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REVIEW ARTICLE

The Emerging Therapeutic Landscape of Advanced Melanoma

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Abstract: Melanoma is the deadliest form of skin cancer being responsible for 80% of skin cancer deaths. Furthermore, the incidence of metastatic melanoma has increased over the past three decades with a mortality rate that continues to rise faster than most of all other cancers. The last few years have witnessed an unparalleled change in treatment options for patients with metastatic melanoma by the development of new therapeutic strategies like targeted therapies and immunotherapies that highly improved the patient's prognosis. Despite the paradigm-shifting success of these novel treatments, their effectiveness is still limited by intrinsic or acquired resistance. The objective of this review is to provide an overview of the new available treatment modalities, criteria to select patients who might benefit from a specific therapy, mechanisms of innate and acquired resistance to these treatments and to discuss strategies to overcome drug resistance.

Keywords: Melanoma, chemotherapy, drug resistance, immunotherapy, targeted-therapies, BRAF, MEK.

1. INTRODUCTION

Melanoma is the most dangerous form of skin cancer and represents less than 5% of all skin cancers, yet is responsible for 80% of skin cancer deaths [1, 2]. The World Health Organization (WHO) estimates that each year there are 2-3 million new cases of skin cancer across the world, being 132000 in the most dangerous form [3-5]. The incidence of melanoma is increasing worldwide, especially in white populations, related to the excess of sun exposure. In Europe, the incidence rate has grown to 10-25 new cases per 100000 inhabitants, although countries such as Australia and United States of America have rates of 50-60 new cases/100000 inhabitants and 20-30 new cases/100000 inhabitants, respectively [6, 7].

Melanoma is a malignant tumor that arises from melanocytes, which are specialized pigmented cells, derived from neural crest and are found predominantly in the skin and hair follicles. Melanoma appears mostly in the skin but can also affect other tissues with melanocytes such as the eye (uvea, conjunctiva and ciliary body), meninges and various mucosal surfaces. It is considered one of the most aggressive human cancers, and although melanomas are usually heavily pigmented, they can also be amelanotic, having a great tendency to metastasize, thus contributing to its aggressive potential and unfavorable prognosis [6, 7]. About 90% of melanoma cases are diagnosed as primary tumors without evidence of metastasis and their 10-year survival is between 75 and 80% [7]. As mentioned before, melanocytes derived from neural crest cells. These cells undergo Epithelial-Mesenchymal-Transition (EMT) in order to migrate and exit from the neural tube. In a similar way, melanoma cells are able to undergo EMT in the initial events of metastasis to dissociate from surrounding keratinocytes [8]. In fact, metastases are the main cause of the death in melanoma patients [8, 9]. The route of metastasis can be either lymphatic or haematogenous. Around 2/3 of all metastases are originally confined to the drainage area or regional lymph nodes. The metastasis can be classified as regional or satellite if distances up to 2 cm from the primary tumor; as in-transit, if it is located between the primary tumor and the first lymph node at a distance of 2 cm (both with a 10 year survival of 30-50%); as micrometastasis if present on a sentinel lymph node biopsy (10 year survival rate of 30-70%); and as clinically recognizable regional lymph node metastases (10 year survival of 20-40%) [10].

For centuries, the main treatment for melanoma patients in the early stages was surgical resection and for metastatic melanoma chemotherapy and high doses of Interleukin 2 (IL2). Over 30 years ago, dacarbazine was approved by USA Food and Drug Administration (FDA) as the first chemotherapeutic, and was considered for a long time, along with high dose IL2 (approved by the FDA in 1992), the standard treatment in advanced melanoma. However, response is only achieved in about 10-15% of all cases of advanced melanoma, and is usually short in duration [11]. A study from University of Pittsburgh Cancer Institute including 243 melanoma patients showed that the overall and the complete response rates were 18,1 % and 8% respectively [12]. High dose IL2 is able to induce durable responses in some patients improving their long-term survival [13].

New treatments have emerged, targeting specific mutations or blocking negative immune checkpoints showing a greater impact on patient overall survival (OS) [13]. New treatment options include drugs targeting different members of the MAPK pathway, like vemurafenib and dabrafenib as BRAF inhibitors and trametinib, cobimetinib and binimetinib as MEK inhibitors. Alternative treatments include, ipilimumab and nivolumab which are immune checkpoint inhibitors targeting CTLA4 and PD-1, respectively [10, 14]. Other agents that inhibit activating mutations in the base of oncogenic processes, as B-RAF and c-KIT promise to show immediate responses and a very clear effect on tumor regression [14, 15].

Alternative therapies use oncolytic viruses (OVs) to induce tumor debulking, through the infection and lysis of oncogenic cells or by triggering acute vascular-disrupting effects and the induction of antitumor immunity. The most advanced agents in clinical development include talimogene laherparepvec (T-VEC, Amgen,

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herpes simplex virus, Phase III trial for melanoma), pexastimogene devacirepvec (Pexa-Vec, SillaJen Biotherapeutics and Transgene S.A., an oncolytic vaccinia virus, Phase IIb trial for hepatocellular carcinoma) and pelareorep (Reolysin, Oncolytics Biotech, a virus that belongs to Reoviridae family Phase III trial in combination with chemotherapy in head and neck cancer) [16-18].

The numerous studies showing efficacy of OVs, have granted the FDA approval of T-VEC in the treatment of advanced melanoma, which was a major development for the field [16]. Throughout this review, we intend to give an overview of the existent treatment options for advanced melanoma and evaluate what the future holds.

2. MELANOMA TREATMENT OPTIONS

According to the USA National Cancer Institute, melanoma therapy options are chosen according to the stage of the lesion (Table **1**), overall patient health, age, and treatment side effects [19, 20]. Surgical resection has been the first option in the management of early melanoma (stage I-III), although it has a minimal impact in the treatment of melanoma harboring regional or distant metastases [21].

2.1. Chemotherapy

Cytotoxic chemotherapy has been used for the treatment of metastatic melanoma (stage IV) for the last decades. Chemotherapy is based on the inhibition of the division of rapidly growing cells, which is a characteristic of cancerous cells, but it is also a characteristic of normal cells with fast proliferation rates, such as the bone marrow, skin cells, gastrointestinal tract cells and hair follicles cells. The fact that chemotherapeutic agents non-specifically target cells that are dividing rapidly is the major reason for their toxicity [22-25].

2.1.1. Dacarbazine and Temozolomide

The first chemotherapeutic agent used to treat advanced melanoma was dacarbazine (DTIC), an alkylating agent (covalently binds to an alkyl group to the DNA bases forming an adduct, thereby preventing multiplication of rapidly growing cells). DTIC has an overall response rate ranging between 10-20% and only allow a complete remission in 5% of patients [26-28]. DTIC is a pro-drug that requires conversion in the liver to its active compound (MTIC). Temozolomide (TMZ) is also a pro-drug that converts to the active compound MTIC, but it does not require liver conversion. TMZ also belongs to the alkylating agents group but has some advantages over many alkylating agents because of its unique chemical structure and pharmacokinetic properties. Its small weight allows the compound to cross the blood brain barrier. This drug has shown efficacy in the treatment of malignant brain tumors and metastatic melanoma [29-31]. Randomized trials that compared DTIC to TMZ showed that there was no significant difference in the overall survival or response rate [32]. Even though, TMZ appears to show advantages over DTIC; TMZ crosses the intact blood-brain barrier, only then converting to MTIC, having a potential in enhanced activity against brain metastasis. Objective response rate (ORR) is defined by the proportion of patients with tumor regression of a predefined amount, which, according to FDA, is determined by tumor assessments from radiological tests or physical examinations. Unfortunately, the ORR of metastasis originated from melanoma to TMZ is low, though there are some results showing that treatment with TMZ leads to a lower progression of brain disease [33].

DTIC has been used as an active comparator arm in clinical trials since 1992, and since then, more than 1000 patients were treated with it, showing an overall response rate of 13.4% and median survivals from 5.6 to 11 months [29]. However, in randomized phase III trials, it has failed to show improvement in overall survival. TMZ has shown similar results [30, 32]. Multiple schedules of DTIC and TMZ have been trialed and published, although, none has shown a greater efficacy than others [32].

2.1.2. Limb Perfusion and Electrochemotherapy

The clinical management of the metastatic lesions can be extremely difficult due to location and the nature of widespread. In-transit metastasis results from the presence of melanoma cells in cutaneous or subcutaneous lymphatic vessels between the primary tumor and the draining lymph nodes (without affecting them) [34]. Patients with in-transit metastasis are classified as stage III, and have a worse prognosis due the probability to invade lymph nodes and evolve for a distant metastatic disease [34, 35]. The first treatment choice for in-transit metastasis is surgery if the number of lesions is limited and no serious morbidities are predicted [36]. When surgery is not possible, Isolated Limb Perfusion (ILP) and Electrochemotherapy are considered, once they allow high doses of anticancer drugs to be delivered to the tumor, minimizing associated systemic drug toxicity. ILP has shown positive results in patients who have advanced melanoma confined to a limb, which usually results in a great number of satellite and/or in-transit metastasis. These patients frequently respond poorly to systemic therapies, since systemic doses of medication have to be greatly smaller due to systemic toxicity [37, 38]. LP offers a new opportunity to improve life quality and long-term survival of these melanoma patients who would otherwise require amputation [39].

The amount of drug that reaches the tumor mass is also limited by the drug pharmacokinetics (absorption, distribution, metabolism

Table 1. Treatment options, according to the staging of the lesion.

Stage (TNM Staging Criteria)	Standard Treatment Options
Stage 0 melanoma	Excision
Stage I melanoma	Excision +/− lymph node management
Stage II melanoma	Excision +/− lymph node management +/- Interferon adjuvant therapy
Resectable Stage III melanoma	Excision +/− lymph node management +/- Interferon adjuvant therapy Adjuvant therapy and immunotherapy
Unresectable Stage III, Stage IV, and Recurrent melanoma	Intralesional therapy Immunotherapy Signal-transduction inhibitors Chemotherapy Palliative local therapy

and elimination) [40, 41]. While in systemic chemotherapy drug doses are dependent on the systemic toxicity, ILP has the advantage to increase the drugs administration dose, reaching levels of 10 to 100-fold times higher, with few systemic effects and organ toxicity. Another advantage is that the drug is directly administrated into the tumor, avoiding hepatic and renal metabolism [42]. ILP provides an alternative to reach high drug concentrations on a limb affected by an unresectable tumor, since circulation of the limb is isolated from the systemic circulation. Hyperthermia is a key component in IPL. The main reason is that high temperatures are able to sensitize melanoma to cytotoxic therapy such as melphalan and Tumor Necrosis Factor (TNF), by increasing blood flow, membrane permeability, local metabolism, and consequently, drug uptake [38].

The most used chemotherapeutic agent in ILP is melphalan (L-phenylalanine mustard (LPAM)), which is a bifunctional alkylating agent, whose function relies on the use of phenylalanine in melanin synthesis (melphalan should provide selective toxicity to melanocytes and melanoma cells in which melanin is synthesized). Systemic melphalan is relatively ineffective because the maximally tolerated dose is much lower than the effective dose. This limitation is overcome in ILP, that enables the toleration of a much higher dose locally [42]. Other drugs are still under clinical trial for its use in ILP, either alone or in combination with melphalan, such as TNF-α, ADH-1/Melphalan, Sorafenib/Melphalan, Temozolomide, Cisplatin and Ipilimumab [43].

Although ILP has had great results, it also comes with major disadvantages, mostly connected with the technique, which is highly invasive and complex. Repetition of the procedure after recurrence is also highly debatable, since it may result in major complications related to the scar tissue from the previous surgical approach of the vessels [37].

Electrochemotherapy (ECT) is a local treatment modality that allows the treatment of cutaneous and subcutaneous metastasis in melanoma patients. This method combines electroporation, the use of short electric pulses to destabilize cell membrane creating a transient increased permeability and chemotherapy: the increased permeabilization allows the entrance of chemotherapeutic drugs. ECT increases drug effectiveness by facilitating the interaction between drugs and its intracellular targets [36, 44]. This technique is considered highly effective in the treatment of melanoma metastasis once it potentiates the cytotoxicity of chemotherapeutic drugs. Bleomycin and cisplatin are considered the most effective drugs for ECT [45]. In fact, using ECT the cytotoxic effect of bleomycin was demonstrated to be increased 8000 times, and for the cisplatin ~80 times [46]. A meta-analysis for ECT presented a complete response of 59% of the patients [47].

2.1.3. Polichemo-Immunotherapy

The use of both chemo and immunotherapy is being studied based in high responses in a small phase II studies. In a phase II study, the administration of IFN-α with IL2 increased the response rate of 20% to 40% [48-51]. Based in these studies, and in the fact that IL2 has promising results when administrated as a single agent, the European Organization for Research and Treatment of Cancer (EORTC) investigated if the addition of IL2 to dacarbazine, cisplatin and IFN-α had an impact on patient's survival. This phase III trial revealed that although IL2 alone or in combination with IFN-α had positive results, when IL2 is combined with chemo-immunotherapy it has no clinically relevant results. Moreover, chemo-immunotherapy caused an increased toxicity when compared to chemotherapy alone [48]. The same group, EORTC, investigated whether it would be possible to identify the melanoma patients that could benefit from intense chemo-immunotherapy after two cycles of dacarbazine monotherapy. In this phase III study, the authors show that the prior treatment with two cycles of dacarbazine was not a good method to select the patients who could benefit from intense chemo-immunotherapy [52]. Several studies show the same conclusion: that chemo-immunotherapy has no benefits over chemotherapy alone [53-56].

2.2. Oncolytic Viruses

Therapy with oncolytic viruses has been considered as a promising therapeutic approach in cancer treatment. Oncolytic viruses are genetically engineered or naturally made viruses that can select and replicate inside neoplastic cells, leaving the normal cells unharmed, increasing simultaneously the host immune tumor cells and oncolysis exposed viral antigens recognition [57, 58]. Oncolytic viruses are classically divided in two categories: (a) viruses that replicate preferentially in cancer cells and are non-pathogenic to humans, given their high sensitivity to innate antiviral signaling or dependence on oncogenic signaling pathways, including autonomous parvoviruses, reovirus, myxoma virus, among others; (b) genetically-manipulated viruses used as vaccine vectors, such as poliovirus, vaccinia virus, measles virus, and genetically-engineered viruses with increased replication in cancer cells, including adenovirus, herpes simplex virus and others [59, 60].

Mechanisms of protection against viral infection (for example, interferon-beta signal pathway) are compromised in most oncologic cells, allowing viral replication in a greater extent in cancer cells than in normal cells. The greatest problem to overcome is making a virus that has cancer cell-specific replication, but does not replicate in normal cells [61].

Talimogene laherparepvec was the first oncolytic virus to demonstrate response improvement in a phase III trial, and became the first FDA approved oncolytic immunotherapy [62]. Talimogene is a herpes simplex virus type 1 (HSV-1)-based intralesional oncolytic immunotherapy that is able to increase antigen presentation and accomplish selective tumor lysis [63]. The increased anti-tumor immune response is attained by the insertion and expression of the human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene, resulting in local production of GM-CSF that leads to the induction of tumor-specific T-cell responses [64]. The FDA approval was based on a phase III trial which had 436 patients with unresectable stage IIB melanoma, showing a significantly increased durable response rate, when talimogene was compared to subcutaneous GM-CSF (16.3% against 2.1%, respectively), and an improved overall survival, of 23.3 versus 18.9 months, also from the comparison of talimogene and GM-CSF [65].

2.2.1. The Role of Oncolytic Viruses in Adjuvant Therapy

The purpose of adjuvant therapy is to reduce the risk of relapse caused by occult disease and is offered to patients after surgical treatment has removed all detectable disease [66]. High-dose interferon was approved by the FDA as adjuvant therapy in melanoma, since the majority of all published studies and meta-analyses showed improvement in relapse-free survival [66, 67].

Recent studies provided evidences of the potential role of oncolytic viruses combined with immunotherapeutic agents in adjuvant therapy. An open label-phase II study compared Talimogene laherparepvec in combination with ipilimumab (an immune checkpoint inhibitor, discussed below) and ipilimumab alone [68]. This study was performed in one hundred and ninety-eight patients with advanced melanoma, in which ninety-eight were treated with Talimogene laherparepvec (21.1 weeks) plus ipilimumab (9.1 weeks) and one hundred of the patients with only ipilimumab (9.1weeks) [68]. The overall survival rate was increased in patients that received both therapies (39%) compared to the ones that were only treated with ipilimumab (18%). The combination of Talimogene laherparepvec plus ipilimumab seemed to have increased efficacy, when compared to the immune checkpoint inhibitor alone [68].

2.3. Immunotherapy

Immunotherapy is defined as the use of the immune system to treat cancer. This approach, including cytokine and vaccine treat-

ments is an alternative to conventional chemotherapeutic drugs [69-71]. One of the first used "immunotherapeutic tools" in the 90's was Interleukin 2 (IL2). Treatment of IL2 results in tumor cell death *in vitro*, by stimulation of NK and CD8+ cells that acquire cytolytic properties IL2 was approved by the FDA in 1998, based on the durable complete response in 8 phase I and II studies [72]. Initial studies revealed that IL2 is able to induce tumor regression in melanoma and other malignancies such as colon-cancer and renal cancer [73, 74]. Yet, IL2 has shown some degree of toxicity mainly associated with vascular leak syndrome, a phenomenon characterized by an increased vascular permeability along with protein and fluid extravasation, resulting in interstitial edema and organ failure [73, 75].

2.3.1. Checkpoint Inhibitors

Recently, three new immunotherapeutic drugs have been approved by the FDA to treat melanoma. Ipilimumab, an antagonist monoclonal antibody to CTLA-4 (approved in 2011), Pembrolizumab and Nivolumab, both antagonist monoclonal antibodies to PD-1 (approved in 2014) (Fig. **1**) [71, 73, 76]. All of these options have shown a positive impact in the patient's overall survival. In fact, when combined, these drugs can produce response rates above 50%. Several studies demonstrate that these drugs are more efficient and less toxic when compared to IL2 [73, 77, 78].

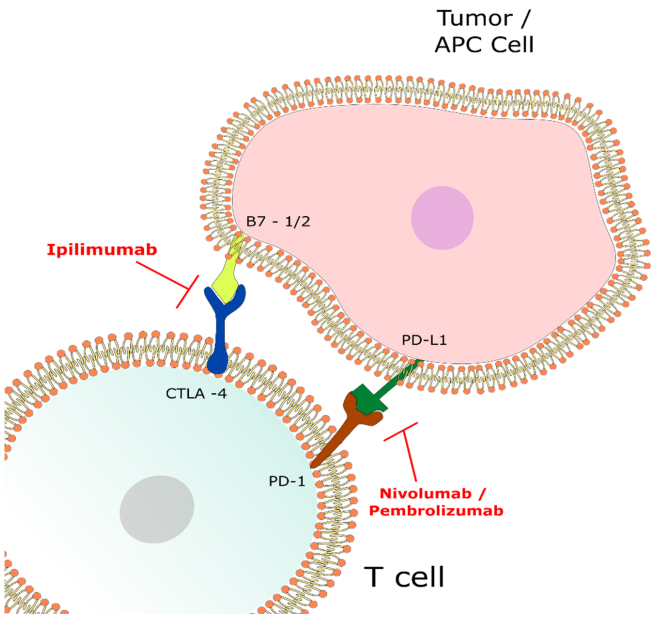

Fig. (1). Immune checkpoint blockade: Ipilimumab (against CTLA-4) blocks the immunosuppression induced by the interaction between the B7 family and CTLA-4 proteins. Nivolumab or Pembrolizumab (against PD-1) block the interaction of PD-L1 ligand to its receptor. The inhibition of these immune checkpoints allows the immune system to target cancer cells.

The primary effector cells of the adaptive immune response against cancer are the T lymphocytes that include helper T cells and cytotoxic T lymphocytes [73, 77]. Instead of attempting the direct stimulation of cytotoxic T cell response, recent trials aimed at the inhibition of immune checkpoints, with more positive results. Immune checkpoints regulate negatively T-cell activation and modulate T-cell activity towards the maintenance of self-tolerance. Nonetheless, when there is chronic antigen exposure, they are responsible for exhaustion of T-cells, and their mechanisms can be co-opted by cancers as a mean of immune evasion [73, 77].

Ipilimumab (Yervoy; Bristol-Myers Squibb, Princeton, NJ) is a human monoclonal antibody that up-regulates T-cell activation, proliferation and effector function, by the blockage of CTLA-4 (cytotoxic T lymphocyte antigen-4 - protein belonging to the immunoglobulin superfamily mainly expressed in activated lymphocytes and conduced with T-cell–mediated cytotoxicity), which is overexpressed in many cancers, including melanoma. It is currently the only FDA-approved checkpoint inhibitor that targets the CTLA-4 pathway [79, 80]. Ipilimumab was approved by the FDA in 2011, based on two prospective, randomized, international trials, one in untreated patients and the other in previously treated patients that showed clinical benefit by prolonging overall survival [80, 81].

The trial with patients that were previously treated had 676 patients, with unresectable stage III or stage IV melanoma. Nearly four hundred of those patients randomly received ipilimumab with glycoprotein (gp) 100 peptide vaccine, 137 were only administered with ipilimumab and 136 only received the gp 100 vaccine. The median overall survival was 10 and 10.1 months with only ipilimumab and ipilimumab plus the vaccine, respectively, compared to 6.4 months with de gp 100 vaccine alone. One year after the treatment, around 45% of the patients from both groups treated with ipilimumab and ipilimumab plus the vaccine were alive, and only 25% of the patients treated with only the vaccine still lived [82, 83]. In 2011, a second phase III trial was designed to compare Ipilimumab to DTIC in 502 patients with untreated metastatic disease. Nearly half of the patients (250) were randomly assigned to receive Ipilimumab plus DTIC while the other 252 received DTIC plus placebo. The median overall survival was 11.2 months for the ipilimumab-DTIC group, and 9.1 months for the placebo-DTIC group. Estimated survival rates at 1 year were 47.3% for the ipilimumab plus DTIC group, and 36.3% for the placebo plus DTIC group [82, 83].

Although Ipilimumab increases activity against tumor cells, it may cause adverse effects by deregulating the immune tolerance to self and cause autoimmune side effects, such as dermatitis, colitis, hepatitis, hypophysitis and thyroiditis. These Immune Related Adverse Events (irAEs) may be controlled and are responsive to systemic corticosteroid therapy or other immune suppressive agents, and curiously, tumor responses may occur even after treatment is stopped to initiate immunomodulatory therapy [73, 83].

PD-1 was first discovered in a T-cell hybridoma undergoing T-cell receptor activation–induced cell death, (giving it its name programmed death 1), and apparently causes cell death by diminishing cell growth factors and survival signals [84]. PD-1 receptor is an inhibitory receptor, and it's signaling inhibits T-lymphocyte activation, reducing its proliferation, production of cytokines and T-cell cytolyses [84].

Nivolumab, a human antibody, and Pembrolizumab, a humanized antibody, are the anti PD-1 antibodies at most advanced stages of development used in melanoma. Binding of these antibodies to PD-1 receptor, prevents its interaction with ligands on tumor cells, leading to the abolishment of the signal that otherwise would lead to inhibition of T-cell proliferation and cytokine production, also promoting immune activation [80]. Accelerated approval of Nivolumab was based on a trial with previously treated patients, where 120 patients were assigned in a proportion 2:1 to nivolumab or the investigator's choice of chemotherapy (DTIC or combination of carboplatin plus paclitaxel). Patients were required to have unresectable or metastatic melanoma that had progressed after treatment with ipilimumab and, in the presence of a BRAF V600 mutation, it was added a BRAF inhibitor. ORR, was observed between 19% and 41%, and stable disease lasting 24 weeks or more was observed in 6% [79]. A trial with 418 previously untreated patients with unresectable stage III or stage IV melanoma not harboring a ***BRAF*** mutation were randomly assigned to receive nivolumab and a DTIC-matched placebo or DTIC with a nivolumab-matched placebo. The trial showed a significant difference in overall survival in favor of nivolumab, with OS rate of 1 year of 72.9% in the nivolumab group and 42.1% in the dacarbazine group [85].

The FDA label provides recommendations for suspected immune related adverse events (irAEs), including withholding the drug and administering corticosteroids [79].

A total of 173 patients with unresectable or metastatic melanoma with disease progression within 24 weeks of the last dose of ipilimumab and, if ***BRAF*** V600 mutation positive, previous treatment with a ***BRAF*** inhibitor, were randomly assigned to one of two doses of pembrolizumab—2 mg/kg or 10 mg/kg. Overall response rate was 26% in both arms. The approved dose was 2 mg/kg administered as an intravenous (IV) infusion for 30 minutes every 3 weeks [86].

A trial with previously untreated and treated patients randomly assigned 834 patients with metastatic melanoma to receive pembrolizumab or four cycles of ipilimumab. Overall survival results showed that the pembrolizumab group was superior to the OS than in the ipilimumab group. The estimated survival rate after 12 months treatment was 74% for patients receiving pembrolizumab and 58% for patients treated with ipilimumab [87].

The FDA label provides recommendations for suspected irAEs, including withholding the drug and administering corticosteroids. Overall, immune checkpoint inhibitors have increased the outcome of melanoma patients when compared to conventional chemotherapy. A meta-analysis comparing patients treated with conventional drugs and patients treated with anti-CTLA4 and anti-PD1 revealed that the latter had better outcomes compared to conventional chemotherapy treatment [88]. However, there are still reports of patients that show intrinsic resistance to immunotherapies [89]. One example is that tumors can express proteins with few molecular changes, making the immune system unable to recognize these antigens as foreign. It is also possible that, with tumor development, cancer cells lose a proportion of its non-silent mutations, producing lower ratio of antigenic epitopes allowing the tumor to adapt and escape immune surveillance, a phenomenon called the immunoadaption of tumors [89]. Moreover, cancer cells have developed mechanisms to escape the immune system resulting in a less efficient therapy [69, 70, 90, 91]. The fact that melanoma cells are able to acquire resistance to chemotherapeutic agents and have the ability to escape immune surveillance makes it urgent to identify reliable biomarkers capable of predicting the response to negative immune checkpoints inhibitors and to develop new effective treatments. In the past few years much attention has been focused on the development of targeted therapies [5, 90].

2.3.2. Immunotherapy Combinations

The combination of CTLA-4 and PD-1 blockade has shown synergism in preclinical mouse models, leading to the clinical development of this dual therapy [92]. A phase III trial, with 945 previously untreated patients with advanced melanoma, confirmed these results. In this particular study, patients were randomly assigned to receive nivolumab alone, nivolumab in combination with ipilimumab or ipilimumab alone. Response rates of monotherapies were similar to previous studies, but the combination of nivolumab and ipilimumab showed an improvement in response and in progression free survival (median of 11.5 months versus 6.9 months with nivolumab and 2.9 months with ipilimumab) [93].

2.3.3. Immunotherapy as an Adjuvant Therapy

Recently, Ipilimumab has been tested for adjuvant treatment. Eggermont *et al*, demonstrated in a randomized phase III study with 950 resected stage III melanoma patients that Ipilimumab treatment significantly improved overall survival and metastasis free survival compared to the placebo treatment [94]. Ipilimumab treatment reduced death risk in 28% compared to the placebo and also the risk of distant metastasis in 24% [94]. Later, in 2015, FDA approved Ipilimumab as adjuvant therapy based on another phase III trial with resected stage III melanoma patients, in which the overall survival rate during the 5 years follow up with ipilimumab was 65.4% compared to the placebo, 54,4% [95]. A different study compared ipilimumab and nivolumab (another immune checkpoint inhibitor) as adjuvant treatments. This study included 906 melanoma patients in stage III or IV that were randomly assigned to receive nivolumab or ipilimumab. Results revealed that 12 months recurrence free survival was higher with nivolumab, 70,5% compared to ipilimumab 60,8% [96].

2.4. Targeted Therapies

Targeted therapies interfere with disease-specific proteins involved in tumorigenesis [22, 97]. These therapies are considered to be the future of cancer treatment and much attention has been focused on developing inhibitors for Mitogen Associated Protein Kinase (MAPK) signaling pathway. MAPK pathway is often hyperactivated in melanoma due to mutations in BRAF and NRAF (two thirds of melanomas) [3, 98, 99]. Activation of the MAPK pathway, a gene regulator of growth and proliferation of normal cells, seems to be critical in the pathogenesis of most melanomas [100]. Extracellular ligands bind to specific membrane-bound receptor tyrosine kinases to initiate the MAPK signaling. Subsequently, there is recruitment and activation of the guanosine triphosphatase RAS resulting in phosphorylation of the serine/threonine kinases RAF. RAF phosphorylates MAPK kinase (MEK) and causes activation of extracellular signal-regulated kinase (ERK) as summarized in Fig. **2**. Constitutive signaling of this pathway leads to oncogenic cell proliferation and avoidance of apoptosis [101].

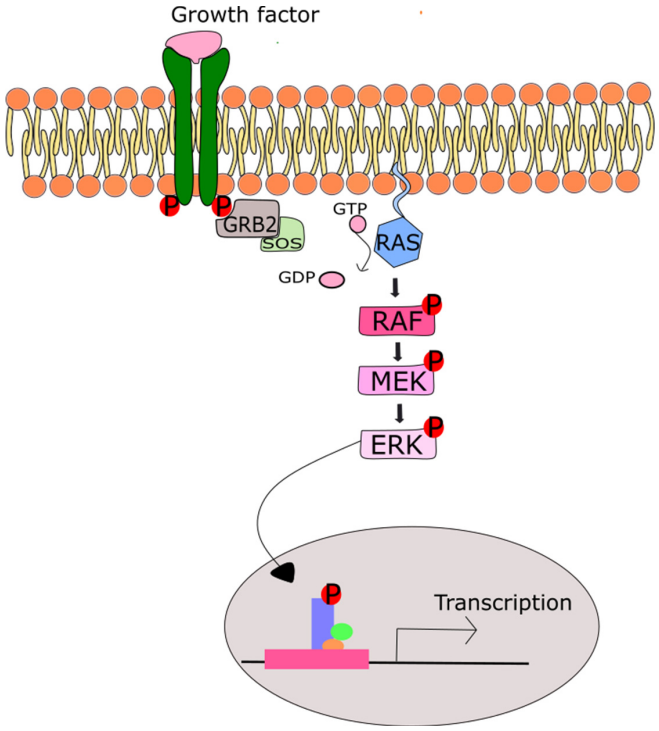

Fig. (2). The MAPK signaling pathway. Growth factors bind to the tyrosine kinase receptor, which brings SOS into close proximity. GDP-RAS is converted into GTP-RAS and phosphorylates RAF. RAF phosphorylates MEK, and MEK phosphorylates ERK. ERK translocates into the nucleus and stimulates transcription of target genes.

MAPK and PI3K pathways are key regulators of cell proliferation in melanoma. The most common mutations in melanoma occur in the *BRAF* (~50%), *PTEN* (30-50%) and *NRAS* loci (10-20%) [102]. Despite recent therapeutic advances in the treatment of advanced melanoma, targeting RAS has not been so successful. NRAS mutations are commonly found in codon 12, 13 and 61 [103]. These mutations lead to an increased activity of RAS being

associated with a poor prognosis. The hyperactive RAS can activate both MAPK and PI3K pathways leading to tumor progression and cancer cell survival [103]. Although, much effort has been put into developing drugs capable of inactivating *NRAS*, to date no effective anti- RAS therapies have been successfully made into the clinic [103-106].

2.4.1. BRAF Inhibitors

BRAF, one of the downstream effector of NRAS, is one of the three human Raf genes (together with A-raf and C-raf). BRAF is a serine/threonine kinase that is frequently hyper activated due to somatic mutations. This is one of the most common mutated genes in melanoma (50-70%) being the most common mutation a substitution of a glutamic acid for a valine at position 600 (V600E) [107]. Mutations in the BRAF gene are associated with activation of the MAPK pathway, leading to a 10-fold increase of the activity of the mutated protein compared to the wild-type BRAF, inducing proliferation and angiogenesis, preventing apoptosis and therefore enhancing the oncogenic activity of melanoma [11, 100].

Sorafenib was the first nonselective BRAF inhibitor that was tested in clinical trials, due to its efficacy in *in vitro* studies and melanoma xenograft models. However, it did not show clinical benefit, either in monotherapy or combined therapy with other chemotherapeutic agents, such as DTIC, carboplatin and paclitaxel, in patients with metastatic melanoma [11, 108-110]. The limited activity of sorafenib in tumors harboring BRAF mutations lead to the development of novel and more selective RAF inhibitors, such as vemurafenib. The latter was the first molecularly targeted therapy for melanoma patients approved by the FDA in 2011. This drug has shown potent anti-proliferative effects in several preclinical models, including the ones harboring the V600E mutation. The mechanism of action involves selective inhibition of the mutated BRAF V600E kinase, which leads to reduced MAPK signaling activity [71, 105, 111].

Pre-clinical studies showed that it was able to inhibit the activity of mutated BRAF, with consequent cell-cycle arrest and apoptosis induction in melanoma cells [112]. Based on clinical data, a new phase I trial was initiated to evaluate efficacy, safety and pharmacokinetics of vemurafenib. Maximum tolerated daily dose was established, and the overall response rate was 80%. A phase II trial was initiated with similar positive results as in the phase I. Squamous cell carcinoma was seen in some patients as a secondary effect from the therapy with vemurafenib, explained by the reactivation of the MAPK pathway in non-melanoma BRAF wild-type cells. Median overall survival was 15.9 months [113]. In the phase III trial, vemurafenib was compared to intravenous DTIC in previously untreated patients with the mutated BRAF and metastatic melanoma. Close to 700 patients were assigned to receive vemurafenib or DTIC, until disease progression or unacceptable toxicity. Overall survival at 6 months and overall response was 84% and 48% in the vemurafenib group and 64% and 5.5% in the DTIC group, respectively. The positive results led to a precocious ending of the clinical trial, and the FDA approval of vemurafenib for the treatment of metastatic or unresectable melanoma with the BRAF mutation V600E [114]. In 2013, FDA approved dabrafenib for the treatment of BRAF V600-mutated metastatic melanoma. A phase II trial dose induced responses in 69% of patients with either BRAF V600E or V600K mutated melanoma. Additionally, it showed activity in brain melanoma metastases. The response rate of 59% was better in the group harboring the V600E mutation when compared to 13% response rate of the patient group harboring the V600K mutation. Progression free and overall survival also showed better results in the patients with the V600E mutation [11]. Dabrafenib was then compared with DTIC in a phase III trial, and showed a significant reduction of 70% in the risk of disease progression in patients treated with dabrafenib, that were previously untreated, and had advanced melanoma with mutated BRAF [100].

Despite the huge success of BRAF inhibitors, there are reports of resistance by re-activation of MAPK signaling, changes in ERK1/2 regulated cell cycle events, activation of alternative signaling pathways and chromatin-regulating events [115]. Re-activation of MAPK signaling can emerge due to mutations on RAS, which promotes C-RAF dimerization and activation and due to ERK mutations. In fact, a study has demonstrated that elevated expression of C-RAF was associated with a mutant BRAF melanoma cell resistance to AZ628, a RAF inhibitor [106, 115, 116].

Considering these complications, a good strategy is the development of new inhibitors for downstream effectors of BRAF, such as MEK. Nowadays, selective MEK inhibitors represent a promising new therapeutic option in BRAF and NRAS mutated melanomas. Some studies demonstrate that preclinical models with BRAF and NRAS mutations are sensitive to MEK inhibitors [106, 117].

2.4.1.1. Combined Targeted Therapies

A trial with dual BRAF and MEK inhibition showed increased apoptosis and delayed of onset resistance compared to BRAF inhibitors alone [118]. Also, a common mechanism of resistance to BRAF inhibitors is MAPK pathway reactivation, so it was hypothesized that the dual therapy with BRAF and MEK inhibitors would eventually overcome such resistance [119]. One of the earliest phase III trials proved that dual therapy with dabrafenib and vemurafenib had clinical benefits when compared to a single agent dabrafenib or vemurafenib strategy, improving patient´s median overall survival [120].

This and other studies with other BRAF or MEK inhibitors demonstrated better response rates, meaningful improvement in overall survival and a manageable toxicity profile, for melanoma patients with the BRAF V600 mutation, treated with the dual BRAF/MEK inhibition [121].

2.4.2. MEK Inhibitors

MEK 1 and 2 are downstream kinases of RAF and are promising therapeutic targets in tumors harboring NRAS and BRAF mutations. Selective MEK inhibitors represent a promising new therapeutic option in BRAF and NRAS mutated melanomas. Some studies demonstrate that preclinical models with BRAF mutations are sensitive to MEK inhibitors [112, 117]. Patients harboring NRAS mutations were found to be partially sensitive to MEK inhibitors [112,117]. The same happened in BRAF mutated melanoma murine xenografts, where MEK inhibitors contributed to tumor regression through increased apoptosis and reduced angiogenesis and proliferation [117, 122, 123]. MEK inhibitors can be classified in two major classes: ATP competitive or non-ATP competitive inhibitors [124]. The ATP competitive inhibitors bind to the ATP binding site of MEK, preventing MEK to be phosphorylated. Most of MEK inhibitors are non-ATP competitive, which means that they bind to an allosteric binding site close to the ATP binding site preventing MEK activation. The MEK1/2 binding sites are relatively unique making the non-ATP competitive inhibitors highly specific [124, 125]. Trametinib is an orally available, small molecule, non-ATP competitive MEK inhibitor that induces cell cycle arrest, reducing tumor grow. It was proven to be clinically effective in the presence of BRAF and NRAS mutations. Therefore, it was accepted by FDA as a single agent for the treatment of patients with V600E BRAF and in combination with dabrafenib [117, 126]. In a phase III trial, 322 patients with BRAF V600E/K-mutant metastatic melanoma were randomly assigned to receive trametinib or conventional chemotherapy (either DTIC or paclitaxel). Progression free survival and overall survival was greater in the trametinib group at 4.8 months and 81%, compared to the chemotherapy group, that was 1.4 months and 67%, respectively. As result, FDA approved trametinib in 2013 for the treatment of patients with unresectable stage IIIC or metastatic melanoma harboring BRAF V600E/K mutations [127].

2.4.3. c-KIT Inhibitors

KIT is a type III receptor tyrosine kinase, and along with its ligand, stem cell factor (SCF), also known as c-kit ligand, are essential elements in the development of melanocytes in vertebrates, regulating its growth, migration, survival and differentiation [128]. Earlier studies showed that KIT protein is lost during progression of melanoma, based on its levels of expression in benign nevi compared to primary and metastatic melanoma, suggesting that it might have a tumor-suppressing role in melanoma [129, 130].

Imatinib is a KIT inhibitor, previously approved and with well-established efficacy in other types of cancer, such as gastrointestinal stromal tumors and dermatofibrosarcoma protuberans. In a phase II trial, 295 patients were tested for the presence of KIT mutation and amplification. The patients that scored positive for the mutation were treated with imatinib, with overall response at 16%, median time to progression of 12 weeks and median overall survival of 46.3 weeks [131].

Other phase II trial with a group of 43 patients with metastatic melanoma and c-Kit mutations and amplification, was given imatinib on a daily dose, unless they showed signs of intolerable toxicity or disease progression. Median progression-free survival was 3.5 months and the 6-month PFS rate was 36.6%. One-year overall survival rate was 51% [132].

Due to the low prevalence of KIT mutations in melanoma, other trials are required to understand the clinical implications of each individual mutation, as well as resistance mechanisms [131].

CONCLUSION

Melanoma is the deadliest form of skin cancer, being responsible for 80% of skin cancer deaths. Chemoresistance and the high rate of metastasis are the main reasons for treatment failure [4, 5, 26].

Surgery has been used for many centuries for the treatment of melanomas found in the early stages. Until the last decade the standard care for metastatic melanoma included chemotherapy and high IL2 doses [5]. The fact that chemotherapeutic agents non-specifically target cells that are dividing rapidly is the major reason for their toxicity. The high doses of IL2 were also associated with significant toxicity. The resistance to conventional chemotherapeutic agents in melanoma leads to an extremely poor prognosis. In the last decade two new therapeutic strategies emerged: immunotherapies based on negative immune checkpoint inhibitors and targeted therapies including BRAF and MEK inhibitors [5, 90]. These treatments revolutionized the standard care for melanoma patients. However, resistance is still a major concern [5, 90]. The main deregulated pathways in melanoma are PI3K and MAPK pathways [102]. The development of BRAF inhibitors, such as vemurafenib, improved the outcome of melanoma patients, but again most of the patients who initially respond, eventually acquire resistance to vemurafenib [115]. Considering these complications, a promising strategy is the development of new inhibitors for downstream effectors of BRAF, such as MEK. Trametinib and cobimetinib, both MEK inhibitors were approved by the FDA, and proven to be clinically effective in the presence of BRAF and NRAS mutations [112, 117]. It is believed that the future of melanoma treatment resides in combined targeted-therapies, mainly the ones directed to MAPK and PI3K pathways and immunotherapies. Immunotherapies highly improved the long-term survival in melanoma patients although some factors can have an impact on this therapy such as patient's immune system and the possibility of adverse reactions as well as the patient financial status. It has been proved that the re-activation of PI3K or MAPK signaling pathways contributes to therapy resistance culminating in tumor progression and a very poor prognosis [133]. Some studies show that the response rate to these therapies is higher compared to standard chemotherapy [134]. Yet, new research will aid elucidating the resistance mechanisms underlying the use of this novel targeted therapies. Another promising strategy is the use of this dual inhibition combined with immunotherapy including IL2, interferon, anti-CTLA4 and anti-PD1 [133, 135]. Future research may be focused on improving the risk-benefit of targeted therapies and immunotherapies by understanding and establishing biomarkers in order to provide information about the patient response to the treatment. Hopefully, the ability to distinguish patients that may benefit from these treatments may improve the clinical outcome of melanoma patients [136].

In summary, despite the advances in the field of melanoma treatment in the past few years, there are still significant obstacles to be overcome that should be treated as a priority in melanoma treatment. Understanding the resistance mechanisms to therapeutic agents can certainly improve the outcome of current therapies and contribute to the development of new therapeutic approaches.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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