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TESI DI DOTTORATO DI RICERCA

IMMUNE PROFILES IN SEQUENTIAL MELANOMA METASTASES: INSIGHTS FOR THERAPEUTIC SUCCESS

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INTRODUCTION

MELANOMA

Malignant melanoma is a very aggressive, therapy-resistant malignancy due to transformation and uncontrolled growth of melanocytes, the pigment-producing cells that reside in the basal epidermal layer of the skin (*Figure 1*) (1). The first accredited report of melanoma is found in the writings of Hippocrates (born c. 460 BC), where he described "fatal black tumors with metastases." In 1960s paleopathologists discovered diffuse bony metastases and round melanocytic masses in the skin of Peruvian mummies, radiocarbon dated to be approximately 2400 years old (2). However, it was not until 1806, when René Laennec described "la mélanose" to the Faculté de Médecine in Paris, that the disease was characterized in detail and named (3). General practitioner William Norris suggested that melanoma may be hereditary in an 1820 manuscript describing a family with numerous moles and several family members with metastatic lesions (4). Molecular insights over the past 20 years have confirmed Norris's theory of a significant genetic contribution to the etiology of melanoma. The first formal acknowledgment of advanced melanoma as untreatable came from Samuel Cooper in 1840. He stated that the only chance for benefit depends upon the early removal of the disease. More than one and a half centuries later this situation remains largely unchanged.

Melanoma is the fifth most common cancer in men and the seventh in women and comprises 4% of all cancers in the United States (5). Since the mid 1960s, melanoma incidence has risen by 3–8% per year in most people of European background, with the greatest increases in elderly men (6). Although melanoma accounts for only 3% of all dermatologic cancers, it is responsible for 65% of skin malignancy-related deaths, and the 5-

year survival of metastatic cutaneous melanoma patients is only 7-19% (7;8). Around 160,000 new cases of melanoma are diagnosed worldwide each year, (9;10) and, according to the 2006 WHO report, about 48,000 melanoma-related deaths occur each year. Incidence rates in Southern and Eastern European countries were fairly low, ranging between 2 and 8 per 100,000 in most countries (11).

More than 95% of tumors are found in the skin, however melanoma is not exclusively a skin cancer: sites of primary extracutaneous melanoma include ocular, mucosal, gastrointestinal, genitourinary, leptomeninges (12).

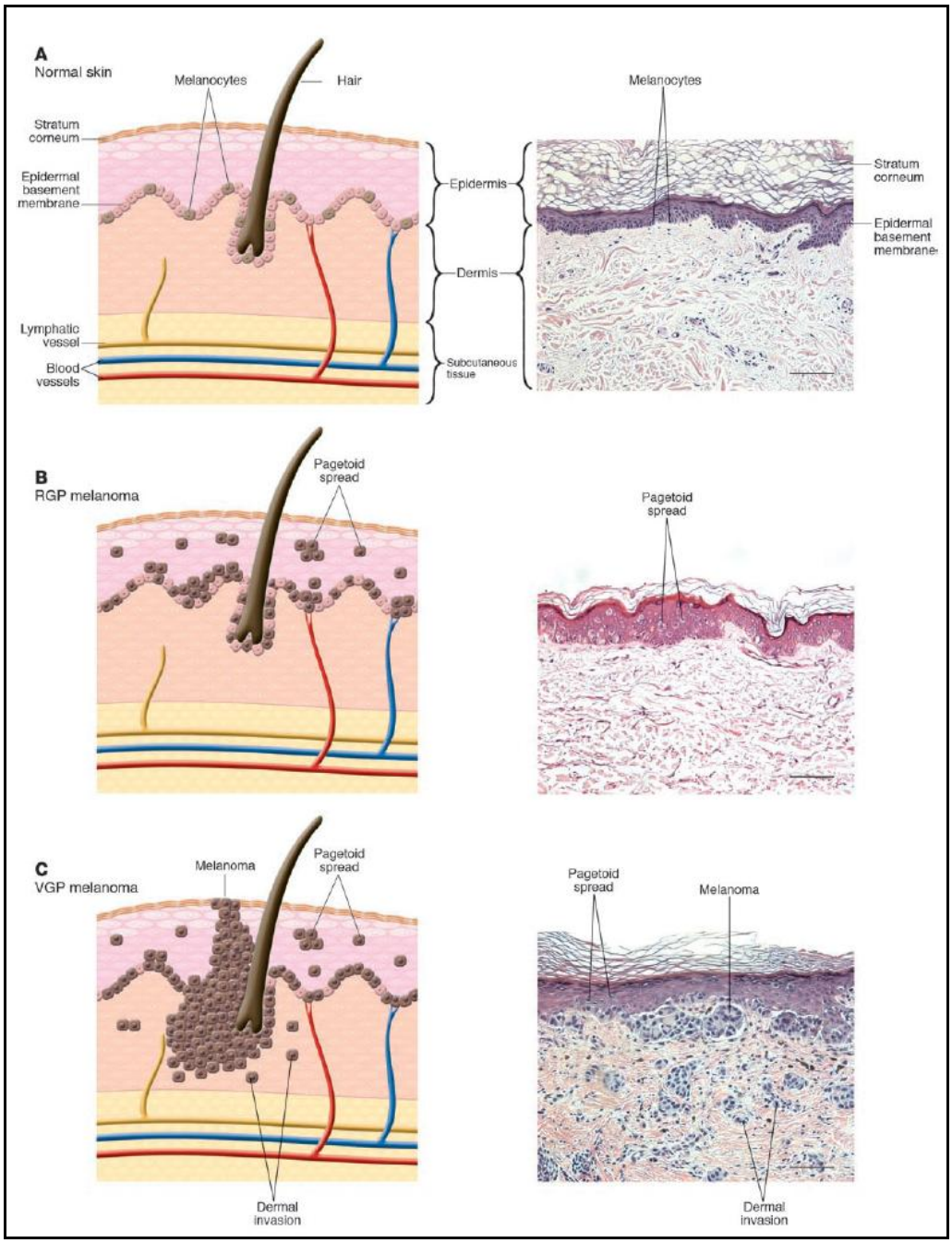

Figure 1: **Phases of histologic progression of melanocyte transformation (taken from (13)).**
Hematossilin-Eosin (H&E) stained histologic sections and corresponding pictorial representation. (A) Normal skin. There is even distribution of normal dendritic melanocytes in the basal epithelial layer. (B) RGP in situ melanoma. Melanoma cells have migrated into the upper epidermis (pagetoid spread) and are scattered among epithelial cells in a "buckshot" manner. Cells have not penetrated the epidermal basement membrane. Melanoma cells show cytologic atypia, with large abundant cytoplasm and increased overall size compared with normal melanocytes. Nuclei are enlarged and hyperchromatic. Commonly, there is more junctional melanocytic hyperplasia (nests of tumor cells at the basement membrane zone) in RGP melanoma than portrayed in the histologic example. (C) VGP malignant melanoma. Melanoma cells show pagetoid spread and have penetrated the dermal-epidermal junction. Melanoma cells show cytologic atypia. Cells in the dermis cluster or individually invade. Magnification, ×20. Scale bar: 20 µm.

Epidemiology

Risk factors that contributes to the genesis of melanoma are divided in two groups: environmental and intrinsic factors (14;15). The most relevant environmental factor is intermittent sun exposure, while intrinsic factors are represented by individual's family history and inherited genotype.

Epidemiologic studies suggest that exposure to ultraviolet radiation (UVA and UVB) (16) is one of the major contributors to the development of melanoma. UV radiation promotes damages to the skin by having direct mutagenic effects on DNA, by reducing cutaneous immune defenses, and by inducing the formation of reactive oxygen species of melanin that cause DNA damage and suppress apoptosis (17). Solar radiation also increases the local production of growth factors, such as stem cell factor, fibroblast growth factor and transforming growth factor α. Resulting signals are traduced via the Ras/RAF pathway, ultimately triggering the transcription of genes involved in cellular proliferation and migration *(Figure 2)* (6). Possible significant elements in determining the risk of developing melanoma include the intensity and duration of sun exposure, the age at which sun exposure occurs, and the degree of skin pigmentation. Individuals with blistering or peeling sunburns (especially in the first two decades of life) have a significantly greater risk for developing melanoma in their adulthood. Fair and red-headed people, persons with multiple atypical nevi or dysplastic nevi and persons born with giant congenital melanocytic nevi have an increased risk (18). Melanoma is more common on the back in men and on legs in women (areas of intermittent sun exposure). The risk appears to be strongly influenced by socio-economic conditions rather than indoor versus outdoor occupations; it is more common in professional and administrative workers than unskilled workers (19;20).

Intrinsic factors include mutations in or total loss of selected Tumor Suppressor Genes (TSGs). Familial melanoma patients tend to have an earlier age at first melanoma diagnosis, thinner tumors, and a higher frequency of multiple primary melanomas (MPMs) than patients who have sporadic melanoma (21). A family history of melanoma greatly increases a person's risk because of mutations in three high-penetrance and one low-penetrance melanoma predisposing genes. The best-characterized high-penetrance gene locus is on chromosome 9p21, CDKN2A (cyclin-dependent kinase inhibitor 2A). By using different first exons, 1a and 1b, respectively, CDKN2A encodes two unrelated proteins, p16INK4A (commonly referred to as p16) and p14ARF (alternative reading frame), which are both tumor suppressors involved in cell cycle regulation. This genetic locus is frequently alterated in melanoma and germline mutations, in one or both of these gene products, are inherited in certain melanoma-prone families (22). When defective, p16 is unable to inactivate CDK4 and CDK6, which phosphorylate Rb, releasing the transcription factor E2F and leading to cell cycle progression. Mutations in p14ARF allows the release of hdm2, resulting in degradation of p53 gene, central to protection against DNA damage by triggering cell cycle arrest or apoptotic cell death *(Figure 2)* (6). Relative risk of carrying a CDKN2A mutation for melanoma patients was demonstrated to significantly increase with the presence of familial occurrence of melanoma, MPMs, and early age of onset. In Italy, the prevalence of CDKN2A germline mutations may vary widely among patients with different geographical origins: its frequency is higher in patients from Northern Italy in comparison to those from Southern Italy (23). The third high penetrance melanoma predisposition gene, CDK4 (cyclin dependent kinase 4), is located on 12q13. Mutations in these gene products are inherited in an autosomal dominant fashion and confer ‘extremely high risk’ (24).

Conversely, MC1R, the melanocortin receptor gene, located on 16q24.3, is a low-penetrance gene. This gene encodes the G-protein coupled receptor for α-melanocyte stimulating hormone (α-MSH) (25). Binding of α-MSH to the receptor increases the expression of enzymes involved in the production of melanin in melanocytes. Light-skinned people often carry germ-line polimorphisms in the MCR1 gene, that reduces the activity of the receptor. (17). MC1R variants seem to increase melanoma risk in families with CDKN2A mutations. Recently, a synergistic relationship between germline MC1R variants and somatic v-raf-murine sarcoma viral oncogene homolog B1 (BRAF) mutations has been suggested, whereby MC1R variant genotypes seem to confer a significantly increased risk of developing BRAF-mutant melanoma in skin not damaged by sunlight. It has been hypothesized that intermittent sun exposure may indirectly induce BRAF mutations through the impairment of MC1R and an increased production of free radicals (23). Worldwide it has been estimated that approximately 20%-40% of kindreds that have familial melanoma are related to germline CDKN2A mutations (21), and only 1%-2% of known melanoma family carry mutations in CDK4 or ARF-only genes (24). The incidence of melanoma has increased in the recent years, but it is not clear to what extent changes in behavior, in the environment, or in early detection are involved (26;27).

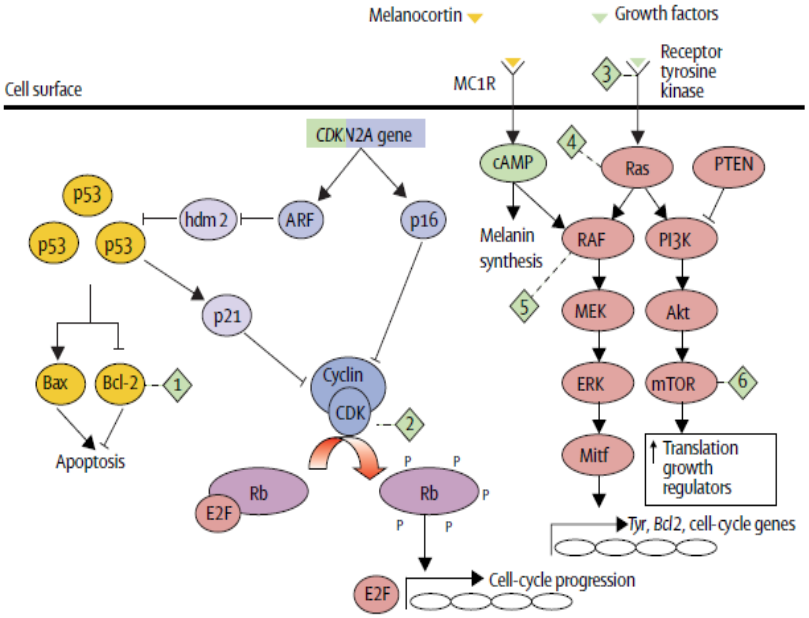

Figure 2: **Selected prominent molecular pathways in the genesis and regulation of melanoma (taken from (6)).**
Targets undergoing experimental inhibition in melanoma therapy (green diamonds): (1) antisense oligonucleotide to Bcl-2 (oblimersen); (2) CDK inhibitors (flavopiridol); (3) receptor tyrosine kinase inhibitors; (4) farnesyl transferase inhibitors; (5) RAF inhibitors (BAY 43-9006); (6) mTOR inhibitors. Barred lines indicate inhibition. SCF=stem cell factor. FGF=fibroblast growth factor. Ras=rous avian sarcoma homologue. BRAF=v-raf murine sarcoma viral oncogene homologue B1. MEK=mitogen-activated protein kinase kinase (MAP2K). ERK=extracellular signal-regulated kinase. MAPK=mitogen-activated protein kinase. Mitf=microphthalmia transcription factor. PI3K=phosphatidylinositol-3 kinase. Akt=murine v-akt oncogene homologue. mTOR=mammalian target of rapamycin. CDKN2A=cyclin dependent kinase inhibitor-2A. CDK=cyclin dependent kinase. Rb=retinoblastoma protein. p16=16.000 MW protein. ARF (p14ARF)=14.000 MW alternate reading frame protein. cAMP=cyclic AMP. Bcl=B-cell lymphoma derived protein. hdm=human double minute chromosome-associated protein. E2F: E2F cell cycle regulated transcription factor. MSH=melanocyte stimulating hormone (melanocortin). MC1R=melanocortin-1 receptor.

Clinical classification

Historically, malignant melanoma was classified first by Wallace Clark and coworkers (28), and later by Richard Reed, who added another type of melanoma (29). Nowadays the classification comprises: *Superficial Spreading Melanoma* (SSM), that can occur at any site and at any age (relative incidence: 50-70%); *Nodular Melanoma* (NM), that has no radial growth phase and could be nodular, polypoid, or peduncolated (relative incidence: 15%-

35%); *Lentigo Maligna Melanoma* (LMM), that occurs on the sun-exposed skin, face and upper estremities of elderly patients (relative incidence: 5-15%), *Acral Lentiginous Melanoma* (ALM), that is common on palmar, plantar and ungula skin of Black and Japanese people (relative incidence: 5-10%), *Desmoplastic melanoma* (DM) (uncommon), miscellaneous group (rare) (13).

Prognostic factors

Adverse prognostic factors in melanoma comprises: pathological, clinical and other factors, including genetic alteration.

The first group includes increase of tumor thickness in millimeters (Breslow's depth), presence of ulceration, depth related to skin structures (Clark level), absent or non-brisk tumor infiltrating lymphocytes, presence of microscopic satellites (30), mitotic rate, lymphovascular invasion, angiotropism, neurotropism, histologic signs of tumor regression, tumor volume, cell type, local recurrence, presence of vertical growth phase and an elevated concentration of LDH in serum (for stage IV melanoma patients) (31).

Clinical adverse factors include increasing age, male sex, tumor location on the trunk, head or neck, and regional or distant metastasis (13). When there is distant metastasis, melanoma is generally considered incurable. The five year survival rate is less than 10% (7). Most patients with metastatic disease confined to the skin, subcutis, lymph nodes and lungs have a better prognosis (median survival: 12 months), whereas metastases to brain, bone and liver are associated with a worse prognosis (median survival: 4-6 months) (6). In some cases, however, patients may live many months or even years with metastatic melanoma. Treatment is palliative, focusing on life-extension and quality of life.

Development and progression

Melanoma has been hypothesized to result from the transformation of a cutaneous melanocyte through nevus and dysplastic nevus stages to *in situ* and eventually invasive melanoma. The traditional model suggests that melanomas initially develop in the epidermis and then invade the dermis. A recent research reveals that melanocytes can be transformed with oncogenes and acquire malignant/invasive characteristics (32). Melanoma arises due to accumulation of mutation in genes critical for cell proliferation, differentiation, and cell death. As additional mutations accumulate, melanoma cells acquire the ability to initiate and sustain angiogenesis, invade across tissue planes, and metastasize. Moreover, melanomas are largely growth factor-independent, presumably due to mutations, such as NRAS and BRAF activation or phosphatase and tensin homolog (PTEN) inactivation, that promote proliferation and survival (33) or to autocrine signaling loops deriving from activation of pathways leading to the production of their own growth factors. This independence from homeostatic pathways allows the tumors to thrive in non-epidermal environments (34). The initiation and progression of melanoma are accompanied by a series of histologic changes, which are described in the Clark model. The five distinct stages in this model include *(Figure 1 and 3)*:

1) nevus, a benign lesion characterized by an increased number of nested melanocytes;

2) dysplastic nevus, characterized by random and discontiguous cytologic atypia;

3) radial-growth phase (RGP) melanoma, where the cells acquire the ability to proliferate intraepidermally;

4) vertical-growth phase (VGP) melanoma, where the cells acquire the ability to penetrate through the basement membrane into the underlying dermis and subcutaneous tissue;

5) metastatic melanoma, characterized by the spread of cells to other areas of the skin and other organs.

The most critical event along this progression line is probably the transition from RGP to VGP, which involves the escape from keratinocyte-mediated growth control, consistent with tumor thickness being one of the strongest predictors of metastatic disease and adverse clinical outcome (35).

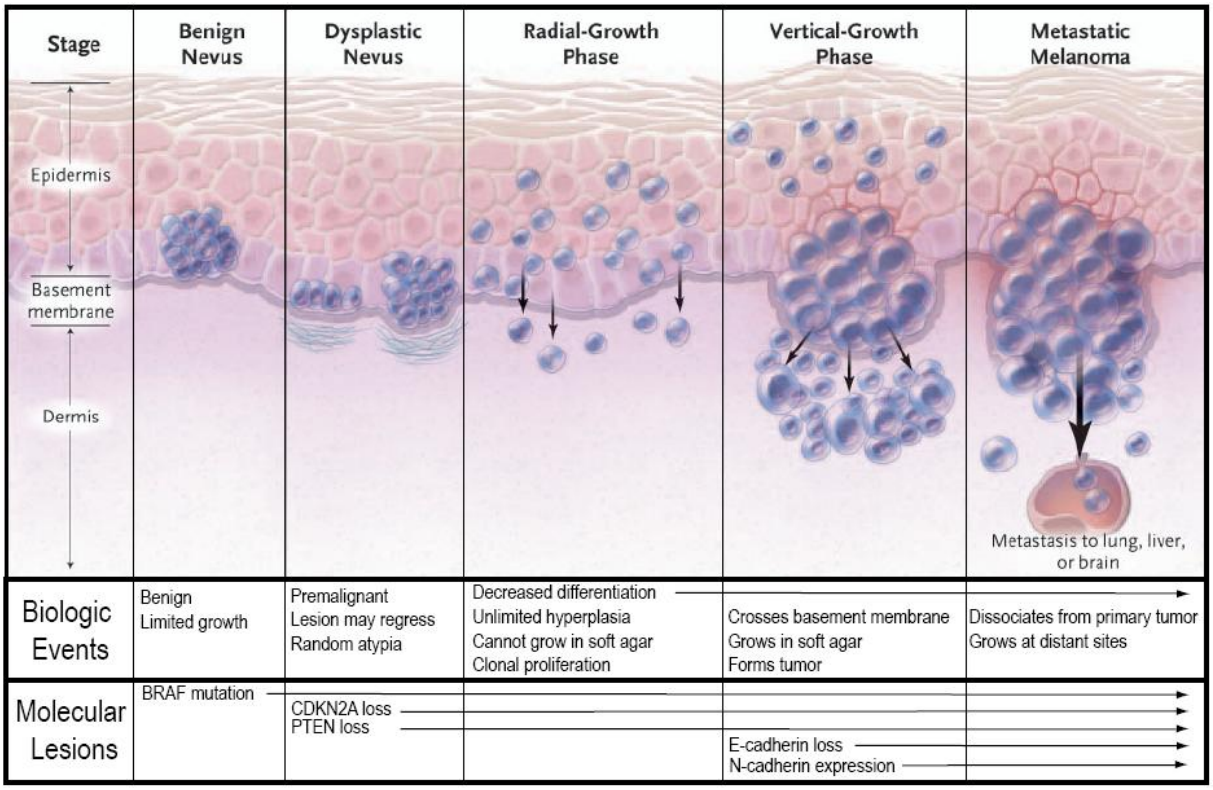

***Figure 3*: Biologic Events and Molecular Changes in the Progression of Melanoma (adapted from (17)).**
At the stage of the benign nevus, *BRAF* mutation and activation of the mitogen-activated protein kinase (MAPK) pathway occur. The cytologic atypia in dysplastic nevi reflect lesions within the cyclin-dependent kinase inhibitor 2A (CDKN2A) and phosphatase and tensin homologue (PTEN) pathways. The vertical-growth phase and metastatic melanoma are notable for striking changes in the control of cell adhesion. Other changes include the loss of E-cadherin and increased expression of Ncadherin.

The pathogenetic mechanisms underlying melanoma development are still largely unknown, nevertheless, several metabolic pathways (e.g. mitogen-activated protein kinase (MAPK), PI3K–AKT, β-catenin, senescence) have been shown to be targeted by classical

genetic alterations such as mutation, deletion and amplification, and to play an important role in melanoma biology. The activation of MAPK pathway through mutations of BRAF appears to be the most common. Notably, BRAF mutations have been found in up to 82% of benign nevi, thus suggesting that activation of the MAPK pathway is a necessary, but not sufficient, event for melanoma development (36;37). Although the activation of the MAPK signaling has been recognized as a critical event, Khavari and colleagues have demonstrated that activation of BRAF–MAPK requires concomitant PI3K–AKT pathway alteration to affect melanoma development (1;32). Consistent with this data, over 60% of human melanomas exhibit activated AKT (38), and mutational inactivation and/or deletion of the PI3K negative regulator PTEN occurs in 30–40% of melanoma cell lines (39;40).

BRAF mutations

Since its discovery through a genome-wide cancer resequencing effort (41), mutations in BRAF have been detected in a variety of tumor types, with the highest incidence in melanoma (ranging from 27% to 70%) (15;42;43). BRAF is a component of the MAP kinase pathway, a signal transduction pathway that transmits mitogenic signals from activated cell surface growth factor receptors, under normal physiologic conditions, through the binding of BRAF with its substrate MAP kinase kinase (MEK) (44). Activating BRAF mutations are the most frequent (60%–80%) somatic genetic event in human melanoma (41;45). 80% of these mutations are found at exon 15, at a single amino acid residue, usually a substitution for valine by glutamic acid, V599E (now referred to as V600E). This mutation costitutively activates BRAF, provoking increased signaling through the MAP kinase pathway and causing activation of the Brn-3 transcription factor, which leads to unregulated cell

growth (46-49). Surprisingly, BRAF oncogenic mutation occurs at a similar high frequency (62–72%) in benign melanocytic nevi as well as in VGP, metastatic melanoma, and melanoma cell lines (45;50). In striking contrast, Dong et colleagues found BRAF lesions in only 10% of the earliest stage or RGP melanomas and concluded that BRAF mutations could be involved in the initiation of the great majority of melanomas but instead might be associated with disease progression (51).

Metastatic melanoma

Metastatic melanoma is an incurable disease with high mortality rate. Patients with metastatic disease have an average survival of <1 year. This high mortality rate is largely the result of the resistance to chemotherapy and radiotherapy (52). Various effectors, which up- or downregulate different molecular pathways, appear to be involved in progression of normal melanocyte to metastatic malignant cells (53). Numerous studies using tissue specimens, cell lines, and xenografts to discover the mechanism(s) behind this transformation, invasiveness, and metastasis are in progress. Alterations of cell cycle proteins (e.g., cyclin D1, pRb, and p16) have a role in transformation and progression in melanocytic tumors. It has been shown that progressive loss of p16 can result in transformation of benign nevi to melanoma and to metastatic melanoma. Progressive increase in expression of cyclin D1 and pRb is associated with progression to melanoma cells; however, cyclin D1 and pRb show relative decrease in thick melanoma and metastatic melanoma (54). Protein Kinase C (PKC) mediates signals for cell growth and is a target of tumor-promoting phorbol esters in malignant transformation (55). Higher expression of protease-activated receptor-1 (PAR-1) is seen in melanoma cell lines and tissue specimens.

Upregulation of PAR-1 mediates high levels of gap junctional intracellular communication molecule connexin (Cx-43) expression, a molecule involved in tumor cell diapedesis and attachment to endothelial cells (56). Type I collagenase and PAR-1 activating functions of matrix metalloproteinase-1 (MMP-1) are required for melanoma progression. Highly expressed MMP-1 is suggested to be involved in progression of noninvasive melanoma to invasive vertical growth phase by degrading type I collagen of skin (57). Downregulation of E-cadherin and upregulation of N-cadherin may be seen in melanoma cells *(Figure 3)*. Such shift of cadherin profile may have a role in uncontrolled proliferation, invasion, and migration (58). A recent study, based on the fact that angiogenesis is one of the factors required for progression and melanoma metastasis, demonstrated that vascular endothelial growth factor (VEGF) and its receptors (VEGF-R1, VEGF-R2, and VEGF-R3) are higher in melanomas and advanced melanomas than in benign nevi. VEGF-R2 shows higher expression in metastatic melanomas than in primary melanoma (59). Using immunoistochemistry on human tissue, it has been shown that there is significantly higher cortactin (a multidomain actin-binding protein important for the function of cytoskeleton) expression in melanomas than in nevi and higher expression in metastatic melanoma than in invasive primary melanomas (60). Moreover, major histocompatibility complex (MHC) molecule overexpression in earlier stages of melanoma and downregulation in metastatic malignant melanoma have been observed (61). Melanoma chondroitin sulfate proteoglycan (MCSP) facilitates the growth, motility, and invasiveness of tumor cells. MCSP expression is associated with increased expression of c-Met, resulting in growth and motility of melanoma cell lines (62).

MELANOMA THERAPEUTICS

Treatment of advanced malignant melanoma is generally performed by a multidisciplinary approach.

Surgery and radiotherapy

Surgery is the first choice therapy for localized melanoma. Surgical treatment of melanoma employs complete surgical excision with adequate margins and assessment for the presence of detectable metastatic disease. Local recurrences and in-transit metastases are treated by surgical excision. Surgery can also be valuable for patients with evident metastatic disease in regional lymph nodes, or with surgically resectable disease in visceral sites. (6). Melanoma has long been considered relatively resistant to radiotherapy. However, surgical resection and radiation therapy also offer palliation in specific circumstances and radiotherapy remains the treatment of choice for treatment of multiple cerebral metastases, and of non-resectable bone metastases, with some success in controlling symptoms (63).

Chemotherapy

Among traditional chemotherapeutic agents, only dacarbazine (DTIC) is Food and Drug Administration (FDA) approved for the treatment of advanced melanoma (64). Combination chemotherapy regimens (such as cisplatin, vinblastine, and dacarbazine) are often employed, though no clinical trials have demonstrated a survival advantage for combination therapy over optimal single-agent therapy (64). Temozolomide is an oral alkylating agent approved for the treatment of malignant gliomas with activity comparable to that of dacarbazine in melanoma. While there is not yet supportive clinical trial data,

temozolomide has been substituted in many combination regimens and is under investigation in combination with radiation therapy for patients with melanoma metastatic to the brain (65). Nevertheless, these therapies are associated with response rates of only 10 to 20% and a small percentage of complete responses; neither is thought to improve overall survival (66). In randomized trials, the median survival among patients treated with dacarbazine was less than 8 months (67).

Targeted therapy

Targeted therapy consists of a novel group of antibodies and small-molecule kinase inhibitors that specifically target proteins that are involved in growth signalling pathways in cancer cells. The identification of somatic mutations in BRAF in the majority of melanoma leads to the development of various targeted-therapies for this disease. Studies with BRAF inhibitors are currently ongoing but, despite good responses, patients with BRAF mutant tumors eventually develop resistance and disease progression. The first RAF kinase inhibitor entering early clinical trials was the oral diphenyl urea, sorafenib (Bay 43-9006) *(Figure 2)*. It has minimum activity on its own, but in combination with the cytotoxic drugs carboplatin and paclitaxel it gave partial response in phase II trials (68). Further research led to the development of second generation more selective RAF inhibitors, which are currently in clinical trials. XL281 (famotidine) is an orally administered inhibitor of the wt BRAF (IC50 4.5 nM), CRAF (IC50 2.5 nM) and mutant $BRAF^{V600E}$ (IC50 6nM) kinases and demonstrated potent inhibitory effect in human melanoma xenograft models (69). At the moment, the best two BRAF inhibitors tested in clinical studies are: PLX4032 and GSK2118436 *(Figure 4)*, which are highly selective and provoke effects on proliferation and apoptosis only in cells harboring

BRAF mutations (44). In a phase II clinical trial for GSK2118436 has been reported an overall response rate of 77% and responses were seen in many sites, including brain (23). In a phase I/II study with PLX4032, in patients with tumors that carry the $BRAF^{V600E}$ mutation, a complete or partial tumor regression was observed in the majority of patients (70). Noteworthy, results from a very recent phase III clinical trial, comparing PLX4032 (Vemurafenib) with dacarbazine, in 675 metastatic melanoma patients, showed that Vemurafenib improved the rates of response and of both progression-free and overall survival, as compared to dacarbazine alone, in patients with metastatic melanoma carrying BRAFV600E mutation (71). Based on these evidences, the prospect of using a number of targeted inhibitors in combination with chemotherapy provides some optimism.

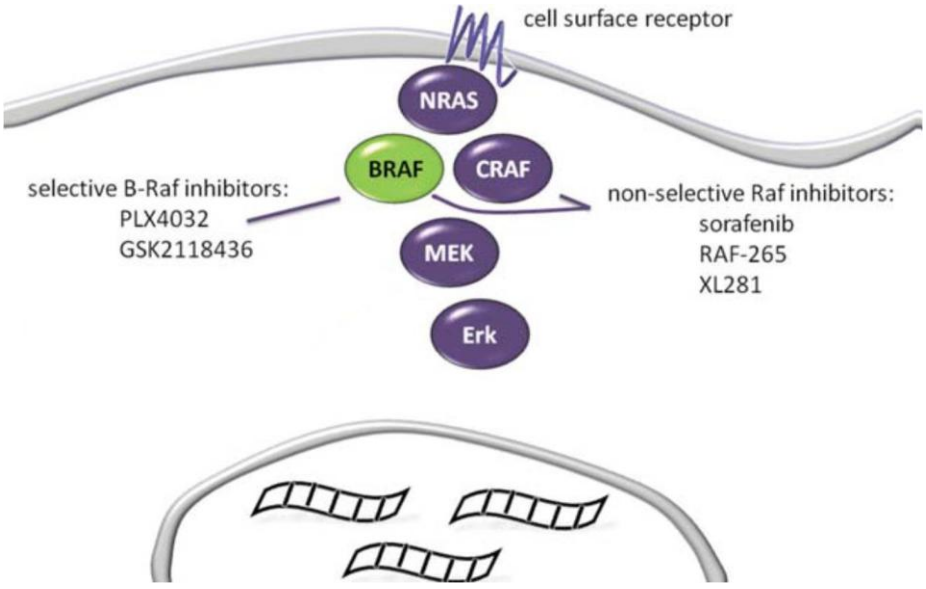

Figure 4: **Inhibitors of the mitogenactivated protein (MAP) kinase pathway that currently are in clinical development (adapted from (44)).**
Nonselective Raf inhibitors have potency for v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1) that is greater than or equal to their potency for v-raf murine sarcoma viral oncogene homolog B1 (BRAF). MEK indicates MAP kinase kinase; NRAS, neuroblastoma RAS viral oncogene homolog; CRAF, proto-oncogene c-RAF; Erk, extracellular signal-regulated kinase.

Immunotherapy

New and innovative approaches and more effective therapies for advanced melanoma are needed. One approach that holds promise is immunotherapy.

Cancer immunotherapy targets the induction or augmentation of anticancer immune-response. The main premise is stimulating the patient's immune system to attack the malignant tumor cells that are responsible for the disease. This can be done either through the immunization of the patient by therapeutic vaccines, in which case the patient's own immune system is trained to recognize tumor cells as targets to be destroyed, or through the administration of therapeutic antibodies as drugs, in which case the patient's immune system is recruited to destroy tumor cells by the therapeutic antibodies. The phenotypic characterization of neoplastic cells and the definition of their functional interactions with host's immune system have provided a strong rationale for designing and applying in the clinic new strategies of cancer immunotherapy. A well known therapy in melanoma is based on the use of biologic agents that stimulate the immune system.

A large, randomized multicenter study in high risk melanoma patients performed by the Eastern Cooperative Oncology Group (ECOG) showed significant improvements in relapse-free and overall survival with postoperative adjuvant IFN-α-2b therapy, compared with standard observation (ECOG 1684) (72). The outcome of this study led to FDA approval of IFN-α-2b for treatment of melanoma. This study was performed on patients with deep primary tumors without lymph node involvement and node-positive melanomas. In other studies, little antitumor activity has been demonstrated in IFN-α-2b–treated metastatic stage IV melanoma. Furthermore, while a large follow-up study (Intergroup E1690) (72) confirmed relapse-free survival with high-dose IFN-α-2b, it did not show an increase in

overall survival. Toxicities of high-dose IFN include constitutional (flulike) symptoms and neuropsychiatric (depression, suicidal intention), hematologic, and hepatic effects (72). Other studies of low-dose IFN versus either observation or high-dose IFN have not shown a statistically significant increase in overall survival (72). Interleukin-2 (IL-2) has been used for the treatment of melanoma with modest success for several decades. In recent trials, IL-2 has been combined with other cytokines, such as granulocyte, macrophage, colony stimulating factor (GM-CSF). However, clinical response rates were not increased by adding GM-CSF to IL-2. There are many acute toxicities associated with Interleukin therapy and extremely close monitoring is essential for safe administration (73).

In addition, a positive Phase III vaccination study was presented in metastatic melanoma, in which a melanocyte protein PMEL (also known as gp100) vaccine that was combined with high-dose IL‑2 was compared with high-dose IL‑2 alone; a statistically significant benefit in progression-free survival and a borderline significant benefit in overall survival was observed for the combination therapy (74). A recent approach to manipulate immune responses against neoplastic cells involves targeting by monoclonal antibodies (mAb) of cytotoxic T-lymphocyte antigen-4 (CTLA4), a key immune checkpoint molecule from the CD28:B7 family. Ipilimumab and Tremelimumab represent the prototypes of this growing class immunostimulating mAb. Both mAb were extensively tested in metastatic melanoma with highly promising results. In this regards, durable responses and disease stabilization have been demonstrated for ipilimumab in phase II clinical trials in advanced melanoma, with 3-year survival rates ranging from 23% to 34% (75-77). In the phase III, randomized, controlled trial MDX010-20, ipilimumab demonstrated a statistically significant improvement in overall survival in patients with previously treated, advanced melanoma

(78). Ipilimumab clinical activity appears to be independent of a number of negative prognostic factors (79), and response to treatment may follow a period of apparent disease progression (78;80). Noteworthy, the therapeutic activity of Ipilimumab was most recently studied also in a phase III study, in combination with dacarbazine, in patients with previously untreated metastatic melanoma (81). A significant improvement in overall survival was observed in the group receiving Ipilimumab plus dacarbazine, than in group receiving dacarbazine plus placebo (81).

Other mechanisms of stimulating the immune system are currently under investigation in clinical trials and include active specific immunotherapeutic approaches, also defined as therapeutic vaccination. In particular, the identification of tumor-associated antigens (TAA) recognized by antibodies and/or cytotoxic T lymphocytes (CTL) (82), together with progresses in the knowledge on the molecular mechanisms regulating tumor-host interactions (16), prompted the development of different pre-clinical and clinical studies of specific immunotherapy. Among others, the cancer vaccines mostly utilized in the clinical setting are based on whole cancer cells, anti-idiotypic antibodies, DNA, recombinant proteins, peptides, autologous heat shock proteins, and peptide-/tumor-pulsed dendritic cells (82-84).

TAA-based immunotherapy is emerging as a promising and additional therapeutic option that is rapidly integrating in the overall treatment of cancer patients.

According to their pattern of expression in neoplastic and normal tissues, TAA thus far identified can be classified in 9 groups including:

a) *Cancer Testis antigens (CTA)*, expressed in tumors of various histological origin but not in normal tissues other than testis and placenta (MAGE, SSX, NY-ESO families: GAGE,PAGE, XAGE superfamilies);

b) *Differentiation antigens*, expressed in normal and neoplastic cells of the same lineage (e.g., Melan-A/MART-1, tyrosinase, gp-100);

c) *Over-expressed antigens*, expressed at higher levels in malignant compared to benign tissues (e.g., HER2/neu, EpCAM);

d) *Tumor-specific unique antigens*, arising from point mutations of normal genes, like β-catenin and CDK-4, whose molecular changes often accompany neoplastic transformation or progression;

e) *Fusion proteins*, deriving from translocation of chromosomes, resulting in the fusion of distant genes, and that characterize each type of disease (e.g., bcr-abl and pml-RARβ in chronic myelogenous leukemia and acute promyelocytic leukemia, respectively);

f) *Oncofetal antigens*, expressed in fetal, neoplastic and specific benign adult tissues in quantitatively varying levels, often used as diagnostic or prognostic markers for malignancies (e.g., α-1-fetoprotein and carcinoembryonic antigen)

g) *Glycolipids and glycoproteins* (i.e., high molecular weight-melanoma associated antigen (HMW-TAA), gangliosides and mucins), expressed at high levels and/or with an aberrant glycosylation status in malignancies of different histotype;

h) *Splice variants of known genes*, identified by SEREX and found to be immunogenic in cancer patients (e.g., NY-CO-37/38);

i) *Cancer-related autoantigens*, also identified by SEREX, are expressed ubiquitously and at a similar level in healthy and malignant tissues (e.g., CEBPγ). The encoding genes are not

altered in tumor samples; however, they elicit antibody responses in cancer patients but not in healthy individuals (85).

CTA

CTA are a large family of tumor-associated antigens expressed in human tumors of different histological origin, but not in normal tissues except for testis and placenta. This tumor-restricted pattern of expression, together with their restricted presentation by the HLA machinery on tumor cell surface (16;86;87), their shared expression by neoplasia of different histotype (88), and their ability to generate a spontaneous humoral and cellular immune response (82;89), identified CTA as ideal targets for tumor-specific immunotherapeutic approaches. Further support to the clinical important role of CTA, in cancer immunotherapy, derives from the correlation between the presence of anti-CTA humoral immune response and a more favorable clinical course of cm patients treated with ipilimumab (90). Among different CTA, recombinant proteins or HLA class I and HLA class II-restricted antigenic peptides of MAGE, NY-ESO and SSX gene families and GAGE/PAGE/XAGE super-families have been utilized for cancer vaccination in clinical trials, resumed in *Table 1* (91). The clinical results emerged from the initial clinical trials indicated that CTA-based vaccination is a safe therapeutic procedure and that complete responses, as well as prolonged disease free survival, were observed in different solid tumors (82;84;92-94). Even if clinical results are encouraging, several issues are still to be optimized to improve the efficacy of CTA-based immunotherapy. In fact, clinically effective CTA-based vaccination of cancer patients faces two major obstacles: 1) the expression of a given therapeutic CTA in a limited percentage of cancer patients, (95) and 2) the highly heterogeneous intratumoral

distribution of CTA within distinct neoplastic lesions (95). The magnitude of the latter aspect appears evident when considering immunohistochemical data reporting that the majority of CTA-positive neoplastic lesions show CTA staining in less than 50% of neoplastic cells (96). This feature of CTA poses a major obstacle for the clinical success of CTA-based vaccination since CTA-negative cancer cells within CTA-positive neoplastic lesions should be able to circumvent vaccination-induced CTA-specific immune recognition, allowing the outgrowth of a CTA-negative malignant lesion.

Table 1: **Ongoing clinical trials with CTA in melanoma (adapted from (91)).**

CTA	Sponsor	Phase	Combination	Identifier
MAGE-A3 ([a]GSK2132231A)	Glaxo Smith Kline	I	Dacarbazine	NCT00849875
MAGE-A10, NY-ESO-1, Melan-A	Ludwig Institute for Cancer Research	I	a) Montanide + Melan-A analogue peptide b) Montanide + Melan-A analog peptide + NY-ESO-1 analog peptide + MAGE-A10 peptide c) Montanide + CpG-7909/PF 3512676+Melan-A analog peptide + NY-ESO-1 analog peptide + MAGE-A10 peptide d) Montanide + CpG-7909/PF-3512676 + Melan-A native and analog peptides + NY-ESO-1 long peptide + MAGE-A10 peptide e) Montanide + CpG-7909/PF-3512676 + Melan-A native and analog peptides + NY-ESO-1 long peptide + MAGE-A10 peptide + low dose IL-2	NCT00112242
MART-1, [b]tyr, gp100, MAGE-A3	Duke University	I	Proteasome siRNA and tumor antigen RNA-transfected dendritic cells	NCT00672542
NY-ESO-1/gp100/MART-1	H. Lee Moffitt Cancer Center and Research Institute	I	Poly IC:LC, emulsified with Montanide ISA 51 with escalating doses of CP 870893	NCT01008527
MART-1 /gp100/NY-ESO-1	H. Lee Moffitt Cancer Center and Research Institute	I	MART-1:26-35 peptide vaccine, NY-ESO-1 peptide vaccine, anti-PD-1 human monoclonal antibody MDX-1106, gp100:209-217 peptide vaccine, gp100:280-288 peptide vaccine, Montanide ISA 51 VG	NCT01176461
MAGE-A3, Melan-A, survivin	Dermatologische Klinik MIT Poliklinik-Universitaetsklinikum Erlangen	I, II	KLH, therapeutic autologous dendritic cells	NCT00074230
MAGE-A3, NA17.A2	Cliniques universitaires Saint-Luc-Université Catholique de Louvain	I, II	Vaccine MAGE-3.A1 peptide, or the NA17.A2 peptide + IL-2, IFN-α and GM-CSF, imiquimod.	NCT01191034
MAGE-A1 /MAGE-A3, tyr/MART-1/gp100	Maria Sklodowska-Curie Memorial Cancer Center, Institute of Oncology	I, II	a) HLA-A1-binding MAGE-A1/MAGE-A3 multipeptide-pulsed autologous dendritic cell vaccine b) HLA-A2-binding tyr/MART-1/gp100 multipeptide-pulsed autologous dendritic cell vaccine c) autologous melanoma lysate-pulsed autologous dendritic cell vaccine d) autologous melanoma lysate/tracer antigen KLH-pulsed autologous dendritic cell vaccine e) dendritic cell-idiotype-KLH	NCT01082198
MART-1/gp100/tyr/NY-ESO-1	H. Lee Moffitt Cancer Center and Research Institute	I, II	Dendritic cell vaccine therapy, therapeutic autologous lymphocytes, fludarabine phosphate	NCT00313508
MAGE-A3 (GSK1203486A)	Glaxo Smith Kline	II		NCT00706238
gp100, MAGE-A3	M.D. Anderson Cancer Center	II	R848 gel	NCT00960752
MAGE-A3 (GSK2132231A)	Glaxo Smith Kline	III	Placebo	NCT00796445

Regulation of CTA expression

Based on the therapeutic limitations posed by their heterogeneous expression, significant efforts have been made to clarify the molecular mechanism(s) regulating CTA expression in neoplastic cells and the factors that influence their distribution in tumor tissues. The extent of methylation of selected CpG dinucleotides in CTA promoters appeared to be the main restricting factor for their expression. In fact, studies performed at the promoter level in neoplastic cells and tissues from melanoma, sarcoma, lung carcinoma, and gastric carcinoma patients (97) (98) (99) (100) revealed a direct correlation between the expression of different members of the MAGE family and the presence of hypomethylated CpG dinucleotides in their promoters. This correlation has been recently confirmed at the single cell level, demonstrating that the intratumoral heterogeneity of MAGE-A3 expression derives from the methylation status of specific CpG dinucleotides within the MAGE-A3 promoter (101). Two major experimental evidences further support the key role of the methylation status of CTA promoters in determining their pattern of expression in neoplastic tissues: 1) the availability of transcription factors required for CTA expression in CTA-negative neoplastic as well as in normal cells (97), and 2) the demonstration that in vitro methylation of CTA promoters is sufficient per se to block their transcriptional activity in cancer cells constitutively expressing high levels of CTA (98)

HLA antigens

Despite the adequate expression of therapeutic TAA on neoplastic cells, their efficient recognition and targeting by TAA-specific cytotoxic T lymphocytes (CTL) relies on the presence and levels of HLA class I antigen expression on the cell surface. Different

abnormalities in the expression of HLA class I antigens have been described, including total HLA class I antigen loss, total HLA class I antigen downregulation, HLA class I locus-specific loss or downregulation (102), and selective loss or downregulation of distinct HLA class I allospecificities (103).

Regulatory and structural defects in genes involved in the biosynthesis of the peptide/β2-microglobulin/HLA class I heavy-chain complex appear to be the most frequent causes for the impaired expression of HLA class I antigens in cancer cells (103). However, recent evidence shows that epigenetic phenomena contribute to the generation of an altered pattern of HLA class I antigen expression in cancer cells. In fact, hypermethylation of CpG islands located in HLA-A, -B, and -C promoters correlated with downregulated mRNA and protein expression of HLA class I antigens in human esophageal squamous cell carcinomas, representing the major mechanism of transcriptional inactivation of HLA class I genes in this neoplasm (104). DNA hypermethylation also was found to be responsible for the downregulated expression of HLA class I antigens in metastatic melanoma (105), while its role in generating total HLA class I antigen loss phenotypes in malignant melanocytic lesions is debated (106).

Immunomodulatory activity of epigenetic drugs

Epigenetically silenced genes can be reactivated using specific inhibitors of the enzymes that establish/maintain epigenetic patterns. Among these, DNMT1 exerts a major role in maintaining DNA methylation patterns throughout cell replication (107), and the inhibition of DNMT1 activity leads to the synthesis of hypomethylated DNA (108). Among inhibitors of DNMT1, one of the most widely applied is 5-aza-2'-deoxycytidine (5AZA-CdR),

which is an aza-analogue of cytosine that is incorporated into DNA during the S-phase (108)of the cell cycle and covalently traps DNMT1, resulting in the synthesis of heritably demethylated DNA (108).

The exclusive role of DNA methylation in controlling their expression makes CTA ideal targets for 5-AZA-CdR–based epigenetic treatments aimed to induce or potentiate CTA-restricted immune recognition of cancer cells. In fact, 5-AZACdR proved effective in both inducing (96) (97) (98) (109)CTA expression in neoplastic cells, in a lineage-independent fashion. More importantly, 5-AZA-CdR was able to establish a homogeneous level of CTA expression among single cell clones derived from a primary culture of metastatic melanoma cells, which constitutively expressed heterogeneous levels of MAGE-A3. These findings demonstrated the ability of 5-AZA-CdR to homogenize CTA expression within neoplastic lesions to be targeted by CTA-specific vaccination (101). The modifications of CTA expression induced by 5-AZA-CdR are considerably durable; in fact, drug-induced de novo expression of CTA was still detectable in cancer cells of different histotype cultured for 60 days after the end of 5-AZA-CdR treatment (105). Furthermore, when examining the effect at single cell level, CTA expression achieved through 5-AZA-CdR-induced promoter demethylation was found to become a constitutive feature of the cell, being inherited indefinitely throughout cell replications (110). This evidence is in accordance with the physiologic heritability of DNA methylation patterns in somatic cells (108). The imunomodulatory activity of DNA hypomethylating agents (DHA) is not restricted to the induction/upregulation of CTA but also influences several components involved in the presentation of TAA to the immune system, and in the recognition and lysis of TAA-positive neoplastic cells by CTL. In this respect, a key role in both presentation and sensitization to CTL lysis is played by HLA class I

antigens, which are frequently downregulated in cancer cells (103). 5-AZA-CdR was demonstrated to be an extremely efficient and reliable drug for upregulating HLA class I antigens and HLA-A1 and -A2 allospecificities in a large panel of metastatic melanoma cells (105). This drug-mediated upregulation of HLA class I antigens was persistent as compared to the short-lived activity of interferon- γ: after 5-AZA-CdR withdrawal, 32 days were required for HLA class I antigens expression to return to the baseline levels, whereas 12 days were sufficient for the reversal of interferon-γ effects (105).

The expression of accessory/costimulatory molecules involved in T-cell activation is also affected by DHA, indicating a broader immunomodulatory activity of these drugs. The upregulation of intercellular adhesion molecule-1 (ICAM-1) expression by 5-AZA-CdR was shared among neoplastic cells of different histologies, having been observed in melanoma (105), ovarian cancer (111), and renal cell carcinoma (RCC) cells (112); in contrast, the upregulation of leukocyte function-associated antigen-3 (LFA-3) was found to be restricted to melanoma cells (105).

Of prospective clinical relevance, the complex phenotypic modifications induced by exposure of neoplastic cells to epigenetic drug were shown: i) to significantly improve the recognition of cancer cells by antigen-specific cytotoxic T lymphocytes (CTL) (113); ii) to abrogate the intratumoral heterogeneity of CTA expression by neoplastic cells thus allowing for their homogeneous CTL recognition (101); iii) to increase the immunogenicity of transformed cells (113).

Further supporting the immunomodulatory role of DHA, systemic administration of low-dose 5-AZA-CdR induced a persistent *de novo* expression of the CTA MAGE-A1, SSX 1-5 and NY-ESO-1 in peripheral blood and bone marrow mononuclear neoplastic cells of patients with

acute myeloid leukemia or myelodysplastic syndrome (114), and of MAGE-A3 and NY-ESO-1 in tumor biopsies from patients with thoracic malignancies (115). These phenotypic changes associated with the development of a humoral response against NY-ESO-1 in selected individuals (115).

Altogether these evidences provide the scientific rationale for the use of DHA in the clinical setting to improve the efficacy of cancer immunotherapy.

The melanoma methylome

Melanoma is thought to arise through a series of genetic and epigenetic events. This most likely involves numerous irreversible changes occurring within the human genome, such as chromosomal deletions, amplifications, and gene mutations. Recently, however, epigenetic events have attracted more attention due to their central involvement in cancer development and potential of therapeutic intervention. Neoplastic transformation is accompanied by a complex deregulation of the cellular DNA methylation homeostasis, resulting in both gene-specific hypermethylation and genome-wide hypomethylation (116).

Aberrant DNA hypermethylation is a frequent event in melanoma and acts as an alternate and/or complementary mechanism to gene mutation or deletion, resulting in the inactivation of specific gene expression and function of TSGs. To date, more than 50 genes have been identified to be aberrantly hypermethylated during different phases of melanoma progression and metastasis, as summarized in *Table N*. Although the exact function of most of these aberrantly silenced genes and their contribution to melanoma progression is still unknown, many studies suggest that they possess different gene functions, such as cell cycle control, apoptosis, cell signaling, tumor cell invasion and metastasis, angiogenesis, and

immune recognition (117). RAR-β2, which mediates growth arrest, differentiation and apoptotic signals triggered by retinoic acids (RA), together with RASSF1A, which promotes apoptosis and growth arrest, and MGMT, which is involved in DNA repair, are the most frequent and well characterized hypermethylated genes in melanoma, being methylated respectively in 70%, 55% (118-120) and 34% of melanoma lesions (118) *(Table 2)*. Notably, a very high incidence of promoter methylation has been observed for genes involved in the metabolic activation of chemotherapeutic drugs (i.e., *CYP1B1*, methylated in 100% melanoma lesions (121), and *DNAJC15*, methylated in 50% melanoma lesions (121), which might contribute to the well-known resistance of melanoma cells to conventional chemotherapy. The list of genes hypermethylated in melanoma is continuously expanding, and it is including new genes that are hypermethylated in virtually all lesions examined (e.g., *QPCT*, methylated in 100% MELANOMA; *LXN*, methylated in 95% melanoma (121), though their function/role in melanoma progression has still to be addressed. Interestingly, some genes, such as *RAR-β2*, are found methylated with similar frequencies in primary and metastatic melanoma, suggesting their methylation as being an early event in melanoma, while others have higher frequencies in advanced disease (e.g., *MGMT, RASSF1A, DAPK*), suggesting the implication of their aberrant hypermethylation in disease progression (118).

Besides TSG hypermethylation, genome-wide hypomethylation might contribute to tumorigenesis and cancer progression by promoting genomic instability, reactivating endogenous parasitic sequences and inducing the expression of oncogenes (122). Recent studies demonstrated that extensive DNA hypomethylation in tumors occurs specifically at repetitive sequences, including short and long interspersed nuclear elements and LTR elements, segmental duplication and subtelomeric regions (123). These findings further

validate the role of DNA methylation in maintaining the stability of the human genome and the suppression of transposable elements in mammalian cells. In this contest, Tellez et al measured the level of methylation of the long interspersed nucleotide element-1 (LINE-1) and Alu repetitive sequences to estimate the genome wide methylation status of melanoma cell lines. With this approach they were able to demonstrate that the extent of repetitive elements hypomethylation inversely correlated with the number of TSGs aberrantly inactivated by promoter hypermethylation (124). On the other hand, genome-wide demethylation has been associated with the *de novo* expression of tumor associated antigens belonging to the CTA family (97;98;101;109). Expression of CTA genes is repressed in normal human skin melanocytes, primarily due to heavily methylated promoter regions in a cell lineage-specific manner. Conversely, these same genes can exist in a demethylated state and are aberrantly re-expressed in subsets of melanoma cells. The biological significance of this gene re-expression continue to be poorly understood. A central question is whether the CTA expression contributes to tumorigenesis or is only a functionally irrelevant by-product of the process of cellular transformation due to global genome hypomethylation. Current evidence would suggest that reactivation of these genes may contribute to overall tumorigenesis. It is clear that the expression of these tumor antigens can result in their recognition and possible destruction by the host immune system. Importantly, recent data suggest that expression of MAGE genes in cancer cells contributes directly to the malignant phenotype and response to therapy (125).

Table 2: **Genes with an altered DNA methylation status in human melanomA (adapted from (126))**

PATHWAY	GENE	METHYLATION STATUS IN	%	FREQUENCY	SOURCE	MODULATED BY 5-AZA-CdR
APOPTOSIS	*DAPK*[b]	methylated	19	16/86	tumors	ND[c]
	HSPB8	methylated	69	11/16	tumors	YES
	TMS1	methylated	50	5/10	tumors	YES
	TRAILR1	methylated	80	8/10	cell lines	YES
	XAF1	methylated	NA	NA	cell lines	YES
	RASSF1A	methylated	NA	NA	cell lines	YES
		methylated	63	26/41	sera	NA
		methylated	28	13/47	sera	NA
		methylated	19	6/31	sera	NA
		methylated	36	9/25	tumors	YES
		methylated	55	24/44	tumors	YES
		methylated	57	49/86	tumors	YES
CELL CYCLE	*CDKN1B*	methylated	9	4/45	tumors	ND
	CDKN1C	methylated	35	7/20	tumors	YES
	CDKN2A	methylated	76	31/41	sera	NA
		methylated	10	3/30	tumors	YES
		methylated	19	11/59	tumors	ND
	TSPY	methylated	100	5/5 male patients	tumors and cell lines	YES
CELL FATE DETERMINATION	*MIB2*	methylated	19	6/31	tumors	ND
	APC	methylated	17	9/54	tumors	YES
	WIF1	methylated	NA	NA	cell lines	YES
DEGRADATION OF MISFOLDED PROTEINS	*DERL3*	methylated	23	3/13	cell lines	NO
DIFFERENTIATION	*GDF15*	methylated	75	15/20	tumors	YES
	HOXB13	methylated	20	4/20	tumors	YES
DNA REPAIR	*MGMT*	methylated	63	26/41	sera	NA
		methylated	19	6/31	sera	NA
		methylated	31	26/84	tumors	ND
		methylated	34	29/86	tumors	YES
DRUG METABOLISM	*CYP1B1*	methylated	100	20/20	tumors	YES
	DNAJC15	methylated	50	10/20	tumors	YES
EXTRACELLULAR MATRIX	*COL1A2*	methylated	80	16/20	tumors	YES
	MFAP2	methylated	30	6/20	tumors	YES
IMMUNE RECOGNITION	*BAGE*	demethylated	83	10/12	cell lines	YES
	HLA class I	methylated	NA	NA	cell lines	YES

PATHWAY	GENE	METHYLATION STATUS IN	%	FREQUENCY	SOURCE	MODULATED BY 5-AZA-CdR
	HMW-MAA	methylated	NA	NA	tumors and cell lines	YES
	MAGE-A1	demethylated	NA	NA	cell lines	YES
	MAGE-A2, -A3, -A4	demethylated	NA	NA	tumors	YES
INFLAMMATION	*PTGS2*	methylated	20	4/20	tumors	YES
INVASION/ METASTASIS	*CCR7*	no CpG island	NA	NA	cell lines	YES
	CDH8	methylated	10	2/20	tumors	YES
	CXCR4	methylated	NA	NA	cell lines	YES
	DPPIV	methylated	80	8/10	cell lines	YES
	EPB41L3	methylated	5	1/20	tumors	YES
	SERPINB5	methylated	100	7/7	cell line	ND
		methylated	13	5/40	tumors	YES
	SYK	methylated	30	6/20	tumors	YES
	TFPI-2	methylated	29	5/17	tumors	YES
	THBD	methylated	60	12/20	tumors	YES
PROLIFERATION	*WFDC1*	methylated	20	4/20	tumors	YES
SIGNALING	*ERα*	methylated	24	26/109	Sera	NA
		methylated	51	55/107	tumors	ND
	PRDX2	methylated	8	3/36	tumors	YES
	PTEN	methylated	62	23/37	sera	YES
	RARB	methylated	13	4/31	sera	NA
		methylated	20	5/25	tumors	YES
		methylated	70	74/106	tumors	YES
	SOCS1	methylated	76	31/41	sera	NA
	SOCS2	methylated	44	18/41	sera	NA
	SOCS3	methylated	60	3/5	tumors	YES
	UNC5C	methylated	23	3/13	cell lines	NO
VESCICLE TRANSPORT	*Rab33A*	methylated	100	16/16	tumors and cell lines	YES
TBD	*BST2*	methylated	50	10/20	tumors	YES
TBD	*FAM78A*	methylated	8	1/13	cell lines	NO
TBD	*HS3ST2*	1\	56	14/25	tumors	ND
TBD	*LRRC2*	methylated	5	1/20	tumors	YES
TBD	*LXN*	methylated	95	19/20	tumors	YES
TBD	*PCSK1*	methylated	60	12/20	tumors	YES
TBD	*PTPRG*	methylated	8	1/13	cell lines	NO
TBD	*QPCT*	methylated	100	20/20	tumors	YES
TBD	*SLC27A3*	methylated	46	6/13	cell lines	NO

[a], methylation status of the gene found in melanoma as compared to that found in normal tissue;
[b], gene symbol: APAF-1, Apoptotic Protease Activating Factor 1; APC, adenomatous polyposis coli; BAGE, B melanoma antigen; BST2, bone marrow stromal cell antigen 2; CCR7, chemokine (C-C motif) receptor 7; CDH1, cadherin 1;CDH8, cadherin 8; CDH13, cadherin 13; CDKN1B, cyclindependent kinase inhibitor 1B; CDKN1C, cyclin-dependent kinase inhibitor 1C; CDKN2A, cyclin-dependent kinase inhibitor 2A; COL1A2, alpha 2 type I collagen; CXCR4, chemokine (C-X-C motif) receptor 4; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; DAPK, death-associated protein kinase; DDIT4L, DNA-damage-inducible transcript 4-like; DERL3, Der1-like domain family, member 3; DNAJC15, DnaJ
homolog, subfamily C, member 15; DPPIV, dipeptidyl peptidase IV; ENC1, ectodermal-neural cortex-1; EPB41L3, erythrocyte membrane protein band 4.1-like 3; ERα, Estrogen Receptor alpha; FAM78A, Family with sequence similarity 78, member A; GDF15, growth differentiation factor 15; HAND1, heart and neural crest derivatives expressed 1; HLA class I, human leukocyte class I antigen; HMW-MAA, high molecular weight melanoma associated antigen; HOXB13, homeobox B13; HS3ST2, heparan sulfate (glucosamine) 3-O-sulfotransferase 2; HSPB6, heat shock
protein, alpha-crystallin-related, B6; HSPB8 heat shock 22 kDa protein 8; LRRC2, leucine rich repeat containing 2; LOX, lysyl oxidase; LXN, latexin; MAGE, melanoma-associated antigen, MFAP2, microfibrillar-associated protein 2; MGMT, O-6-methylguanine-DNA methyltransferase; MIB2, mindbomb homolog 2; MT1G, metallothionein 1G; NKX2-3, NK2 transcription factor related, locus 3; NPM2, nucleophosmin/nucleoplasmin 2; OLIG2, oligodendrocyte lineage transcription factor 2; PAX2, paired box 2; PAX7, paired box 7; PCSK1, proprotein convertase subtilisin/kexin type 1; PGRβ, progesterone receptor β; PPP1R3C, protein phosphatase 1, regulatory (inhibitor) subunit 3C; PRDX2, Peroxiredoxin; PTEN, Phosphatase and tensin homologue; PTGS2, prostaglandin-endoperoxide synthase 2; PTPRG, Protein tyrosine phosphatase, receptor type, G; QPCT, glutaminyl-peptide cyclotransferase; RARB, Retinoid Acid Receptor β2; RASSF1A, RAS associacion domain family 1; RIL, Reversion-induced LIM; RUNX3, runt-related transcription factor 3; SERPINB5, serpin peptidase inhibitor, clade B, member 5; SLC27A3, Solute carrier family 27; SOCS, suppressor of cytokine signaling; SYK, spleen tyrosine kinase; TFPI-2, Tissue factor pathway inhibitor-1; THBD, thrombomodulin; TIMP3, tissue inhibitor of metalloproteinase 3; TMS1, Target Of Methylation Silencing 1; TNFRSF10C, tumor necrosis factor receptor superfamily, member 10C; TNFRSF10D, tumor necrosis factor receptor superfamily, member 10D; TP53INP1, tumor protein p53 inducible nuclear protein 1; TPM1, tropomyosin 1 (alpha); TRAILR1, TNF-related apoptosis inducing ligand receptor 1; TSPY, testis specific protein, Y-linked; UNC5C, Unc-5 homologue C; WFDC1, WAP four-disulfide core domain 1; WIF1, Wnt inhibitory factor 1; XAF1, XIAP associated factor 1. [c], NA, not applicable; ND, not done; TBD, to be determined.

AIM OF THE STUDY

Metastatic melanoma is a very aggressive disease, characterized by substantial unresponsiveness to conventional therapies and high mortality rates world-wide. Thus, new therapeutic options are urgently needed, especially for the advanced phase of the disease. Improved understanding of the biology of melanoma has led to the identification of molecular targets and to the recent development of novel therapies, which are showing very encouraging clinical results. Among the new therapeutic options with highly encouraging clinical results are targeted therapy with BRAF inhibitors and immunotherapy with therapeutic vaccines and immunomodulating monoclonal antibodies.

More than 60% of human melanomas harbor BRAF mutations, especially the V600E (41), for which different selective BRAF inhibitors are being developed in clinical trials (68), showing impressive response rates in metastatic melanoma patients (ranging from 48% to 81%) (70;71). On the other side, melanoma expresses a wide range of TAA. Among these, CTA represent ideal targets for active specific immunotherapy of patients with melanoma. Indeed, their frequent expression in melanoma cells, their limited expression in normal tissues and their *in vivo* immunogenicity (85), render CTA essentially tumor-specific therapeutic targets.

The ultimate effectiveness of systemic targeted therapy based on BRAF inhibitors and of CTA-specific immunotherapy clearly relies on the consistency of activating BRAF mutations and of CTA-expression across metastatic lesions of a given patient. In fact, wild-type BRAF genotype or absence of therapeutic CTA make neoplastic cells unresponsive to treatment, and can be expected to drive clinical resistance to therapy and eventual progression of the disease. As far as immunotherapy, besides TAA expression, an additional

requirement for the effectiveness of treatments is the expression of adequate amounts of different “immune molecules” (e.g., HLA class I and class II antigens, accessory/costimulatory molecules) on melanoma cells. In this scenario, a major role in determing long-lasting therapeutic success of both targeted-therapies and immunotherapies is played by the maintenance of the target genotypic and phenotypic characteristics along with the clinical course of the disease, which ensures, in principle, the sustained responsiveness to treatment.

In light of these notions, and to provide new molecular insights to possibly improve the clinical effectiveness of novel therapeutic options for melanoma patients, the present study aimed to characterize in-depth the presence of different promising therapeutic targets on melanoma cells along with the clinical course of the disease. The study took advantage from the availability of a large panel of short-term cell cultures obtained from metachronous lesions of metastatic melanoma patients. Tumor cell cultures were utilized in place of tumor tissues, to minimize the interference of contaminating normal cells, usually present in surgically removed neoplastic lesions, thus obtaining melanoma specific genotypic and phenotypic data.

In particular the biomolecular characteristics investigated in melanoma cultures under study were: i) the BRAF mutational status; ii) the expression profiles of a large panel of TAA (CTA and differentiation antigens); iii) and of additional “immune molecules”(HLA class I, HLA class II, β-2 microglobulin, ICAM-1, LFA-1, LFA-3, HMW-MAA).

Additionally, in light of the important role of altered DNA methylation in the biology of melanoma cells and in their immune recognition, further aspects evaluated in this study were: i) the maintenance of DNA methylation levels across the course of disease; ii) the

effect of DHA on gene expression profiles (GEP) and on the expression of immune molecules in melanoma cell cultures under study.

The results obtained allow a more comprehensive characterization of the genotypic, epigenetic and phenotypic features of melanoma cells along with the course of the disease, providing the scientific background to design novel and potentially more effective therapeutic strategies for melanoma patients.

MATERIALS AND METHODS

Patients, tissues and cells

63 sequential metastatic melanoma lesions derived from 24 melanoma patients referred for surgery at the National Cancer Institute of Aviano (Italy) were processed after surgical removal and used to generate primary cultures of melanoma cells at the 6th-7th ex vivo passage. For the generation of primary cultures of melanoma cells, cells obtained by mechanical mincing and enzymatic digestion of tumor specimens were seeded in T25 tissue culture flasks in RPMI Medium 1640 (Biochrome KG, Berlin, Germany) complete supplemented with 20% heat inactivated FBS, 2 mM L-glutamine and 100 μg/mL penicillin (Sigma Chemical Co.), 100 μg/mL streptomycin (Bristol-Myers Squibb S.r.l). From 2 to 5 metastatic lesions of each patient (mean: 2.6) derived from the 24 melanoma patients were snap-frozen in liquid nitrogen and stored at −80°C until RNA and DNA extraction was performed.

$BRAF^{V600E}$ mutation quantification

In the $BRAF^{V600E}$ lesions, we assessed the percentage of alleles carrying the mutation by pyrosequencing analysis, as previously reported by Edlundh-Rose et al (127). To perform this assay, which is based on 'sequencing by synthesis' principle, PCR amplifications were carried out with the following primers:

5'-biotin-TGAAGACCTCACAGTAAAAATAGG-3' (forward)

5'-TCCAGACAACTGTTCAAACTGAT-3' (reverse).

Samples were denaturated at 94°C for 5 min, amplified for 35 cycles consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s and elongated at 72°C for 7 min. The 5'-biotinylated PCR

products of the region including codon 1799 were immobilized onto streptavidin-coated paramagnetic beads (GE Healthcare), denaturated by 0.1 mol/l NaOH and released according to the manufacturer's instructions using PyroMark Vacuum Prep Workstation (Biotage, Uppsala, Sweden). These reactions were performed in a 96-well plate using Pyro Gold Reagents (Biotage). The primed single-stranded DNA templates were subjected to real-time sequencing of the region including codon 600 by using the reverse primer 5'-TGATTTTGGTCTAGCTACA-3'. A titration series with different dilutions of a sample containing the V600E mutation was set up to evaluate the linear correlation between the height of the peaks and the percentage of mutant alleles in the heterozygous lesions. These percentages were calculated by using PSQ96MA software (Biotage) for allelic quantification.

Indirect immunofluorescence (IIF)

Monoclonal antibodies, conventional antisera and reagents.

The anti-HLA class I mAb TP25.99 and the anti-DR HLA class II mAb Cl413 were kindly provided by Dr. Soldano Ferrone (Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY). The anti- intercellular adhesion molecule-1 (ICAM-1) mAb Cl203 and the anti-LFA-3 mAb TS2/9 were purchased from Becton Dickinson. The anti-HMW-MAA mAb 763.74 was purchased from Serotec. FITC-conjugated F(ab')2 fragments of rabbit anti-mouse immunoglobulins (Ig) were purchased from DAKO. ChromePure mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc.

IIF analysis.

IIF was performed by incubating cells ($1X10^5$) with an excess of mAb at 4°C for 30 min. After three washings with PBS supplemented with 0.5% BSA and 0.01% (wt/vol) NaN3

(PBSBSA-AZ), cells were incubated for an additional 30 min at 4°C with FITC-F(ab')2 fragments of goat anti-mouse IgG (H + L) antibodies. After three washings with PBS-BSA-AZ, cells were resuspended in PBS-BSA-AZ and analyzed for surface fluorescence utilizing a FACS Calibur flow cytometer (Becton Dickinson Co., Mountain View, CA) and data were analyzed by the Cell Quest Software (Becton Dickinson). A sample was classified as positive when the mean value of fluorescence intensity was higher than 20. Mean value of fluorescence intensity obtained with isotype-matched mouse Ig was lower than 20 on all cell lines tested.

Isolation of total RNA

Total RNA was extracted by TRIzol Reagent (GIBCO BRL Co., USA). Cells were pellet by centrifugation at 1400 rpm for 5 minutes and then lysed by adding 1 ml TRIzol per 5 x 10^6 cells. Following a 5 minute incubation at room temperature, 0.2 ml of chloroform per ml of TRIzol reagent was added to each tube and the resulting mixture shaken vigorously by hand. After centrifugation at 12000 rpm at 4°C for 15 minutes, the RNA-containing aqueous phase was transferred to a fresh tube and precipitated by adding 0.5 volumes isopropanol per ml TRIzol. The aqueous phase and isopropanol were mixed, kept at room temperature for 10 minutes and then centrifuged at 12000 rpm at 4°C for 10 minutes. The supernatant was discarded and the RNA pellet washed in 1 volume of 75% ethanol per ml TRIzol to remove salt. The pellet was then air dried at room temperature and resuspended in DEPC H2O. RNA concentration and quality were determined using the NanoDrop ND-1000, a full spectrum (220-750 nm) spectrophotometer that measures 1 µl samples with high accuracy and reproducibility.

Digestion of RNA with DNASE I

In competitive RNA-PCR studies, contaminating DNA can produce incorrect results because of its potential to act as a second competitor, so total RNA was digested with RNase free DNase I (Roche Diagnostics) to remove contaminating genomic DNA. 1 µl of DNase I reaction buffer (200 mM Tris-HCl pH 8, 500 mM KCl, 20 mM MgCl2) and 10 units of DNase I RNase-free (10U/µl) (Roche) were added per µg of RNA sample in 10 µl reaction volume. After incubation at 37°C for 30 minutes, DNase I was inactivated by heating at 65°C for 10 minutes in presence of 25 mM EDTA (1 µl/µg of RNA sample).

Retrotrascription

2 µg of total RNA was used as a template for the reverse transcription (RT) in 20 µl reaction volume using Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen) and random hexamer primers (Promega). RNA, 1 µl random hexamer primers (0.5 µg/µl) (Promega), 4 µl 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH) (Invitrogen) and H2O added to 11 µl were incubated at 65°C for 5 minutes and quick chilled on ice with brief centrifugation. 4 µl 1st Strand buffer 5X (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl2) (Invitrogen), 2 µl 0.1 mM Ditiotreitol (DTT) (Invitrogen) and 2 µl RNase Inhibitor (40U/µl) (Invitrogen) were then mixed with contents of the tube and incubated at 25°C for 10 minutes. Afterwards, 1 µl of M-MLV RT (200 U/µl) (Invitrogen) was added and the tube was transferred to a 37°C water bath for 50 minutes. The reaction was inactivated by heating at 70°C for 15 minutes and the volume was then adjusted to 100 µl with distilled water.

RT-PCR analysis

Every PCR amplification was performed starting from 5 µl of cDNA in a total volume of 50 µl containing:

PCR buffer 1x (100 mM Tris-HCl pH 8.3, 500 mM KCl) (Takara)	5 µl
MgCl2 25 mM (Takara)	3 µl
dNTPs 10 mM (Takara)	4 µl
primers (sense+antisense) 100 pmoles/µl (Invitrogen)	1 µl
Taq DNA polimerase (5U/µl) (Takara)	0.25 µl

The oligonucleotide primer sequences and gene-specific PCR amplification programs used were reported in *Table 3*. The integrity of RNA and corresponding cDNA was confirmed through the amplification of β-actin housekeeping gene. Primers sequences, Temperature of annealing (TA), numbers of cycles and amplification length (bp) were reported in *Table 3*.

Table 3:

Antigen	PCR Amplicon Length	Primers	T_A	Cycles n°
ß-actin	615 bp	F 5'-ggcatc gtg atg gac tcc g-3' R 5'-gct gga agg tggaca gag a-3'	68°C	21
MAGE-A1	421 bp	F 5'-cgg ccg aag gaa cct gac cca g-3' R 5'-gct gga acc ctc act ggg ttg cc-3'	72°C	30
MAGE-A2	230 bp	F 5'-aag tag gac ccg agg cac tg-3' R 5'-gaa gag gaa gaa gcg gtc tg-3'	67°C	30
MAGE-A3	725 bp	F 5'-tgg agg acc aga ggc ccc c-3' R 5'-gga cga tta tca gga ggc ctg c-3'	72°C	30
MAGE-A4	446 bp	F 5'-gag cag aca ggc caa ccg-3' R 5'-aag gac tct gcg tca ggc-3'	68°C	30
MAGE-A6	725 bp	F 5'-tgg agg acc aga ggc ccc c-3' R 5'-cag gat gat tat cag gaa gcc tgt-3'	72°C	30
GAGE 1-6	239 bp	F 5'-aga cgc tac gta gag cct-3' R 5'-cca tca gga cca tct tca-3'	55°C	30
NY-ESO-1	379 bp	F 5'-cac aca gga tcc atg gat gct gca gat gcg g-3' R 5'-cac aca aac ctt ggc tta gcg cct ctg ccc tg-3'	69°C	35
SSX 1-5	663 bp	F 5'-acg gat ccc gtg cca tga acg gag acg ac-3' R 5'-ttg tcg aca gcc atg ccc atg ttg gtg a-3'	67°C	35
MART-1	602 bp	F 5'- ctgaccctacaagatgccaagag -3' R 5'- atcatgcattgcaacatttattgatggag -3'	60°C	24
Tyrosinase	284 bp	F 5'- ttggcagattgtctgtagcc -3' R 5'- aggcattgtgcatgctgctt -3'	60°C	36

The integrity of each RNA and random hexamer primers -synthesized cDNA sample was confirmed by the amplification of the β-actin housekeeping gene. 10 µl of each RT-PCR sample were run on a 2% agarose gel and visualized by ethidium bromide staining.

Quantitative RT-PCR

SYBR-green quantitative RT-PCR reactions were performed on 20 ng of retrotranscribed total RNA in a final volume of 20 µl SYBR-green Universal Master Mix (Applyed Biosystems) at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and at 60°C for 1 min, followed by dissociation performed at 95°C for 15 s, 60°C for 20 s and 95°C for 15 s. SYBR-green primers sets were summarized in *Table 4*:

Table 4:

Antigen	Primers	PCR Amplicon Length
ß-actin	F 5'-cga gcg cgg cta cag ctt-3'	59 bp
	R 5'-cct taa tgt cac gca cga tt-3'	
MAGE-A1	F 5'-gcc aag cac ctc ttg tat cct g-3'	83 bp
	R 5'-gga gca gaa aac caa cca aat c-3'	
MAGE-A3	F 5'-tgt cgt cgg aaa ttg gca gta t-3'	74bp
	R 5'-caa aga cca gct gca agg aac t-3'	
NY-ESO-1	F 5'-tgc ttg agt tct acc tcg cca-3'	136 b
	R 5'-tat gtt gcc gga cac agt gaa-3'	

Measurement of gene expression was performed utilizing the ABI PRISM 7000 Sequence Detection System (Applyed Biosystems, Milan, Italy) and the copy number of specific antigen and of the reference gene β-actin was established in each sample by extrapolation of the standard curve. The number of selected antigen cDNA molecules in each sample was then normalized to the number of cDNA molecules of β-actin.

LINE-1 bisulfite pyrosequencing analysis

Genomic DNA was extracted from short-term cultures of melanoma cells by proteinase K treatment followed by standard phenol/chloroform extraction and ethanol precipitation (128). Bisulfite conversion was carried out on 500 ng genomic DNA using EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's protocol. Methylation analysis of the LINE-1 elements was performed as previously described (128), with minor modifications. LINE-1 elements were amplified using 50 pmol each of forward primer 5'-TTTTTTGAGTTAGGTGTGGG-
3' and reverse biotinylated primer 5'-TCTCACTAAAAAATACCAAACAA-3' in a 50 µL reaction volume containing 2.5 ng of bisulfite-treated DNA, 1× PCR buffer, 1.5 mM MgCl2 and 1.25 U of Platinum Taq DNA polymerase (Invitrogen, Milan, Italy). PCR thermal amplification profile consisted of an initial denaturation step of 5 min at 95°C, followed by 50 cycles of 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C. The PCR product was purified using Streptavidin Sepharose High Performance beads (Amersham Biosciences, Uppsala, Sweden) and denatured using 0.2 mol/L of NaOH solution. Next, 0.3 µmol/L of the sequencing primer (5'-GGGTGGGAGTGAT-3') was annealed to the purified singlestranded PCR product and the Pyrosequencing reaction was performed using the PSQ HS 96 Pyrosequencing System (Pyrosequencing, Inc., Westborough, MA). The level of methylation for each of the 3 analyzed CpG sites (CpG1, CpG2, CpG3) was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines (Figure 1). Within- and between run variations for the determination of LINE-1 methylation through the pyrosequencing assay utilized have been previously described (128).

Treatment with 5-AZA-CdR

Melanoma cell lines were seeded at a density of 2.5 X 10_5 cells/ml in a T75 tissue culture flask. Once cells became firmly adherent to plastic, medium was replaced with 8 ml of fresh medium, containing 1 µM 5-AZA-CdR, every 12h for 2 days (4 pulses). Flasks were wrapped in aluminum foil to avoid light exposure. At the end of treatment, medium was replaced with fresh culture medium without 5-AZA-CdR, cells were cultured for an additional 48 h, and used for phenotypic and molecular assays.

Gene expression profile

The quantity and the quality of RNA, extracted as previous described, was assessed with NanoDropR ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). *In vitro* transcription, labeling and purification of dye-labeled cRNA were performed using the Quick Amp Labeling Kit, one-color (Agilent Technologies, Santa Clara, CA, USA) following manufacturer's guidelines. Gene expression profiling was performed by a One-Color strategy using Cy3-labeled aRNA from 6 metastases derived from 3 patients (Mel 195, Mel 201 and Mel 458) (Quick Amp Labeling, Agilent Technologies, Santa Clara, CA, USA). A mixture of 1650 ng of Cy3-labeled reference cRNA, Blocking Agent and Fragmentation Agent was hybridized to Whole Human Genome (4x44K) oligo microarray platform (Agilent Technologies, Santa Clara, CA, USA). Hybridization was performed for 17 hours at 65°C in 2x GEx Hybridation Buffer HI-RPM (Agilent Technologies, Santa Clara, CA, USA), using Agilent's Hybridization Oven at 10 rpm. Following washing, slides were analyzed by Agilent Microarray Scanner. Feature Extraction Software provided by Agilent (version 9.5.3) was used to

quantify the intensity of fluorescence images and to normalize results by subtracting local background fluorescence according to the manufacturer's instruction. Differentially expressed genes between treated and untreated melanoma cell cultures were selected with a cut-off set ≥3 for the Fold Change.

RESULTS

Analysis of BRAF mutations in sequential melanoma metastases

To evaluate whether the BRAF mutational status undergoes evolution with disease progression in melanoma, we assessed the activating $BRAF^{V600E}$ mutation in primary cell cultures generated from initial metastatic lesions surgically removed from 14 cutaneous melanoma patients and 18 subsequent metastases (*Table 5*).

Pyrosequencing analysis revealed that genomic DNA of the sequential melanoma metastases, removed from different body districts of individual patients at a median time interval of 302 days (range 26-1318), stabilized for the $BRAF^{V600E}$ mutated status. Indeed, the original heterozygous $BRAF^{V600E}$ status was consistently retained in 10 out of 14 patients investigated, while 2 out of 14 patients acquired a homozygous mutated status as compared to an initial heterozygous condition (*Table 5*). In contrast, no $BRAF^{V600E}$ mutation was acquired in 2 patients with an initial homozygous wild-type BRAF genotype (*Table 5*).

Table 5: **$BRAF^{V600E}$ mutation genotyping of sequential metastatic melanoma lesions**

Patient	Metastasis # [a]	Site of metastasis [b]	Days [c]	Percentage of mutant alleles [d]	$BRAF^{V600E}$ [e]
Mel 90	I	M	0	57.5	WT/MT
	II	M	71	58.9	WT/MT
Mel 91	I	LN	0	55.5	WT/MT
	II	LN	238	58.8	WT/MT
Mel 120	I	LN	0	55.7	WT/MT
	III	LN	672	64.2	WT/MT
Mel 140	I	SC	0	61.4	WT/MT
	II	SC	411	70.4	WT/MT
	III	SC	728	92.9	**MT/MT**
Mel 146	I	SC	0	66.3	WT/MT
	II	LN	254	67.7	WT/MT
	III	SC	554	66.7	WT/MT
Mel 195	I	LN	0	54.6	WT/MT
	II	SC	91	46.8	WT/MT
Mel 201	I	LN	0	57.4	WT/MT
	II	P	1318	84.8	**MT/MT**
Mel 255	I	LN	0	58.8	WT/MT
	II	SC	292	52.1	WT/MT
	III	SC	299	55	WT/MT
Mel 261	I	SC	0	42.4	WT/MT
	II	SC	305	35.9	WT/MT
	III	SC	721	33.6	WT/MT
Mel 435	I	LN	0	7.2	WT/WT
	II	SC	139	7	WT/WT
Mel 458	I	SC	0	0	WT/WT
	II	SC	26	0	WT/WT
Mel 532	I	SC	0	58.2	WT/MT
	II	SC	364	46.8	WT/MT
Mel 554	I	SC	0	45.3	WT/MT
	II	LN	118	44.9	WT/MT
Mel 592	I	LN	0	64.5	WT/MT
	II	LN	337	65	WT/MT

[a] Short-term cell cultures were established from initial (I) and subsequent sequential metastatic lesions removed from melanoma patients referred for surgery at the National Cancer Institute of Aviano (Italy).
[b] M, muscle; LN, lymph node; SC, subcutaneous; P, pancreas.
[c] Time frame of metastasis excision since the surgical removal of the first analyzed lesion.
[d] Percentage of $BRAF^{V600E}$ mutant alleles was determined by pyrosequencing assay.
[e] WT/WT, wild-type BRAF homozygote (% $BRAF^{V600E}$ < 25); WT/MT, $BRAF^{V600E}$ heterozygote (% $BRAF^{V600E}$ 25 – 75); MT/MT, $BRAF^{V600E}$ homozygote (% $BRAF^{V600E}$ > 75).

Expression of "immune molecules" in sequential melanoma metastases

To determine whether the expression of "immune molecules" is stable or undergoes alteration during disease progression, primary cell cultures from sequential metastatic lesions of 18 melanoma patients, were assessed for their antigenic phenotype by indirect immunofluorescence (IIF) analysis followed by flow cytometry.

Results reported in *Table 6* demonstrated that the antigenic profiles of neoplastic cells of the same patient were mostly unstable through the course of the disease. Indeed, 16 out of 18 patients examined were characterized by alterations ≥50% of the mean fluorescence intensity values of 2 or more antigens investigated during disease progression. Noteworthy, data did not identify any univocal trend for variation of "immune molecules" in the course of the disease. In fact, though up-regulation of antigen expression levels along with tumor progression appeared to be more frequent (10 out of 18), down-regulation was also present (6 out of 18). Besides, mixed patterns showing concomitant up- and down-regulation of different antigens in the same patient were frequently observed (11 out of 18), suggesting that independent mechanisms of the antigens examined may affect the patients (*Table 6*). Noteworthy, changes in antigen expression did not appear to correlate neither with the time-intervals to develop new metastatic lesions nor by anatomic site of metastasis. Among all investigated immune molecules, HLA class I was the less maintained in sequential metastases (2/15, 13.3%), while β2-microglobulin, ICAM-1 and HMW-MAA were the most maintained (7/18, 38.9%) (*Table 6*).Importantly, however, our data demonstrated that the majority of the antigens analyzed (HLA class I, β2-microglobulin, ICAM-1, LFA-1, LFA-3 and HMW-MAA) were frequently and highly express in the majority of patients, with the only exception of HLA class II, not expressed in all sequential lesions of 5 out of 19 patients (*Table 6*).

Table 6: **Indirect immunofluorescence analysis of the antigenic profile of sequential metastatic melanoma cell cultures**

Pts	MTS #[a]	days[b]	MTS site	HLA class I	b2m	HLA class II	ICAM-1	LFA-1	LFA-3	HMW-MAA	Trend[d] M	Trend[d] U	Trend[d] D
120	I	0	LN	385[c]	497	316	49	77	5.7	534			
	III	672	LN	516	683	577	390	39.5	58.5	489	4/7	3/7	
140	II	411	C/SC	43.7	133	19.5	130	73	30.9	132			
	III	728	C/SC	128	163	171	120	47.8	13.9	125	4/7	2/7	2/7
146	I	0	C/SC	230	277	298	42	52	22	59			
	III	554	C/SC	508	614	143	192	52.4	62	206	1/7	5/7	1/7
195	I	0	LN	137	149	60.2	12.3	30.4	94.4	205			
	II	91	C/SC	242	277	149	106	46	18	173	1/7	5/7	1/7
201	I	0	LN	971	984	160	151	63	62	309			
	II	1318	PA	270	531	52.7	129	74.7	514	447	4/7	1/7	2/7
203	I	0	C/SC	464	708	305	69.4	7.8	12.1	64.2			
	III	468	C/SC	791	1140	21.8	131	60.6	31.5	148		6/7	1/7
	IV	478	C/SC	520	813	3.6	122	69.3	21.9	145	6/7		1/7
255	I	0	LN	423	526	97	395	108	55	382			
	II	292	C/SC	156	216	11.5	40.2	38.4	34.3	250	2/7		5/7
256	I	0	LN	385	661	8.5	176	182	62	984			
	II	89	LN	824	1194	23.4	73.4	279	59.4	293	1/7	4/7	2/7
261	I	0	C/SC	99.2	108	31.1	294	44.3	34.8	120			
	II	305	C/SC	478	803	134	466	32	11.9	214		5/7	1/7
	III	721	C/SC	498	616	11.6	165	53.7	13.6	134	4/7	1/7	2/7
318	I	0	LN	95.8	740	143	54.5	119	84.2	148			
	II	713	LN	86.8	820	8.5	87.8	114	80.2	192	5/7	1/7	1/7
	V	1484	LN	241	1082	29.3	88.5	164	28.8	197	4/7	2/7	1/7
335	I	0	C/SC	168	293	28.8	43.6	53.6	29.7	122			
	II	91	C/SC	423	663	20.2	210	87.5	76.6	248	2/7	5/7	
435	I	0	LN	429	664	5.1	194	193	63.1	15.2			
	II	139	C/SC	267	428	4.5	135	128	41.8	33.6	7/7		
	IV	180	M	1409	2134	20	153	227	67.4	7.85	1/7	5/7	1/7
458	I	0	C/SC	227	503	4.2	42.8	67.3	51.9	244			
	II	26	C/SC	113	379	5.1	34	91.1	55.8	116	6/7		1/7
462	IV	0	C/SC	211	398	6.1	238	58	33.3	269			
	V	47	C/SC	173	328	11.3	82.5	51.1	15.1	137	5/7		2/7
	VI	61	C/SC	131	203	40.5	170	94.5	13.5	292	2/7	4/7	1/7
	VII	120	C/SC	160	334	7.1	142	38.2	7.7	262	3/7	1/7	2/7
532	I	0	C/SC	93.8	235	7.5	66.9	89.9	8.6	40.9			
	II	364	C/SC	69.4	122	33.4	14.2	NT	NT	54.1	2/5	1/5	2/5
	IV	607	C/SC	69.8	106.6	10.3	5.7	NT	NT	153.9	2/5	1/5	2/5
550	I	0	LN	527	667	5.1	87	434	11	152			
	II	102	C/SC	NT	403	3.8	76.9	NT	NT	35.6	2/4		2/4
554	I	0	C/SC	528	655	146	59.3	138	9.5	345			
	II	118	LN	NT	540	15.4	54.2	NT	NT	165	2/4		2/4
560	I	0	C/SC	78.8	127	3.5	24.3	89.1	6.5	45.9			
	II	108	L	NT	137	3.3	28.6	NT	NT	62.3	4/4		
NM mean fluorescence[e]				**13/15**	**11/18**	**14/18**	**11/18**	**8/13**	**11/13**	**11/18**			
M mean fluorescence[e]				**2/15**	**7/18**	**4/18**	**7/18**	**5/13**	**2/13**	**7/18**			

[a] Cultures of melanoma cells established from initial (I) and subsequent sequential metastatic lesions, removed from cutaneous melanoma patients referred for surgery at the National Cancer Institute of Aviano (Italy), were sequentially incubated with antigen specific mAbs and with FITC-conjugated F(ab')2 fragments of rabbit anti-mouse Ig. Cells were then analyzed by flow cytometry.

[b] Time frame of metastasis excision since the surgical removal of the first analyzed lesion.

[c] Mean values of fluorescence intensity.
[d] Antigen-expression trend during the progression of the disease. M: maintained expression between sequential metastases in the number of antigens down-reported; U: up-regulated expression between sequential metastases in the number of antigens down-reported; D: downregulated expression between sequential metastases in the number of antigens down-reported.
[e] Number of patients maintaining the antigen expression levels over time. M: variation < 50%; NM: variation ≥ 50%. Abbreviations: LN = lymph node; SC = subcutaneous; C = cutaneous; P= pancreas; M= muscle; L= lung; MTS = metastasis; NT = non tested.

CTA expression profiles in sequential melanoma metastases by RT-PCR analyses

Total RNA of 63 primary cell cultures derived from sequential metastatic lesions surgically removed from 24 melanoma patients were analyzed for their constitutive expression of melanoma differentiation antigens (MART-1 and tyrosinase) and of a large panel of CTA (MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, NY-ESO-1, SSX 1-5, GAGE 1-6) (Table N). From 2 to 5 metastases were analyzed from each patient (mean 2.6), and the median period between surgical removals dates of metastases was 359.5 days (range: 26-3131 days) (*Table 7*).

As expected, RT-PCR analyses showed that the differentiation antigens were expressed in the majority of tumor samples, with percentages of positivity ranging from 74.6% to 93.7% for MART-1 and tyrosinase, respectively. Almost all patients expressed these antigens in at least one lesion: MART-1 being expressed in 20 out of 24 patients, tyrosinase in 23 out of 24 patients (*Table 7*). Concomitant absence of MART-1 and tyrosinase was observed in both metastatic lesions (I and II) of only one patient (Mel 554), and only in the first metastatic lesion of patient Mel 435 (*Table 7*). Results also demonstrated that the expression of MART-1 and tyrosinase was maintained in 19 out of 24 and in 21 out of 24 of patients, respectively. The other patients showed a common trend towards loss of antigen expression during the progression of the disease, with the only exception of patient Mel 435, who presented a negative first lesion for tyrosinase, while the second and the third were positive

(*Table 7*). RT-PCR analyses of CTA expression revealed that MAGE-A4 was the less prevalent (14 out of 24) in the melanoma patients under study, while MAGE-A1 and MAGE-A3 were the most expressed, being present in at least one lesion of all patients (24 out of 24) (*Table 7*). The comparison of sequential metastases from the same patient revealed that the expression of each CTA was maintained in over half of the patients (13 of 24) and it appeared not to be influenced by either temporal range between sequential metastases, nor anatomic site of metastasis (*Table 7*). Among all CTA examined, MAGE-A2 was the most stable among sequential metastases (maintained in 91.6% of patients), while MAGE-A3 and GAGE 1-6 were the less stable (maintained in 75% of patients) (*Table 7*).

Table 7: **RT-PCR analysis of CTA and differentiation antigens expression in sequential melanoma metastases**

Pts	MTS #[a]	days[b]	MTS site	β-actin	CTA								Differentiation antigens	
					MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	NY-ESO-1	SSX 1-5	GAGE 1-6	MART-1	tyrosinase
Mel 90	I	0	M	+	+	+	+	+	+	+	+	+	+	+
	II	71	M	+	+	+	+	+	+	+	+	+	+	+
Mel 91	I	0	LN	+	+	+	+	+	+	+	+	+	+	+
	II	238	LN	+	+	+	+	+	+	+	+	+	+	+
Mel 120	I	0	LN	+	-	+	+	+	-	+	+	+	+	+
	III	672	LN	+	+	+	+	+	-	+	+	+	+	+
	IV	762	SC	+	+	+	+	+	-	+	+	+	-	+
Mel 140	I	0	SC	+	+	+	+	-	+	+	+	+	+	+
	II	411	SC	+	+	+	+	+	+	+	+	+	+	+
	III	728	C	+	+	+	+	+	+	+	+	-	+	+
Mel 146	I	0	C/SC	+	+	+	+	+	+	+	+	+	+	+
	II	254	LN	+	+	+	+	+	+	+	+	+	+	+
	III	554	C/SC	+	-	+	+	+	+	-	+	+	-	+
Mel 195	I	0	LN	+	+	-	-	-	-	-	-	-	+	+
	II	91	C/SC	+	+	+	+	-	-	-	+	+	+	+
Mel 200	II	0	M	+	+	+	+	-	+	-	+	+	+	+
	IV	420	I	+	+	+	+	-	+	-	+	+	+	+
Mel 201	I	0	LN	+	+	+	+	-	+	-	+	+	+	+
	II	1318	P	+	+	+	+	+	+	+	+	+	-	+
Mel 203	I	0	SC	+	+	+	+	+	+	-	+	-	+	+
	II	76	SC	+	+	+	+	+	+	+	+	+	+	+
	III	468	SC	+	+	+	+	+	+	+	+	+	+	+
	IV	478	SC	+	+	+	+	+	+	+	+	-	+	+
	V	504	SC	+	+	+	+	+	+	+	+	+	+	+
Mel 255	I	0	LN	+	+	+	+	-	-	-	+	+	+	+
	II	292	SC	+	+	+	+	-	-	-	+	+	+	+
	III	299	SC	+	+	+	+	-	-	-	+	+	+	+
Mel 256	I	0	LN	+	-	-	-	-	-	-	-	-	+	+
	II	89	LN	+	+	-	+	-	-	+	+	+	-	+
Mel 261	I	0	SC	+	+	+	+	-	+	-	+	+	+	+
	II	305	C/SC	+	+	+	+	-	+	-	+	+	+	+
	III	721	C/SC	+	+	+	+	-	+	-	+	+	+	-
Mel 318	I	0	LN	+	+	+	+	+	+	+	+	+	+	+
	II	713	LN	+	+	+	+	+	+	+	+	+	+	+
	V	1484	LN	+	+	+	-	-	-	+	+	+	+	+
Mel 335	I	0	C/SC	+	+	+	+	-	-	+	+	+	+	+
	II	91	SC	+	+	+	+	-	-	+	+	+	+	+
	III	168	SC	+	+	+	+	-	-	+	+	+	+	+

Pts	MTS[a] #	days[b]	MTS site	β-actin	CTA								Differentiation antigens	
					MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	NY-ESO-1	SSX 1-5	GAGE 1-6	MART-1	tyrosinase
Mel 435	I	0	LN	+	+	+	-	+	+	+	+	+	-	-
	II	139	C/SC	+	+	+	+	+	+	+	+	+	-	+
	IV	180	M	+	+	+	+	+	+	-	+	+	-	+
Mel 458	I	0	C/SC	+	+	-	-	-	-	-	-	+	+	+
	II	26	C/SC	+	+	-	+	-	-	-	+	+	+	+
Mel 462	IV	0	C/SC	+	-	+	-	-	+	-	-	-	+	+
	V	47	C	+	-	+	+	-	-	-	-	+	+	+
	VI	61	C/SC	+	+	+	+	-	-	-	-	-	+	+
	VII	120	C/SC	+	-	+	-	-	+	-	-	-	+	+
Mel 496	I	0	LN	+	+	+	+	+	+	+	+	+	-	+
	III	3131	LN	+	+	+	+	+	+	+	+	+	-	+
Mel 532	I	0	SC	+	+	+	+	-	+	NT	+	+	+	+
	II	364	LN	+	-	-	+	-	+	NT	+	+	-	+
	IV	607	C/SC	+	-	-	+	-	+	-	+	-	-	+
Mel 542	I	0	LN	+	+	+	+	+	+	+	+	+	+	+
	III	674	SC	+	+	+	+	-	-	-	+	+	+	+
Mel 550	I	0	LN	+	+	+	+	+	+	-	-	+	-	+
	II	102	SC	+	+	+	+	+	+	-	-	+	-	-
Mel 554	I	0	SC	+	+	+	+	+	+	+	+	+	-	-
	II	118	LN	+	+	+	+	+	+	+	+	+	-	-
Mel 560	I	0	SC	+	+	+	+	-	-	+	+	-	+	+
	II	108	POL	+	+	+	+	-	-	+	+	-	+	+
Mel 592	I	0	LN	+	+	+	+	+	-	+	+	+	+	+
	II	337	LN	+	+	+	+	+	-	+	+	+	+	+
	III	2092	LN	+	+	+	+	+	+	+	+	+	-	+
CTA expression frequency[c]					24/24 (100%)	22/24 (91.6%)	24/24 (100%)	14/24 (58.3%)	17/24 (70.8%)	16/23 (69.5%)	22/24 (91.6%)	23/24 (95.8%)	20/24 (83.3%)	23/24 (95.8%)
Maintained CTA frequency[d]					19/24 (79.1%)	22/24 (91.6%)	18/24 (75%)	21/24 (87.5%)	21/24 (87.5%)	18/23 (78.2%)	21/24 (87.5%)	18/24 (75%)	19/24 (79.1%)	21/24 (87.5%)

[a] Total RNA was extracted from short-term cell cultures established from initial (I) and subsequent sequential metastatic lesions removed from cutaneous melanoma patients referred for surgery at the National Cancer Institute of Aviano (Italy).

[b] Time frame of metastasis excision since the surgical removal of the first analyzed lesion.

[c] Number and percentage of patients, showing the CTA expression in at least a lesion.

[d] Number and percentage of patients maintaining the CTA expression over time.

Abbreviations: LN = lymph node; SC = subcutaneous; C = cutaneous; I = intestine; P= pancreas; M= muscle; L= lung; MTS = metastasis; NT = non tested.

On the other hand, the expression profiles of investigated CTA resulted heterogeneous among the distinct sequential metastases in 11 out of 24 patients. A clear

pattern of gain or loss of 2 or more CTA along with disease progression has been observed in 4 and 3 patients, respectively (*Table 7 and 8*). Furthermore, 4 patients were characterized by a high instability of CTA phenotype resulting in subsequent gain and loss or *viceversa* of the expression of different CTA over time (*Table 7 and 8*).

***Table 8*: Melanoma patients with heterogeneous CTA expression profile.**

PZ	MTS[a] #	days[b]	β-actin	MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	NY-ESO-1	SSX 1-5	GAGE 1-6	N° CTA with modified expression[c]	Trend[d]
Mel 140	I	0	+	+	+	+	-	+	+	+	+		
	II	411	+	+	+	+	+	+	+	+	+	2	V
	II	728	+	+	+	+	+	+	+	+	-		
Mel 146	I	0	+	+	+	+	+	+	+	+	+		
	II	254	+	+	+	+	+	+	+	+	+	2	L
	III	554	+	-	+	+	+	+	-	+	+		
Mel 195	I	0	+	+	-	-	-	-	-	-	-	4	G
	II	91	+	+	+	+	-	-	-	+	+		
Mel 201	I	0	+	+	+	+	-	+	-	+	+	2	G
	II	1318	+	+	+	+	+	+	+	+	+		
Mel 203	I	0	+	+	+	+	+	+	-	+	-		
	II	76	+	+	+	+	+	+	+	+	+		
	III	468	+	+	+	+	+	+	+	+	+	2	V
	IV	478	+	+	+	+	+	+	+	+	-		
	V	504	+	+	+	+	+	+	+	+	+		
Mel 256	I	0	+	-	-	-	-	-	-	-	-	5	G
	II	89	+	+	-	+	-	-	+	+	+		
Mel 318	I	0	+	+	+	+	+	+	+	+	+		
	II	713	+	+	+	+	+	+	+	+	+	3	L
	V	1484	+	+	+	-	-	-	+	+	+		
Mel 435	I	0	+	+	+	-	+	+	+	+	+		
	II	139	+	+	+	+	+	+	+	+	+	2	V
	IV	180	+	+	+	+	+	+	-	+	+		
Mel 458	I	0	+	+	-	-	-	-	-	-	+	2	G
	II	26	+	+	-	+	-	-	-	+	+		
Mel 462	IV	0	+	-	+	-	-	+	-	-	-		
	V	47	+	-	+	+	-	-	-	-	+	4	V
	VI	61	+	+	+	+	-	-	-	-	-		
	VII	120	+	-	+	-	-	+	-	-	-		
Mel 532	I	0	+	+	+	+	-	+	NT	+	+		
	II	364	+	-	-	+	-	+	NT	+	+	3	L
	IV	607	+	-	-	+	-	+	-	+	-		
Maintained CTA frequency[c]				7/11 (63,9/1%)	9/11 (81.8%)	5/11 (45,5%)	8/11 (72,7%)	9/11 (81,8%)	5/11 (45,5%)	8/11 (72,7%)	5/11 (45,5%)		

[a] Total RNA was extracted from short-term cell cultures established from initial (I) and subsequent sequential metastatic lesions removed from cutaneous melanoma patients referred for surgery at the National Cancer Institute of Aviano (Italy).
[b] Time frame of metastasis excision since the surgical removal of the first analyzed lesion.
[c] Number and percentage of patients maintaining the CTA expression over time.
[d] CTA-expression trend during the progression of the disease.
Abbreviation: Pts = patient; MTS= metastasis; G = gain; L = loss; V= variable; NT = not tested.

Quantitative evaluation of CTA expression levels in sequential metastases

To confirm data obtained by qualitative RT-PCR, and to characterize the quantitative expression of CTA in sequential metastases, mRNA specific for MAGE-A1, MAGE-A3 and NY-ESO-1 was measured by quantitative RT-PCR analysis on primary melanoma cell cultures from metachronous metastases of patients showing variable (Mel 146, Mel 195, Mel 203 and Mel 458) or maintained (Mel 261) CTA expression profile over time by qualitative RT-PCR (*Figure 5*). A complete agreement was observed between qualitative and quantitative RT-PCR results, positivity by qualitative RT-PCR being associated with levels of expression $\geq 10^{-5}$ CTA mol /l β–actin mol.

Furthermore, quantitative RT-PCR revealed that, beyond the qualitative changes in terms of presence/absence of CTA, melanoma patients with variable CTA expression profile among metachronous metastases showed also quantitative variations exceeding 2-folds (range: 2.02 -3874.78) among CTA-positive metastases. In contrast, no quantitative variation ≥ 2-folds was observed in CTA expression levels among sequential metastases of Mel 261, which was characterized by stable CTA profile in qualitative RT-PCR analyses (*Figure 5*).

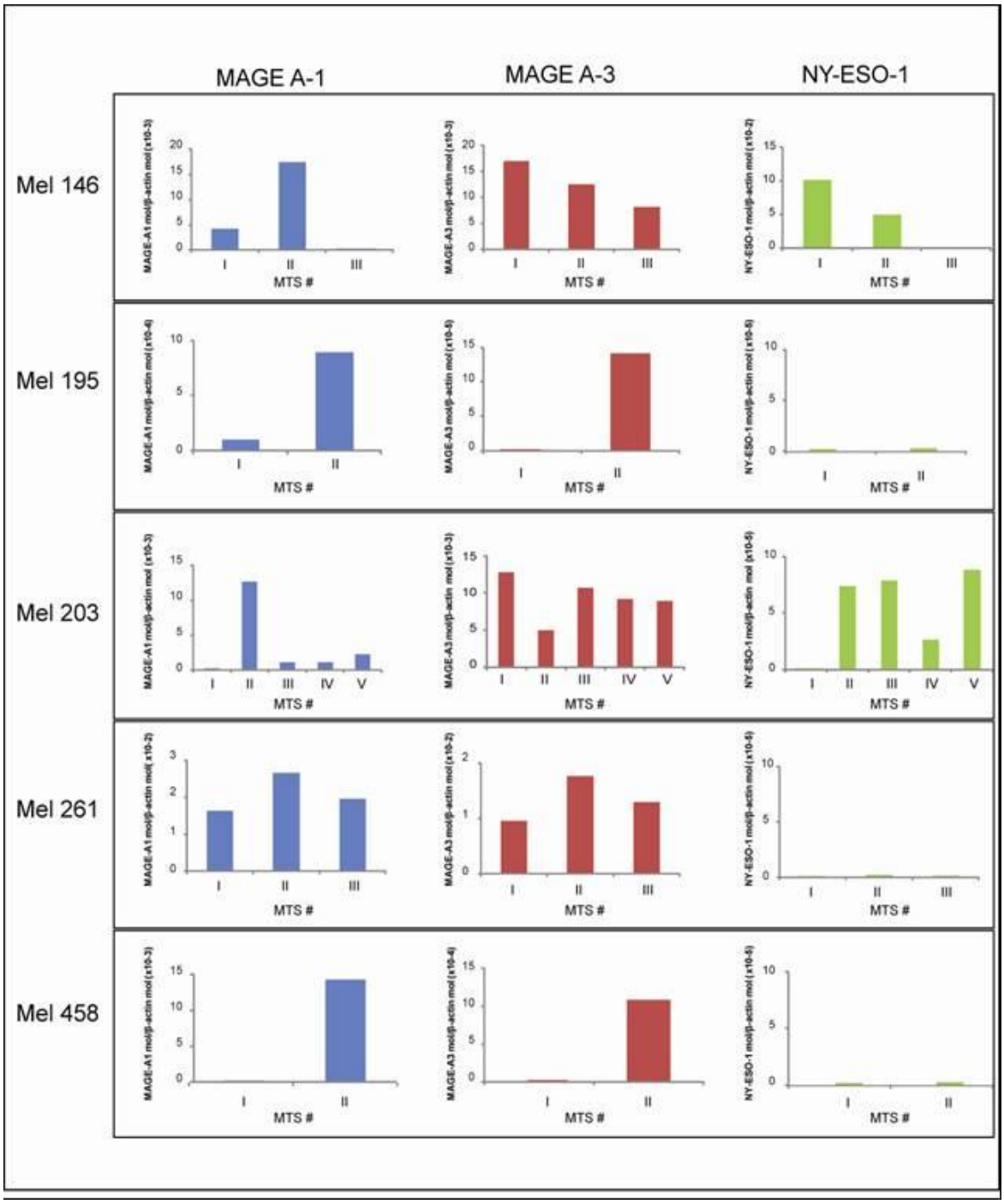

Figure 5: **Quantitative RT-PCR analyses of CTA expression in sequential metastases from 5 melanoma patients.** Retrotranscribed total RNA from primary cell cultures generated from metastatic lesions of each melanoma patient was subjected to SYBR green quantitative PCR reactions using MAGE-A1, MAGE-A3, NY-ESO-1 and β-actin-specific primer sets. The expression of each gene was normalized to the expression of the housekeeping gene β-actin, and data were reported as gene cDNA molecules/β-actin cDNA molecules.

Genomic DNA methylation changes in sequential melanoma metastases

Alterations in DNA methylation, including genomic hypomethylation, are a common feature of human cancer and may provide a shared mechanism to generally impact on melanoma cell biology, including immune recognition of cancer cells.

To define whether changes in the overall content of 5-methylcytosine occurr during melanoma progression, the extent of methylation of LINE-1 repetitive elements, used as surrogate of overall genomic DNA methylation, was analyzed in 45 primary cell cultures generated from sequential metastatic lesions surgically removed from 18 melanoma patients.

DNA methylathion level was considered to be maintained when the difference between LINE-1 values of the sequential metastases was less than 5% (*Figure 6*). The mean difference in the percentage of LINE-1 methylation among autologous metachronous metastases was 4.69 (range 1.10-19.65), with both increase and decrease in methylation observed along with disease progression. Accordingly, most patients (10 out of 18) were shown to maintain the DNA methylation profile over time. No common trend towards hypomethylation/hypermethylation during disease progression was observed in the remaining patients.

A decrease in methylation levels during the progression of the disease appeared to be associated with an acquisition of CTA expression, supporting the strong role of methylation in modulating CTA expression. Noteworthy, Mel 195 melanoma patient, who had a major decrease in LINE-1 levels during disease progression (*Figure 6*), showed a global gain of CTA, acquiring the *de novo* expression of 4 CTA (*Table 7*). These data were also supported by Spearman's rank correlation analysis, which showed a significant ($p \leqslant 0.05$) inverse correlation between LINE-1 methylation levels and the number of expressed CTA (rho= -

0.4881014) as well as the expression of MAGE-A3 (rho= -0.4650528), MAGE-A4 (rho= -0.4097523) and NY-ESO-1 (rho=-0.5138287) in the sequential metastases under study.

Interestingly, a marginally significative (p-value= 0.04) correlation was also observed between LINE-1 methylation and HLA class I antigen expression, though the correlation coefficent was low (rho: 0.35).

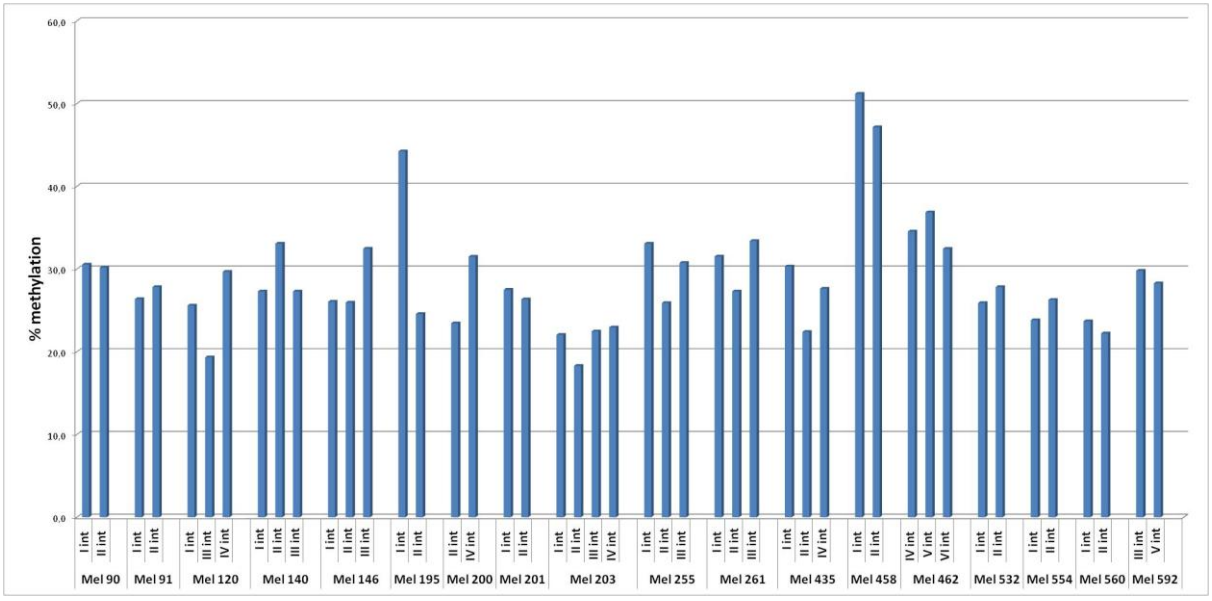

Figure 6: **LINE-1 methylation in sequential metastases.**
Representative histograms for the methylation levels of LINE-1, evaluated by bisulfite pyrosequencing analysis in short-term cultures of sequential melanoma metastases. All cells were analyzed at 6^{th}-7^{th} *in vitro* passage.

Activity of 5-AZA-CdR in sequential metastases

It has been shown that treatment with DHA, like 5-AZA-CdR, can revert the costitutively heterogeneous inter- and intra-tumor expression of therapeutic CTA, suggesting a strategy to augment antitumor immunity in cancer patients. To investigate the possibility to revert the observed costitutive heterogeneity of CTA expression among different sequential melanoma metastases, primary cell cultures derived from 9 metastases of 4 patients, selected according to heterogeneity in CTA profile and LINE-1 methylation levels, were

treated *in vitro* with 5-AZA-CdR. RT-PCR analysis revealed that 5-AZA-CdR induced the expression of all investigated CTA (MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, NY-ESO-1, SSX 1-5, GAGE 1-6) in all CTA-negative melanoma cell cultures, leading to a homogeneous CTA-positive expression profile among autologous metachronous metastases of different patients (*Table 9*).

Table 9: **RT-PCR analysis of CTA expression in sequential melanoma metastases treated with 5-AZA-CdR**

Pts	MTS #	treatment	β-actin	MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	NY-ESO-1	SSX 1-5	GAGE 1-6
Mel 146	I	ctrl	+	+	+	+	+	+	+	+
		5-AZA-CdR	+	+	+	+	+	+	+	+
	II	ctrl	+	+	+	+	+	+	+	+
		5-AZA-CdR	+	+	+	+	+	+	+	+
	III	ctrl	+	-	+	+	+	-	+	+
		5-AZA-CdR	+	+	+	+	+	+	+	+
Mel 195	I	ctrl	+	-	-	-	-	-	-	-
		5-AZA-CdR	+	+	+	+	+	+	+	+
	II	ctrl	+	+	+	+	-	-	+	+
		5-AZA-CdR	+	+	+	+	+	+	+	+
Mel 201	I	ctrl	+	+	+	+	-	-	+	+
		5-AZA-CdR	+	+	+	+	+	+	+	+
	II	ctrl	+	+	+	+	+	+	+	+
		5-AZA-CdR	+	+	+	+	+	+	+	+
Mel 458	I	ctrl	+	+	-	-	-	-	-	+
		5-AZA-CdR	+	+	+	+	+	+	+	+
	II	ctrl	+	+	-	+	-	-	+	+
		5-AZA-CdR	+	+	+	+	+	+	+	+

[a] Total RNA was extracted from treated (5-AZA-CdR) or not (ctrl) short-term cell cultures, established from initial (I) and subsequent sequential metastatic lesions removed from 4 cutaneous melanoma patients referred for surgery at the National Cancer Institute of Aviano (Italy). RT-PCR analysis was performed using CTA-specific primer pairs. PCR products were then separated on a 2% agarose gel and visualized by ethidium bromide staining.

The anlysis was further extended in order to comprehensively characterize the effects of DHA treatment on melanoma cells from metachronous metastases. To this end, Agilent whole human genome microarrays were employed to define the remodelling of gene expression profiles (GEP), induced by 5-AZA-CdR on primary cell cultures, established from metachronous metastases of Mel 195, Mel 201 and Mel 458 patients.

Data analysis demostrated that, on average, the expression of 3890 genes (range 409-4421) was modulated of at least 3 folds in metachronous melanoma cells under study, following 5-AZA-CdR treatment. To elucidate the biologic significance of the genes representing the expression signature of 5-AZA-CdR-treated *vs* untreated melanoma cells, the gene identifiers for the differentially expressed genes of all sequential metastases were linked to the "Onto-Express" web-based bioinformatic tool for global analysis of their function. As summarized in *Table 10*, among the highest scoring GO terms (considering $p \leq 0.05$ and number of associated genes ≥ 10) the most common biological processes modulated by 5-AZA-CdR treatment in melanoma cell cultures under analyses were: i) immune response (in 6 out of 6 metastases); ii) regulation of transcription (in 5 out of 6 metastases) ; iii) cell adhesion (in 5 out of 6 metastases); iv) signal transduction (in 4 out of 6 metastases); v) trascription (in 4 out of 6 metastases); vi) multicellular organismal development (in 4 of 6 metastases). Moreover, a consistent up-regulation of several CTA genes (belonging to MAGE-A, MAGE-B, MAGE-C, SSX and GAGE families), which are well-known as immune-related genes but are not included in the immunologic GO bioprocesses classes yet, was observed following 5-AZA-CdR treatment in all investigated cell cultures from autologous sequential melanoma metastases.

***Table 10*: Genes modulated by 5-AZA CdR in sequential metastases of 3 melanoma patients.**

Pts	MTS #	Biological process	genes #	p-value
Mel 195	I	signal transduction	84	0,002232
		regulation of transcription, DNA-dependent	43	6,51E-06
		immune response	39	4,14E-07
		transport	38	3,16E-04
		cell adhesion	35	0,003206
		transcription	32	5,85E-06
		cell-cell signaling	27	4,42E-06
		inflammatory response	25	4,80E-06
Mel 195	II	**immune response**	20	4,66E-07
		inflammatory response	14	1,99E-06
		cell differentiation	14	0,00266
		transport	14	0,009713
		G-protein coupled receptor protein signaling pathway	13	0,021904
		spermatogenesis	12	3,74E-05
		cell-cell signaling	10	0,002126
		apoptosis	10	0,030934
Mel 201	I	signal transduction	148	1,50E-09
		multicellular organismal development	91	7,43E-07
		regulation of transcription, DNA-dependent	64	5,44E-08
		cell adhesion	61	6,21E-07
		immune response	55	6,41E-09
		cell differentiation	51	6,00E-06
		G-protein coupled receptor protein signaling pathway	51	3,22E-04
		ion transport	51	5,45E-04
Mel 201	II	signal transduction	131	1,69E-04
		multicellular organismal development	99	6,99E-08
		regulation of transcription, DNA-dependent	76	2,53E-06
		cell adhesion	62	2,08E-06
		immune response	55	4,82E-08
		proteolysis	54	2,64E-06
		transcription	53	9,46E-08
		ion transport	52	0,001174
Mel 458	I	regulation of transcription, DNA-dependent	80	9,93E-04
		multicellular organismal development	69	0,011538
		cell adhesion	60	4,26E-07
		transcription	48	3,11E-07
		ion transport	48	0,001371
		immune response	42	1,37E-04
		proteolysis	39	0,00557
		cell-cell signaling	30	1,41E-04
Mel 458	II	signal transduction	247	3,07E-04
		regulation of transcription, DNA-dependent	218	0,008199
		multicellular organismal development	197	-1,70E-11
		transcription	157	5,73E-05
		cell adhesion	132	1,30E-11
		cell differentiation	102	1,22E-08
		ion transport	94	0,00189
		immune response	75	0,001173

DISCUSSION

In this study we reported for the first time the concomitant characterization of metachronous melanoma lesions for the presence of novel attractive and promising therapeutic targets for the treatment of patients with melanoma, namely the targeted-therapy target mutated BRAF and the immunotherapy target CTA. This was complemented by the characterization of metachronous melanoma cells for their expression of additional "immune molecules" required for their efficient immune recognition, their overall level of genomic DNA methylation and their sensitivity to GEP remodeling by DHA.

The analysis of the activating $BRAF^{V600E}$ mutation in metachronous metastases of melanoma patients, revealed that, once melanoma reaches the metastatic stage, its $BRAF^{V600E}$ mutational status remains substantially unchanged in subsequent melanoma metastases, regardless of the time-intervals to develop new metastatic lesions and site(s) of further metastatization. These data are in line with a recent study by Lin et al, who reported a marked degree of intratumor heterogeneity for activating BRAF mutations in primary melanomas, on the contrary of a major stability and homogeneous presence of mutated BRAF in metastatic lesions of melanoma patients. The lack of further modifications in the BRAF mutational condition, once melanoma has metastasized, can possibly result from absent/limited intratumor heterogeneity for the mutation in the metastatic disease. This hypothesis has been supported by our recent study, which demonstrated that single cell clones generated from the short-term metastatic melanoma cultures of 2 patients retained the heterozygous $BRAF^{V600E}$ mutated and homozygous wild-type genotypes of the parental metastatic cells, respectively (129). The accurate definition of the "evolutionary biology" of activating BRAF mutation in melanoma progression bears important practical implications

since BRAF mutation in metastatic cutaneous melanoma is the molecular hallmark to select patients for treatment with the highly effective BRAF kinase inhibitors under active clinical development (70). Based on the data available, it appears that activating BRAF mutations are positively selected during melanoma progression until reaching the metastatic stage when the BRAF mutational status stabilizes. This finding, together with the likely limited intra-tumor heterogeneity of BRAF mutations, suggests that the metastatic stage of BRAF mutated melanomas represents the most appropriate therapeutic setting for BRAF inhibitors. The highly stable BRAF status identified among metachronous melanoma metastases also bears important practical implications. In fact, any metastatic accessible tissue, either fresh or archival, regardless of the timing of metastasis and site of melanoma progression, being representative of the final BRAF mutational status of disease in a given individual, could be safely utilized to identify patients who are candidate to BRAF inhibitors. In clinical trials, treatment with selective BRAF inhibitors results in very high initial response rates which are, however, usually limited in time, with only a small proportion of patients who maintain a response for more than 12 months (68). The molecular mechanisms of melanoma resistance to BRAF inhibitors are being investigated, in order to develop therapeutic strategies for overcoming or preventing resistance. Initial data suggest that resistance to BRAF inhibitors is independent from the outgrowth of BRAF wild-type melanoma lesions (130). Being the BRAF mutational status stable over time in metastatic melanoma, it is reasonable to speculate that overcoming the BRAF inhibitors-induced intrinsic resistance of melanoma cells, could allow to continue or re-challenge patients with these highly promising therapeutic agents.

On the other hand, preliminary evidence suggests that oncogenic BRAF may contribute to immune escape through down-regulation of melanocyte differentiation

antigens on tumor cells, and that blocking BRAF activity via inhibitors leads to increased expression of these antigens and to an improved recognition of melanoma cells by antigen-specific CTL (131). Thus, combinations of BRAF inhibitors with immunotherapy may be another rational direction to pursue in the melanoma therapy, in order to take advantage both of the rapid and high response rates of BRAF-inhibitors and of the long-lasting disease control of immunotherapeutic approaches (131).

Among different targets of immunotherapy, CTA represent ideal candidates for tumor-specific vaccination strategies, because of their restricted expression in neoplastic cells but not in normal cells, except in testis and placenta (two immune-privileged organs without MHC-I expression) and their ability to elicit a spontaneous humoral and cellular immune-response (132). Further emphasis on CTA as important targets for melanoma immunotherapy came from most recent data demonstrating that clinical response to immunotherapy with anti-CTLA4 monoclonal antibodies correlate with cellular and/or humoral immune responses against CTA (79).

Despite the above reported advantages of CTA as therapeutic targets, an effective and durable clinical response to anti-CTA immunotherapies requires their homogeneous expression among all metastases of a given patient. In this context, very limited and discordant literature data are available about CTA-expression in synchronous and metachronous metastatic lesions of melanoma patients. In particular, Dalerba et al. demonstrated that CTA and melanoma differentiation antigens expression was homogeneous in the 82% of autologous metastatic lesions, collected from melanoma patients (133). Accordingly with this data, Sigalotti et al. have reported a maintained expression pattern of CTA and differentiation antigens among concomitant or sequential

metastases surgically removed from melanoma patients (98). Discordant results derived from other studies (134), which showed an unstable MAGE-A3 expression profile between sequential metastases (134) and reported that MAGE-A1 and MAGE-A4, but not NY-ESO-1, were acquired with advancing disease (135).

Our studies aimed at shading light on these contrasting literature data, taking advantage of early *in vitro* passage autologous neoplastic cells from metachrounous melanoma lesions, which allowed precise evaluation of melanoma-specific CTA profile without the interference of the contaminating normal cells that are present at different extents in whole tumor tissues. Analyzing a wide panel of CTA, we showed that only 13 out of 24 melanoma patients analyzed maintained the profile of CTA expression among sequential autologous metastases. The remaining presented variable changes in CTA expression patterns along with disease progression, resulting in gain or loss of CTA without a common trend. Similar variations were observed also for the quantitative levels of CTA expressed by autologous metachronous lesions. The absence of a univocal trend of variation of CTA expression along with disease course remarks the current lack of knowledge of the role of CTA in melanoma progression, which may depend of different factors, including: i) the cellular contest of action; ii) the interaction of CTA-positive neoplastic cells with the host. Besides CTA, it appears that melanoma progression is accompanied by a general instability of the expression of molecules involved in the immune recognition of cancer cells, as evidenced by the variations in the levels of HLA class I, HLA class II, β2-microglobulin, ICAM-1, LFA-1, LFA-3, and HMW-MAA observed among subsequent metastases. This overall instability of immune phenotype of melanoma cells along with disease progression clearly poses a major obstacle in long term systemic efficacy of melanoma immunotherapies.

In this context, the known role of DNA methylation in regulating the expression of different molecules involved in the immune recognition of cancer cells, strongly support the use of DHA to revert the heterogeneous expression of immune molecules among metachronous melanoma lesions, allowing their efficient targeting by the immune system. Support to this notion comes from our present findings demonstrating the ability of 5-AZA-CdR to generate a homogeneous CTA-positive phenotype among all primary cell cultures from metachronous melanoma lesions of investigated patients. Besides, a more comprehensive immunomodulatory activity of DHA on metachronous metastases could be recognized based on the finding that GEP profiling identified “immune response” as a shared biological process modulated by 5-AZA-CdR in autologous cell cultures. The clinical potential of DHA in improving immune targeting of melanoma cells is further sustained by pre-clinical *in vivo* studies demonstrating that systemic 5-AZA-CdR persistently induces and/or up-regulates the expression of CTA and HLA class I antigens on human melanoma xenografts (113). Along this line, initial studies on cancer patients showed that 5-AZA-CdR administration was able to induce long-lasting expression of CTA in both hematologic (114)and solid malignancies (115). Supporting the *in vivo* immunomodulatory activity of DHA, anti-CTA humoral immune responses were elicited in about 30% of patients in which de novo expression of CTA in the autologous neoplastic tissues was observed following treatment (115). Furthermore, a recent study showed that modifications of GEP induced by 5-AZA-CdR were preferentially restricted to tumour tissues and did not significantly affect normal tissues (Coral et al, unpublished). This represents a key learning in light of the clinical use of DHA as immunomodulatory agents. In fact, by preferentially targeting the sole tumour cells, DHA

should neither induce immune reaction(s) against normal tissues when utilized alone, nor increase that of immunotherapeutic agents in combination strategies (91) (136).

Overall, our present results provide a comprehensive characterization of promising therapeutic targets currently evaluated for the therapy of melanoma along with tumor progression, and provide insights on potential strategies to improve therapeutic success. Among these, the broad immunomodulatory activity of DHA strongly support their use to improve effectiveness and applicability of CTA-based immunotherapies as well as of non-specific immunotherapeutic strategies, such as immunomodulating antibodies (137), that are emerging as powerful therapeutic tools in solid tumours of different histotypes (81).

PUBBLICATIONS

Coral S., Covre A., Nicolay H.J.M.G., **Parisi G**., Rizzo A., Colizzi F., Fonsatti E., Fratta E., Sigalotti L., and Maio M. Epigenetic remodelling of gene expression profiles of neoplastic and normal tissues: immunotherapeutic implications. Submitted

Coral S., **Parisi G**., Covre A., Colizzi F., Nicolay H.J.M.G., Rizzo A., Fratta E., Sigalotti L., and Maio M. Immunomodulatory activity of SGI-110, a 5-aza-2'-deoxycytidine-containing demethylating dinucleotide. Submitted.

Sigalotti L*, Fratta E*, **Parisi G**, Coral S, and Maio M . Stability of BRAF V600E mutation in metastatic melanoma: new insights for therapeutic success?. *LS and EF equally contributed to this work. Br J Cancer. 2011 Jul 12;105(2):327-8.

Fratta E, Sigalotti L, Covre A, **Parisi G**, Nicolay HJ, Coral S, and Maio M. Epigenetic Mechanisms in Cancer Formation and Progression, in Cancer Epigenetics: Biomolecular Therapeutics for Human Cancer (eds A. Giordano and M. Macaluso), John Wiley & Sons, Inc., Hoboken, NJ, USA. doi: 10.1002/9781118005743.ch10.

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