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Chapter 16

Melanoma: Treatments and Resistance

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Additional information is available at the end of the chapter

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1. Introduction

In the past two decades, it has been observed an increased incidence of skin cancer around the world [1-4]. This increase is particularly important in melanoma [5]. Latin-American data have shown both an increase in incidence rates of skin cancer [6] and in mortality from malignant melanoma [7]. The number of melanoma cases worldwide is increasing faster than any other cancer. Although early detection, appropriate surgery, and adjuvant therapy have improved outcomes, the prognosis of metastatic melanoma remains very poor. Advanced melanoma is still associated with an extremely poor median survival, ranging from 2 to 8 months, with only 5% surviving more than 5 years and remains one of the most treatment-refractory malignancy [8]

2. Treatments

The only way to cure a malignant melanoma is early detection and appropriate surgical treatment, because once it reaches an advanced stage, is highly resistant to conventional radiotherapy and chemotherapy [9]. The median survival for patients with metastatic disease is approximately 8 months [10], and chemotherapy has so far failed to improve survival. Treatment options include radiation therapy, chemotherapy, immunotherapy and biochemotherapy which are summarized below.

2.1. Radiotherapy

The use of adjuvant radiotherapy (RT) in melanomas has been controversial. *In vitro* studies have shown that melanoma cells possess a broad shoulder on the cell survival curve and

thus have a large capacity for DNA repair. As a result, hypofractionated RT schedules have been developed to counteract this perceived radioresistance, producing excellent locoregional control rates of 85% and higher [11,12]. Radiation Therapy Oncology Group (RTOG) Trial 83-05 was a prospective randomized study comparing hypofractionation to conventional fractionation. The results showed no difference in partial or complete response rates between the two schedules, and the overall response rates were approximately 70% [13]. The role of adjuvant radiation therapy (RT) following nodal surgery in malignant melanoma remains controversial. Despite the high incidence of distant metastases, loco-regional control remains an important goal in the management of melanoma. Surgery and adjuvant RT provides excellent loco-regional control, although distant metastases remain the major cause of mortality.[14]

2.2. Chemotherapy

Chemotherapeutic agents are cytotoxic anticancer drugs which aim is impair the cell division, resulting in the death of rapidly dividing cells. They are widely used in the treatment of malignancies; however, melanomas are resistant to many forms of traditional chemotherapy.

2.2.1. Chemotherapy with single drugs in melanoma

Several antitumoral drugs have been used to treat the melanoma. One of the most known is dacarbazine. In 1975, dacarbazine (DTIC) became the first US Food and Drug Administration (FDA) approved chemotherapeutic agent for the treatment of metastatic melanoma. The response rates with dacarbazine were 15–25%, with median response ranging from 5 to 6 months, but with less than 5% of complete responses [17-19]. Long-term follow-up of patients treated with DTIC alone shows that less than 2% of the patients could survive for 6 years [15,16]. In a meta-analysis comparing two or three-drugs combination regimens with DTIC alone, Huncharek et al. [20] concluded that there was no advantage for the combination in terms of response or survival. Since survival was not improved by the use of single or combination chemotherapy for metastatic melanoma, treatment decisions remain controversial, and quality of life and toxicity issues from treatment assume greater importance.

An orally analogue of DTIC is temozolomide whose activity has been tested in several clinical studies as single agent in metastatic malignant melanoma [18,21,22]. A randomized phase III trial comparing TMZ to DTIC on patients with advanced melanoma demonstrated a statistically significant increase in progression-free survival (1.9 months vs 1.5 months) when TMZ was administered [18].

Fotemustine (FTMU) is the most active nitrosourea used against the metastatic melanoma. It has been widely tested in Europe and has shown overall response of 20–25% including 5–8% of complete response rates and it was the first drug to show significant efficacy in brain metastases [23,24]. However, at conventional doses, little or no activity was observed against melanoma brain metastases [25].

Platinum-based drugs are widely used in the treatment of cancer. In patients with melanoma, cisplatin was shown to induce a 15% response rate with a short median duration of 3

months. Doses up to 150 mg/m^2 in combination with amifostine produced tumor responses in 53% of patients. However, all of those responses were partial, and the median response duration was only 4 months [26]. Regarding carboplatin, in a study on 26 chemotherapy-naive metastatic melanoma patients, a response rate of 19% with 5 partial responses was reported and thrombocytopenia was the dose-limiting toxicity [27].

The vinca alkaloids, especially vindesine and vinblastine, have induced responses in approximately 14% of melanoma patients and they are usually used in combination with other drugs [28]. Docetaxel or paclitaxel, do not have a significant activity in melanoma [29-32]. The role of tamoxifen (TAM) as single agent at standard or high-doses in the treatment of melanoma is negligible with a response rate ranging between 0% and 10%. Currently all of these drugs are rarely used as single agent therapy in metastatic melanoma.

2.2.2. Chemotheraphy with combined drugs in melanoma

In a phase II study, Lattanzi et al. [33] reported their experience with the addition of TAM to the three-drug combination regimen of cisplatin, carmustine and dacarbazine (the Dartmouth regimen) and showed high response rates (55%) with a 20% complete response. Since then several randomized clinical trials have been conducted to confirm the therapeutic benefit of TAM in combination with chemotherapy.

Cocconi et al. [34] published a small phase III trial demonstrating an improvement of response and survival with the addition of tamoxifen to dacarbazine compared to dacarbazine alone. However, two large randomized trials with low and high-dose tamoxifen in combination with either dacarbazine alone or the Dartmouth regimen failed to demonstrate an advantage to the addition of tamoxifen [35,36].

The efficacy of the combination of paclitaxel and carboplatin in the treatment of metastatic melanoma was reported some years ago. Although originally tested in two small phase II clinical trials and deemed not sufficiently clinically active, this evidence suggests that the combination of paclitaxel and carboplatin may be worth further consideration [37].

2.3. Immunotherapy

Immunotherapy in melanoma consists of various approaches leading to specific or non-specific immunomodulation. Immunotherapies are being used for melanoma patients in stage II–III patients in the adjuvant setting, where only a fraction of patients have widespread (microscopic) disease with the aim to prevent relapse of disease, prolong relapse-free survival and, ideally, prolong overall survival (OS). In patients with stage IV disease, there is a need for adequate systemic therapies as median OS for this patient group is only 6–9 months [38]. However, for the first time in >30 years, prospective randomized trials in patients with distant metastatic melanoma demonstrated an OS benefit [39].

Some agents used in the treatment against the melanoma are ipilimumab and tremelimumab, fully human IgG1 and IgG2 monoclonal antibodies, respectively. They block cytotoxic T-lymphocyte- associated antigen 4 (CTLA-4), a negative regulator of T cells, and thus aug-

ment T-cell activation and proliferation [40,41]. A phase-III trial was completed first and its results were reported in 2010 [39]. This trial compared ipilimumab alone or in combination with a gp100-peptide vaccine, compared to the vaccine alone in patients who had failed prior therapy or therapies. Melanoma patients receiving ipilimumab and ipilimumab + vaccination had a significantly better survival outcome than those receiving the vaccine alone. Ipilimumab was combined with high-dose IL-2 in 36 patients in the surgery branch of the NCI, with some remarkable observations. There were six patients (17%) with long-lasting complete response, all over 5 years, and none of the patients relapsed. Moreover, there was no increased toxicity as compared to high-dose IL-2 alone [42]. Other study showing a combination of tremelimumab with high-dose interferon yielded a high overall response rate of 30% in 33 melanoma patients, with three complete responses and seven partial responses, all long-lasting responses. Again, there was no increased toxicity compared to high-dose IFN therapy alone [43].

Interferon-α (IFN-α) has been approved in the adjuvant setting for the treatment of high-risk melanoma based on clinical trials in the early 1990s [44,45]. In a metastatic situation, melanoma patients treated with the single agent IFN-α showed approximately 15% of responses, with less than 5% of complete response rates and median response duration between 6 and 9 months with a maximum of 12 months for the best studies [46]. These response rates, while encouraging, were not significant enough to lead to its widespread use in the treatment of metastatic melanoma. However, observations that patients with non-visceral disease were more likely to respond suggested that the use of IFN-α may demonstrated a grater impact in patients with micrometastasis [46, 47]. Other combination studied was IL-2 with IFN-α. This association did not seem to achieve better results (median response rate of 18% with three complete responses) than if these agents were given alone [48-50]. By contrast, in a small randomized phase III trial comparing continuous infusion IL- 2 plus interferon vs. continuous infusion decreasing IL-2 plus interferon, Keilholtz and colleagues [51], demonstrated improved response rates and reduced toxicity with decreasing doses of IL-2.

2.4. Biochemotherapy

Because chemotherapy and cytokines have different and synergistic mechanisms of action and in order to improve response rates and durable remissions, several groups developed in the early 1990s the concept of biochemotherapy, a combination of chemotherapy and biologic response modifiers.

Dacarbazine/IFN-α is one of the most evaluated combinations in metastatic malignant melanoma. In a randomized phase II trial, Falkson et al. [52] reported that the association of IFN-α with dacarbazine resulted in an encouraging response rate (53% vs. 20% for dacarbazine alone) and a higher duration of response (8.9 months vs. 2.5 months) but IFN-α significantly increased the toxicity. However, a follow up of a large randomized trial demonstrated no benefit for the addition of IFN-α to dacarbazine and significantly more severe toxic events occurred with treatments containing IFN-α [36].

The other approaches of biochemotherapy have involved sequential chemotherapy (cisplatin, vinblastine, and dacarbazine, CVD) followed by biologic response modifiers (continuous infu-

sion of 9 MIU/m2 of IL-2 + IFN-α) because of concern of toxicity when drugs were given simultaneously or concurrent with chemo-immunotherapy. Both approaches have produced promising results with overall response rates between 40% and 60% and a long-term remission rate of about 9%. The sequential approach was compared to chemotherapy alone in a randomized trial conducted at the MD Anderson Cancer Center. Although both response rate and time to progression were improved in the sequential biochemotherapy group, the survival difference was at borderline significance and the toxicity was very high [53]. The results of the largest phase III trial (ECOG/Intergroup E3695 trial) and most definitive test for biochemotherapy comparing concurrent CVD-Bio to CVD alone showed that biochemotherapy produced slightly higher response rates and significantly longer median progression-free survival than CVD alone, but once again failed to show any improvement in either overall survival or durable responses. Considering the extra toxicity and complexity, this concurrent biochemotherapy regimen should not be recommended for patients with metastatic melanoma [54].

2.5. Signal transduction inhibitors

In the past decades, no significant impact on survival has been made in spite of increased response rates achieved with combinations of chemotherapeutics or with the combination of chemotherapy and cytokines such as interferon (IFN) or interleukin-2 (IL-2). However, great advances have been made in a very short time, both in terms of targeted drugs that kill melanoma cells.

Sorafenib was designed to inhibit tyrosine kinase activity of CRAF, but this drug inhibits both the wild-type RAF protein as the V600E mutant protein. Subsequently, it was shown that sorafenib is actually a multikinase inhibitor, can inhibit many other molecules such as VEGFR2 and 3, PDGFR, p38 MAPK, FLT3, c-Kit and RET [55]. Although preclinical experiments, both *in vitro* and in animal models, seemed to be encouraging, the results of clinical trials have not confirmed the efficacy of sorafenib for the treatment of disseminated melanoma [56]

After the failure of sorafenib in melanoma, was synthesized a more specific BRAF inhibitors, in particular against the protein with the V600E mutation: PLX4032, a low molecular weight drug, for oral administration. In the first clinical trial published in 2010 [57], the objective response was observed in 81% of the $BRAF^{V600E}$ melanoma patients with 2 complete responses and 24 partial responses. Responses occurred in patients with visceral metastases in locations usually resistant to treatment such as liver, intestine and bone. However, despite having achieved a good response, relapses occur early, usually in a period of 8-12 months after treatment [58].

The possibility that c-Kit was a therapeutic target in melanoma has long since shuffled. In fact, c-Kit is a protein that acts as a receptor for a growth factor essential for epidermal melanocytes and has a role in the differentiation and migration of melanocytic cells during embryonic development [59]. In 2011, a phase-II study from China reported 20–30% response rates and prolongation of progression-free survival with imatinib treatment [60].

From 15 to 30% of melanomas have mutations of NRAS. RAS activation mutations stimulate MAP kinase pathway, but also the route of PI3K/AKT among others. A phase II trial using

the RAS inhbitor Tipifarnib was performed; however, it was closed for lack of response. None of the patients was selected based on the presence of mutations of NRAS [61].

MEK is a protein of the MAP kinase pathway, located downstream BRAF. Several MEK inhibitors (PD0325901, AZD6244, GSK1120212, and E6201) have been synthesized. Bases on some results, it appears that these pharmacological agents may be effective as single agents in the treatment of melanoma. However, there are many preclinical studies suggesting that it would be a good alternative to the combined treatments, both to avoid resistance in the use of drugs directed against BRAF/V600E mutation, as for the treatment of BRAF mutations other than V600E or mutations of NRAS, especially if associated with inhibitors of PI3K/AKT pathway [62-65]

Different derivatives of rapamycin (CCI-779 or temsirolimus) have been used as inhibitors of the PI3K/AKT pathway. These inhibitors act on mTOR molecule downstream AKT/PKB. There are also dual inhibitors of PI3K and mTOR, PI3K and AKT [66]. Although clinical outcomes of these drugs in phase II trials have not been good, there are several authors proposing their use in combined therapies especially with drugs that inhibit the MAP kinase pathway [62, 63, 65, 67] or even, simultaneous inhibition via PI3K/AKT [68].

3. Resistance to the treatments in melanoma

Simultaneous resistance to several structurally unrelated drugs that do not necessarily have a common mechanism of action is called multidrug resistance phenomena. An important principle in multidrug resistance is that cancer cells are genetically heterogeneous. Although the process results in uncontrolled cell growth for clonal expansion of cancer, tumor cells exposed to chemotherapeutic agents will be selected by their ability to survive and grow in the presence of cytotoxic drugs. Therefore, in any population of cancer cells that are exposed to chemotherapy, more than one mechanism of multidrug resistance may be present [69]. Different types of multidrug resistance mechanisms have been described in cancer cells. Natural resistance to hydrophobic drugs sometimes known as classical multidrug resistance, usually results in the expression of efflux pumps with an ATP-dependent drug broad specificity. These pumps belong to a family of conveyors called ABC transporters (ATP-binding cassette) that show sequence and structural homology [70]. The resistance is caused by increased output by lowering the intracellular concentration of the drug. Resistance may also occur due to reduced entry of the drug. Water-soluble drugs, which are returned by carriers that are used to carry nutrients into the cell, or agents that enter through endocytosis, could fail without evidencing of increased output. Examples of this kind of drugs include the antifolate methotrexate, nucleotide analogues such as 5-fluorouracil and 8-azaguanine, and alkylating agents such as cisplatin [71,72]. Multidrug resistance can also result from the activation of coordinated systems of detoxification, such as DNA repair systems and cytochrome P-450 [73]. In another hand, resistance can also result from a defective apoptotic pathway. This can occur because of malignant transformation, such as in cancer, or as a result of non-functional mutant p53 [74]. Alternatively, cells may acquire apoptotic pathways

changes during exposure to chemotherapy and changes in the levels of ceramides [75] or changes in the cell cycle machinery, which triggers checkpoints and prevent initiation of apoptosis. Below we present several mechanisms of resistance to the treatments that have been described in melanoma.

3.1. Antipoptotic characteristics in melanoma

Melanocytes and their stem cell precursors are activated to secrete melanin and protect neighboring keratinocytes and other epidermal cells from further damage [76]. Thus, melanocytes should be programmed to survive. Keratinocytes promote melanocyte expression of Bcl-2 by secreting neuronal growth factor (NGF) and stem cell grow factor (SCF). NGF binds to its receptors in the melanocyte membrane and increases the levels of Bcl-2 [77]. SCF interacts with its receptor c-KIT on the membrane and leads to the activation of transcription factor Mitf, which induces proliferation and differentiation of melanocyte precursors [78]. Tumorigenic melanoma cells may take advantage of high endogenous Bcl-2 levels to survive under adverse environmental conditions that they may encounter during metastatic progression and, given the connection between apoptosis and drug sensitivity, bypass the effects of chemotherapeutic drugs. Similarly, BclxL and Mcl-1, other anti-apoptotic members of the Bcl-2 family, are strongly expressed in normal melanocytes, benign nevi, primary melanoma and melanoma metastases, and may contribute to melanoma resistance to therapy [79,80]

In melanoma, two members of the IAP family, survivin and ML-IAP, have been associated with tumor progression, as they become detectable in melanocytic nevi and further overexpressed in invasive and metastatic melanomas [81,82]. Survivin is abundantly expressed, and its subcellular localization varies depending upon tumor thickness and invasiveness. Survivin overexpression has been shown in squamous cell carcinoma (SCC), and it is involved in UVB-induced carcinogenesis. The presence of survivin both in the nucleus and in the cytoplasm throughout the epidermal layers of psoriatic lesions suggests the involvement of this protein in the keratinocyte alterations typical of this disease [81]. Similarly, suppression of survivin can increase the sensitivity of melanoma cells to chemotherapeutic agents [83,84]. ML-IAP is also upregulated in melanoma cell lines and absent in normal melanocytes [85]. ML-IAP's effects on the mitochondrial pathways are considered to be related to a direct inhibition of the pro-apoptotic factor Smac/Diablo, and the caspases 9 and 3 [86]. The role of ML-IAP on melanoma chemoresistance has not been proven yet, but the overexpression of ML-IAP in breast cancer cell lines (MCF-7) or in HeLa cells protects against the drug Adriamycin and other apoptotic inducers, including TNF-α, FADD or BAX [86,87].

3.2. p53 pathway

p53 suppresses tumor development through multiple activities including induction of growth arrest, apoptosis, senescence, and autophagy [88,89]. Environmental agents such as UV that induce cellular damage activate the p53 tumor suppressor and p53 activation results in p53-dependent programmed cell death (apoptosis) in many cell types. Melanocytes are resistant to UV-induced apoptosis suggesting that p53 activity is somehow blocked

(non-functional p53), a state shared with melanoma cells [90], which are resistant to conventional modes of chemotherapy that aim to stimulate p53-dependent apoptosis.

Melanoma is one of a number of tumor types where p53 is still wild type, indicating that other events are contributing to p53 inactivation, in fact p53 function could be disabled by lesions that disrupt other components of the pathway. Studies using mouse models of melanoma have shown that disruption of the upstream p53 regulator $p14^{ARF}$ can functionally replace p53 loss during melanomagenesis [91]. Analogous to the human situation, tumors arising in these mouse models present wild type p53 [91]. Moreover, the abnormal phosphorylation of p53 by Chk2 kinase may contribute to the resistance of melanoma cells to radiotherapy [92]. Disruption of apoptosis downstream of p53 may alleviate pressure to mutate p53 and simultaneously decrease drug sensitivity [93]. For example, Apaf-1 and caspase 9 can be essential downstream effectors of p53-induced apoptosis and their disruption can facilitate oncogenic transformation of cultured fibroblasts [94]. In melanomas, Apaf-1 protein and mRNA expression are frequently downregulated in metastatic cell lines and tumor specimens [95]. Interestingly, Apaf-1 protein levels can be restored by addition of the methylation inhibitor 5-aza-2′-deoxycytidine (5azaCdR), suggesting that DNA methylation contributes to suppression of Apaf-1 levels. Whether methylation blocks Apaf-1 mRNA expression directly by interfering with the recruitment of transcription factors at the Apaf-1 promoter or by affecting a regulator of Apaf-1 expression remains an open question. In any case, Apaf-1 downregulation compromises the apoptotic response of melanoma cells in response to p53 activation [95] or E2F-1 [96]. Restoring physiological levels of Apaf-1 through gene transfer or 5aza2dC treatment enhances chemosensitivity, alleviating cell death defects associated with reduced Apaf-1 expression [95].

In tumor cells, the selective pressure to delete or inactivate p53 is very high. This primarily occurs through mutations in p53, amplification/overexpression of its inhibitors like Mdm2, Mdm4 (Mdm2 family member) [97]. The key molecule in the p53 regulatory network is Mdm2, an E3 ubiquitin ligase with potentially oncogenic activity. Dynamic fine-tuning of the Mdm2-centered network dictates the proper rapidity, intensity, and duration of a p53 response, resulting in the appropriate biological outcomes [98]. Although p53 is one of the most frequently mutated tumor suppressor genes in cancer, it is mutated in only about 13% of uncultured melanoma specimens [99-101]. The absence of p53 mutations in melanoma has been attributed to the epistatic loss of ARF [101] or amplification of HDM2 [102], both of which lead to a functionally debilitating interaction between HDM2 and p53. Ji et al. have provided important data that HDM2 antagonism can effectively restore p53 function, suppress melanoma growth, and synergize with MEK inhibition [103].

3.3. Signaling pathways in melanoma

In malignant melanoma, the PI3K/AKT signaling pathway is frequently constitutively activated [104]. Several studies indicate that only a combinatorial inhibition of PI3K/AKT and MAPK signalling induces apoptosis in melanoma cells efficiently [105,106]. On the other hand, inappropriate activation of survival signaling pathways such as those mediated by mitogen-activated protein kinase (MEK)/extracellular-regulated kinase (ERK) and phosphoi-

nositide 3-kinase (PI3K)/AKT, either as consequences of genetic alterations or resulting from environmental stimulations, is known to play a central role in the resistance of melanoma to apoptosis [107,108].

One-third of primary melanomas and about 50% of metastatic melanoma cell lines showed reduced expression of PTEN as a result of allelic deletion, mutation or transcriptional silencing [109,110], suggesting that inactivation of PTEN is a late, but frequent, event on melanomagenesis [111,112]. Multiple lines of evidence point to the PI3K/AKT/PTEN pathway as a putative candidate for therapeutic intervention in melanoma because PTEN overexpression can revert the invasive phenotype of human and mouse melanoma cell lines [113,114] and elevated PTEN activity may sensitize cells to chemotherapeutic drugs [115].

Recent progress in the identification of genes relevant for melanomagenesis was made, revealing the importance of several signaling pathways. Sinnberg et al. [116] suggest that the oncogenic transcription factor Y-box binding protein-1 (YB-1) play a pivotal role in melanoma cells. YB-1 could be a key player, activated by the signalling pathways MAPK and PI3K/AKT. Indeed, was demostrated that both signaling pathways are able to increase S102-phosphorylation and nuclear translocation of YB-1. It is known that S102-phosphorylated YB-1 can induce the expression of the catalytic subunit of PI3K and by this increases PI3K activity [117].

In melanoma cells, the NF-kB pathway can be altered by upregulation of the NF-kB subunits p50 and RelA [118,119] and downregulation of the NF-kB inhibitor IkB [120,121]. Consequently, downstream NF-kB targets like c-myc, cyclin D1, the anti-apoptotic factor TRAF2, the invasion-associated proteins Mel-CAM or the pro-angiogenic chemokine GRO are also frequently upregulated in melanoma [122]. Recent studies have highlighted that some components of NF- kB family, such as p50 and p65/ RelA proteins, are overexpressed in the nuclei of dysplastic nevi and melanoma cells compared to those of normal nevi and healthy melanocytes, respectively [123]. Other data show that a hyperactivation of NF-kB can be also caused by an increased expression of other factors involved indirectly in NF-kB pathway. Recent studies on the gene expression profile of melanoma cells have shown an increased expression of Osteopontin (OPN) [124], a secreted glycophosphoprotein that induces NF-kB activation through enhancement of the IKK activity based on phosphorylation and degradation of IkBa [125]. Indeed, OPN induces AKT phosphorylation and, in turn, phosphorylated AKT binds to IKKa/b and activates IKK complex [125]. Mutational activation of BRAF, common in human melanomas, has been also associated with an enhanced IKK activity and a concomitant increase in the rate of IkBa ubiquitination and its subsequent degradation. This process overall entails a constitutive induction of NF-kB activity and an increased survival of melanoma cells [126]. Combination of these data with others reported in literature strongly suggests that the enhanced activation of NF-kB may be due to deregulations occurring in upstream signaling pathways such as RAS/RAF, PI3K/AKT and NIK [121].

Oncogenic mutations on Ras-family members, RAS and B-RAF, have been shown to impinge at multiple levels on AKT/NF-kB, RAF/MAPK and RAL/Rho signaling pathways [127] producing survival signals to disengage cell cycle checkpoint controls, favor metastasis and

block pro-apoptotic stimuli. In support of this hypothesis, overexpression of N-RAS in human melanoma cells enhances Bcl-2 expression and contributes to a higher tumorigenicity and drug resistance in mouse xenotransplant models (i.e. subcutaneous injections) [128]. Chin and collaborators have generated melanomas in the context of a specific genetic background (INK4a/ARF deficiency) by conditional overexpression of H-RAS in melanocytes. Once the tumors were formed, downregulation of H-RAS expression led to a marked tumor regression by enhanced apoptosis of the tumor cells and also on the host-derived endothelial cells [129]. High-throughput analyses of genetic alterations in human cancers demonstrate that specifically, B-RAF, a RAS effector, was found to be mutated in 66% of human melanomas. Mutations are restricted to a few single amino-acid changes (primarily on V599) that render a constitutive active kinase with transforming properties in NIH3T3 cells [130]. Interestingly, previous studies indicate that wild-type B-RAF may inhibit programmed cell death downstream of cytocrome C release [131].

Although >50 mutations in BRAF have now been described, the most common BRAF mutation in melanoma, accounting for 80% of all of the BRAF mutations, is a valine to glutamic acid (V600E) substitution [130,132]. Acquisition of a V600E mutation in BRAF destabilizes the inactive kinase conformation switching the equilibrium towards the active form, leading to constitutive activity [132]. Mechanistically, mutated BRAF exerts most of its oncogenic effects through the activation of the MAPK pathway [133]. MAPK activity drives the uncontrolled growth of melanoma cells by upregulating the expression of cyclin D1 and through the suppression of the cyclin dependent kinase inhibitor $p27^{KIP1}$. Pre-clinical studies have shown that introduction of mutated BRAF into immortalized melanocytes leads to anchorage independent growth and tumor formation in immunocompromised mice [133]. Conversely, downregulation of mutated BRAF using RNAi causes cell cycle arrest and apoptosis in both *in vitro* and *in vivo* $BRAF^{V600E}$ mutant melanoma models [133]. Although it has been suggested that the acquisition of the $BRAF^{V600E}$ mutation is an early event in melanoma development, with 80% of all benign nevi showing to be BRAF mutant, the available evidence indicates that mutant BRAF alone cannot initiate melanoma [134,135].

3.4. DNA Mismatch Repair (MMR) proteins

Late et al. [136] determined that melanoma cells exhibiting resistance to cisplatin, etoposide and vindesine present a reduction of 30 to 70% in the nuclear content of each of the DNA mismatch repair (MMR) proteins hMLH1, hMSH2 and hMSH6. A decreased expression level of up to 80% of mRNAs encoding hMLH1 and hMSH2 was observed in drug-resistant melanoma cells selected for cisplatin, etoposide and fotemustine. In melanoma cells that acquired resistance to fotemustine, the activity of *O*6-methylguanine-DNA methyltransferase (MGMT) was considerably enhanced. The data of this group indicate that modulation of both MMR components and MGMT expression level may contribute to the drug-resistant phenotype of melanoma cells.

DNA mismatch repair (MMR) deficiency and increased *O*6-methylguanine-DNA methyltransferase (MGMT) activity have been related to resistance to *O*6-guanine methylating agents in tumour cell lines. However, the clinical relevance of MMR and MGMT as drug

resistance factors is still unclear. In a retrospective study, the expression levels of the MMR proteins, hMSH2, hMSH6 and hMLH1, Ma et al. [137] analysed by immunohistochemistry in melanoma metastases from 64 patients, who had received dacarbazine (DTIC) based chemotherapy. All tumours showed positive nuclear staining for hMLH1. The response rates were similar in patients with hMSH2 and/or hMSH6 positive tumours to these in patients with negative tumours. In other retrospective study, Ma et al. [138] analysed the levels of the DNA repair protein *O*(6)-methylguanine-DNA methyltransferase (MGMT) in melanoma metastases from patients receiving dacarbazine (DTIC) either as a single drug or as part of combination chemotherapy regimens, and related the expression levels to the clinical response to treatment. DTIC as single agent was given to 44 patients, while 21 received combination chemotherapy. Objective responses to chemotherapy were seen in 12 patients, while 53 patients failed to respond to treatment. The expression of MGMT was determined according to the proportion of antibody-stained tumor cells, using a cut-off level of 50%. In 12 of the patients, more than one metastasis was analyzed, and in seven of these cases, the MGMT expression differed between tumours in the same individual. Among the responders a larger proportion (six out of 12, 50%) had tumors containing less than 50% MGMT-positive tumor cells than among the non-responders (12 out of 53, 23%). These data are consistent with the hypothesis that MGMT contributes to resistance to DTIC-based treatment. The conclusion that can be drawn from the fact that the development of drug resistance in melanoma cells is accompanied by down modulation of certain components of the MMR system and by an increase in MGMT activity when *O*6-alkylating agents are applied has several far-reaching implications regarding primary and acquired clinical resistance to these drugs. Furthermore, reduction or deficiency in MMR may increase the mutation rate in affected cells leading subsequently to an increased rate of development of resistance to other drugs having different targets. In addition, an enhanced mutation rate may contribute to increased phenotypic variation and therefore the clinical aggressiveness of melanomas and their metastases.

Recently, Li et al. [139] demonstrated the expression of DNA repair genes ERCC1 and XPF is induced by cisplatin in melanoma cells and that this induction is regulated by the MAPK pathway, with the role of DUSP6 phosphatase being particularly important. This induction contributes to increased drug resistance, which is one of the major obstacles to melanoma treatment, suggesting that ERCC1 or XPF inhibitors could be used to enhance the effectiveness of cisplatin treatment.

3.5. Multidrug Resistance Proteins (MRP)

The intrinsic multidrug resistance and sensitivity in melanomas and in pigment-producing cells involves multiple ABC transporters and melanosome biogenesis [140]. Melanoma cells express a group of ABC transporters, including ABCA9, ABCB1, ABCB5, ABCB8, ABCC1, ABCC2, and ABCD1 [140,141].

ABCC1 was shown to cooperate with glutathione S-transferase M1 to help melanoma cells escape the cytotoxicity of vincristine [141]. Have been described too that B16 melanoma

(B16M) cells presenting high ABCC1 and GSH content show high metastatic activity and high multidrug and radiation resistance [142]. Elevated expression of ABCC2 was shown to cause cisplatin resistance by reducing nuclear DNA damage, decreasing cell cycle G2-arrest, and increasing reentry into the cell cycle [4].

Has been reported that ABCB5 and ABCB8 mediate doxorubicin resistance in melanoma cells [143, 144]. ABCB5 shares 73% of sequence homology with the classic and the most studied multidrug resistance protein ABCB1 (P-gp, MDR1) [145,146] and was firstly detected in tissues derived from the neuroectodermal lineage including melanocyte progenitors [145], melanoma cell lines and patient specimens [143,146-148]. In melanoma, ABCB5-expressing cells are endowed with self-renewal, differentiation and tumorigenicity abilities [149,150]. Their abundance in clinical melanoma specimens correlates positively with the neoplasic progression suggesting that ABCB5 expression is associated with tumor aggressiveness. Moreover, the growth of melanoma xenografts in mice was delayed when the animals were treated with a monoclonal anti-ABCB5 antibody [149]. As a member of the ABC transporter family, ABCB5 is thought to play a role in drug efflux. This was supported by experiments measuring the intracellular accumulation of Rhodamine 123 [145]. These data suggest that ABC proteins may be important molecular targets for the reversal of multidrug resistance in melanoma cells.

4. Does oxidative stress contribute to the resistance in melanoma?

Free radicals are implicated in the pathogenesis of a multistage process of carcinogenesis. They can cause DNA base alterations, strand breaks, damage to tumor suppressor genes and enhanced expression of proto-oncogenes. The burst of reactive oxygen species (ROS) and the reactive nitrogen species (RNS) has been implicated in the development of cancer [151,152]. Excessive production of ROS can be harmful to both normal and cancer cells. High levels of ROS cause damage to lipids, DNA and cellular proteins, disrupting their normal function. However, some cancer cells can develop mechanisms that use ROS for purposes such as mitogenic upregulation of the expression of antioxidant enzymes [153-155]. Several studies have investigated the role of antioxidant enzymes in cancer and it has been shown that these enzymes play a significant role in regulating cancer growth and survival [156,157]. The carcinogenic effect of oxidative stress is attributed primarily to the genotoxicity of ROS in various cellular processes [158]. For example, hydroxyl radicals can react with purines andor pyrimidines as well as chromatin proteins, resulting in base modifications and genomic instability which can cause alterations in gene expression [159]. These data have suggested the accumulation of ROS as a common phenomenon in many cancer cells. Such accumulations can cause direct damage to DNA by increasing the cellular mutation and/or promoting and maintaining the tumorigenic phenotype by activating a second messenger in intracellular signaling cascades [160]. In addition, ROS have been determined to cause epigenetic alterations that affect the genome and play a major role in the development of carcinogenesis in humans [161]. More specifically, the production of ROS is associated with alterations in DNA methyla-

tion patterns [162, 163]. In particular, hydroxyl radicals that produce DNA lesions, such as 8-hydroxyl-2-deoxyguanosine, 8-hydroxyguanine, 8 -OHdG [164-166], and damage to the single strand of DNA [167] have been shown to decrease DNA methylation by means of interfering with the ability of DNA to function as a substrate for the DNA methyltransferases (DNMTs) and thus resulting in global hypomethylation [168].

Oxidative stress may play different roles in the pathogenesis of melanoma and non-melanoma skin cancer. It is likely that in non-melanoma skin cancers, a diminished antioxidant defense caused by chronic UV exposure contributes to the occurrence of mutations and carcinogenesis, whereas melanoma cells are equipped with a high antioxidant capacity and might use their ability to generate ROS for damaging surrounding tissue and thus supporting tumour progression and metastasis [169]. Gidanian et al. showed that melanosomes derived from melanoma cells in comparison to melanocytes actively produce excessive amounts of ROS [170]. Higher intracellular levels of ROS in melanoma cells were also detected by the studies by Meyskens et al. [171]. They furthermore showed that due to these elevated levels of ROS, melanin itself becomes progressively more oxidized and starts to function as a pro-oxidant [172]. They also showed that oxidation of melanin can be further increased by binding of metals, such as iron. These melanin-metal complexes can be converted by the Fenton reaction thereby producing even more ROS [173]. There is supportive evidence that sustained oxidative stress is related to oxidative DNA damage [174]. Atypical melanocytes have increased levels of oxidative stress and oxidative DNA damage [175, 176]. In line with these observations, Leikam et al. found that ROS production was accompanied by enhanced DNA damage [177].

4.1. Oxidative stress by antitumoral treatments

The cytotoxicity of some antitumoral drugs like actinomycin-D (AMD), adriamycin (ADR), cisplatin (Cis-Pt), vincristine (VCR), cytosine arabinoside (Ara-C) and dacarbazine (DTIC) are, to a greater or lesser extent, linked to the generation of free radicals and/or to the antioxidant defense of the cells. AMD and ADR are xenobiotics, which, in the cell, enter to cycles of oxidation and reduction, generating ROS [178,179]. Cis-Pt does not produce ROS; however, during its detoxification the level of glutathione (GSH) decreases [180]. In the case of DTIC, it has been shown that the resistance of melanoma cells to that drug is also partly linked to changes in the level of GSH [17,181]. ROS generated by mitochondria intensify the apoptosis induced by cytosine arabinoside [182].

Radiotherapy is a cornerstone in the treatment of several cancers. Ionic irradiation exposes all cells to high levels of oxidative stress, thus resulting in the formation of ROS, increasing DNA damage and ultimately leading to cell death. Another mechanism of the action of radiotherapy is to alter cellular homeostasis, thus modifying the signal transduction pathways and predisposing to apoptosis [183]. However, there are conflicting reports on the effect of radiotherapy on oxidative stress. Some studies have reported increased oxidative stress after radiotherapy [184], while others have reported decreased oxidative stress after radiotherapy in cancer patients [185, 186].

4.2. Transcription factors Nrf1 and Nrf2 are regulators of oxidative stress signaling

Nrf1 (NF-E2 related factor-1) and Nrf2 (NF-E2 related factor-2) nowadays are known as two oxidative stress sensitive transcription factors that belong to the CNC/bZIP family of transcription factors consisting of NF-E2, Nrf1, Nrf2, Nrf3, BACH1, and BACH2 [45–48]. Both Nrf1 and Nrf2 are responsible for regulating the expression of many antioxidant genes including peroxiredoxin-1 (Prx-1), thioredoxin-1 (Txn-1), GCLC (Glutamate cysteine ligase catalytic subunit - an enzyme responsible for catalyzing the formation of glutathione), glutathione peroxidase (GPX-1), drug metabolizing enzymes (cytochrome P-450s), and several ATP Binding Cassette (ABC) transporters that are responsible for drug efflux [187-190]. All of these genes are essential for the maintenance of oxidative homeostasis and contain an Electrophile Response Element (EpRE) to which Nrf1 and Nrf2 bind (also known as the Antioxidant Response Element). Both Nrf1 and Nrf2 are essential to the cellular response to oxidative stress and several studies have shown that knockdown of Nrf1 and/or Nrf2 expression sensitizes cells to oxidative stress [191-193]. It has also been suggested that Nrf2 responds to inducible oxidative stimuli and that Nrf1 regulates oxidative stress [194]. Increased oxidative stress has been shown to promote tumor proliferation and survival through deregulation of redox-sensitive pathways [153,195,196]. Nrf2 resides predominantly in the cytoplasm where it interacts with the actin-associated cytosolic protein INrf2, which is also known as Keap1 (Kelch-like ECH-associated protein 1). INrf2 functions as a substrate adaptor protein for a Cul3/Rbx1-dependent E3 ubiquitin ligase complex to ubiquitinate and degrade Nrf2, thus maintaining a steady-state level of Nrf2 [197].

Data from tumor cell lines isolated and profiled from human patients have indicated that many tumors have adapted to exploit the cytoprotective actions of Nrf2 both *in vivo* and *in vitro* through mutations of Keap1 and Nrf2, which lead to the constitutive upregulation and permanent activation of Nrf2-signaling to enhance the tolerance of the cancer cells to toxins and thereby limit the efficacy of chemotherapeutic agents. The loss of INrf2 (Keap1) function is shown to lead to nuclear accumulation of Nrf2, activation of metabolizing enzymes and drug resistance [198]. Studies have reported mutations resulting in dysfunctional Nrf2 in lung, breast and bladder cancers [199-203].

In a study carried out by Matundan et al. [204], they demonstrated the basal Nrf2 expression pattern in human melanoma was increased in 7 of 8 human melanoma cell lines. Immunoblots of Nrf2 showed over-expression in 6 of 8 metastatic melanoma cell lines and they determined that Nrf2's contribution was protective against redox stress in melanoma, and that decreased Nrf2 activation sensitizes melanoma cell lines to existing chemotherapeutics [204].

4.3. NRF2 are related with the expression of multidrug resistance proteins

Ogura and colleagues reported previously that Nrf2 binds within the *ABCB1* promoter's -126 and -102 regions, which contain the ATTCAGTCA motif. They have purified Nrf2 from the nuclear extract of K562/ADM cells, a multidrug-resistant cell line derived from human myelogenous leukemia K562 cells. This group determined that ATTCAGTCA motif is a positive regulatory element of MDR1 gene and that the motif is important for Nrf2 binding.

These results suggest that Nrf2 may be involved in the positive regulation of the *ABCB1* gene transcription [205].

Maher and collaborators examined the possibility that Nrf2 is also involved in the expression levels of ABCC1 in mouse embryo fibroblasts. The constitutive expression levels of Mrp1 mRNA and protein were significantly lower in Nrf2 (-/-) cells compared with those in wild type cells. In addition, significant induction by diethyl maleate was observed in wild type, but not in Nrf2 (-/-) cells, suggesting the involvement of Nrf2 in both the constitutive and inducible mRNA and protein expression of ABCC1. In addition, the uptake of [^{3}H]2,4-dinitrophenyl-S-glutathione, a typical substrate of ABCC1, into isolated membrane vesicles also demonstrated that Nrf2 regulates the transport activity of glutathione conjugates in mouse fibroblasts [206]. In another hand, Maher evaluated whether oxidative conditions (that is, the disruption of hepatic GSH synthesis) or the administration of nuclear factor-E2-related factor-2 (Nrf2) activators (oltipraz and butylated hydroxyanisole) can induce hepatic ABC transporters and whether that induction is through the NRF2 transcriptional pathway. Livers from hepatocyte-specific glutamate-cysteine ligase catalytic subunit-null mice had increased nuclear NRF2 levels, marked gene and protein induction of the Nrf2 target gene NAD(P)H: quinone oxidoreductase 1, as well as ABCC2, ABCC3, and ABCC4 expression. The treatment of wild type and Nrf2-null mice with oltipraz and butylated hydroxyanisole demonstrated that the induction of ABCC2, ABCC3, and ABCC4 is NRF2-dependent. In Hepa1c1c7 cells treated with the Nrf2 activator tert-butyl hydroquinone, chromatin immunoprecipitation with Nrf2 antibodies revealed the binding of NRF2 to antioxidant response elements in the promoter regions of mouse ABCC2 [-185 base pairs (bp)], ABCC3 (-9919 bp), and ABCC4 (-3767 bp). In this way, the activation of the Nrf2 regulatory pathway was shown to stimulate the coordinated induction of hepatic ABCs [190].

4.4. NRF2 represses the p53 pathway

You et al. [207] confirmed that Nrf2 is directly involved in the basal expression of Mdm2 through the antioxidant response element, which is located in the first intron of this gene. This linkage between Nrf2 and Mdm2 appears to cause the accumulation of p53 protein in Nrf2-deficent MEFs. They also showed that ovarian carcinoma A2780 cells silenced for Nrf2 by shRNA displayed higher levels of p53 activation in response to hydrogen peroxide treatment, leading to increased cell death. Collectively, those results suggest novel evidence that the inhibition of Nrf2 can suppress Mdm2 expression, which may result in p53 signaling modulation. Thus, forced inhibition of Nrf2 expression in cancer cells may be lead to activation of apoptosis response through the activation of p53 signaling.

4.5. Nrf2 and signalling pathways

The functional interaction between the Keap1-Nrf2 pathway and PTEN-PI3K-AKT pathway has been reported in several studies using cell lines. The pharmacological inhibition of the PI3K-AKT pathway represses the nuclear translocation of Nrf2 [208, 209]. In another hand, Beyer et al. showed that AKT phosphorylation was robustly augmented in the P/K-Alb mice in Nrf2-dependent manner, which is consistent with the previous report that Nrf2 positively

regulates the activation of AKT [210]. Recently, Mitsuishi et al. [211] demonstrated a contribution of Nrf2 to cellular metabolic activities in proliferating cells, and the positive feedback loop between the PTEN-PI3K-AKT and Keap1-Nrf2 pathways, which appears to be one of the most substantial mechanisms for promoting the malignant evolution of cancers. It should be noted that Nrf2 accumulation, which is achieved by the functional impairment of Keap1 combined with the sustained activation of PI3K-AKT pathway, allows Nrf2 to get involved in the modulation of metabolism under pathological conditions. In contrast, temporary accumulation of Nrf2 at a low level is sufficient for Nrf2 to exert the cytoprotective function under physiological conditions [211].

Su et al. [212] reported the first evidence that Nrf2 is phosphorylated by MAPKs *in vivo*, however the nuclear accumulation of Nrf2 was slightly enhanced by its phosphorylation. This group concluded that direct phosphorylation of Nrf2 by MAPKs has a limited contribution in regulating the Nrf2-dependent antioxidant responses.

4.6. Nrf2 and anti-apoptotic features

Nrf2 resides predominantly in the cytoplasm where it interacts with the actin-associated cytosolic protein INrf2, which is also known as Keap1 (Kelch-like ECH-associated protein 1). INrf2 functions as a substrate adaptor protein for a Cul3/Rbx1-dependent E3 ubiquitin ligase complex to ubiquitinate and degrade Nrf2, thus maintaining a steady-state level of Nrf2 [197]. A study conducted by Niture et al. demonstrated that INrf2, in association with Cul3/Rbx1, ubiquitinates and degrades Bcl-2 [213]. However they recently demonstrated that Nrf2 binds to Bcl-2 ARE and regulates expression and induction of the Bcl-2 gene. Nrf2 mediated the up-regulation of Bcl-2, down regulated the activity of pro-apoptotic Bax protein and caspases 3/7, and protected cells from etoposide/radiation-mediated apoptosis that leads to drug resistance. Thus, they demonstrate that Nrf2-mediated up-regulation of Bcl-2 plays a significant role in preventing apoptosis, increasing cell survival, and drug resistance [214].

5. Conclusion

Melanoma continues to increase in incidence in many parts of the world, but there is currently no curative treatment once the disease has spread beyond the primary site because of the absence of effective therapies. This is believed to be largely due to the resistance of melanoma cells to induction of apoptosis by available chemotherapeutic drugs and biological reagents. Drug resistance is likely not only a primary consequence of acquired genetic alterations selected during or after therapy, but rather inherent to the malignant behavior of melanoma cells at diagnosis. Data support the existing hypothesis that talks about melanoma cells are “born to survive”. Their aggressive behavior stems from intrinsic survival features of their paternal melanocytes nourished by additional alterations acquired during tumor progression. These inherent survival mechanisms may be partly caused by the oxidative stress to which melanoma cells are exposed. Nrf2 is a transcription factor that is consid-

ered a double-edged sword because it participates in the regulation of oxidative stress, however has been shown that overexpression of Nrf2 is a common phenomenon in several cancer types, participating in chemoresistance and tumor survival. We assume that this phenomenon also overlaps in melanoma, thus the intrinsic or extrinsic resistance produced in melanoma cells is partly due to overexpression of Nrf2, which can promote cell survival through mechanisms already reviewed in this chapter. Although these mechanisms presented in the last part of this chapter were not studied in melanoma, we believe that future studies endorse our theory. The knowledge about melanoma treatment has been widespread in recent years, but still is not enough, hence we must deepen in this area in order to improve the existing treatments and create effective targeted therapeutic target against this disease.

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