTHER RESEARCH

8.1. General discussion and conclusion

Cutaneous malignant melanoma is the most aggressive form of skin cancer and from a clinical perspective presents many challenges. Its incidence continues to increase in many countries and consequently this malignancy has attracted a lot of research interest as it became a significant health problem in several countries. The exact mechanisms by which melanoma occurs and develops remain unclear but it is well established that genetic as well as environmental factors such as UV exposure play a major role in the development of melanoma. Loss of heterozygosity deletions on different chromosomal regions such as 1p, 6q, 9p, 10q and 11q are observed during the development and progression of CMM, these cytogenetic regions are believed to contain critical tumour suppressor genes central to melanoma development. When TSG function is inactivated, the control of normal cell division may be lost contributing to the development of CMM.

Genetic alteration of chromosome 9p appears to be a critical and frequent event in CMM development. Chromosomal aberrations such as LOH and homozygous

deletion in various malignant cell lines including melanoma are regularly observed. Data suggest that critical loci which are distinct from the main TSG involved in melanoma CDKN2A, are believed to reside on the chromosomal region 9p21 adjacent to the INK4 locus (Parris *et al.*, 1999; Pollock *et al.*, 2001). Hence, the aim of this study as stated in chapter 1 was to identify novel tumour suppressor genes at chromosome 9p21 which may be important in CMM development

The functional complementation technique of microcell mediated chromosome transfer technique was central to the research presented herein, and was used to construct cell hybrids by the transfer of a single chromosome 9 carrying a deletion at the INK4 locus (chromosome 9a) into the UACC-903 human malignant melanoma recipient cell line. In this way the effect of potential tumour suppressor loci on chromosome 9p21 could be examined independently of the presence of the CDKN2A gene P16. The constructed hybrids were afterwards analysed and tested for anchorage independent growth in a semi-solid medium as described in chapter 4. Two types of hybrids were obtained: (a) segregant (tumourigenic) hybrids in which there was no suppression of anchorage independent growth in soft agar and which were classified as non suppressor hybrids and, (b) suppressor hybrids (suppression of tumourigenicity) that displayed no growth or a reduced capacity for anchorage independent growth in soft agar.

To determine if differences in gene expression of loci located at chromosome 9p21 were responsible for altered tumorigenicity in the segregant and suppressor

hybrid cell lines, gene expression analysis of 23 functional genes that are located in the chromosomal region 9p21 was conducted. Gene expression analysis of these gene in both suppressor and non suppressor hybrids as well as the UACC-903 parental cell line and three other control cell lines, resulted in the identification of the two genes IFNE1 and IFNA1 as potential tumour suppressor genes. This is justified by the fact that these two genes exhibited expression in all the suppressor hybrids, whereas expression of these two genes was often absent in non suppressor hybrids, suggesting that the loss of these genes could be associated with a loss of tumour suppressor activity. Thus, chapter 6 presents an accurate approach to measure the levels of expression of IFNE1 and IFNA1 in the constructed hybrids. The findings derived from this approach suggest that the IFNE1 is not a candidate tumour suppressor gene because no significant difference was detected when the level of expression of this gene in suppressor and non suppressor hybrids was examined. The IFNE1 gene is a member of the type I interferon family and it is highly expressed in the brain but its biochemical and biological functions are poorly understood (Peng *et al.*, 2007). In addition, the same authors reported that the anti-viral and anti-proliferative activities of the recombinant human IFNE1 gene were lower than other members of type I IFN such as IFNA2 (Peng *et al.*, 2007).

In the case of interferon alpha 1 gene, real-time PCR analysis revealed a difference in the level of expression of the IFNA1 gene between the suppressor and non suppressor hybrids. The levels of IFNA1 expression reflect the amount of cDNA of the gene in the suppressor versus non suppressor cell hybrids.

In Figure 6.12 it can be seen that the expression of IFNA1 in the suppressor hybrids is generally higher than the levels of expression in the non suppressor hybrids. Therefore, examination of the IFNA gene in this study provides evidence that it may be a potential tumour suppressor gene involved in the development of cutaneous malignant melanoma.

Other studies have demonstrated that this gene is implicated in the induction of expression of p53 by boosting p53 responses to stress signals (Takaoka *et al.*, 2003). In addition, other studies confirmed that IFNA1 can directly inhibit the proliferation of tumour cells by down-regulating expression of oncogenes and inducing tumour suppressor genes such as p53, which can contribute to the antiproliferative activity of the IFN α cytokine (Ferrantini *et al.*, 2007).

These observations lend weight to the findings presented in this study and confirm that IFNA1 may be a potential tumour suppressor gene itself or it has a critical role in the activation of other tumour suppressor genes implicated in the progression of cutaneous malignant melanoma.

The type I interferon family are an important class of genes that encodes for cytokines which play a role in activation of the immune system, inhibition of viral replication and suppression of cell proliferation (Stark *et al.*, 1998). These properties have given the type I IFN important roles in infections and cancer and a number of interferons are being used in therapies for many clinical conditions (Hardy *et al.*, 2004). One of the applications of type I interferon for cancer therapy is for the treatment of melanoma. The interferon IFN α 2b is an approved

therapy for melanoma, but has only shown to be clinically effective as adjuvant therapy of high risk melanoma cases (reviewed in Moschos and Kirkwood, 2007). Also, IFN α alone, even at high doses, does not cure all of the tested patients (Kirkwood *et al.*, 1996). Currently, data from 12 randomized controlled trials using IFN α are available; they all failed to demonstrate a clear impact of IFN α therapy on overall survival in melanoma patients (Lens, 2006). These remarks suggest that this might depend on the genetic profile of individual patient's including the type of tumour and its stage and grade. However, the better understanding of the direct and the indirect anti-tumour roles of type I interferons in cancer in general and particularly melanoma, as well as the identification of all the mechanisms of type I interferons signalling and the mediation of immunosurveillance, will provide important information for the design of future treatments based on the use of interferons.

8.2. Research limitations and future work

As described and justified in the different chapters of the studies presented in this thesis, the collection and data interpretation used several cellular and molecular methodologies. The methods used have provided useful results where the application of the functional complementation technique of MMCT together with genes expression analysis has permitted the identification of a candidate TSG for CMM. But this research method has some limitations and as an example of these limitations, animal studies would have provided a more robust way to confirm the

type of the constructed hybrids as segregant or suppressor. *In vivo* tumourigenicity of the monochromosome hybrids consists in the inoculation of the suspended cells from various hybrids into nude mice followed by measurement of the tumours formed as described in Parris *et al.*, 1999. However, this method was not used during this project because of the costs of animal studies as explained previously in chapter 3. Although anchorage independent assays using soft agar cloning is regarded as a reliable method for analysing tumourigenicity in a variety of tumour cell types (Shin *et al.*, 1975).

Moreover, it would have been interesting to examine the melanoma tumour cells for cytogenetic studies like FISH to retrieve the introduced chromosome 9 or its fragments and maybe carry out genotyping using microsatellite analysis to determine the deleted loci. Also, another possibility can be used to confirm the involvement of IFNA1 in the development of melanoma and which consists of the use of siRNA knock down against IFNA1 and its effects on tumourigenicity. If IFNA1 knock down results on increase proliferation and rapid tumour growth, it will confirm the tumour suppression properties of IFNA1 in CMM. Furthermore, testing IFNA1 protein using western blotting in cells taken at different stages of melanoma progression would provide an indication of changes of expression occurring in CMM.

Initially, this study was carried out using only the highly tumourigenic malignant melanoma cell line UACC-903, which can be considered as a limitation. Further work to confirm the role of IFNA as a potential TSG for melanoma should involve the analysis of the expression of this gene in a number of tumorigenic

melanoma cell lines and melanoma tumour samples of different stage and grade. Additionally, it will be useful to construct hybrids using different recipient cell lines. In doing so, a range of hybrids will be available which will provide a larger opportunity to map and clone the candidate genes.

Finally, to minimise the aforementioned limitations and in order to confirm the suppression effect of the IFNA1 gene in melanoma cell lines, the transfection of the cDNA of the IFNA1 gene into the UACC-903 and other melanoma cell lines is required and currently underway in our laboratory as explained in Chapter 7. This approach consists of a stable transfection. Melanoma cell lines stable transfected with the IFNA cDNA will be analysed for suppression of tumourigenicity using standard approaches described in this study.

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