Universidade de Aveiro Secção Autónoma de Ciências da Saúde
Ano 2011

Sandra Raquel de Oliveira Tavares

Identificação de biomarcadores terapêuticos em Melanoma

Identification of therapeutic biomarkers in Melanoma

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Paula Soares, Professora Auxiliar da Faculdade de Medicina da Universidade do Porto.

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agradecimentos

A realização desta tese de mestrado, em tão prestigiada instituição, só pode ser possível graças à Professora Doutora Paula Soares. Pelo acolhimento no seu grupo, pela atenção que dedicou ao meu trabalho e pelo seu apoio em todo este processo lhe estarei sempre muito grata.
Agradeço também à Helena Pópulo pelo rigor e paciência. Foi por estes que atingi um nível mais elevado de autonomia no laboratório.
Terei que agradecer também a todo o grupo de Oncobiologia do IPATIMUP, pelo apoio, amizade e entreajuda que senti desde o primeiro momento.
Agradeço ao Professor Michael Schrader pela disponibilidade e valiosas indicações na elaboração desta tese.
Por último, gostaria de agradecer à minha família e ao Ricardo pelo apoio e encorajamento para terminar esta etapa da minha formação científica.

palavras-chave melanoma, biomarcador, BRAF, GNAQ, GNA11, via de sinalização do mTOR, RAD001

resumo

O melanoma deriva da transformação maligna de melanócitos e é frequentemente encontrado na pele e na região ocular. A identificação de marcadores moleculares que inequivocamente indiquem a agressividade do tumor ou que possam prever a resposta/resistência a uma terapia é uma prioridade da comunidade científica que estuda esta patologia. Mutações do gene do BRAF, maioritariamente V600E e mutações do gene do NRAS têm sido implicadas no desenvolvimento de melanoma cutâneo. Enquanto, mutações nos genes do GNAQ e GNA11 são encontradas com elevada frequência em melanomas uveais. Todas estas mutações podem levar à activação da via de sinalização das MAP cínases, conduzindo ao aumento da proliferação celular

O objectivo deste estudo foi a identificação de mutações genéticas frequentes como indicadores de prognósticos ou de previsão de resposta a uma terapia de inibição da via do mTOR e avaliar o potencial de efectores desta via como biomarcadores terapêuticos em melanomas cutâneos e oculares.

A existência de alterações genéticas nos genes do BRAF e NRAS foi determinada em 13 casos de melanoma cutâneo. O estado mutacional dos genes GNAQ e GNA11 foi avaliado em 34 casos de melanoma ocular. A análise mutacional foi realizada com recurso a PCR e sequenciação. A associação entre o estado mutacional e as características clínico-patológicas foi também estudada. Um conjunto de linhas celulares de melanoma com diferentes perfis genéticos foi tratado com um fármaco inibidor do mTOR, RAD001. A proliferação celular e apoptose foram avaliadas com os ensaios SRB e TUNEL, respectivamente, a expressão de efectores da via do mTOR foi detectada por immunoblotting.

G361, uma linha celular derivada de melanoma cutâneo com mutação BRAFV600E, apresentou a maior inibição de crescimento após tratamento com RAD001. RAD001 não induziu um aumento de apoptose em nenhuma das linhas celulares utilizadas neste estudo, sugerindo que o RAD001 não induz alterações no processo de apoptose.

Este estudo apresenta a mutação BRAFV600E como um possível marcador terapêutico do fármaco, RAD001, para pacientes com melanoma cutâneo mas não para pacientes com melanoma ocular, podendo no futuro ser uma ferramenta útil na selecção de pacientes para uma terapia baseada na inibição do mTOR.

keywords melanoma, biomarker, BRAF, GNAQ, GNA11, mTOR pathway, RAD001

abstract

Melanoma results from the malignant transformation of melanocytes and is frequently found in the skin and ocular region. The identification of molecular biomarkers, which unequivocally indicate the aggressiveness of the tumour or predict the response/resistance to a therapy, is of high priority in the field of melanoma research.

BRAF gene mutations, predominantly V600E, and mutations in the NRAS gene have been implicated in the development of cutaneous melanoma. Mutations in the GNAQ and GNA11 genes are found frequently in ocular melanoma, particularly in the uveal subtype. These mutations can lead to the activation of the MAPK pathway, which can lead to growth-promoting activities.

The aim of this study was to identify frequent mutations that can be used as prognosis or predictive markers for mTOR pathway inhibition. In addition, the potential of mTOR pathway effectors as therapeutic biomarkers in cutaneous and ocular melanoma was also addressed.

Genetic alterations in the BRAF and NRAS genes were determined in 13 cutaneous melanoma tumour samples and GNAQ and GNA11 genes mutational status were evaluated in a series of 34 tumour samples of ocular melanoma. Mutational analysis was done by PCR/Sequencing. The mutational status and its correlation to clinical-pathologial features was then assessed. A panel of melanoma cell lines with different BRAF, GNAQ and GNA11 mutational status were treated with RAD001, an mTOR pathway inhibitor. Cell viability and apoptosis were assessed by SRB assay and TUNEL assay, respectively, mTOR pathway effectors expression levels were detected by immunoblotting.

G361, a cutaneous melanoma-derived cell line, which harbours BRAFV600E, showed the highest level of cell growth inhibition after treatment with RAD001. RAD001 did not induce increased levels of apoptosis in any of the cell lines treated, suggesting that RAD001 does not induce alterations in the apoptosis process.

This study supports BRAF mutational status as a possible predictive biomarker to RAD001 treatment in cutaneous melanoma but not in ocular melanoma patients and as a promising tool to select patients to mTOR inhibition therapy.

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2 Abbreviation list

4E-BP1	eIF4E Binding Protein 1
AKT	Protein kinase B
ALM	Acral Lentiginous Melanoma
ARF	Alternative reading frame
BCL-2	B-cell lymphoma 2
BRAF	v-Raf murine sarcoma viral oncogene homolog B
CDKN	Cyclin-dependent kinase inhibitor 2
CM	Cutaneous Melanoma
DAG	Diacylglycerol
eEF2K	Eukaryotic Elongation Factor-2 Kinase
EGFR	Epidermal Growth Factor Receptor
FBS	Fetal Bovine Serum
FDA	Food And Drug Administration
FGF-2	Fibroblast growth factor-2
FGFR	Fibroblast Growth Factor Receptor
GAP	GTPase-Activating Protein
GNAQ	Guanine Nucleotide-binding protein G(q) subunit alpha
GPCR	G protein-Coupled Receptor
Gq	Q Class of Heterotrimeric GTP Binding Protein
HIF-1	Hypoxia-inducible factor 1
IL-8	Interleukin 8
INK4	Inhibitor of CDK4
IP3	Inositol Trisphosphate
IRS-1	Insulin Receptor Substrate 1
LDH	Lactic Dehydrogenase
LMM	Lentigo Maligna Melanoma
MAP4K3	MAP Kinase Kinase Kinase Kinase-3
MAPK	Mitogen-Activated Protein Kinase

MC1R	Melanocortin receptor-1
MITF	Microphthalmia-associated transcription factor
mLST8	Mammalian lethal with SEC13 protein 8
mTOR	Mammalian Target of Rapamycin
mTORC1	mTOR Complex 1
mTORC2	mTOR Complex 2
MYC	Myelocytomatosis viral oncogene
NF-kB	Nuclear factor-kB
NM	Nodular Melanoma
NRAS	Neuroblastoma RAS viral (v-Ras) oncogene homolog
PBS	Phosphate buffered saline
PDCD4	Programmed Cell Death 4
PDGFR	Platelet-Derived Growth Factor Receptor
PDK-1	3′Phosphoinositide-Dependent Kinase 1
PH	Pleckstrin Homology
PIP2	Phosphatidylinositol-4,5-Bisphosphate
PI(3,4)P2	Phosphatidylinositol-3,4-Bisphosphate
PIP3	Phosphatidylinositol-3,4,5-Triphosphate
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidylinositol Bisphosphate
PKC	Protein Kinase C
PLCb	B-Isoforms Of Phospholipase C
PRAS40	Proline-Rich AKT Substrate 40
PTEN	Phosphatase and Tensin Homolog
RSK	90 kDa Ribosomal Protein S6 Kinase
RTK	Receptor Tyrosine Kinase
S6K1	S6 Kinase 1
SSM	Superficial Spreading Melanoma
TGF-α	Transforming growth factor alpha (TGF-α)

TSC 1/2	Tuberous Sclerosis Complex 1/2
UM	Uveal Melanoma
UVR	Ultraviolet Radiation
VEGFR	Vascular Endothelial Growth Factor Receptor

3 Introduction

3 Introduction

Malignant melanoma derives from the transformation of the pigment-producing cells (melanocytes) that are found in the skin, ocular region, gastrointestinal and genito-urethral mucosal surfaces and in the meninges.[1]

3.1 History of Melanoma

Probably, the first mention to melanoma was made by the ancient Greek physician Hippocrates in the 5th century B. C., referring to it as "black herpetic type lesions".[2, 3] However, the first description of the disease was made several centuries later by René Laënnec, the inventor of the stethoscope. He presented melanoma as a disease entity to the Faculté de Médecine in Paris in 1806. Despite this, the word "Melanoma" was used by him only 6 years later when he reported a case of a disseminated melanoma. About five decades later, William Norris published a report in which he stated some principles about the clinical management and epidemiology of melanoma. Norris proposed for the very first time, an hereditary disposition for this disease, several years before the genetic paradigm articulated by Mendel in 1866. In his report, Norris also suggested a possible relationship between moles and cutaneous melanoma.[4]

3.2 Cutaneous and Ocular Melanoma: incidence and risk factors

Cutaneous melanoma

According to the Portuguese Association of Cutaneous Cancer (APCC), it is estimated that in Portugal the incidence of cutaneous melanoma is of 10 new cases per 100,000 habitants per year, which means about 1000 new cases per year (http://www.apcc.online.pt/ - 21/06/2011). This incidence is similar to that observed in countries in Southern Europe (Figure 1).

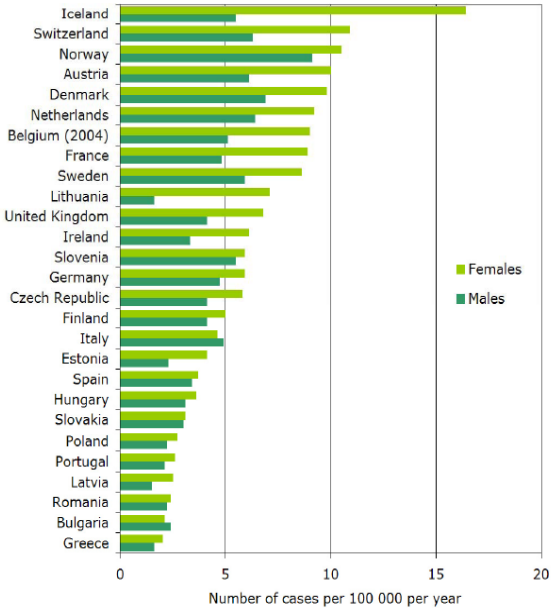


Figure 1: Age-standardized incidence of melanoma in people aged under 55 years in selected European countries in the year 2000. Taken from WHO (2009)

Melanoma has proven to be disproportionately lethal considering its potential for cure in the early stages and its relatively low incidence compared with non-melanoma skin cancers. Since the 1960's, cutaneous melanoma (CM) incidence has increased by 3-8% per year in most European countries, with the greatest increases observed in elderly men.[5] Several environmental and endogenous conditions had been proved to correlate with higher risk of developing CM such as:

Environmental

- Severe childhood sunburn despite sun exposure control in later life;
- High levels of childhood sun exposure despite absence of sunburn;
- Adult exposure particularly in individuals with non-acclimatised skin;
- Sunbed and sunlamp exposures.[3]

Endogenous

- The fair, freckled and Caucasian skin types;
- Genetic predisposition;
- Familial history of melanoma.

Melanoma development involves the accumulation of genetic abnormalities in known signalling pathways. These pathways can be induced by Ultraviolet Radiation (UVR), which can damage the cells' DNA leading to mutations, and by the production of reactive oxygen species that will also result in DNA damage and can suppress apoptosis or reduce the cutaneous immune defences.[5, 6]

The lag-time between the exposure to UVR and the development of melanoma can be decades apart, making it harder for prevention campaigns to succeed (primary prevention). Therefore, these initiatives need to be persistent and reiterative, keeping in mind that their impact will only be recognised several years later. The secondary prevention of CM focuses on the diagnosis of thinner tumours. Early diagnosis and excision of thin *in situ* lesions are the best hope for short term mortality reduction , while primary prevention may provide long-term results.[5]

The pattern of geographic distribution of CM shows that its incidence is increasing as the latitude decreases , in other words, CM incidence rates in Caucasian populations increase with proximity to the Equator.[5, 7] Interestingly, this relationship is not observed in Western Europe (Figure 1) where mortality from melanoma is four to six times higher in Nordic than in the Mediterranean countries (in which Portugal is included). This may be due to the combination of a light skin type with excessive intermittent exposure to UVR, during periods spent in Southern Europe on the Summer.[5, 7]

Ocular melanoma

Primary ocular melanoma can occur in four tissues of the ocular region: in the uveal tract (uvea), conjunctiva, eyelid and orbit.[8] Although ocular melanoma is very uncommon compared with CM, it is deadly as well. UM (Uveal Melanoma) is the most common ocular melanoma in the western world, with 7 new cases per 1 million individuals per year.[9] In the USA, it develops in approximately 2500 North Americans annually and conjunctival melanoma produces 200 new cases per year.[8] The incidence of UM has been kept relatively stable in recent decades.

The most important factor in the development of UM is the presence of congenital ocular melanocytosis, also known as nevus of Ota, when the eyelid skin is affected. White individuals with this condition have a 1 in 400 chance for the development of uveal melanoma in their lifetime. Another risk factor for the development of UM is the presence of an uveal nevus. The incidence of uveal melanoma increases with age, reaching its maximum between the 6th and 7th decade of life. It is slightly more common in males and uncommon in children and dark-skinned individuals. Unlike CM, the inexistence of a geographic pattern for the distribution of UM indicates that chronic and occupational UVR exposures are non-significant risk factors in this case. More studies are required to identify environmental risk factors that trigger the development of UM.[10] A possible genetic predisposition is implied by the number of patients with bilateral uveal melanoma, which is greater than would be predicted by chance alone.[8, 10]

Conjunctival melanoma is a rare form of ocular melanoma, accounting only for 2% - 3% of all diagnosed cases. In contrast to UM, environmental exposure to UVR has been pointed as a possible explanation to an increasing incidence of conjunctival melanoma from 0.24 to 0.8 per million per year in Caucasians.[11] A higher incidence was identified in individuals with mean age of presentation of the pathology between 50 and 60 years. Although, conjunctival nevus and primary acquired melanosis are predisposing factors, the development of malignant melanoma of the conjunctiva can occur without a precursor lesion.[8, 11]

3.3 Clinical aspects of Cutaneous and Ocular Melanoma

Cutaneous melanoma

Cutaneous melanoma can be classified according to clinical, histopathological, epidemiological and molecular criteria. Clinically, there are four distinct subtypes: Superficial Spreading Melanoma (SSM), Nodular Melanoma (NM), Acral Lentiginous Melanoma (ALM) and Lentigo Maligna Melanoma (LMM). Table 1 shows their major differences regarding frequency, common body areas, sun exposure, age of diagnosis and morphologic features.[12] The most common subtypes are associated with intermittent sun exposure, with SSM being the most common (70%) followed by NM (30%). Although both types develop in common body areas, which are the most exposed to UVR (trunk and legs), they have distinct morphologic features.[13-15]

Table 1: Clinical classification of cutaneous melanoma. Adapted from McGovern (1982); Chin et al. (1998) and Ghosh et al (2009).

Subtype	Frequency	Common Body Areas	Sun Exposure	Median Age of Diagnosis	Morphologic features
SSM	70%	▪ Trunk (men) ▪ Legs (women) ▪ Proximal extremities	Intermittent	44	▪ Spreading pigmented macule ▪ Flat or very slightly raised
NM	10-30%	▪ Trunk (men) ▪ Legs (women)	Intermittent	53	Polypoid or dome-shaped
ALM	Rare	▪ Palms ▪ Nail beds ▪ Soles of the feet	Absent	65	Spreading pigmented patch
LMM	< 5%	▪ Face ▪ Head ▪ Neck	Chronic	65	Darkly pigmented raised papule or nodule

Histological classification is performed based on the location and/or depth of involvement. Melanoma progression occurs by the evolution of an *in situ* growth that starts to get thicker and invades vertically (the underlying skin layers), spreads to regional lymph nodes and finally, metastasizes.[15] As can be seen in Figure 2, CM progression can begin with the development of either dysplastic or benign nevi. These can then evolve to *in situ* melanoma, which is characterized by a radial growth pattern and its primary confinement to the epidermis. At this stage the cells are still dependent on growth factors and are not tumourigenic. The vertical growth phase of malignant melanoma, which denotes a transition to a more aggressive and lethal condition, is characterized by tumour invasion into the underlying dermis, subcutis and upper epidermis. At this stage, cells are not dependent of growth factors and often metastasise to distant sites, such as liver, lung, brain, bone or small intestine.[14, 15]

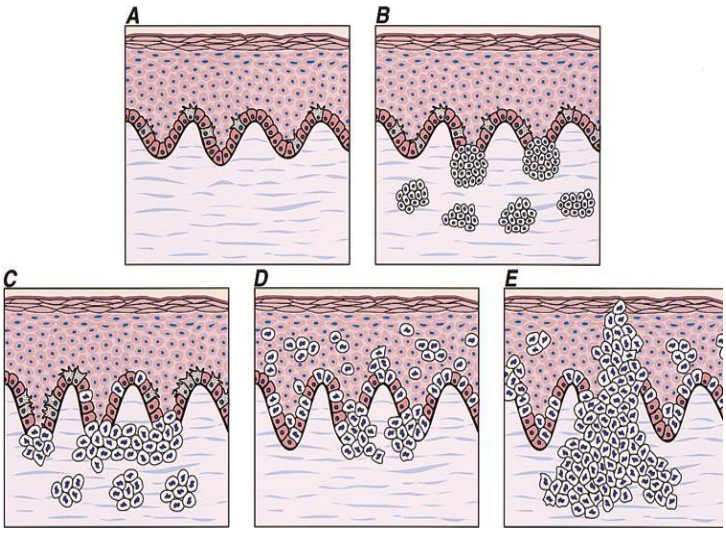


Figure 2: Stages of histopathologic progression in melanocyte transformation. (*A*) Normal skin. The dendritic melanocytes are evenly distributed throughout the basal layer. (*B*) Benign proliferation of melanocytes. Nevoid melanocytes are organized into uniform nests in a compound nevus. (*C*) Melanocyte dysplasia. Dysplastic nevus has irregular and bridging nests of large atypical melanocytes. (*D*) *In situ* melanoma, radial growth phase (RGP). Single cells are in the upper layer of the epidermis. (*E*) Malignant melanoma, vertical growth phase (VGP). Taken from Chin et al. (1998).

Ocular melanoma

Conjunctival melanoma

Conjunctival melanoma presents substantial clinical variability that comprises asymptomatic raised pigmented plaque, maculae, or tumours ranging from small lesions to higher dimension tumour masses.[8, 16] The clinical features pointing to melanoma include large size, variegated appearance, lack of mobility in relation to the sclera, extension onto cornea, presence of large feeder vessels and evidence of canalicular obstruction.[11] The colour ranges from light to dark brown and in rare cases can be amelanotic. An association between increased risk of conjunctival melanoma development in patients with a genetic predisposition for the development of cutaneous melanoma has not been established.[11]

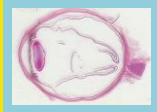
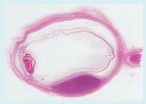
Histologically, conjunctival melanoma can be composed by a single cell type or by different proportions of four cell types: small polyhedral cells, epithelioid cells, balloon cells, and spindle cells. The subtypes are classified as malignant when significant cytologic atypia, including large nuclear size, prominent nucleoli or mitotic activity, is observed. Additionally, the cell type involved may confer a higher or lower level of malignancy, for instance tumours composed uniquely by spindle cells have been classified as less aggressive, while the presence of epithelioid cells have been used as an indication of poor prognosis.[11]

Uveal melanoma

The uvea is a vascular layer within the eye that is divided into three anatomic compartments, the iris, ciliary body and choroid. The majority of UM cases occur in the choroid, representing 90% of intraocular melanoma. Iris melanoma, the rarer type of UM, is associated with a more benign diagnosis when compared to choroidal and ciliary body melanomas.[8-10, 17] Their main features are summarized in Table 2.[10]

Patients with uveal melanoma may have visual loss, but many do not present symptoms and the condition is frequently only discovered on routine ocular examination.

Table 2: **Clinical classification of uveal melanoma**. Adapted from Laver et al. (2010) and van den Bosch (2010).

Subtype	Location	Clinical Variations	Clinical Presentation
Iris Melanoma	Inferior portion of the iris 	Circumscribed	▪ Variable pigmentation. ▪ Well-defined mass in the iris stroma.
		Diffuse	▪ Clinical picture of acquired hyperchromic heterochromia and secondary glaucoma.
		Tapioca	▪ Multiple hard nodules giving a surface appearance of tapioca pudding.
		Trabecular-meshwork	▪ Diffuse growth around the anterior chamber angle without producing a distinct mass. ▪ Presentation of ipsilateral glaucoma.
Ciliary Body Melanoma		Iridociliary (growth into the iris) Ciliochoroidal (growth into the choroid)	▪ Possibility of large size tumour. ▪ Dome-shaped mass in the affected area or diffuse circumferential growth pattern, known as ring melanoma.
Choroidal Melanoma	Under the retina 		▪ Sessile or dome-shaped mass. ▪ Surface orange pigment at the level of the retinal pigment epithelium. ▪ Retinal detachments can be seen secondary to the tumour growth. ▪ Absence or presence of pigmentation. ▪ Possibility of diffuse growth pattern with only minimal tumour thickness.

3.4 Prognostic Factors of Cutaneous and Ocular Melanoma

Cutaneous melanoma

Over time, dermatopathologists developed systems to determine the prognosis for non-metastatic CM, establishing melanoma thickness, location, histological type, gender and ulceration as important indicators of patient prognosis.[5]

In 1970, Breslow developed a system in which the best independent determinant, among several factors assessed, is the total tumour thickness. Breslow's system measures tumour thickness from the upper layer of the epidermis to the innermost depth of tumour invasion (Figure 3). Due to its simplicity, reproducibility and objectivity, it became the classification system of choice. Wallace Clark also developed a classification system. In his system, he established a correlation between anatomic level of invasion, mitotic index and prognosis (Figure 3). In comparison with Breslow's system, this system provides more information but may lack on reproducibility.[14]

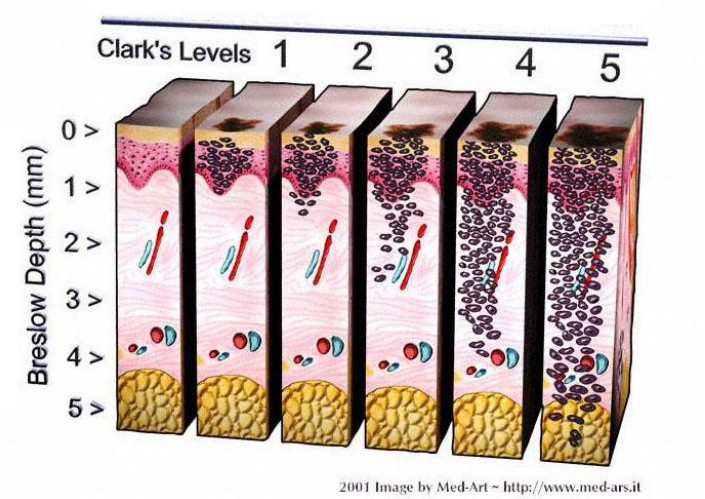


Figure 3: Pathological classification of melanoma by Clark's levels and Breslow depth. Taken from www.med-ars.it (21/06/2011)

In 2009, the American Joint Committee on Cancer Staging System for Cutaneous Melanoma was published, in which the TNM categories were defined according to the prognosis of the patients (Table 3).[18] The tumour thickness (measured in milimeters), the mitotic rate per mm^2 and the presence or absence of ulceration are the primary criteria of T classification. In 2009, primary tumour mitotic rate was added as a new covariate after a multifactorial analysis, which revealed that proliferation of the primary melanoma was the second most determinant predictor of survival, after tumour thickness. Melanoma

ulceration is defined as the traumatic disruption of the epidermis overlying a major portion of the primary melanoma. A higher risk for metastasis is predicted by melanoma ulceration, therefore, its presence worsens the prognosis in these patients, compared with those who have melanomas of similar thickness without ulceration.[18, 19]

The number of metastatic nodes is primarily defined by the N category. Within this category, there are two patient groups with significantly different survival rates: the patients with micrometastasis and the patients with macrometastasis. The first group does not have clinical or radiologic evidence of lymph node metastasis but has pathologically documented nodal metastasis. In contrast, patients with macrometastasis have both clinically detectable nodal metastasis and pathologic examination that confirms the number of nodal metastasis.[19]

The M categories were defined using as main criteria the number and site(s) of distant metastasis, and the elevated serum levels of lactic dehydrogenase (LDH), being attibuted the worst prognosis to patients with higher number of distant metastasis, with metastasis not located in the skin, subcutaneous tissue or distant lymph nodes and with high levels of serum LDH.[19]

Table 3: Cutaneous Melanoma TNM Classification. Table from Balch et al. (2009).

T classification	Thickness	Ulceration Status
T1	≤1.0 mm	a: without ulceration and mitosis < 1/mm²
		b: with ulceration and mitosis ≥ 1/mm²
T2	1.01 – 2.0 mm	a: without ulceration
		b: with ulceration
T3	2.01 – 4.0 mm	a: without ulceration
		b: with ulceration
T4	≥ 4 mm	a: without ulceration
		b: with ulceration
N classification	**No. of Metastatic Nodes**	**Nodal Metastatic Mass**
N0	0	Not applicable
N1	1	a: micrometastasis
		b: macrometastasis
N2	2-3	a: micrometastasis
		b: macrometastasis
		c: in transit met(s)/satellite(s) without metastatic nodes
N3	4 or more metastatic nodes, or matted nodes, or in transit met(s)/satellite(s) with metastatic node(s)	
M classification	**Site**	**Serum Lactate Dehydrogenase**
M0	No distant metastasis	Not applicable
M1a	Distant skin, subcutaneous, or nodal metastasis	Normal
M1b	Lung metastasis	Normal
M1c	All other visceral metastasis	Normal
	Any distant metastasis	Elevated

Table 4: Prognostic indicators for cutaneous melanoma. Taken from de Vries (2004).

Prognostic factor	Most favorable when:
Breslow thickness	Thin (< 1,51 mm)
Histology	SSM
Age	Young
Gender	Female
Ulceration	Absent
Mitotic activity index	Low

In summary, Table 4 shows the best prognostic factors in CM. As already mentioned, the most important prognostic indicator of survival is thickness. Age is another factor, as younger people show better prognostics compared with older people even when tumour thickness is the same. If age and tumour thickness are the same,, the female gender has better prognosis over the male gender. SSM generally have better prognosis than the other histological subtypes, because they usually have a thin Breslow thickness. The absence of ulceration and a low mitotic index are also indicators of a good prognosis.[5]

Ocular Melanoma

Conjunctival melanoma

In conjunctival melanoma there is no established system to ascertain the prognosis. Worse outcome may be indicated by: nonbulbar (fornix, palpebral) location, involvement of the caruncle and skin (rich in lymphatic tissue), invasion into the eye or brain, local recurrence, and large tumour size. Conjunctival melanoma of any thickness can metastasize due to the close proximity of lymphatic channels to the superficial *substantia propria*. Fatal conjunctival melanoma is associated with metastasis to the liver, lung, brain, skin, and peritoneum.[11]

A pagetoid growth pattern, mitotic activity greater than 5 mitotic figures per 10 high-power fields, cell type morphology different from spindle cell and the absence of an inflammatory response were all suggested to be associated with a poorer prognosis.[8, 11]

Uveal melanoma

Metastatic uveal melanomas are typically resistant to therapy and within a year of the onset of the systemic symptoms patients die. Therefore, there is much interest in the development of an accurate predictive testing that may allow systemic prophylaxis in high-risk patients.[17]

The most important factors (histopathologic and genetic) for predicting metastatic disease are: basal tumour diameter, tumour height, ciliary body involvement, transcleral extension, epithelioid melanoma cytomorphology, high mitotic rate, extravascular matrix patterns such as closed loops, microvascular density, chromosome 3 deletion and chromosome 8q gain.[17]

From the parameters presented, tumour size is the most suitable for prediction of metastatic disease, considering that mortality rate gradually increases with increasing tumour thickness (Table 5).[10]

Table 5: Uveal melanoma classification and prognosis based on tumour size. Table based in Laver et al. (2010).

Classification	Thickness	Diameter	5-year Mortality
Small	≤ 3mm	<10mm	16%
Medium	3 - 5 mm	10 - 15 mm	32%
Large	> 5 mm	>15 mm	53%

The cell type of UM also relates to prognosis. Currently, there is a cytologic classification of UM dividing it into 3 main tumour types: spindle-cell, mixed-cell and epithelioid-cell type (Table 6).[10] UM with low mitotic activity have better prognosis; while tumour infiltration by lymphocytes has been associated with decreased survival. There are other histopathologic parameters that can be evaluated, namely the size of nucleoli, specific extracellular matrix patterns and expression of various cell surface markers, such as metalloproteinases, gangliosides, adhesion molecules, and immunologic markers.[10]

Table 6: Cytologic classification of uveal melanoma. Table based in Laver et al. (2010)

Classification	Cell Growth	Prognosis
Spindle-cell	▪ Compact cohesive fashion. ▪ Surrounded by a dense reticulin framework.	Better prognosis
Epithelioid-cell	▪ Less cohesively fashion. ▪ Not surrounded by a network of reticulin.	Worse prognosis
Mixed-cell	▪ Mixture of the two previous cell types.	Worse prognosis

3.5 Genetic Alterations in Melanoma

Tumourigenesis is a multistep process and some of these steps reflect genetic alterations that progressively drive normal cells to transform into highly malignant ones.

In 2011, in "The Hallmarks of Cancer: the next generation", Hanahan and Weinberg suggest ten essential alterations in cell physiology that all together condemn normal cell to malignant growth (Figure 4):

1. Insensitivity to growth-inhibitory signals;
2. Deregulation of cellular energetic;
3. Self-sufficiency in growth signals;
4. Avoidance of immune destruction;
5. Limitless replicative potential;
6. Tumour promotion of inflammation;
7. Evasion of programmed cell death;
8. Tissue invasion and metastasis;
9. Sustained angiogenesis;
10. Genome instability and mutation.

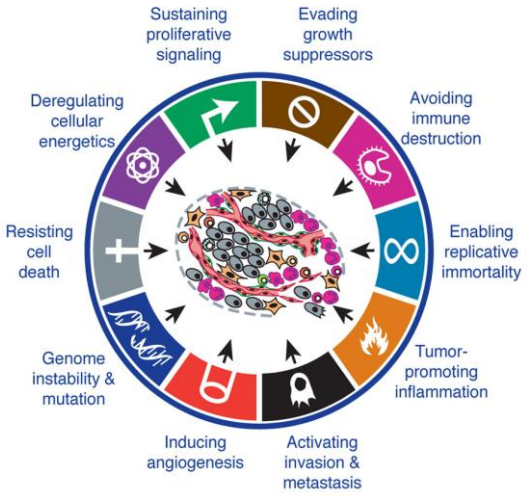


Figure 4: **Acquired capabilities of cancer.**
Figure from Hanahan and Weinberg (2011).

To achieve each of these novel capabilities, cells have to successfully overcome anti-cancer defense mechanisms established in the cells and tissues.[20]

Some of these generic cancer cell features are altered in a similar fashion in CM and UM. For instance, both tumours are highly metastatic, which is illustrated by the early initiation of metastasis.[9, 10] The chromosomal regions frequently observed to be amplified or deleted in both of these types of melanocytic tumours are similar even though the exact frequencies in which they occur differ. As an example, loss of chromosome 3 is much more frequent in UM than in CM, gain of 8q is found in 40% of UM and in 25% of the CM cases and in both UM and CM genetic alterations (gain or loss) were reported in chromosome 6. On the other hand, monosomy of chromosome 10 is more frequently encountered in CM than in UM, 60% and 27%, respectively[9, 10, 21] Other

similarities between UM and CM include gene expression status: many of the genes found to be frequently overexpressed or underexpressed in CM are also observed in UM (for example, MYC, BCL-2 and PTEN).[9] Although some of the genetic changes underlying the development of CM and UM have been characterized, the role of oncogenes or tumour suppressor genes in the pathogenesis of UM is less well established. Alterations in the CDKN2A familial melanoma locus (which encodes for INK4A and ARF) (CM exclusive), receptor tyrosine kinase (RTK) function, activation of MAPK pathway components (commonly at the levels of BRAF and NRAS particularly in CM), and activation of the PI3K-AKT pathway through loss of PTEN are the main genetic changes that have been identified through genomic structure and sequence analysis.[15]

Mutations affecting progression of cell cycle

CDKN2A

The establishment of familial history of melanoma as a strong predictor of melanoma development led to the identification of 9p21 as an important locus in melanoma, with loss of heterozygosity or mutation at 9p21 occurring in melanoma prone families. Within this region are the CDKN2A and CDKN2B genes. CDKN2A encodes two tumour suppressor proteins, INK4A (p16INK4A) and ARF (p14ARF in humans and p19ARF in mice). [15] INK4A is a cyclin-dependent kinase inhibitor that activates the tumour suppressor retinoblastoma gene (RB) via negative regulation of Cdk4/6. The INK4 proteins ensure that RB remains in a complex with the E2F transcription factor. This complex promotes and represses transcription of target genes, leading to G1 arrest.[22] 25–40% of familial melanoma forms have mutations in the INK4A coding region.[23] The role of this genetic alteration in UM development has not been established.[9, 15, 24] The p14ARF protein acts on the p53 cell cycle control pathway by stabilization of p53 and so allowing cell cycle arrest at the G1/G2 phase. Loss-of-function INK4A /p14ARF mutations occur in 30-70% of the sporadic cutaneous melanomas.[25, 26]

Mutations involved in cell signalling cascades deregulation

According to Hanahan and Weinberg, one of the ten essential alterations in cell physiology for cancer development is the self-sufficiency in growth signals.[20] While a growth factor may offer a degree of signalling stimulation, robust activation of signalling can arise from the activation of individual signalling molecules. Therefore, the disruption in these signalling pathways may result in aberrant cell proliferation and/or apoptosis, and eventual tumour development. Two major signalling cascades have been linked to melanoma development: the RAS/RAF/MEK/ERK and the PI3K pathways.[27]

MAP Kinase and PI3K Signalling

The RAS/RAF/MEK/ERK mitogen-activated protein (MAP) kinase pathway has been the most directly linked to the development of melanoma due to its growth-promoting outputs. MAPK signalling is initiated by extracellular signals through their binding to receptor tyrosine kinases (RTKs), and subsequent activation of RAS, a membrane-bound GTPase. The RTKs that interact with RAS, or other members of the RAS superfamily are several and include: epidermal growth factor receptor (EGFR), c-KIT, platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) and fibroblast growth factor receptor (FGFR).[1, 15, 28-30]

RAS activation occurs when it is localized in the plasma membrane and binds to GTP. RAS can initiate signalling via different proteins/pathways, being RAF and phosphatidylinositol-3-kinase (PI3K) the downstream effectors better described. Activation of PI3K by RTKs or by RAS leads to phosphorylation of phosphatidylinositol-4,5-biphosphate (PIP2) and subsequent generation of phosphatidylinositol-3,4,5-

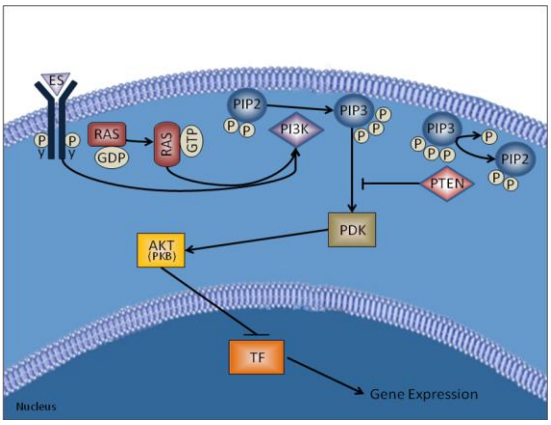


Figure 5: Overview of PI3K signalling. Based in Fecher et al (2008). ES – Extracellular signal; TF – Transcription Factor

triphosphate (PIP3) and results in the activation of AKT (protein kinase B) (Figure 5). This pathway is involved in cellular survival, apoptosis, cytoskeletal rearrangement and tumour cell chemo-resistance.[29]

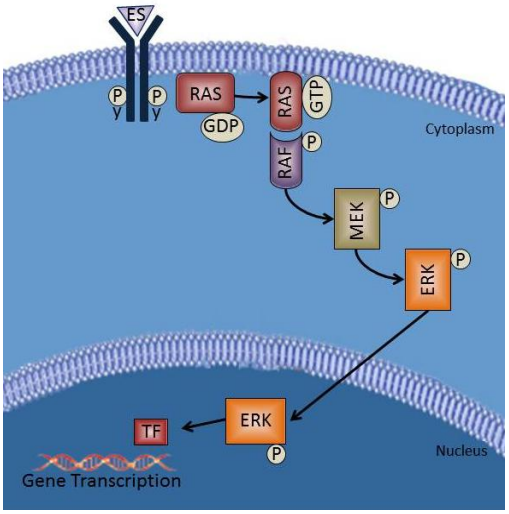


Figure 6: Overview of MAPK signalling. Based in Fecher et al (2008). ES – Extracellular signal; TF – Transcription Factor

BRAF, a member of the RAF family of serine/threonine protein kinases is recruited to the membrane by activated RAS. Once at the cell membrane, BRAF is phosphorylated and in turn, phosphorylates and activates MEK. Activated MEK phosphorylates and activates ERK. ERK by phosphorylation propagates this signal through cytoplasmic and nuclear targets (Figure 6). In melanoma, the broad downstream effects of activated ERK include transcription of genes involved in melanoma cell proliferation (e.g. FGF-2, IL-8, and HIF-1a), down-regulation of cyclin-dependent kinases, actin organization and cell motility, increased survival and protection against FAS induced apoptosis, invasion and metastasis due to extracellular matrix remodelling, and angiogenesis.[29]

A sustained hyper-activation of the pathway can theoretically be achieved by activating mutations of any signalling mediator upstream of ERKs and there are clear tumour type specific patterns of mutational activation.[23-25]

Receptor tyrosine kinases

Hyperactive extracellular signal-regulated kinases (ERKs) are common in various cancers. The RAS/RAF/MEK/ERK pathway is activated by various receptors, including EGFR, c-KIT and c-MET. These molecules are possible targets to alterations leading to changes in the associated signalling cascades.[1, 15]

The EGFR can be activated by EGF, TGF-α, amphiregulin and heparin-binding EGF. Such binding can then activate the MAPK and PI3K signalling cascades. The EGFR gene is located on chromosome 7 and copy number gains of chromosome 7 have been observed in the later stages of CM.[1, 15]

The c-KIT gene encodes a RTK that binds to stem cell factor. The KIT receptor can activate the MAPK, PI3K and phospholipase C signalling cascades. In melanocytes, it has been associated with migration, survival, proliferation and differentiation processes. This receptor has been used to support the potential utilization of the status of a gene to define a subpopulation of melanomas. Therefore, there are some reports correlating the frequency of mutation in c-KIT gene and in cutaneous melanoma subtypes. In 2006, a screen for mutations in melanoma subtypes found that 39% of mucosal, 36% of acral and 28% of the melanomas on chronically sun-damaged skin have KIT mutations, whereas melanomas on intermittent exposure sites did not.[31] In choroidal melanoma, c-KIT expression was found in most of the tumours studied, although no correlation to parameters such as cell type, largest macroscopic tumour dimension, scleral invasion or pigmentation were observed.[32]

The c-MET is a multifaceted regulator of growth, motility and invasion in a number of cell lineages. It is involved in three main pathways: MAPK signalling, responsible for proliferation; PI3K signalling involved in scattering; and STAT signalling (in association with the previous two signalling pathways).[1] In cutaneous melanoma, no mutations have been identified in c-MET gene but its importance in melanoma progression was showed by the upregulation of the receptor expression in metastatic melanoma.[15]

RAS Family

The RAS genes are among the most frequently mutated genes in human cancers, but different malignancies display different frequencies and spectra of mutations in NRAS, HRAS and KRAS. So, in contrast to other solid tumours, activating mutations of RAS genes are relatively infrequent in CM ranging from low to 15–25% incidence.[9]

The RAS family members have distinct roles in melanoma. While activated HRAS expression on an INK4A, ARF or p53 mutation background promotes nonmetastatic melanoma in a mouse model, NRAS expression in INK4A/ARF deficient mice promotes metastasis of cutaneous melanoma with high penetrance and short latency.[23-25, 28-29] CM presents mutations almost exclusively in NRAS, with 90% of mutations localizing to codon 61.[28] Activating NRAS mutations have been correlated with chronic sun damage and nodular lesions and are rarely found in dysplastic nevi, one of the potential starting points for CM.[9] In UM, the absence of NRAS mutations was reported by Zuidervaardt and co-workers[30] and Pópulo and co-workers.[33]

Oncogenic HRAS point mutations and genomic locus 11p amplification have only been identified in Spitz nevi, a benign lesion that does not progress to melanoma.[34] Mutations of KRAS gene have not been reported in human melanocytic lesions.[1]

RAF Family

RAF family proteins lie downstream of RAS and mediate its signal transduction. The RAF family is composed by the cytosolic serine-threonine kinases ARAF, BRAF and CRAF.[1, 15]

BRAF can regulate various aspects of cell growth and survival, while its inhibition sensitizes cells to apoptosis.[35] In a systematic genetic screen, BRAF mutations were identified in a variety of tumour cell lines, with the highest incidence in CM derived-cell lines.[36] Mutations in BRAF are found most frequently in melanomas at sites with intermittent UV exposure[37] and occur in approximately 11% of lentigo maligna melanomas (arising from chronically sun-exposed areas).[15, 30] BRAF mutation is associated with germline variants of the MCR1 gene, which promotes melanin production.[38] The melanocortin receptor 1 (MC1R) is the receptor for α-melanocyte-stimulating hormone,

which is stimulated by UV radiation, suggesting a degree of interplay between BRAF and UV exposure. In this regard, it is interesting that BRAF mutations are the most prevalent in melanomas associated with intermittent sun exposure. Alterations in BRAF seem to be an early somatic event. Mutations in BRAF are not commonly found in familial melanoma, neither in UM.[9] The role of BRAF in melanoma is unclear as demonstrated by the fact that BRAF mutations are also frequently found in benign and dysplastic nevi. These nevi often remain growth-arrested for their lifetime and rarely progress to melanoma.[28-30]

Sequencing revealed that up to 67% of CM samples had a BRAF mutation, being the amino acid substitution V600E, by far, the most commonly found in both cell lines and tumour samples.[36] It has been suggested that BRAFV600E-induced checkpoint mechanisms operate to constrain malignant transformation. In fact, congenital nevi that sustained BRAFV600E expression are positive for the senescence marker β-galactosidase and for INK4A. The BRAF effect is an example of oncogene-induced senescence – a mechanism by which premalignant lesions are inhibited to progressing. Therefore, it is believed that BRAFV600E expression alone is not sufficient to transform human melanocytes, requiring the cooperation of other determinants to drive melanoma formation.[39]

The majority of BRAF mutations occur in areas of the protein with defined biochemical functions. The single phosphomimetic substitution of V600E inserts a negatively charged residue (Valine → Glutamic Acid), in the kinase activation domain. This mutation renders the enzyme constitutively active.[36] BRAFV600E stimulates constitutive ERK signalling and directly and/or indirectly regulates the expression and function of several genes critical to proliferation and survival of melanoma cells. These include transcription factors, such as microphthalmia-associated transcription factor (MITF), NF-kB, and the cell cycle regulators Cyclin D1, INK4A, and p27Kip1.[1]

Of note, BRAF and NRAS mutations are mutually exclusive in melanoma, and in fact, the occurrence of mutations in one gene or the other may be specific to certain subtypes of melanoma.[37] This suggests that there are innate differences in the roles that BRAF and NRAS may play in melanoma and this may be reflected in shifts in signalling.[15]

In BRAF mutant cells, BRAF is required for ERK signalling. However, in melanoma cells, in the context of NRAS mutation, a switch in signalling from BRAF to CRAF accompanied by disruption in cyclic AMP signaling occurs.[40]

GNAQ

The q class of heterotrimeric GTP binding protein (Gq) family members (Gq, G11, G14, and G15/16) are composed of three subunits that switch between inactive and active states in response to guanine nucleotides.[41] GNAQ encodes the α subunit of Gq that mediates signals between G-protein-coupled receptors (GPCRs) and stimulates all b-isoforms of phospholipase C (PLCb) to initiate inositol lipid signalling. Nearly 40% of GPCRs rely upon Gqα family members to stimulate inositol lipid signalling. These include more than 50 subtypes of receptors responsive to a range of hormones, neurotransmitters, neuropeptides, chemokines, autocrine and paracrine molecules.[41]

PLCb enzymes catalyze the hydrolysis of the PIP2, releasing inositol trisphosphate (IP3) and diacylglycerol (DAG). These second messengers propagate and amplify the Gα-mediated signal through stimulation of protein kinase C (PKC). As a result of phospholipase C stimulation, protein kinase C is activated and can then activate RAF/MEK/ERK (Figure 7) and other signalling pathways.[42]

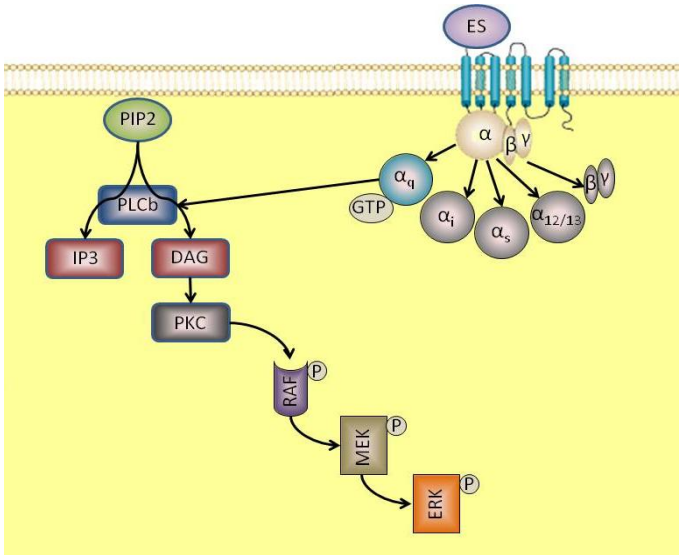


Figure 7: Activation of RAF/MEK/ERK signalling pathway by Gq protein. ES – Extracellular sinal. Based on Raamsdonk et al (2009).

GNAQ has been suggested to be a regulator of cell growth through a RAS independent or RAS dependent signalling mechanism involving the PKC-dependent ERK pathway because of the ability of this mechanism to mimic growth factor signalling stimulation and, in the absence of BRAF and NRAS mutations, to trigger the MAPK pathway implying this pathway as an early

event in neoplasms.[41, 42] GNAQ is also linked to endothelin signalling, which is essential for melanocyte survival early during development and is also required for the migration of melanoblasts. Gq signalling may also contribute to the association observed for melanoma invasion and metastasis.[41, 42]

GNAQ mutations were reported to occur in 83% of blue nevi, 50% of "malignant blue nevi", and 46% of uveal melanomas. All mutations in GNAQ lead to constitutive activation and occur almost exclusively at codon 209.[42] In this position, within the RAS-like domain of GNAQ, the glutamine amino acide is substituted by a proline or a leucine causing the constitutive activation of the molecule. This scenario resembles the loss of GTPase activity in the corresponding residue of RAS (residue 61).[42]

In UM, activating GNAQ mutation at codon 209 was found in 49% of primary UMs, 22% of iris melanomas and 54% of posterior UMs. GNAQ mutation was not associated with any clinical, pathologic or molecular features associated with tumour progression (e.g. age, gender, cytological type, pT group, mitotic rate, largest tumour diameter and scleral extension), reinforcing the idea that the GNAQ mutation may be an early event in UM development.[43, 44] Additionally, in 2009, Bauer and co-workers showed that disease-free survival did not significantly correlated with GNAQ mutation status. Therefore, GNAQQ209L are not suitable to predict the clinical outcome in uveal melanoma.[45]

The establishment of GNAQ as the first oncogene in uveal melanoma lead Lamba and co-workers (2009) to study the presence of mutations in exon 5 of GNAQ and GNA11, which encodes a G-protein, from the same class as GNAQ (Gq class), that harbours a residue equivalent to Q209 of GNAQ, in other human tumours. No mutations of GNAQ exon 5 were found in any tumour types studied, other than blue nevi. Lamba and co-workers also did not detected mutations in exon 5 of GNA11.[41] More recently, GNA11 somatic mutations were found to be well represented in UM. Somatic mutations in both GNA11 and GNAQ, affecting Q209 (exon 5) and R183 (exon 4) were found in a mutually exclusive pattern. Although, there is no difference in survival and disease-free survival between tumours with GNAQ mutations and tumours with GNA11 mutations, but more studies are required in larger series of tumours for this to be confirmed.[46]

Due to the high frequency of GNAQ mutations, targeting either the mutated protein or the oncogenic signalling pathway controlled by GNAQ may open up new therapeutic possibilities in UM.

PTEN

Phosphatase and tensin homologue (PTEN), a genuine tumour suppressor, is another important genetic alteration in signal transduction molecules in melanoma.

The lipid phosphatase PTEN opposes the activity of PI3K by dephosphorylating phosphoinositides. The lipid phosphatase activity of PTEN and its ability to act as an "off" switch for PI3K signalling, suggests that PTEN functions as a tumour suppressor by directly antagonizing the activity of the PI3K signalling pathway. Therefore, expression of PTEN can inhibit AKT phosphorylation, promote apoptosis and inhibit growth. Loss of PTEN activity (by mutations, deletions or promoter methylation) is often registered in primary and metastatic human cancers.[15, 28, 47] In metastatic melanoma samples, the frequency of PTEN mutation ranges from 7% to 19%.[48, 49] However, melanoma cell lines have a high frequency of PTEN mutations or PTEN loss. Loss of heterozygosity of 10q, where the PTEN gene is located, has been observed in various cancers, including CM and UM.[9]

NRAS and PTEN overlapping functions have been suggested, because it was verified that cell lines or uncultured melanoma tumours can carry a mutation in NRAS, or in PTEN, or neither, but not in both.[1, 15, 28] At variance BRAF and PTEN mutations are found concurrently in 20% of CM.[1] PTEN loss is correlated with increasing Breslow depth and tumour progression.[15, 28, 47]

3.6 The Mammalian Target of Rapamycin (mTOR) Signalling in Melanoma

mTOR is a cytoplasmic serine-threonine kinase that acts as a sensor of mitogens, energy and nutrients. Its pathway has a pivotal role in cell growth, protein translation, autophagy, motility and metabolism. mTOR is composed by two different multiprotein complexes: mTOR complexes 1 and 2 (mTORC1 and mTORC2).[50]

mTORC1 consists of mTOR, mammalian LST8 (mLST8), proline-rich AKT substrate 40 (PRAS40), and Raptor. Its activity depends on the availability of nutrients such as glucose, oxygen and amino acids.[50] mTORC1 signalling cascade is activated through the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Figure 8). PI3K, is a lipid kinase that can be activated by multiple mechanisms, such as binding of growth factors to receptor tyrosine kinases, activation of G-protein-coupled receptors, and by oncogenes such as Ras. Activated PI3K phosphorylates phosphoinositides, generating the biologically active lipids phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) and PIP3.[50]

PIP3 binds to the pleckstrin homology (PH) domain of the serine-threonine kinase AKT, promoting its translocation to the cell membrane. AKT is then activated by sequential phosphorylation at Thr308 and Ser473 residues. Phosphorylation at Thr308 is mediated by PDK-1 (3'phosphoinositide dependent kinase 1), which itself is activated by the binding of PIP3 to its PH domain and subsequently translocated to the cell membrane. The activation of AKT is complete after phosphorylation of the Ser473 residue by mTORC2. Active AKT phosphorylates PRAS40 and tuberous sclerosis complex 2 (TSC2) at Ser939 and Thr1462. TSC2, phosphorylated at these sites and in a heterodimeric complex formed with TSC1, inhibits the GAP (GTPase-activating protein) activity of this complex. The TSC2 carboxy terminus, which has a conserved GAP domain, suppresses the activity of the Ras-related GTPase Rheb, a selective activator of mTORC1. Therefore, TSC2 inhibition by AKT results in activation of mTORC1 (Figure 8).[50, 51]

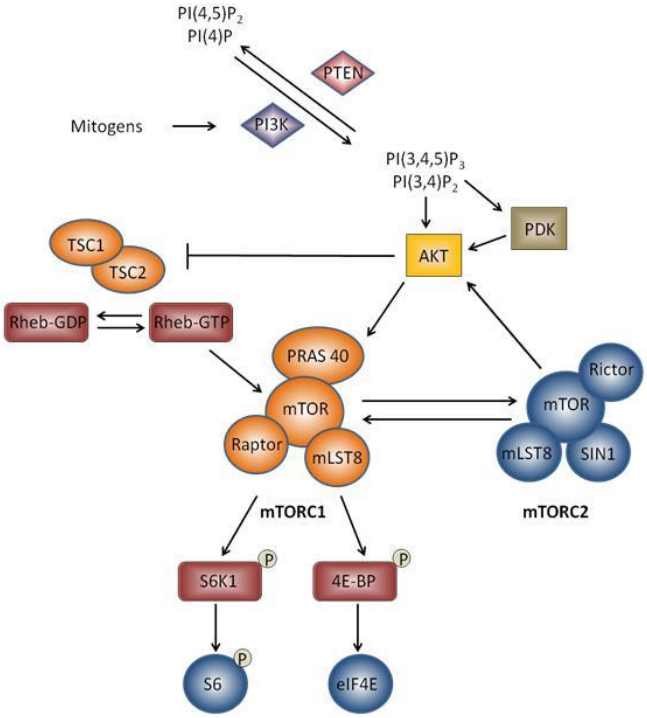


Figure 8: mTOR regulation by the PI3K/AKT pathway.

Based on Memmott et al (2009).

The Ras/MAPK signalling is another pathway that activates mTOR through the direct inhibition of TSC2. TSC2 is a direct substrate of ERK (Ser664) and is also a substrate of the downstream ribosomal protein S6 kinase (RSK; Ser1798). ERK-dependent phosphorylation negatively regulates TSC2 function by blocking its interaction with TSC1. RSK-dependent phosphorylation inhibits the ability to deactivate Rheb.[50, 52]

Additionally, TSC2 is regulated by the cellular energy sensor AMPkinase. When cellular energy stores are reduced, due to hypoxia, nutrient deprivation or increase of AMP levels, AMPK is activated, phosphorylating TSC2 and causing its activation. TSC is phosphorylated in Ser1227 and Ser1345, which are distinct from the sites phosphorylated by Akt/ERK. Activated TSC2 inhibits mTOR signalling, reducing protein synthesis.[50]

There are multiple mechanisms by which amino acids, in particular leucine and isoleucine, activate the mTOR pathway. One of them is the increase of the MAP kinase kinase kinase kinase-3 (MAP4K3) activity, which correlates with increased phosphorylation of S6 kinase 1 (S6K1).[40] Another mechanism by which amino acids

stimulate the mTOR pathway is related to the class III PI3K, hVps34. This mechanism is independent of TSC2 or Rheb and also leads to activation of S6K1.[50, 53]

mTORC1 activation results in phosphorylation of its effectors: eIF4E binding protein 1 (4E-BP1) and S6K1. A specific motif (the TOS motif) found in both 4E-BP1 and S6K1 mediates direct binding of these proteins to Raptor, allowing them to be phosphorylated by the mTORC1 complex. 4E-BP1 hyperphosphorylation leads to inhibition of 4E-BP binding to eukaryotic initiation factor 4E (eIF4E), activating translation. eIF4E enhances cell proliferation, survival, and angiogenesis through selective translation of mRNA such as cyclin D1, Bcl-2, Bcl-xL and vascular endothelial growth factor (VEGF) as well as the nucleocytoplasmic transport of selected mRNA such as cyclin D1. S6K1 is a key regulator of cell growth. It phosphorylates ribosomal protein S6 and, in some models, enhances the translation of mRNAs possessing a 5′ terminal oligopyrimidine tract. S6K1 also phosphorylates other important targets, including insulin receptor substrate 1 (IRS-1), eIF4E, programmed cell death 4 (PDCD4), eukaryotic elongation factor-2 kinase (eEF2K), glycogen synthase kinase 3, and S6K1 Aly/REF-like target.[44] Both eIF4E and S6K1 overexpression has been linked to poor cancer prognosis (Figure 9) due to their implication in cellular transformation.[54, 55]

mTORC2 is composed of mTOR, Rictor, mSin1, PRR5 (protor) and mLST8(GßL). mTORC2 can regulate cell polarity and the spatial control of cell growth by the assembly of the actin cytoskeleton in response to mitogenic signals through phosphorylation and activation of PKCα. mTORC2 also phosphorylate AKT at Ser473, giving mTOR a dual role as a substrate and as an effector in the AKT signalling pathway. Because AKT promotes cell proliferation, survival and inhibits apoptosis, activation of AKT by mTORC2 could be another important mechanism by which mTOR promotes tumourigenesis.[51, 56]

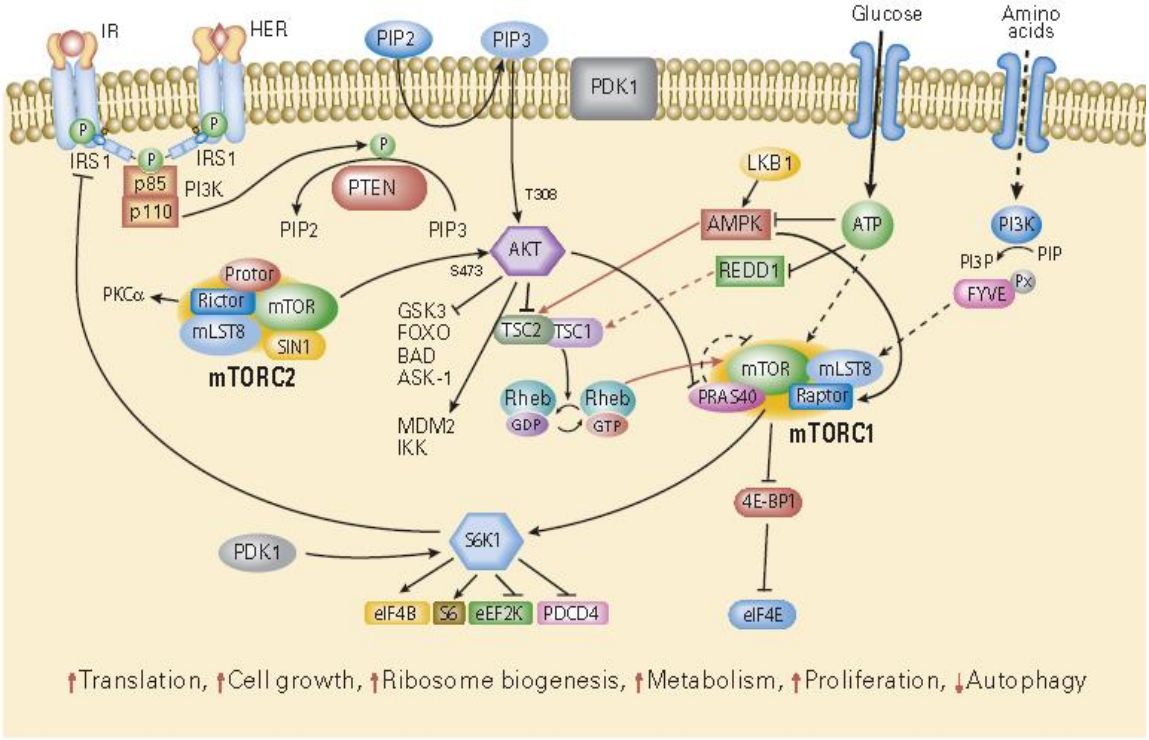


Figure 9: mTOR signalling network. Taken from Meric-Bernstam (2009).

In the literature, there are several reports showing a strong association between mTOR activation and malignant melanoma. For instance, Pópulo and co-workers (2010) reported an overexpression of the mTOR pathway effectors and their phosphorylated forms in most of the cases of ocular melanomas, indicating a role for the mTOR pathway activation in the pathogenesis of ocular melanoma.[57] Karbowniczek and co-workers (2007) showed that mTOR activation occurs during the pathogeneses of the majority of cutaneous melanomas. In addition, hyperphosphorylation of ribosomal protein S6 is more associated with malignant versus benign melanocytic lesions than any other single marker.[58] Recently, Pópulo and co-workers (2010) found an association between the expression of several proteins of the mTOR pathway and a poorer prognosis in CM. This association suggests a relationship between mTOR pathway activation and aggressive CM, being pS6 linked to several clinicopathological parameters of the disease.[33] Currently, a high number of reports exist that support the importance of mTOR signalling in melanoma development and the role for mTOR pathway as a therapeutic target.

3.7 Inhibitors of mTOR signalling pathway

mTOR, as the name implies, is inhibited by rapamycin. Rapamycin is a natural antibiotic derived from the organism *Streptomyces hygroscopicus* found on Easter Island (also known as Rapa Nui).[47]

Rapamycin and its analogs bind to FK506 binding protein, and this complex binds to mTOR, inhibiting downstream signalling. Rapamycin has several effects. It has been shown to be capable of inducing cell cycle arrest in a variety of tumour types and, in addition to its antitumour properties, rapamycin also inhibits endothelial cell proliferation, hypoxia inducible factor 1 and VEGF expression, angiogenesis and vascular permeability.[51]

The two complexes that form mTOR have different responses to rapamycin exposure. mTORC1 is rapamycin-sensitive, and rapamycin causes the dephosphorylation of 4E-BP1 and S6K1. In contrast, mTORC2 was originally thought to be rapamycin-insensitive. However, rapamycin regulates rictor phosphorylation, indicating that components of mTORC2 may be regulated by rapamycin. Further, long-term treatment of mammalian cells with rapamycin reduces mTORC2 levels and inhibits AKT.[51]

Despite the importance of mTOR in the biological processes, rapalogs have been generally well tolerated in the oncologic clinical trials performed, leading predominantly to disease stabilization rather than tumour regression. The higher doses administrated in some studies were the cause of the toxicity reported. Toxicity includes asthenia, mucositis, nausea, cutaneous toxicity, diarrhea, hypertriglyceridemia, thrombocyopenia, hypercholesterolemia, elevated transaminases, hyperglycemia and pneumonitis. mTOR was accepted, by Food and Drug Administration (FDA) in 2007, as a valid therapeutic target for renal cell carcinoma. Rapalogs have also been evaluated in clinical trials in other cancer types, including melanoma.[51]

Given its lack of positive results in tumour regression for most tumour types, mTOR-targeted therapies will likely be used in combination therapy. This strategy aims to induce a cytotoxic rather than a cytostatic response, and subsequent tumour regression.

For example, the combination of rapamycin with chemotherapy has shown to enhance apoptosis *in vitro* and to enhance antitumour efficacy *in vivo*.[51]

There is a number of mTOR inhibitors developed, including: Temsirolimus (CCI-779; Wyeth-Ayerst, Madison, NJ), Everolimus (RAD001, Novartis, Basel, Switzerland) and AP-23573 (Ariad Pharmaceuticals, Cambridge, MA).[59] RAD001 is esterified and thus an orally available rapamycin derivative. It was already shown to act as an inhibitor of mTOR, being capable of inhibiting the proliferation and the growth of a broad spectrum of tumour cell lines and tumours. Studies have also demonstrated the drug's potential for inhibiting endothelial proliferation, thus emphasizing its potential anti-angiogenic activity.[47, 60]

There are new mTOR-targeted therapies being developed, such as dual inhibition and the use of AMPK activators. The main difference of dual inhibition by rapalogs is the capability to inhibit both mTORC1 and mTORC2. The inhibition will affect the AKT activation, making these agents dual PI3K/mTOR inhibitors. These agents may bypass feedback loops, potentially increasing their efficacy compared with rapalogs. BEZ235 (Novartis, East Hanover, NJ) and EX147 (Exelixis, San Francisco, CA) are examples of this new class of therapeutic agents. The tolerability and efficacy of these agents are currently being tested in clinical trials. There is another type of therapy being evaluated in clinical trials and based on downregulation of mTOR signalling, which is the use of the antidiabetic drug metformin—an activator of AMPK.[50, 51]

4 Aims

4 Aims

The most critical biological questions to be answered by the melanoma research include:

- Which are the environmental factors that contribute to and/or modulate the risk of melanoma development?
- Which are the genetic events that underlie melanoma development, progression and metastasis?
- Which are the biological features (biomarkers) in early lesions that can be used to predict the propensity for metastasis?
- Which are the genetic alterations that can be used to predict therapeutic response?
- Are there biological or molecular pathways/networks that might prove amenable to therapeutic intervention?

Focusing on the last two questions, the general aims of this study were the identification of frequent mutations as prognosis or as predictive markers and evaluation if mTOR pathway inhibition can be used as therapeutic tool in patients (or subsets of patients) suffering from malignant melanoma.

For that purpose, we approached the following specific aims:

- In order to determine if specific genetic alterations can lead to the activation of mTOR pathway, we established the genetic profile of 2 series of human malignant melanomas (cutaneous and ocular melanomas).
- To evaluate the impact of an mTOR inhibitor analogous of rapamycin, RAD001, on the growth of melanoma-derived cell lines, their proliferation and apoptosis were assessed. We also evaluated the expression of elements of mTOR pathway, using several melanoma-derived cell lines treated with different concentrations of RAD001 at different time points.
- Finally, we intended to verify if there is any correlation between the genetic profile of primary and metastatic melanoma cell lines, the sensitivity to RAD001 and the expression of mTOR pathway effectors.

5 Methods

5 Methods

5.1 Cell lines and reagents

The human-derived cell lines Mewo and G361 cutaneous melanoma cell lines were kindly provided by Dr. Marc Mareel, from the Department of Radiotherapy and Nuclear Medicine, Ghent University Hospital, Belgium. 92.1[61], OCM-1[62], OMM-1[63], OMM-2.3[64] and Mel 285[65] uveal melanoma cell lines were kindly provided by Dr. Martine Jager, from the laboratory of Ophthalmology, Leiden University, The Netherlands. The antibodies used were as follows: anti-PTEN, anti-phospho-AKT (Ser473), anti-phospho-mTOR (Ser2448), anti-phospho-4E-BP1 (Thr37/46), anti-phospho-S6 (Ser 235/236), anti-Raptor, anti-phospho-ERK1/2 (Thr202/Tyr204) (all from Cell Signalling Technology, Beverly, MA, USA), anti-GNAQ, anti-BRAF, anti-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-rictor (Abnova, Taipei City, Taiwan). RAD001 (Everolimus) was kindly provided by Novartis Pharma AG, Basel, Switzerland, and was dissolved in DMSO to yield a stock solution of 5 $x10^{-3}$ M, which was stored at −20°C. On the day of experiments and immediately before treatment of the cells, the stock solutions were diluted with medium to the final concentrations. The controls were prepared as appropriate, using the same vehicle as for the drug.

5.2 Cell cultures

The cells were grown at 37°C, in its appropppriate medium (Table 7) and maintained in a humidified atmosphere (5% CO_2). The cells were grown in T25 tissue culture flasks and passaged once every 7–10 days on a ratio of 1:5. Routinely, media were supplemented with 10% fetal bovine serum (FBS) (Gibco/BRL – Invitrogen, San Diego, CA, USA), 100U/mL of Penicillin, 100ug/mL Streptomycin (Gibco/BRL - Invitrogen, San Diego,CA, USA) and 0.5% Fungizone (Gibco/BRL - Invitrogen, San Diego, CA, USA).

Table 7: Cell lines and appropppriate media (Gibco/BRL - Invitrogen, San Diego, CA, USA).

Cell Line	Medium
Mewo	DMEM
G361	McCoy
92.1	RPMI
Mel285	RPMI
OCM-1	RPMI
OMM-1	RPMI
OMM-2.3	RPMI

5.3 Clinicopathological features

Clinical pathological data regarding the cutaneous melanomas were retrieved from the files from Hospital de São Marcos, Braga, Portugal. The procedures were in accordance with the institutional ethical standards. 13 cutaneous melanomas were formalin-fixed and paraffin-embedded. The gender, subtype, Breslow thickness, Clark's Level, mitotic rate, epidermal ulceration, necrosis and pT staging[19] of the cases are summarized in Table 8.

After mutational analysis, a possible correlation was determined between each feature and the mutational status of the studied genes.

The ocular melanoma patient series used in this study has been previously described.[33] Briefly, 34 enucleated ocular melanomas (6 located in the conjunctiva and 28 located in the uvea) and the clinicopathological data were retrieved from the Department of Pathology and the Oncology Registry of Hospital S João (HSJ), Porto, Portugal. The procedures were in accordance with institutional ethical standards. The median age, gender, subtype, cytological type (epithelioid, spindle or mixed) of the tumours, pT staging (according to AJCC[66]), mitotic rate, median/range of the largest diameter of the tumour and sclera extension of the cases are summarised in Table 9. None of the cases had clinicopathological evidence of lymph node involvement and/or distant metastasis at diagnosis.

Table 8: Clinicopathological features of cutaneous melanomas

Clinicopathological features	Cutaneous Melanoma
Number of cases, *n*	13
Gender, *n* (%)	
Male	8 (61.5)
Female	5 (38.5)
Subtype, *n* (%)	
Acral	2 (15.4)
Lentigno Maligna Melanoma	2 (15.4)
Superficial Spreading Melanoma	8 (61.6)
Breslow thickness, *n* (%)	
≤ 1.0 mm	4 (36.4)
1.01-2 mm	3 (27.3)
2.01-4 mm	2 (18.2)
≥ 4.01 mm	2 (18.2)
Clark's Level, *n* (%)	
II	5 (45.5)
III	2 (18.2)
IV	1 (9.1)
V	3 (27.3)
Mitotic Rate, *n* (%)	
≤ 1 mitosis/mm^2	6 (60.0)
>1 mitosis/mm^2	4 (40.0)
Ulceration, *n* (%)	
Absent	7 (77.8)
Present	2 (22.2)
Necrosis, *n* (%)	
Absent	12 (92.3)
Present	1 (7.7)
pT, *n* (%)	
pT1	5 (41.7)
pT2	3 (25.0)
pT3	2 (16.7)
pT4	2 (16,.7)

SD, standard deviation

Table 9: Clinicopathological features of ocular melanomas

Clinicopathological features	Ocular Melanomas
Number of cases, *n*	34
Median age (± SD)	59 (20.5)
Gender, *n* (%)	
Male	10 (34.5)
Female	19 (65.5)
Location, *n* (%)	
Conjunctiva	6 (17.7)
Uvea	28 (82.3)
Cytological type, *n* (%)	
Spindle-cell	12 (35.3)
Epithelioid-cell	10 (29.4)
Mixed-cell	12 (35.3)
pT, *n* (%)	
pT1	3 (8.8)
pT2	20 (58.8)
pT3	9 (26.5)
pT4	2 (5.9)
Mitotic Rate, *n* (%)	
≤ 1 mitosis/mm^2	22 (68.8)
>1 mitosis/mm^2	10 (31.3)
Median basal tumour diameter, mm (range) *	11.1 (3-18)
Tumour scleral involvement, *n* (%) *	
Absent	21 (75.0)
Present	7 (25.0)

SD, standard deviation

* Features evaluated exclusively in uveal melanoma

5.4 BRAF, NRAS, GNAQ and GNA11 mutation analysis

Mutation analysis of BRAF, NRAS, GNAQ and GNA11 genes was performed in cutaneous melanoma cell lines (G361 and Mewo) and in ocular melanoma cell lines (Mel 285, OCM-1, 92.1, OMM-1 and OMM-2.3). BRAF and NRAS mutational status was studied in cutaneous melanoma tumours. In ocular melanoma tumours, mutation analysis of GNAQ and GNA11 genes was performed.

Tumour DNA was extracted from manually dissected 10 μm whole sections of paraffin-embedded material using the Invisorb spin tissue mini KIT (Invitek, Berlin, Germany) following the manufacturer's instructions.

Fragments encompassing BRAF exon 15, NRAS exon 2, GNAQ exon 4 and GNA11 exons 4 and 5 were amplified by polymerase chain reaction (PCR) with the primers presented in Table 9. DNA from thyroid cancer cell lines was used as positive control and included in all analyses. PCR consisted of an initial denaturation step for 5 min at 95^0C, followed by 40 cycles of 95^0C for 30 s, annealing temperature (Ta) (Table 10) for 40 s and 72^0C for 45 s, ending with elongation for 10 min at 72^0C.

All PCR products were purified and directly sequenced on an ABI Prism 3130 xl Automatic sequencer (Perkin-Elmer, Foster City, California, USA) using the ABI Prism Dye Terminator Cycle sequencing KIT (Perkin-Elmer). The sequencing reaction was performed in a forward direction, and an independent PCR amplification, in both directions, was performed in samples that were inconclusive or mutated.

Table 10: Primers, sequences and corresponding annealing temperatures.

Gene	Sequence	Ta
GNA11 Q209	5′- gtcctgggattgcagattg -3′ 5′- atacgaccaagtcctggtgg -3′	58^{0}C
GNA11 R183	5′-gtgctgtgtccctgtcctg-3′ 5′-ggcaaatgagcctctcagtg-3′	60^{0}C
GNAQ Q209	5′-ttttccctaagtttgtaagtagtgc-3′ 5′- cgtggagtcagacaatgagg -3′	57.5^{0}C
GNAQ R183	5′-tggtgtgatggtgtcactgacattctcat-3′ 5′-agctgggaaataggtttcatggactcagt-3′	58^{0}C
BRAF V600	5′-tgcttgctctgataggaaaa-3′ 5′- ttgaacagttgtctggatcc -3′	56^{0}C
NRAS Q61	5′-gattcttacagaaaacaagtggttatagat-3′ 5′- aatacatgaggacaggcgaagg -3′	57^{0}C

Ta – annealing temperature

5.5 Measurement of cell viability - Sulphorodamine B (SRB) assay

Due to the optimal range of optic density, a previous determination of most adequate cell concentration had to be performed. For cell viability assays, cells were seeded as triplicates in 96-well plates at a density of 6000 cells per well for cutaneous melanoma cell lines, 8000 cells per well for ocular melanoma cell lines and 2000 cells per well for OCM-1 cell line, in 200 µl medium. The following day, culture medium was replaced by fresh medium with different concentrations of RAD001 for 24h and 48h. RAD001 was dissolved in DMSO and was added to the culture medium. Two different concentrations were tested (20 nM and 50 nM). Melanoma cells incubated in culture medium with DMSO were used as control, whereas culture medium with DMSO alone was used as blank calibration.

Human cultured cell viability for each cell line was assessed in the appropriate medium, containing also 10% FBS. Cells were incubated for 24h and 48h, fixed in 50 µl of cold 50% TCA (Trichloroacetic acid) for 1 h at 4ºC, washed with distilled water, and air dried. 150 µl of a SRB (Sigma-Aldrich, St. Louis, MO, USA) solution at 0.1% in 1% acetic acid was then added. The plates were incubated for 30 min at room temperature and washed with 1% acetic acid and air dried. Finally, 150 µl of 10 mM Tris-base buffer was added, plates were shaken and measured at 560 nm, using a Synergy Mx microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Cell viability was calculated based on the optical density of the solution, as previously described.[66] In mild-acidic condition, SRB binds to basic aminoacids residues of tricloroacetic TCA-fixed cells.

Results were expressed as percentage of the growth relative to the control. All assays were performed in triplicates and were repeated at least three times.

5.6 Measurement of apoptosis - TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay

Cells were plated in 6 cm cell culture dishes and subjected to treatments with 20 nM or 50 nM RAD001 for 24h and 48h. DMSO solution of the same volume and concentration used to dissolve RAD001 was used as control. Cells were fixed with 4% paraformaldehyde (15 min) at room temperature, washed in PBS and permeabilised with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in 0.1% sodium citrate (2 min) on ice. TUNEL analysis was performed using the "In situ cell death detection kit, fluorescein" from Roche, following the manufacturer's instructions. The proportion of TUNEL-positive nuclei was determined from counting at least 500 cells.

5.7 Protein extraction

Cells were seeded in 6 cm cell culture plates and subjected to the indicated treatments with RAD001 for 24h and 48h. Cells were scraped and washed with Phosphate buffered saline (PBS). Whole-cell lysates were prepared by adding RIPA (Radio-Immunoprecipitation Assay) buffer supplemented with protease and phosphatase inhibitors (4% and 1%, respectively) to each well plate, as appropriate, followed by incubation for 15 min at 4º C. All tubes were centrifuged at 14,000 rpm for 10 min at 4^0C and the supernatant was stored at −20^0C. Protein yield was quantified in triplicates using the Bradford protein assay KIT Dc Protein Assay (BioRad, Hercules, CA, USA).

5.8 Immunoblotting (IB) analysis

Total cell lysates (50 μg) were denatured and separated by electrophoresis on an SDS-PAGE gel (7 and 12%, depending on molecular weight of the protein of interest) and electroblotted to a Hybond ECL membrane (Amersham Biosciences, Amersham, UK).

The membrane was subsequently incubated with the primary antibody in 5% non-fat dry milk in PBS/Tween 20, or 5% bovine serum albumin in PBS/Tween 20 (Table 11), overnight at 4^0C. After 3 washes in PBS/Tween 20 (Sigma-Aldrich, St. Louis, MO, USA), the membranes were re-incubated with the secondary antibody for 45 min, as appropriate. Immunodetection of the immunoblots was performed using an ECL

detection solution (Amersham Biosciences, Amersham, UK). Membranes were re-incubated with a goat polyclonal anti-actin antibody that served as a loading protein control.

The optical density of the bands of interest was measured using Bio-Rad Quantity One 1-D Analysis software (4.6.6 version). The optical density of β-actin bands was used as a normalizing factor. For each blot, the normalized values were used for statistical evaluation.

Table 11: List of primary antibodies.

Target	Antibody	IB conditions
PTEN	Ref. 9559 Cell Signalling Technology Rabbit	1:1000 BSA 4ºC, Overnight
phospho-AKT (Ser473)	Ref. 3787 Cell Signalling Technology Rabbit	1:1000 BSA 4ºC, Overnight
phospho-mTOR (Ser2448)	Ref. 2971 Cell Signalling Technology Rabbit	1:1000 BSA 4ºC, Overnight
phospho-4E-BP1 (Thr37/46)	Ref. 2855 Cell Signalling Technology Rabbit	1:1000 BSA 4ºC, Overnight
phospho-S6 (Ser 235/236)	Ref. 4856 Cell Signalling Technology Rabbit	1:1000 BSA 4ºC, Overnight
Raptor	Ref. 2280 Cell Signalling Technology Rabbit	1:1000 BSA 4ºC, Overnight
phospho-ERK1/2 (Thr202/Tyr204)	Ref. 9101 Cell Signalling Technology Rabbit	1:1000 BSA 4ºC, Overnight
Rictor	Ref. H00253260-M01 Abnova Mouse	1:1500 Milk 4ºC, Overnight
GNAQ	Ref. SC-392 Santa Cruz Biotechnology Rabbit	1:350 Milk 4ºC, Overnight
BRAF	Ref. SC-166 Santa Cruz Biotechnology Rabbit	1:500 Milk 4ºC, Overnight
Actin	Ref. SC-1616 Santa Cruz Biotechnology Goat	1:2000 Milk 4ºC, Overnight

5.9 Statistical analysis

The statistical analysis was performed using STAT VIEW-J 5.0 (SAS Institute, Inc., Cary, North Carolina, USA). The possible association between mutational status of the genes studied and clinicopathological features was analyzed by the Fisher's exact test. The data obtained in the experiments with cell lines was analysed by the two-tailed unpaired Student's t-test. A p value < 0.05 was considered statistically significant.

6 Results

6 Results

6.1 BRAF, NRAS, GNAQ and GNA11 mutation analysis

The most frequent gene mutations in cutaneous and ocular melanoma were studied by PCR/sequencing, the sequence primers and corresponding annealing temperature are described in Methods (5.4 - BRAF, NRAS, GNAQ and GNA11 mutation analysis). To evaluate their prognostic biomarker potential, BRAF and NRAS mutational status was studied in cutaneous melanoma tumours. In ocular melanoma tumours, a mutation analysis of GNAQ and GNA11 genes was performed.

The genetic profile of the set of cell lines was established by mutation analysis of BRAF, NRAS, GNAQ and GNA11 in cutaneous melanoma cell lines (G361 and Mewo), and in ocular melanoma cell lines (Mel 285, OCM-1, 92.1, OMM-1 and OMM-2.3).

Only BRAF V600E showed a correlation with a clinicopathological feature in cutaneous melanoma

BRAF

In cutaneous melanoma tumours, gene sequencing was successful for the assessment of BRAF status in 12 tumours. V600E mutation was found in 25% of the 12 cases analysed (Figure 10).

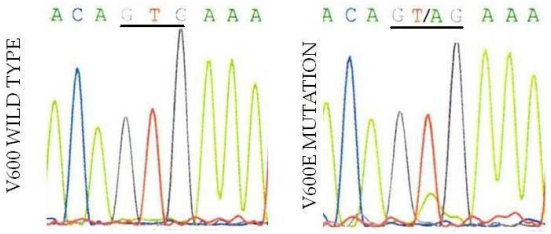


Figure 10: Mutational analysis of BRAF gene. Representative electropherograms of BRAF gene sequencing from a case with wild-type V600 sequence and a case with heterozygous V600E mutation; both were obtained by PCR/Sequencing (Methods).

To evaluate a possible association between BRAF mutational status and prognostic parameters of the cutaneous melanoma cases, a relationship between the presence or absence of BRAF V600E mutation and clinicopathological features was

studied by statistical analysis as described in Methods (5.9 - Statistical analysis). The results are summarised in Table 12.

Gender was the only clinicopathological feature with significant correlation with BRAF mutational status. In the series studied, 3 of 5 (60%) women presented the V600E mutation of BRAF, while all men had the wild-type form of BRAF.

Table 12: Clinicopathological features and BRAF mutational status in cutaneous melanoma.

Clinicopathological features	$BRAF^{wt}$ n (%)	$BRAF^{V600E}$ n (%)	p
Gender			
Male	7 (100.0)	0	0.046
Female	2 (40.0)	3 (60.0)	
Breslow's thickness			
≤ 1.0 mm	3 (75.0)	1 (25.0)	0.854
1.01-2.0 mm	2 (66.7)	1 (33.3)	
2.01-4.0 mm	1 (50.0)	1 (50.0)	
≥ 4.01 mm	2 (100.0)	0	
Mitotic Rate			
<1 mitosis/mm^2	4 (66.7)	2 (33.3)	>0.999
≥1 mitosis/mm^2	3 (75)	1 (25)	
Ulceration			
Absent	5 (71.4)	2 (28.6)	>0.999
Present	2 (100.0)	0	
Necrosis			
Absent	8 (73.7)	3 (27.3)	>0.999
Present	1 (100.0)	0	
pT			
pT1 /pT2	5 (71.4)	2 (28.6)	>0.999
pT3 /pT4	3 (75.0)	1 (25.0)	

wt, wild-type form. Clinicopathological features were evaluated by a pathologist from Hospital de São Marcos, Braga, Portugal. The clinicopathological features chosen to study the correlation with BRAF mutation are recommended by staging classification system.[18] Fisher's exact test was used and differences were considered as statistically significant when $p<0.05$.

NRAS

In cutaneous melanoma tumours, the sequencing for the assessment of the NRAS gene status was successful in 13 tumours. The substitution CAA➔CTA has been identified in only one case (7.8%) (Figure 11). Due to the absence of an adequate number of NRAS mutated cases, a correlation between mutational status and clinicopathological features was impossible to establish.

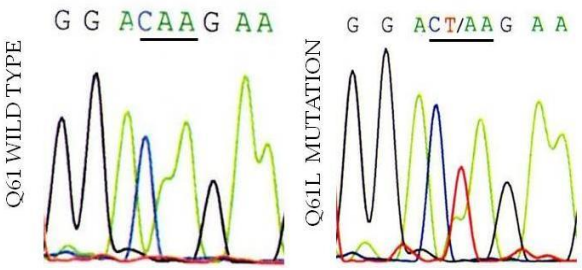


Figure 11: **Mutational analysis of NRAS gene.** Representative electropherograms of NRAS gene sequencing from a case with wild-type Q61 sequence and a case with heterozygous Q61L mutation; both were obtained by PCR/Sequencing (Methods).

Mutation analysis of BRAF and NRAS in cutaneous melanoma tumour samples showed only one correlation between BRAF and clinicopathological feature. No evaluation of correlation of NRAS mutational status was possible to perform.

Neither GNAQ nor GNA11 mutational status correlates to the clinicopathological feature tested of ocular melanoma

GNAQ

In ocular melanoma tumours, the sequencing was successful for the assessment of GNAQR183 status in 28 tumours. None of the studied cases showed GNAQR183 mutations.

GNA11

In ocular melanoma tumours, the sequencing was successful for the assessment of the status of GNA11 exon 4 and exon 5, in 24 and 31 tumours, respectively. The substitution CAG➔CTG has been identified in 4 (12.9%) of the 31 cases (Figure 12). None of the cases studied showed GNA11R183 mutations.

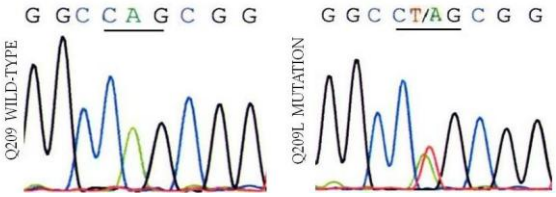


Figure 12: Mutational analysis of GNA11 gene. Representative electropherograms of GNA11 gene sequencing from a case with wild-type Q209 sequence and a case with heterozygous Q209L mutation, both were obtained by PCR/Sequencing (Methods).

Table 13: Clinicopathological features and GNA11 mutational status in uveal melanoma

Clinicopathological features	$GNA11^{wt}$ n (%)	$GNA11^{Q209L}$ n (%)	p
Median age			
< 59	8 (72.7)	3 (27.3)	0.317
≥59	15 (93.75)	1 (6.25)	
Gender			
Male	7 (70)	3 (30)	0.281
Female	16 (94.1)	1 (5.9)	
Cytological type			
Spindle-cell	8 (72.7)	3 (27.3)	0.375
Epithelioid-cell	9 (100)	0	
Mixed-cell	10 (90.9)	1 (9.1)	
pT			
pT1/ pT2	18 (85.7)	3 (14.3)	>0.999
pT3/ pT4	9 (90)	1 (10)	
Mitotic Rate			
<1 mitosis/mm²	15 (78.9)	4 (21.1)	0.2768
≥ 1 mitosis/mm²	10 (100)	0	
Median basal tumour diameter			
<11mm	8 (88.9)	1 (11.1)	>0.999
≥11 mm	14 (82.4)	3 (17.6)	
Tumour scleral involvement			
Absent	16 (80)	4 (20)	0.546
Present	7 (100)	0	

wt, wild-type form. Clinicopathological features were evaluated by a pathologist from Hospital de São João, Porto, Portugal. The clinicopathological features chosen to study the correlation with GNA11Q209L are described in the Introduction Fisher's exact test was used and differences were considered as statistically significative when $p<0.05$.

Since all conjunctival melanoma cases presented the wild-type form of GNA11, only uveal melanoma cases were considered to study a possible association between GNA11 mutational status and prognostic parameters, as described in Methods (5.9 - Statistical analysis). The results, summarised in Table 13, show no significant correlation between the GNA11 mutational status and any clinicopathological feature.

Mutation analysis of GNAQ and GNA11 in ocular melanoma tumour samples showed that none of the genes evaluated correlated to any clinicopathological feature.

Genetic profile establishment of the melanoma cell lines used in this study

BRAFV600E was identified in G361 and OCM-1 cell lines. The 92.1 cell line presented a Q209L mutation of the GNAQ gene. The OMM-1 cell line showed a homozygous Q209L mutation of the GNA11 gene (Figure 13). No alterations were detected in the NRAS gene. The mutational status of the genes studied in the cell lines is summarised in Table 14.

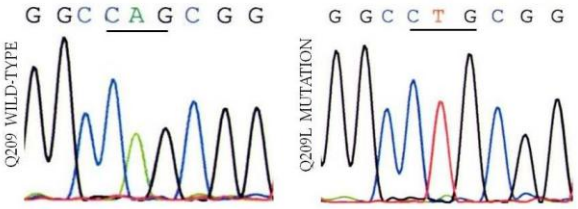


Figure 13: Mutational analysis of GNA11 gene in cell lines. Representative electropherograms of GNA11 gene sequencing from a cell line with wild-type Q209 sequence and a cell line with Q209L homozygous mutation; both were obtained by PCR/Sequencing (Methods).

Table 14: BRAF, NRAS, GNAQ and GNA11 mutational status in melanoma cell lines.

Location	Cell Line	BRAF	NRAS	GNAQ		GNA11	
		V600	Q61	Q209	R183	Q209	R183
Cutaneous Melanoma	G361	V600E	WT	WT	WT	WT	WT
	Mewo	WT	WT	WT	WT	WT	WT
Uveal Melanoma	Mel 285	WT	WT	WT	WT	WT	WT
	OCM-1	V600E	WT	WT	WT	WT	WT
	92.1	WT	WT	Q209L	WT	WT	WT
Sub-cutaneous Metastases of UM	OMM-1	WT	WT	WT	WT	Q209L	WT
Liver Metastases of UM	OMM-2.3	WT	WT	Q209L	WT	WT	WT

WT, wild-type form.

6.2 Identification of predictive biomarkers to RAD001 treatment of melanoma.

The G361 cell line showed the highest level of cell growth inhibition after RAD001 exposure.

In this study, seven melanoma cell lines were used, two cutaneous melanoma cell lines and five ocular melanoma cell lines, with different genetic profiles (Table 14). The effect of RAD001 treatment on growth inhibition of the this panel of cell lines was evaluated by the SRB assay, which indirectly estimates cell number, by staining total cellular protein with the dye SRB (Methods, 5.5 - Measurement of cell viability - Sulphorodamine B (SRB) assay).

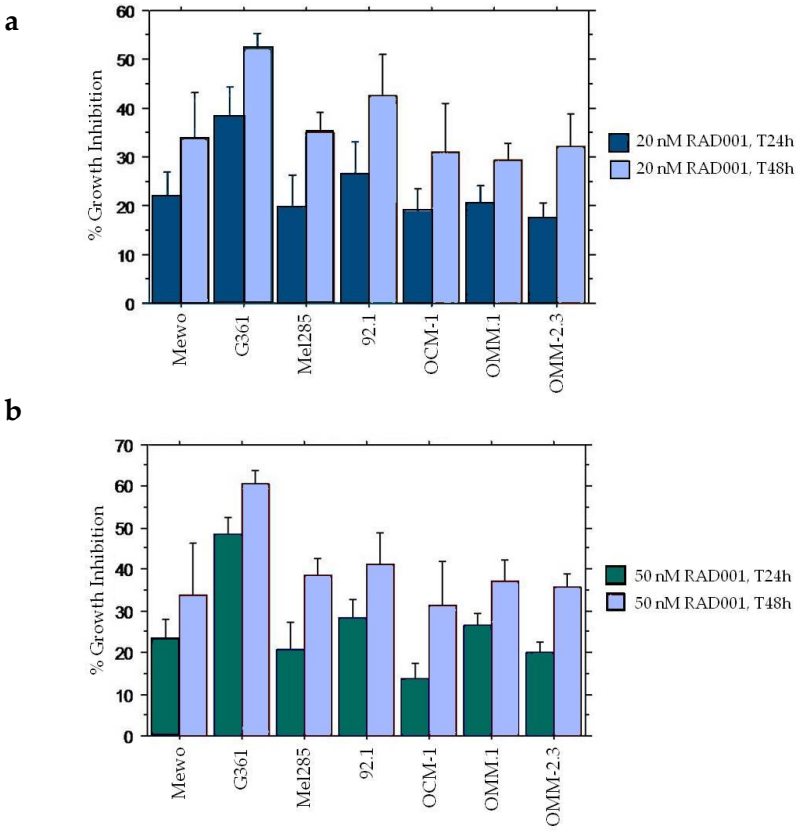


Figure 14: Growth inhibition rates in melanoma cell lines after treatment with RAD001. The percentage of cell growth inhibition relative to control was determined by the SRB assay. Melanoma cell lines were treated with RAD001 at **(a)** 20nM and **(b)** 50 nM. SRB assay was performed 24h and 48h later. Cells in control condition received treatment of DMSO solution of the same volume and concentration used to dissolve RAD001. The assay results shown are the mean (± standard deviation) of three independent experiments. Differences between growth inhibition rates of G361 and the other melanoma cell lines for 20 nM and 50 nM RAD001 after 24h and 48h of exposure to the drug were evaluated by Student's t-test. All p-values were <0.05.

The two concentrations tested, 20 nM and 50 nM, of RAD001 caused different growth inhibition in all cell lines at the time points chosen, 24h and 48h (Figure 14). The cutaneous melanoma-derived cell line G361 presented a growth inhibition rate of 38% after 24h and 52% after 48h of exposure to 20 nM RAD001. Growth inhibition rates of G361 at 24h and at 48h of exposure to 20 nM RAD001 were always significantly higher than the other cell lines tested. With a higher concentration of RAD001 (50 nM), G361 presented again as the most sensitive of the cell lines used in the study. The growth inhibition rates after 24h and 48h of exposure to the drug were 48% and 60%, respectively. Concerning exposure for 24h and 48h periods with this RAD001 concentration, growth inhibition rates of G361 were always significantly higher than the other cell lines studied ($p<0.01$). No differences were found on growth inhibition rates induced by RAD001 in the ocular melanoma cell line OCM-1 presenting the BRAF V600E mutation compared to the other ocular melanoma cell lines.

No significant differences were found between growth inhibition rates of cell lines harbouring Q209L mutations of GNAQ or GNA11 and WT cell lines.

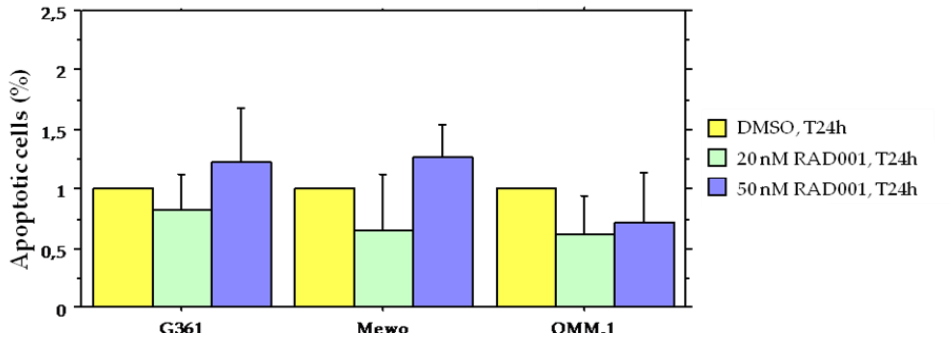
The G361 cell line appeared to be more sensitive to RAD001 than any of the other cell lines tested.

RAD001 did not induced apoptosis in the cell lines studied.

To assess if growth inhibition resulted from cell death, the effect of RAD001 treatment on apoptosis of the panel of cell lines used was evaluated by a TUNEL assay. Due to the higher sensitivity of G361 cells to RAD001, this cell line was an obvious choice for this assay. Mewo cells were chosen as a control, since this cell line harbours no mutation of any of the genes studied. OMM-1 is a cell line isolated from an ocular melanoma skin metastasis and for that reason TUNEL assay was used to verify if RAD001 increased the number of apoptotic cells in a metastatic cell line.

When compared to RAD001 solvent, DMSO, 20 nM and 50 nM of RAD001 caused no significant differences on the number of apoptotic cells, in all cell lines at 24h and 48h of exposure (Figure 15).

a



b

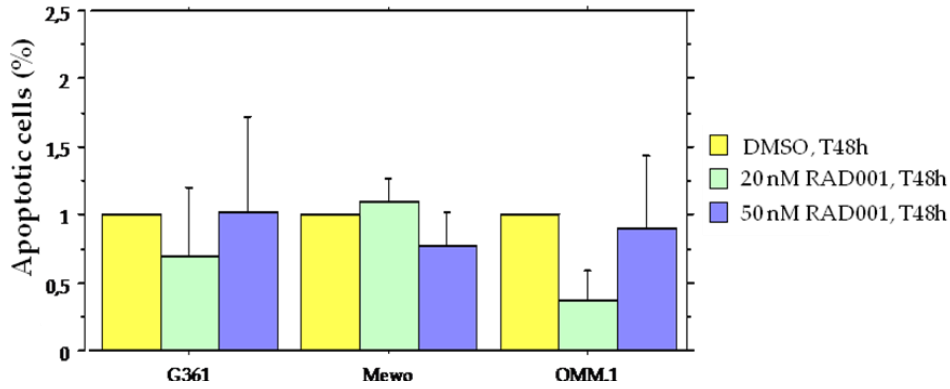


Figure 15: Apoptosis measurement in melanoma cell lines exposed to RAD001. Melanoma cell lines were treated with DMSO, 20 nM RAD001 or 50 nM RAD001. TUNEL assay was performed **(a)** 24h and **(b)** 48h later. Cells in control condition received DMSO solution of the same volume and concentration used to dissolve RAD001. The assay results shown are the mean (± standard deviation) of three independent experiments. Differences of death cell rates between melanoma cell lines for 20 nM and 50 nM RAD001 after 24h and 48h of exposure were evaluated by Student's t-test. All p-values were ≥0.05.

RAD001 altered the expression of mTOR pathway effectors in all cell lines tested.

To evaluate the potential of mTOR pathway effectors as therapeutic markers of RAD001 treatment, the effect of RAD001 exposure in the expression of mTOR pathway effectors was studied in melanoma-derived cell lines treated with two concentrations of the drug for 24h and 48h, by immunoblotting (Methods, 5.8 – Immunoblotting (IB) analysis). The results are presented in Figure 16 and Figure 17 – section 3, Appendix. Information about statistical significances can be found in Figure 17.

In all cell lines, PTEN, an inhibitor of the mTOR pathway[15], Rictor, a constituent of the mTORC2[51] and BRAF, which activation leads to mTOR pathway activation[33], showed no alteration in their expression after RAD001 treatment, with both RAD001 concentrations, in two time points. Expression of Raptor, one of the elements of mTORC1[51], was not altered in 92.1, G361 and Mewo cell lines, but it was increased in Mel 285 cells at 24h and 48h with 50 nM and 20 nM RAD001, respectively, it was also increased in both concentrations after 48h of exposure, in OMM-1 cells. The expression of p-mTOR was decreased in all cell lines, but was significantly inhibited by RAD001 in G361 and Mewo cells after 24h and in 92.1, Mel285 and OMM-1 cells after 48h. The phosphorylation of two downstream effectors of mTORC1, S6 and 4E-BP1, was assessed by the level of expression of their phosphorylated forms. There was an efficient inhibition of the phosphorylation of S6 in all cell lines and conditions, while partial inhibition of phosphorylation of 4E-BP1 was observed at 24h in OMM.1 and at 48h in Mel 285 with both RAD001 concentrations. mTORC2 phosphorylates AKT at Ser473[50], therefore an antibody anti-p-AKT(Ser473) was used to assess the level of AKT specifically activated by the mTOR pathway. Treatment with RAD001 induced an increase in expression of p-AKT(Ser473) after 24h in the cell line G361, and after 48h in 92.1 and Mewo cells. The effect of RAD treatment in the activation of the MAPK pathway was evaluated by the phosphorylated forms of ERK1/2 expression. An increase in p-ERK1/2 was observed in Mel285, at 48h, after treatment with 20 nM RAD001; in G361, at 24h, after 20 nM RAD001 treatment and in OMM-1, at 48h, after treatment with both RAD001 concentrations.

The results showed that RAD001 treatment induced alterations in expression levels of most of the mTOR pathway effectors studied.

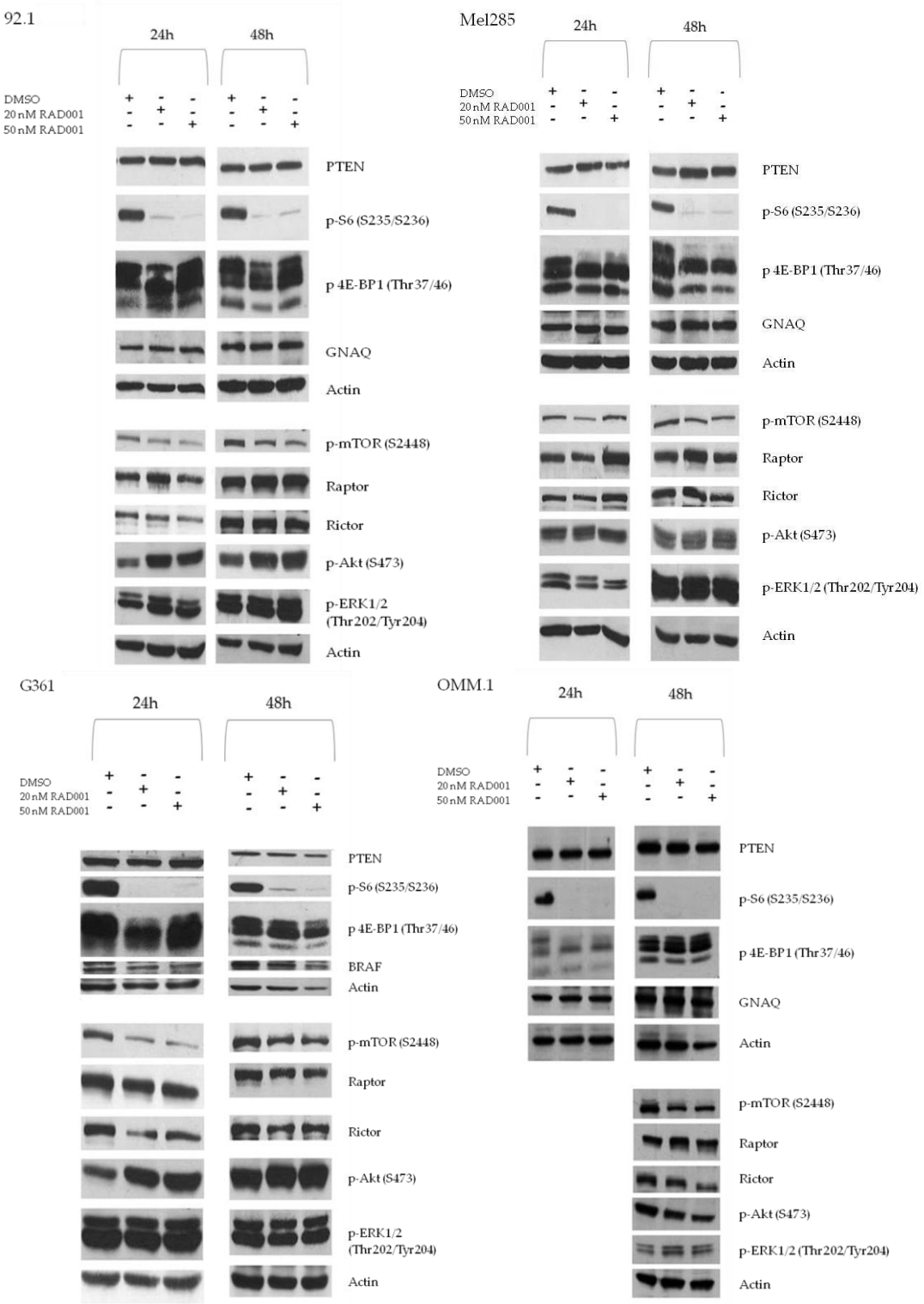


Figure 16: Expression of mTOR pathway effectors in 92.1, Mel285, G361 and OMM-1 cell lines after RAD001 exposure. Cells were exposed to 20 nM and 50 nM of RAD001 for 24 h and 48h. In control condition, cells received treatment of DMSO solution of the same volume and concentration used to dissolve RAD001. Total cell lysates (50 μg) were separated by SDS–PAGE, blotted to nitrocellulose membranes and probed with antibodies. β-Actin served as a loading control. Due to the similarity between Mewo's and G361 cells results, image correspondent to Mewo cells was omitted. The lack of results to the expression of some proteins at 24h, in OMM-1 cell lines, was caused by technical problems that could not be solved in the period of time given to develop the study.

7 Discussion

7 Discussion

7.1 BRAF, NRAS, GNAQ and GNA11 are not suitable prognostic biomarkers

Biomarkers are molecules used as indicators of a normal or pathologic biological state (diagnostic/prognosis markers) or as markers of therapeutic response to a pharmacological agent (predictive markers).[67] In the setting of metastatic melanoma, there are few prognostic factors, and those fail to effectively discriminate between the large subpopulation of patients with an aggressive disease course and the small population with a relatively indolent natural history.[68] Therefore, it is of great importance to search for molecular markers of metastatic potential that in combination with other factors would provide the most accurate prognosis possible.

In this study, the mutational status of some genes previously reported as frequently mutated in CM (BRAF [36] and NRAS [69]) and OM (GNAQ[42] and GNA11[46]) was evaluated in two series of melanoma tumour samples. The CM series showed a frequency of BRAF mutation (25% of 12 cases) similar to those reported in two previous studies (27%-28%)[70, 71] but significantly lower than reported in other studies.[36, 71, 72] Correlation analysis between BRAF mutational status and clinicopathological features was performed, though there were some limitations due to the small number of available samples to analyse. The mutation observed (BRAFV600E) showed no correlation to most clinicopathological features, being gender the only exception. BRAFV600E appeared to have a higher incidence in the female gender (60%, p=0.046). The absence of a significant association of BRAF mutation with clinicopathological parameters, including clinical stage at diagnosis, subtype of melanoma, presence of epidermal ulceration, presence of necrosis, Clark's level and tumour thickness has also been reported by Pópulo and co-workers (2011)[33], Shinozaki and co-workers (2004)[73] and Saldanha and co-workers (2006)[74]. The other gene which its mutational status was evaluated in cutaneous melanoma tumour samples was NRAS. BRAF and NRAS activate the same signalling pathway and that is the reason why mutations in these two genes did not coexist in any of the cases. Although we were expecting this mutually exclusive behaviour, in this series of cutaneous melanoma tumour samples, NRAS mutation revealed and unexpected lower frequency of mutation

(7.8%), since in the literature the reported frequencies were considerably higher.[69, 75] As there was only one case with NRAS mutation identified in this study, it was not possible to perform a correlation analysis between NRAS mutational status and clinicopathological features.

The genes chosen to be studied in the ocular melanoma tumour samples were GNAQ and GNA11. These two genes encode members of the q class of heterotrimeric G-protein α subunit and have overlapping functions in melanocytes , possibly due to 91% identity at the amino acid level.[46] Only very recently, mutations in exon 5 of the GNA11 gene have been reported in about 31.9% of uveal melanomas.[46] Interestingly, in our series of uveal melanomas, the frequency of GNA11 mutations identified at exon 5 was considerably lower (15.4%) and none of the cases harboured simultaneously mutations in GNA11 and GNAQ. Like other mutually exclusive mutations, this pattern is probably caused by the overlapping functions of the two proteins. This high similarity between the two proteins may explain the resemblance between the results that Pópulo and co-workers (2010) have reported to this case series about the mutations in codon 209 of GNAQ[44], and the absence of association of mutation in GNA11 gene with any of the clinicopathological features tested (age, gender, cytological type, pT group, mitotic rate, largest tumour diameter and sclera extension) that is described in this study. Van Raamsdonk and co-workers recently described that the frequency of mutations affecting exon 4 of GNAQ and GNA11 was 2.8% and 2.1% of the 145 cases of primary uveal melanoma studied, respectively. These mutations, in R183, revealed to be less prevalent than mutations in Q209.[46] In the present study, no mutations of GNAQR183 or GNA11R183 were observed, possibly due to the smaller number of cases evaluated in comparison to the study of Van Raamsdonk et al.[46]

This series of ocular melanoma has been previously characterized regarding the expression of MAPK and mTOR pathway effectors and no association with the GNAQ mutational status was found (unpublished data). After the evaluation of GNA11 mutational status, the same association study was performed and a similar lack of correlation between levels of expression of MAPK and mTOR pathway effectors and the mutational status of GNA11 was obtained, which may indicate the existence of other

unknown genetic mechanisms that may play a role in MAPK activation in uveal melanoma.

In conclusion, none of the gene mutations studied gave evidence that it could be a useful biomarker to establish a reliable prognosis in CM or OM.

7.2 BRAF is a potential therapeutic biomarker

Besides the difficulty of establishment of accurate molecular prognostic factors, the identification of effective treatments is also a constant problem in the melanoma research. The probability to find a marker for melanoma metastasis or to design a uniform approach to treat such a heterogeneous disease is still very remote. To overcome this, the genetic heterogeneity of melanoma has to be always taken into account.

One of the features shared by ocular and cutaneous melanoma is the constitutive activation of the MAPK pathway. BRAF, in cutaneous melanoma[29], and GNAQ and GNA11, in ocular melanoma[42, 44], can be responsible for this pathway's constitutive activation. The link between MAPK and mTOR pathways has already been established by Li Ma and co-workers (2005)[76]. They demonstrated that MAPK activation leads to phosphorylation of TSC2 by ERK 1/2, which results in TSC1-TSC2 complex dissociation. This dissociation markedly impairs TSC2 ability to inhibit mTOR signalling, indicating that the MAPK pathway functions upstream of the TSC complex and cooperates with the PI3K pathway to modulate mTOR signalling.[76] Very recently the connection between these signalling pathways was identified by our group in a series of CM. In our study, in tumours with BRAFV600E, high levels of mTOR pathway activation were identified by significantly increased expression of pS6 and p4E-BP1 and significantly decreased levels of the mTOR pathway inhibitor, PTEN.[33] Although there was activation of both pathways in a series of ocular melanoma[57], no connection was established between the GNAQ mutational status and mTOR pathway expression effectors (unpublished data). The impact of RAD001, an mTOR pathway inhibitor, on cell proliferation, cell apoptosis and signalling effector expression was studied to determine if any of the genes in question

(BRAF, GNAQ and GNA11) could be used as a tool to predict therapeutic responses and to select patients for the most suitable and effective treatment.

Due to the connection between BRAFV600E and increased mTOR pathway activation in cutaneous melanoma, the result obtained with the G361 cell line that showed the highest growth inhibition after RAD001 treatment, was already expected. The BRAFV600E effect in cells sensitivity to RAD001, was also studied in an ocular melanoma cell line, OCM-1. Even though this cell line harbours a BRAFV600E mutation, it had a growth inhibition similar to BRAF wild-type cell lines. These results indicate that higher level of growth inhibition, after RAD001 treatment, of cell lines harbouring BRAFV600E might be specific to cutaneous melanoma-derived cell lines. Thus, constitutive activation of BRAF may not play a role as relevant in ocular melanoma as it does in cutaneous melanoma.

Cells from primary ocular melanoma harbouring mutations in exon 5 of the GNAQ gene showed no significant differences in growth inhibition when compared to wild-type cells for GNAQ exon 5. These results are coherent with previous findings from our group[44, 57] and suggest that the mutational status of this gene is not related to the response to RAD001 exposure. Growth inhibition of two metastatic cell lines, harbouring GNAQQ209L or GNA11Q209L mutation, was also evaluated after exposure to the same drug. No significantly different responses were observed, indicating that metastatic cells do not show higher sensitivity than cell lines with GNAQ or GNA11 wild-type, neither when compared to cell lines from primary tumours with GNAQQ209L.

The responses to RAD001 by cells with mutations in BRAF or GNAQ genes confirm previous indications of MAPK constitutive activation in both types of melanoma (cutaneous and ocular) and subsequent divergence to different and unknown pathways resulting in different responses to this drug.

After the observations related to growth inhibition after RAD001 treatment, the levels of apoptosis were assessed. In previous reports, RAD001 showed to be unable of induce apoptosis in cell lines from different types of cancers, such as breast cancer, ovarian cancer [77], medullary thyroid carcinoma [60] and melanoma [78-80], being described in some studies an accumulation in G1 resulting from cell cycle arrest due to blocking of

mTOR.[81, 82] Confirming the results described in the literature, [78-81] in this study, the cell lines selected for apoptosis evaluation showed no differences in the number of apoptotic cells over time or in a dose-dependent manner.

Analysis of the expression of mTOR pathway effectors revealed some interesting differences between cell responses to RAD001 treatment. More importantly, all cell lines studied showed a partial or complete inhibition of phosphorylation of S6. Taking into account the results obtained with the SRB assay and the immunoblotting, we can conclude that p-S6 levels can only give an indication of the efficiency of RAD001 in mTOR signalling inhibition and cannot be used to give information of sensitivity to the treatment, since the most sensitive cell line was G361, but the cell line with higher inhibition of phosphorylation of S6 was OMM-1. Similar effects in sensitive and insensitive cell lines have been described.[83] Taken together, the similar levels of inhibition of S6 phosphorylation in all cell lines and the major impact of that inhibition in the impairment of G361 (harbouring BRAFV600E) growth, we can suggest a higher dependence of cell growth in mTOR pathway activation induced by BRAF constitutive activation, in the cutaneous melanoma cell line. This high inhibition of S6 phosphorylation may be caused by the lack of activation of S6K by the mTORC1 complex. Since S6K is related to protein translation, particularly in G1 phase,[84] the inhibition of this protein may explain the absence of increased levels of apoptosis in the cells used in this study and suggest an arrest in G1 phase after RAD001 treatment. However, further studies, including cytometry analysis must be performed to verify this hypothesis, since the lack of protein translation would induce a cell cycle arrest in G1 phase.

4E-BP1 is another downstream target regulated by mTORC1 complex. Although, most of the cell lines show no significant differences in global expression of p-4E-BP1, all of them present, in immunoblotting analysis, many bands representing different stages of phosphorylation, being clear a decrease in the expression of the higher stages. Therefore, like levels of p-S6, levels of p-4E-BP1 will only be useful to assess the inhibition of mTOR pathway, but not to predict responses to RAD001 treatment. To support this, OMM-1 and Mel285 were the only cell lines in which the global levels of p-4E-BP1 decreased after 24h

and 48h, respectively, being this decrease insufficient to increase these cell lines' sensitivity.

In the past, loss of PTEN and activation of Akt have been used as markers with predictive value of responses to mTOR inhibitors.[86] However, in my study, both p-Akt and PTEN showed to be useless to predict melanoma cell line sensitivity. RAD001 induces a relief of the negative feedback loop, that through S6K activation, and degradation of insulin receptor-substract-1, attenuates PI3K signalling, decreasing AKT levels.[81] Due to the disruption of this feedback loop, the presence of the drug induces in CM cell lines, G361 and Mewo an increase in p-AKT levels. Besides AKT, ERK phosphorylation also showed an increase in some of the cell lines tested. In 2008, Wang and co-workers reported an increase of p-ERK levels in some human lung cancer cell lines after prolonged treatment with rapamycin.[85] In our study, after 24h of treatment all cells lines showed increased levels of p-ERK, but only 20 nM RAD001 during 48h of exposure could significantly induce activation of MAPK pathway in Mel285. Together, the increase in p-AKT and p-ERK levels indicate that RAD001 may induce the activation of at least two important survival pathways, MAPK and PI3K, which may explain the relatively low effect in growth inhibition in most of the cell lines studied. Neither AKT nor ERK phosphorylation levels showed specificity, therefore they are not suitable to predict resistance to RAD001 treatment. Instead, these results show the importance of designing and test different combinatorial anticancer therapies. In literature two major strategies were described and encouraging results were obtained: optimal blockade of mTOR signalling pathway by combination of compounds that inhibit different components within this pathway[80] and combined targeting of mTOR signalling pathway and MAPK signalling pathway[85].

Susceptibility of melanoma cells to the inhibition of signaling pathways have been also linked to the micro-environment. Meier and co-workers have showed that when metastatic melanoma cells are grown in tri-dimensional spheroids, they exhibited a resistance to the inhibition of only one pathway, being killed only by the simultaneous inhibition of PI3K and MAPK pathways.[87] Therefore, after confirmation of the predictive value of BRAF mutation, the cytotoxicity should be evaluated in a tri-dimensional model.

In conclusion, from this study a new molecular prognostic marker has not emerged, in the genes selected, but it was possible to identify a candidate genetic marker to predict responses to RAD001 treatment. The obtained results suggest that BRAF mutational status could be used as marker to predict treatment response in CM patients, but not in OM patients. This is possibly a more reliable biomarker than expression levels of mTOR pathway effectors. Since it is a genetic marker, BRAF mutational status will not be affected by previous chemotherapy treatments, as p-S6 or AKT expression levels are.[84] All these features give to BRAF mutation status a high potential of utilization in selection of patients to mTOR inhibition therapies. Further studies *in vitro* and *in vivo* with a broad set of ramapycin analogs and with other CM cell lines harbouring BRAFV600E, should be conducted to confirm these data.

8 References

8 References

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9 Appendix

9 Appendix

Section 1: Frequently used buffers

PBS (pH 7.4):

137 mM NaCl

2.7 mM KCl

12.7 mM $Na_2HPO_4.2H_2O$

1,76 mM KH_2PO_4

1 L H_2O

Sulforhodamine B assay

Tris-base Buffer (10 mM, pH= 10.5):

0.6 g Tris-base

500 mL H_2O

TCA solution:

50% TCA

50% H_2O

SRB 0.1%:

0.1 g SRB

100 mL 1% glacial acetic acid

TUNEL assay

Permeabilization solution:

0.1g Sodium Citrate

100 µL Triton X-100

100 mL H2O

Immunoblotting

RIPA Buffer:

1% NP-40

150 nM NaCl

50 mM Tris (pH 7.5)

2 mM EDTA

Loadding Buffer:

90% Laemmli 4x

5% β-Mercaptoethanol

5% Bromophenol Blue

Running Buffer (pH 8.3):

25 mMTris

192 mM Glycine

0.1% SDS

PBS/Tween 20 0.5%:

995 mL PBS

5 mL Tween 20

Transfer Buffer (pH 8.1-8.4):

25 mM Tris Base

192 mM Glycine

10% Methanol

1.8 L H_2O

Section 2: Equipment

Table 15: Equipment and manufacturers.

Equipment	Company
Synergy Mx microplate reader	BioTek Instruments, Inc., Winooski, USA
ABI Prism 3130 xl Automatic sequencer	Perkin-Elmer, Foster City, California, USA
Centrifuge 5417 F	Eppendorf AG, Hamburg, Germany
Mycycler™ Thermal cycler	BioRad Laboratories Inc,Hercules, California, USA
Immunoblotting system	BioRad Laboratories Inc,Hercules, California, USA

Section 3: Quantitative analysis of Immunoblotting

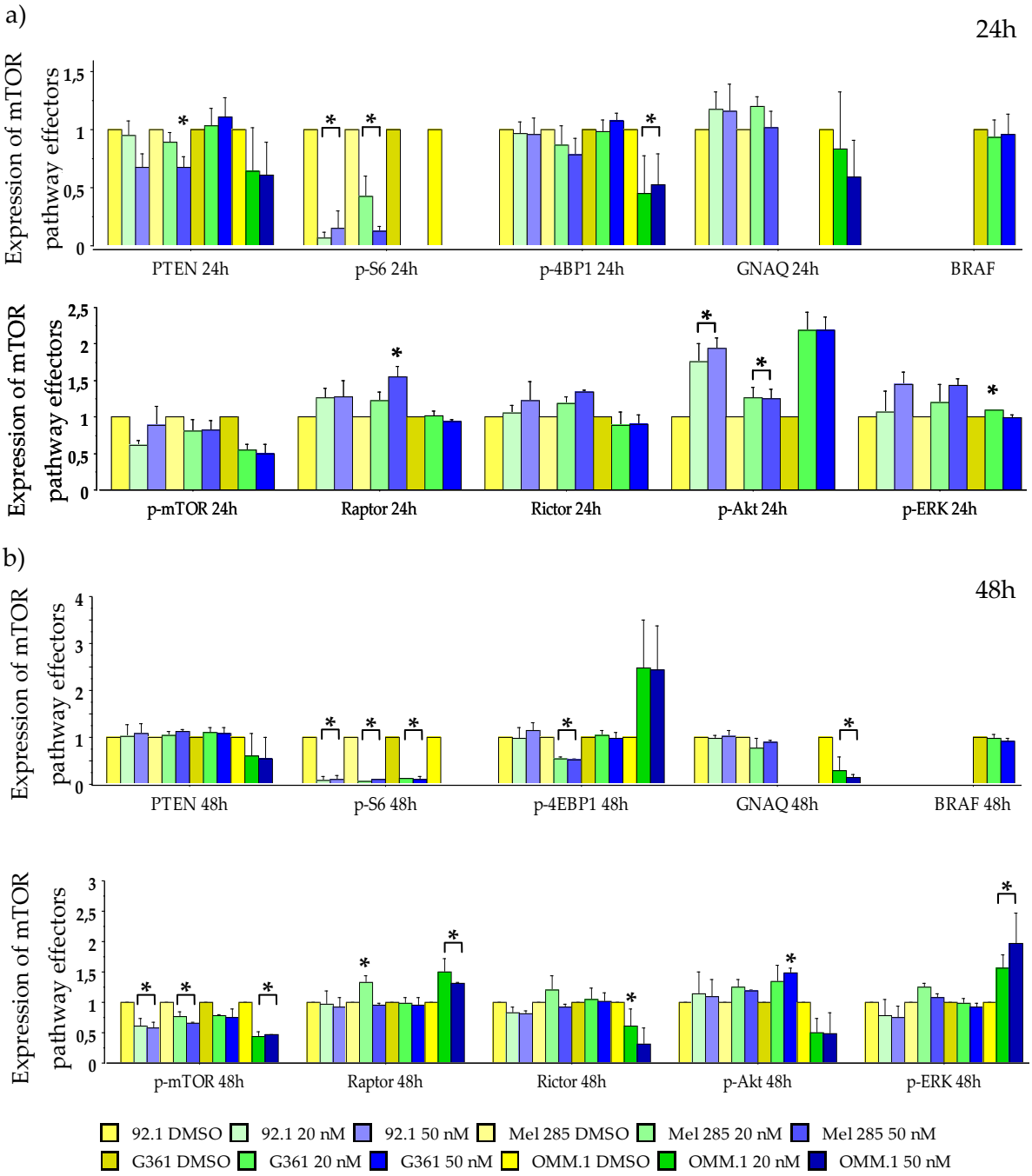

Figure 17: Quantitative analysis of expression of mTOR pathway effectors treatment with DMSO, 20 nM RAD001 or 50 nM RAD001, in 92.1, Mel285, G361 and OMM-1 cell lines. Cells were exposed to 20 nM and 50 nM of RAD001 for **(a)** 24 h and **(b)** 48h. In control condition received treatment of DMSO solution of the same volume and concentration used to dissolve RAD001. Total cell lysates (50 μg) were separated by SDS–PAGE, blotted to nitrocellulose membranes and probed with antibodies. β-Actin served as a loading control. Due to the similarity between Mewo's and G361 cells results, quantitative analysis correspondent to Mewo cell line was omitted. The lack of results to the expression of some proteins at 24h, in OMM-1 cell lines, was caused by technical problems that could not be solved in the period of time given to develop the study. Student's t-test was used to assess statisticall differences in protein expression. * = p-values <0.05.

Section 4: Communication in scientific meeting and publication related to this study

- Pópulo H., Faustino A, **Tavares S**, Lopes J. M., Soares P. (2011), Evaluation of melanoma cell lines sensitivity to RAD001 treatment. XX Porto Cancer Meeting: Drug Resistance in Cancer: from biology to molecular targets and drugs, 28-29th April, IPATIMUP, Porto, Portugal.

- Pópulo H., Faustino A., **Tavares S.**, Lopes J.M., Soares. P. (2011). "Differential activation of mTOR pathway in BRAF and GNAQ human transformed cells." *Submitted to Molecular Cancer Therapeutics.*