

Melanoma Metamorphoses: Advances in Biology and Therapy

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Abstract

Melanoma is a malignancy of melanocytes of cutaneous, uveal or mucosal origins. This review discusses advances in biology and new approaches in staging and advanced disease therapy. The role of UVA light in dark reactions generating dioxetane products and cyclobutane pyrimidine dimers in melanoma genesis is detailed. Utility of screening programs and prevention with enhanced UVA blockers is included. Different staging algorithms for cutaneous, uveal and mucosal melanomas are described. Advances in adjuvant radiotherapy and immunotherapy are noted. Stereotactic radiotherapy for brain metastases and metastasectomy for oligometastatic disease has impacted the natural history of this disease. While combined BRAF and MEK inhibition for BRAF mutant melanoma patients have produced durable remissions, relapse is frequent and due to multiple genetic mechanisms. Immunotherapy with anti-CTLA4 and anti-PD1 immune checkpoint blockers also yields long-term responses, but there remains many patients unresponsive to immune checkpoint blockade or whom develop resistance. Host and tumor-specific resistance mechanisms are explored. Some new areas of melanoma research include nanoparticle fluorescence surgical imaging for regional and metastatic disease, tumor gene expression profiling to predict BRAF inhibitor resistance, combination of ion channel blocker with MEK inhibitor for wild-type BRAF metastatic melanoma, establishment of the innate immune signalling pathway for stereotactic radiotherapy of melanoma, and testing of IDO inhibitors with anti-PD1 inhibitors in metastatic melanoma.

Keywords: Melanoma biology; Melanoma targeted therapy; Melanoma immunotherapy

Melanoma is a malignancy of melanocytes of cutaneous, uveal, or mucosal origins. This review discusses advances in biology and new approaches in staging and advanced disease therapy.

Incidence

Melanoma is occurring with a startling and increasing incidence in the United States, Europe, and Australia. Two percent of all Americans will develop melanoma in their lifetime, and a million Americans are living with a diagnosis of melanoma. With an annual rate of 75,000 cases/year or 22/100,000 people, cutaneous melanoma represents 4.5% of all cancer cases. The incidence of melanoma has tripled between 1975 and 2012 [1]. Annual rates for uveal and mucosal melanomas are 1,200 and 600 cases/year and have not increased over time [2,3].

Risk Factors

Risk factors for cutaneous melanoma have been well described and a partial risk algorithm is available [4]. Higher risk ratios are seen for people with xeroderma pigmentosum (2000x), FAMMM syndrome-133x, previous melanoma-12.4x, multiple nevi-5.5x, tanning bed use-3.9 x, Parkinson's disease-3.6x, blond or red hair-1.8 x, family history of melanoma-1.7x, pale or freckled skin-1.6x, blistering sunburn history-1.3x [5-8]. Interesting correlations have been observed between melanoma and airline pilot/crew occupation, immunosuppression, and living in lower latitudes [9-13]. Dietary factors have been extensively studied with equivocal results- the most recent implicate beneficial effects of vitamin D containing foods, butter, and oregano on melanoma incidence [14,15]. There is a strong interaction between genetic presence of DNA repair errors and UV exposure [16-18].

Etiology

More is known about the photochemistry and genetics leading to cutaneous melanoma than about the origins of uveal or mucosal melanomas. The solar fusion reaction generating helium yields highly energetic photon products that excite the coronal hydrogen electron to

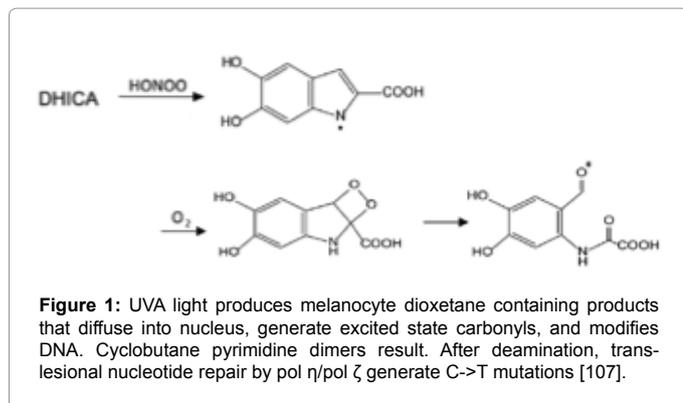
higher energy states. When these electrons return to lower quantum states, light photons are emitted towards the earth including UVA, UVB, UVC and visible light. Water molecules and other particles in the atmosphere block UVC and some UVB. Most UVA and visible light reaches the earth and humans. Primitive humans were protected from excessive photo-damage by hair or eumelanin skin pigmentation. However, human migration, pigmentation gene mutations, cultural dress norms and recreational activities have led to heightened skin exposure to UVA. A long-known paradox was the association of melanoma with UVA rather than UVB light. Only UVB light has the energy necessary to directly induce cyclobutane pyrimidine dimers. These DNA adducts are the precursors for the genetic lesions associated with melanoma. In the last year, Brash and colleagues at Yale discovered a pheomelanin-dependent pathway that converts UVA exposure into melanocyte cyclobutane pyrimidine dimers [19]. UVA induces expression of nitric oxide synthetase, NADPH oxidase and melanin synthetases. These enzymes produce nitric oxide, superoxide, peroxynitrite, and pheomelanin in the cytosol and melanosomes, respectively. These products combine to yield melanin degradation products and dioxetane-containing molecules in the cytosol (Figure 1) [20]. Dioxetane products persist and diffuse to the nucleus where they undergo thermolysis to excited-state triplet carbonyls- $n \rightarrow \pi^*$. This species participates in Dexter triplet-triplet energy transfer to

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converts DNA- π^* to cyclobutane pyrimidine dimers- π^* ->strained σ . Uveal melanomas have been associated with blue light, and mucosal melanomas with formaldehyde exposure [21,22].

Somatic cell DNA sequencing revealed a dominant mutation signature associated with melanoma-Signature 7 due to C>T substitutions on the untranscribed strand. This signature is caused by cyclobutane pyrimidine dimer repair by transcription-coupled nucleotide-excision repair [23]. BRAF mutations occur from error-prone replication repair by pol η /pol ζ from nearby CPD formation [24]. Founder mutations occur in mitogen-activated protein kinase-MAPK proliferation control genes yielding four melanoma subtypes-mutant BRAF, mutant RAS, mutant NF1, and triple wild-type containing KIT, GNAQ/11 [25,26]. Further mutations in cell cycle checkpoint-CDKN2A, immortalization-TERT promoter, and other pathway genes-p53, PTEN, BAP1, ARID1, YAP1 provide a growth advantage [27-29]. In fact, a careful study of multiple biopsies of evolving melanocytic lesions showed progression from BRAF/NRAS/NF1 mutations nevus to pTERT/CDKN2A/ARID1A mutations in *in situ* melanoma to PTEN/P53/YAP1/BAP1 invasive melanomas [30]. Acquisition of a neural crest stem cell phenotype with LOX10 and SETDB1 overexpression further contributes to invasive melanoma [31,32]. GNA11/Q and c-KIT are preferentially mutated in uveal and mucosal melanomas, respectively. Melanoma has the highest mutation burden of any cancer.

Screening and Prevention

Because of the location of cutaneous melanomas, screening for early detection is feasible, non-invasive, and relatively inexpensive. Visual examination focuses on the “ABCDE” mnemonic-asymmetry, border irregularity, color variegation, diameter>6 mm, and evolution with rapid morphologic changes [33]. However, observer training is required to distinguish benign from malignant moles with the best exam sensitivity and specificity achieved by dermatologists. Further, more and less thick lesions are discovered by more frequent examinations with yearly better than biannual better than every five years better than once only. Ophthalmology exams are needed to screen for uveal melanomas [34]. There is no effective screening for mucosal melanomas. Results are improved with addition of supplemental dermatoscopy and total body photography [35]. In a meta-analysis, dermatoscopy increased sensitivity of diagnosis from 74% by naked eye to 90% with the instrument without a significant decrease in specificity. Addition of total body photography led to removal of thinner melanomas (note change in size of lesion as shown below). Further, 40% of discovered lesions were not being followed by visual or dermatoscopic surveillance [36]. There are sobering limitations. Nodular melanomas grow rapidly and

may advance to late stage between examinations complicating periodic monitoring. Amelanotic and desmoplastic melanomas lack most of the ABCDE features often leading to delayed diagnosis. Nevertheless, screening saves lives as demonstrated in the Schleswig-Holstein study with a single standardized whole-body examination where a 48% decline in melanoma mortality was reported compared to unscreened adjacent regions of Germany [37].

Preventive measures centre on reduced UV skin exposure with avoiding sun exposure between 10 AM and 2 PM, wearing light colored, dry, synthetic material, high ultraviolet protection factor-UPF clothing with UV absorbers in laundry detergent, wide-brimmed hats and applying high sun protection factor-SPF UVA-UVB broad spectrum sunscreens in adequate amounts-one teaspoon each for face, neck, and two teaspoons each for abdomen, back and limbs and frequency every 2-3 h [38,39]. UVB blockers include physical barriers-titanium dioxide and chemical absorbers-oxybenzone. UVA blockers include the chemicals-avobenzone/octocrylene, ecamsule, silatrizol, and bemetrizinol. Only the avobenzone/octocrylene is available in U.S [40]. Uveal melanoma protection includes orange/yellow sunglasses and blue light-blocking lens replacements [22].

Diagnosis

A punch or excisional biopsy with histologic and sometimes immunohistochemical analysis is required for cutaneous melanoma diagnosis [41]. Morphological/histological findings of cutaneous melanomas include pagetoid cells or atypical cells with branching dendritic structures, broadened honeycombed pattern or cerebriform clusters [42,43]. Melanomas are assigned to superficial spreading melanoma, lentigo maligna, nodular melanoma and desmoplastic melanoma. Cytologically atypical melanocytes in epidermis and dermis with mitoses and necrosis or inflammation are seen. Desmoplastic melanomas have abnormal melanocytes surrounded by fibrous tissue [44]. Markers for melanocytic differentiation include HMB45, Melan-A/Mart, tyrosinase, MITF and SOX10. Both radial growth-superficial spreading melanoma and vertical growth-nodular melanoma predominant patterns are seen. Uveal melanomas are diagnosed by ophthalmoscopy and scans rather than biopsy. Mucosal melanomas require tissue biopsy confirmation for diagnosis.

Staging

For cutaneous melanomas, patient history, the patient's gender and age are determined. The physical exam and punch biopsy provide evidence of location of disease (extremity or axial), ulceration, tumor thickness, and mitotic rate. Once the biopsy confirms the diagnosis, blue dye + radiocolloid (e.g., ^{99m}Tc tilmanocept) infusion with sentinel node biopsy is performed [45]. For clinically positive nodal metastases and for mucosal or uveal melanomas, PET/CT and brain MRI scans are done to assess visceral metastases. Tumor specimens should be sent for oncogene studies-BRAF or c-KIT for node positive cutaneous melanomas or mucosal melanomas, respectively. Several prognosis algorithms are available online for cutaneous melanomas including <http://melanomaprognosis.org> and www.melanomacalculator.com [46,47]. As noted below, cutaneous melanoma-free survival is shortened by older age, male gender, thicker tumor, tumor ulceration, measurable mitoses/mm², positive sentinel nodes, multiple or macroscopic nodal metastases, any distant metastases-particularly non-lung visceral lesions, and poor prognosis gene expression profile (Figure 2) [48-52]. The 31-gene expression panel of Castle Biosciences combined with sentinel node status was the most accurate predictor of disease-free, metastasis-free and overall survival. Staging provides important

information to patients for planning definitive and adjuvant therapies and deciding on frequency and type of monitoring. Uveal melanoma survival is lower for thick or wide lesions, ciliary body or extraocular extension, bad prognosis gene expression profile or with metastases identified by PET, CT or MRI scans (Figure 3) [53,54].

Mucosal melanoma prognosis depends on tumor size, organ site, and presence of node or visceral metastases. Tumor thickness >1 mm, >3 cm, and >3 cm for female genital tract, head and neck and GI tract, respectively, leads to shortened survival (Figure 3) [55]. Non-vulvar organ site is worse [3]. Positive nodes or visceral metastases portend short survival.

Local Therapy

Cutaneous melanoma local control is achieved by excisional surgery. For cutaneous melanomas ≤ 1 mm thickness, 1 cm margins are sufficient. For melanomas between 1 and 2 mm, 1-2 cm margins are done. For melanomas ≥ 2mm thickness, 2 cm margins are required. These excision margins yield a 4% local recurrence rate that is not improved by wider margins based on multiple randomized trials [56].

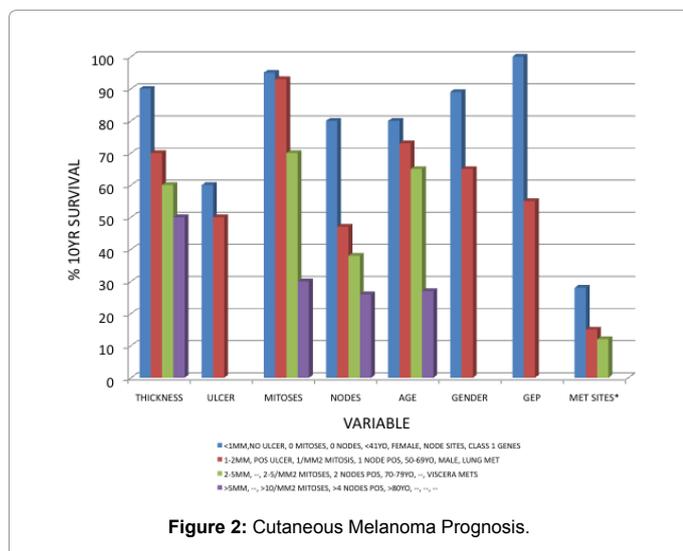


Figure 2: Cutaneous Melanoma Prognosis.

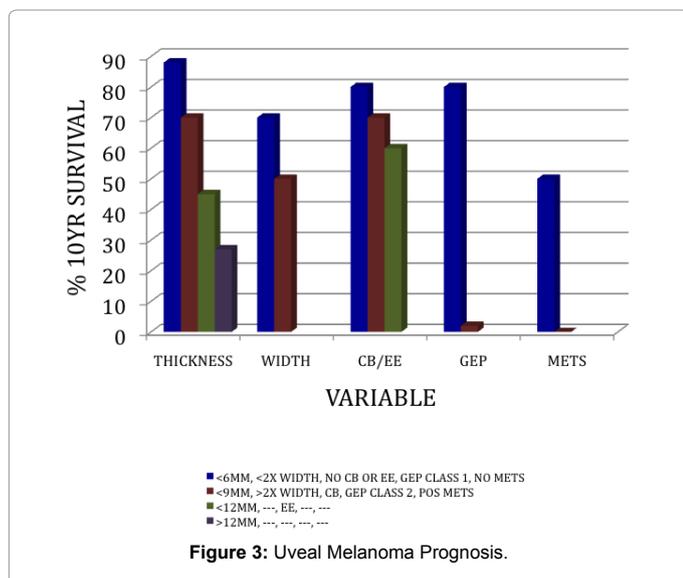


Figure 3: Uveal Melanoma Prognosis.

Uveal melanomas of ≤ 18 mm diameter and ≤ 12 mm thick are treated with plaque brachyradiotherapy, stereotactic radiosurgery or proton beam radiotherapy with retention of eyesight in most cases and 2%, 2% and 5% local recurrence rates, respectively [57,58]. The Collaborative Ocular Melanoma Study established the relative equivalence of ¹²⁵I plaque brachyradiotherapy to enucleation for local and systemic disease control [59]. Some centers consolidate treatment with transpupillary thermotherapy or sector photocoagulation. Intravitreal anti-VEGF or periocular triamcinolone injections may be used to minimize vascular complications. For uveal melanomas >18 mm diameter and >12 mm thickness, enucleation is performed. A minimal manipulation technique is done, and a hydroxyapatite globe inserted.

Mucosal melanoma primary therapy includes local excision with 2 cm margins for disease confined to the vulva [60]. For advanced disease, pelvic exenteration is done. Sinus melanomas are treated with wide local excision followed by external beam radiotherapy (Ming, 2014). Anal melanomas may be treated by either wide excision or abdominoperineal resection without differences in five-year survival [61].

Regional Therapy

Cutaneous melanomas have a 20% frequency of nodal and regional subcutaneous involvement, and efforts to control regional disease are an important part of care. Sentinel node sampling is done for cutaneous melanomas but not uveal or mucosal melanomas. If either positive sentinel nodes or clinically enlarged nodes are found, lymphadenectomy is carried out. Dissections are limited to the target lymph node basin-cervical, axillary, inguinal, iliac, epitrochlear or popliteal. In the setting of microscopically positive sentinel nodes, lymphadenectomy results in improved regional control, and for intermediate thickness primary tumors (1.2-3.5 mm), better overall survival [62]. However, lymphadenectomy is associated with a significant morbidity rate of 23%. Patients experience lymphedema, seroma formation, dysesthesias, and motor dysfunction. The post-adenectomy recurrence rate is 20% [63]. The German DeCOG-SLT trial demonstrated no superiority for lymphadenectomy in sentinel node positive patients and concluded adenectomy was not indicated for ≤ 1 mm micrometastases [64]. An ongoing MSLT-2 randomized clinical trial is comparing immediate lymphadenectomy for sentinel node positive disease versus ultrasound monitoring followed by lymphadenectomy on clinical recurrence. This study will address the value of early lymphadenectomy for all patients. For patients post-lymphadenectomy with positive parotid node or extra-nodal tumor spread or ≥ 3 cm cervical nodes or ≥ 4 cm axillary/inguinal nodes or ≥ 2 involved cervical/axillary nodes or ≥ 3 involved inguinal nodes, adjuvant 48 Gy external beam radiation over 20 fractions over 4 weeks, was well tolerated and reduced regional relapse from 55% to 25% [65]. There is no survival advantage for lymphadenectomy in vulvar, uveal, sinus or anal melanoma patients. Sinus melanoma adjuvant radiotherapy reduces regional recurrence two-fold [66]. Anal melanoma adjuvant radiotherapy to the extended field after wide excision reduces regional recurrence three-fold but does not impact distant relapse and may be associated with severe toxicities including lymphedema and proctitis.

Adjuvant Systemic Therapy

Melanoma-related death is due to systemic metastases present at diagnosis. After local-regional therapy, adjuvant therapy to prolong disease-free survival and improve overall survival has been tested. The only FDA approved agents are interferon α2b and pegylated interferon

$\alpha 2b$. Interferon- $\alpha 2b$ is a secreted glycoprotein that binds cell surface receptors with two distinct subunits. The bound complex phosphorylates Janus kinase 1-JAK1 and tyrosine kinase 2. Phosphorylated JAK phosphorylates the cytoplasmic transcription factors signal transducers and activators of transcription 1 and 2-STAT1/2. STAT1/2-P attaches to interferon-stimulated gene factor 3 and translocates to the nucleus where it reacts with interferon-stimulated response elements in the promoters of interferon-responsive genes and activates transcription promoting a pro-inflammatory state [67]. Meta-analyses of multiple randomized trials showed a one-year improvement in disease-free survival and a 3% benefit in overall survival for interferon versus placebo [68]. The subset of patients with ulcerated melanomas either ≥ 2 mm or with microscopic nodal disease also had a ten months disease-free survival benefit but a 20% benefit in overall survival for interferon [69,70]. Interferon toxicities are fatigue, anorexia, hepatotoxicity and flu-like symptoms and are controlled by dose modification and aggressive symptom control [71,72].

Based on the success of interferon, adjuvant ipilimumab (an anti-CTLA-4 immune checkpoint antibody discussed in more detail below) has been tested in node positive patients in U.S. and European randomized trials compared to interferon and placebo, respectively. Preliminary results of the European study at 2.5 years of follow-up show an ipilimumab nine months improvement in disease-free survival [73]. Similar to interferon, the greatest benefit was in ulcerated primaries with microscopic nodal involvement. Survival data is not yet available. Ipilimumab toxicities led to half of patients discontinuing treatment within three months. The most common side effects were autoimmune hepatitis, colitis and hypophysitis. The FDA approved adjuvant use of ipilimumab 10 mg/kg q 3wk x 4 then q12 wks x 3 years for node positive melanoma post-lymphadenectomy on 10/28/2015. An ongoing SWOG 1404 trial will compare high dose interferon, ipilimumab and pembrolizumab for adjuvant therapy of high-risk, resected stage III/IV melanomas.

Therapy of In-transit/Satellite Metastases

Isolated loco-regional skin metastases occur in 7% of melanoma patients and are associated with a 50% ten-year survival [74,75]. Thus, in-transit or satellite disease has an intermediate prognosis-some patient live decades with only regional recurrences and other patients rapidly progress with systemic metastases and death. The diagnosis is based on location of skin lesions between the primary tumor and draining lymph node basin. Possible causes include unique tumor genetics or lymphatic tumor emboli. Therapies yielding the highest disease control rate include intra-lesional recombinant interleukin-2 or talimogene laherparepvec genetically engineered herpes simplex virus, CO₂ laser lesion ablation, and hyperthermic or normothermic isolated limb perfusion or temozolomide limb perfusion (Andtbaca, 2015;) [76-78]. Durable remission rates are 40%, 16%, 24% and 25%, respectively. Side effects of interleukin-2, herpes simplex virus and CO₂ laser are local inflammation, fevers, and fatigue. Isolated limb perfusion may be associated with muscle injury or, rarely, limb loss. Intra-lesional therapies have been associated with durable control of treated lesions and regression of other metastases suggesting a prolonged, abscopal effect.

Metastasectomy for Oligometastatic Disease

Approximately, one-fifth of metastatic melanoma patients have a few indolent lesions limited to one to three organs [79]. A review of fourteen clinical trials showed of patients with isolated skin/soft tissue/node, lung, or non-lung viscera metastases, 58%, 23% and 15%

of patients, respectively, had disease amenable to metastasectomy. Compared with control patients matched for number of metastases and number of organ systems affected, metastasectomy yielded a median survival of 16 months and a 21% four-year survival versus 7 months and 7% survival in the control population in the MSLT-1 trial [80]. Best results were seen for skin/soft tissue/nodal metastatic patients with a hazard ratio of 0.24 favoring surgery. An international phase III trial (NCT01013623) is randomizing patients with ≤ 6 metastases in ≤ 3 organs to surgery versus systemic medical therapy alone to confirm the survival advantage of metastasectomy and identify patient subsets with maximal benefit.

Radiation Therapy for Metastatic Melanoma to Brain

Close to half of metastatic melanoma patients develop symptomatic brain metastases, and these lesions contribute to death in a fourth of all metastatic melanoma patients [81]. Unless surgery is needed for diagnosis, stereotactic radiosurgery-SRS is the established treatment for single and multiple brain metastases. 20-24 Gy single fraction or 25-30 Gy multiple fractions is administered. SRS has been shown to yield equal overall survival with improved neurocognitive function relative to whole brain radiation therapy-WBRT [82]. Frequent brain MRIs are employed to monitor for recurrence. 45% of SRS-treated patients required further brain radiation-either additional SRS or WBRT [83]. Death due to brain disease occurred in 11% of patients [84]. A graded prognostic assessment tool is available at www.brainmetgpa.com and provides melanoma median survival based on performance status and number of brain lesions [85]. A rare side effect (2%) is radiation necrosis that can be treated with corticosteroids, bevacizumab and/or surgery.

Chemotherapies for Metastatic Melanoma

Cytotoxic chemotherapies selectively target proliferating cells. The rationale is that tumor cells divide more frequently than normal tissues. While likely true for many hematologic malignancies and germ cell tumors, most tumors including melanomas have variable growth fractions. Thus, melanoma chemotherapy remissions are uncommon and normal tissue toxicities including myelosuppression and GI toxicities are frequent. A range of single agents and combination chemotherapies have been tested yielding response rates of 10-26%, progression-free survivals-PFS of 2-7 months, and overall survivals of 6-12 months (Figure 4) [86-90].

Because of the low response rates and minimal impact on overall

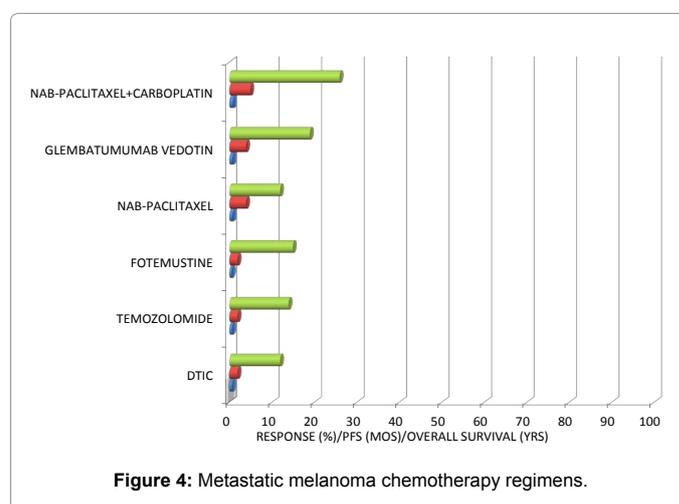


Figure 4: Metastatic melanoma chemotherapy regimens.

survival, efforts have been made to identify predictive biomarkers of response. O⁶-methylguanine DNA methyltransferase enzyme-MGMT repairs DTIC/temozolomide/fotemustine DNA damage and blocks cytotoxicity. Methylation of the MGMT promoter inhibits RNA and protein expression and enhances sensitivity to drug killing. MGMT promoter methylation was an excellent predictor of melanoma temozolomide response [91]. miR-659-3p microRNA expression controls expression of several melanoma anti-apoptosis genes and was associated with response to carboplatin plus paclitaxel [92]. gpNMB protein is the target of glembatumumab and gpNMB expression may predict response to glembatumumab vedotin. Additional confirmatory studies for each will be needed. These may permit better patient selection for chemotherapy agents. Nevertheless, toxicities are significant. Myelosuppression/GI toxicities, neuropathy, and rash were seen with temozolomide/ DTIC/fotemustine/carboplatin, nab-paclitaxel, and glembatumumab, respectively. While greater response rates, progression-free survival rates and overall survival were seen for combination regimens, frequency and severity of side effects worsened. Most of the studies were phase II, and response rates and response durations were less when the same drugs were tested in phase III trials. No evidence has been obtained that any one of the chemotherapy regimens is superior to others or to best supportive care. At present, our first approach at UTSW is to treat first with pathway inhibitors or immune modulators (see below) or enter eligible patients on novel clinical trials and reserve chemotherapy for salvage.

Pathway Inhibitor Therapy for Metastatic Melanoma

All living cells communicate with their environment via hormones and receptors. Mammalian cells possess cell surface receptors that transmit and amplify signals into the cytoplasm by secondary messengers including kinases. The signals modify gene transcription and translation, alter protein structure and change metabolism. Thus, cells are poised to adjust to changes in the extracellular milieu. Mutations in pathways can lead to neoplastic cell proliferation, survival and/or migration. Melanomas frequently have mutations in the mitogen-activated protein kinase-MAPK pathway. Small molecule compounds that block constitutive activation of pathway elements were used successfully in patients with BRAF V600E/K, NRAS and cKIT mutant metastatic melanomas. Further, some clinical benefit has occurred with VEGF inhibition in LDH elevated, non-lung viscera metastatic melanomas.

Half of cutaneous metastatic melanoma patients have BRAF V600E/K and have been treated with combinations of BRAF and MEK inhibitors such as dabrafenib/trametinib, vemurafenib/cobimetinib and encorafenib/binimetinib [93]. Response rates are 75% with PFS of 1 year and median overall survival 2 years [94]. Toxicities are pyrexia, nausea, diarrhea, rash, fatigue, edema, photosensitivity, central serous retinopathy, transaminasemia. The greatest problem is drug resistance due to MAPK reactivation or up-regulation of the PI3K/PTEN/AKT or NF- κ B pathways. MAPK reactivation occurred with MEK1/2 mutations, RAC1 mutation, MAP3K8 or CRAF expression, BRAF amplification, truncated BRAF variants, or NRAS or KRAS mutations [95-98]. Compensatory PI3K/PTEN/AKT pathway activation was seen with PI3KCA, AKT1/3, PIK3R2, PTEN, PHLPP1 mutations, PDGFR β or RhoB expression or stromal HGF expression [99,100]. Numerous *in vitro* and *in vivo* mouse melanoma models have shown synergistic cytotoxicity and anti-tumor efficacy of combining MAPK pathway inhibitors with PI3K/AKT inhibitors and clinical studies of these combinations are ongoing [101-104]. NF- κ B activation and MITF amplification also produced MAPK-independent resistance [105,106].

Twenty percent of cutaneous melanomas have NRAS mutations. The MEK inhibitor binimetinib achieves a 20% response rate and six-month PFS in NRAS mutant patients (Ascierto, 2013). Fifteen percent of acral and mucosal melanomas possess c-KIT mutations. Imatinib mesylate produces a 33% response rate, a 4 month PFS, and a 14 month overall survival in patients with c-KIT exon 8, 11 or 13 mutations [107,108]. Metastatic melanoma patients with non-lung visceral metastases and elevated LDH show increased tumor angiogenesis and improved response to anti-VEGF therapy. Bevacizumab plus carboplatin plus paclitaxel gave a 26% response rate, 6 month PFS, and 12 month OS that was superior in each case to carboplatin plus paclitaxel alone [109]. For all pathway inhibitors, recurrences are due to branched evolution with multiple different resistance genotypes for each metastasis in a patient.

Immunotherapy for Metastatic Melanoma

Several factors triggered an interest in immunotherapy for melanoma. First, metastatic melanoma has the highest rate among cancers of spontaneous durable remissions at 0.2% [110,111]. These regressions are associated with marked tumor lymphocytic infiltration. Second, interferon showed some anti-melanoma activity as noted above. Third, the discovery of the T cell growth factor, interleukin 2-IL2, and its cloning and expression and purification from *E.*

coli facilitated testing in humans. IL2 binds heterodimeric receptors on cytotoxic T cells and NK cells, generates P-STAT5 and promotes differentiation and proliferation [112]. Rosenberg and others tested high doses of IL2 in metastatic melanoma patients [113]. Patients administered 600,000 IU/kg/IV bolus every eight hours for up to 14 doses in cycles separated by 1-4 weeks achieved a response rate of 16% including 6% complete responses-CRs. Remarkably, most of the CRs have remained disease-free for decades. Unfortunately, the treatment is associated with neutrophil paralysis and severe capillary leak syndrome. Toxicities are sepsis, hypotension, uremia, pulmonary edema, hepatic and GI toxicities, thrombocytopenia and 1% deaths. Prophylactic antibiotics and a stringent protocol for dosing, fluid supplements and pressors have reduced side effect severity [114]. Predictors of CR include N-RAS mutation, better pre-treatment performance status, normal LDH and only skin/subcutaneous metastases [115]. Pre-treatment stereotactic body radiotherapy may improve CR rate [116]. Nevertheless, the severe toxicities and limited efficacy discourage most patients from this treatment.

These observations opened the possibility of long-term remissions from expanding cytotoxic T lymphocytes in patients. However, once reaching the tumor, very few remissions were obtained. The immunosuppressive tumor microenvironment was due in part to local immune checkpoints-CTLA4 blocking CD28 co-stimulation and PDL1 inhibiting T cell activation. Human monoclonal antibodies blocking the immune checkpoints were developed--ipilimumab and tremelimumab for anti-CTLA4 and nivolumab and pembrolizumab for anti-PD1. The combination of nivolumab and ipilimumab--FDA approved 9/30/15 produced a 61% response rate and a 12 months PFS in metastatic cutaneous melanoma. Median OS exceeds two years [117]. Toxicities are severe and common but manageable. Autoimmune complications are seen in most patients and consist of colitis, hepatitis, hypophysitis, dermatitis, and are treated with drug discontinuation, steroids and/or infliximab [118]. Pretreatment low serum VEGF and LDH, NRAS mutation, large mutational load or neoantigen load, tumor T cell infiltration, viral defense signature, high tumor interferon γ mRNA levels, lack of IPRES gene signature, oligoclonal circulating and

tumor TCR clonality, absence of Wnt/ β -catenin pathway activation, elevated PD-L1 level, and elevated circulating lymphocyte, monocyte or eosinophil levels predict response (Goldmeyer, 2016) [119-132]. Relapse to anti-PD1 therapy has been linked to loss-of-function mutations in JAK1, JAK2, and beta-2microglobulin [133]. Similar efficacy was observed in acral and mucosal metastatic melanomas, but not in uveal metastatic melanomas [134-136].

Another method to ameliorate the immunosuppressive melanoma microenvironment is intra-lesional injections of modified herpes simplex virus type 1 carrying deletions of virulence genes ICP34.5 and ICP47 and insertion of human granulocyte-macrophage colony stimulating factor, talimogene laherparepvec or T-VEC. T-VEC selectively replicates in melanoma cells and lyses the cells promoting systemic anti-tumor immunity. T-VEC has been given at 10^6 pfu/mL and then 10^8 pfu/mL up to 4 mL at 2 weeks intervals for up to 18 months. For patients with subcutaneous and nodal lesions and limited visceral metastases, the response rate was 23% and median OS was 24 months vs 6% and 19 months with subcutaneous GM-CSF [137]. T-VEC was FDA approved on 10/27/2015. With these exciting developments, the stage is set for exploratory studies to further improve response rates and response duration in different patient subsets.

Novel Areas of Melanoma Clinical Research

Preclinical melanoma research employing *in vivo* models has focused on the syngeneic B16 melanoma in C57BL/6 mice, human melanoma cell lines or patient derived tissue sample xenografts in immunocompromised mice, and genetically engineered mouse models with RAS, p16INK4A, p19ARF, CDK4, p53, RET, BRAF, GNAQ, glutamate receptor GRM1 and/or PTEN mutations [138-140]. The syngeneic model and genetically engineered mice models offer metastatic variants including luciferase transformants for live animal imaging. Each model has advantages and disadvantages with similar patient biology in the patient derived xenografts and relevant immunology in the syngeneic models. In this review, we focused on translational research currently in patient studies.

Visual and palpable clues are used in radical surgery to differentiate melanoma from healthy tissue and are limited to node or tumor deposits of approximately 3-5 mm diameter. Thus, as noted earlier many patients relapse locally in the same organ. Jiming Gao and colleagues synthesized a nanoparticle composed of polyethylene glycol-polyethylpropylaminoethyl methacrylate copolymers with attached fluorescent dye molecules and tertiary amines [141]. In the acid environment of tumor deposits, the micellar particles invert and expose the attached dye. With infrared illumination, tumors deposits as small as 1 mm are detected. Similar or better resolution to that obtained with cetuximab-IRDye800 [142] may occur but with better application for melanoma and other cancers. These nanoprobe will enter clinical trials in 2017 and may improve detection of small metastatic melanoma deposits at lymphadenectomy and metastasectomy.

Staging tests include BRAF mutation analysis to determine patient suitability for pathway inhibitors. However, a third of BRAF mutant melanoma patients are intrinsically resistant to inhibitors. A five-gene expression profile that identifies resistant patients has been reported [143]. Combining gene expression profiles of resistant patient samples and cell lines with RNAi high throughput screening for BRAF/MEK inhibitor resistance and chromatin immunoprecipitation sequencing, a pattern of gene expression in patients was discovered that correlated with primary clinical resistance to inhibitors--Sox10-independent (Figure 5). Further testing patient's tumors with the five-gene profile

and measuring BRAF inhibitor progression-free survival is underway. This will permit better therapeutic decision making in BRAF mutant metastatic melanoma patients.

Staging tests for immunotherapy response have not to date offered methods to overcome resistance. However, a novel finding may yield a low-cost method to improve efficacy. Lida and colleagues in 2013 showed germ-free or antibiotic treated tumor-bearing mice did not respond to immune or chemotherapy regimens whereas their genetically identical siblings with gut microbiota showed excellent responses that were linked to myeloid-derived macrophages that migrated from the gut to the tumor [144]. Vetzou and Sivan observed similar variations in sensitivity to anti-CTLA4 antibodies and anti-PD-L1 antibodies in tumor-bearing mice that depended upon the presence of commensal *Bacteroides* and *Bividobacterium* species, respectively [145,146]. Again, the appropriate gut bacteria induced mature intra-tumoral dendritic cells that in turn recruited and differentiated interferon- γ producing T cells. Pre-treatment human gut microbiota profiles may differ in immune checkpoint responders versus non-responders and is being examined at the University of Texas Southwestern by faecal bacterial DNA metagenomic shotgun sequencing using established techniques [147]. Subsequently, quantitative PCR analyses of key bacterial species will be done and bacterial abundance quantified [148]. These experiments will provide a complete picture of specific microbial populations and how they correlate with immune checkpoint modulator treatment and response. If specific organisms yield immunotherapy sensitivity or resistance, followup intervention studies may be designed.

Sean Morrison and colleagues explored cytotoxic activity of small molecule in melanoma [149]. They found cardiac glycosides reacted with the ATP1A1 sodium/potassium pump overexpressed on melanoma cells leading to cell death. The glycosides inhibited the plasma membrane ion transporter leading to cytosolic acidification. When combined with MEK inhibitor, mitochondrial calcium increased, and mitochondrial membrane potential, ROS, NAD⁺ and ATP levels fell. Melanoma cell death was seen. The digoxin plus trametinib combination yielded melanoma regressions in immunocompromised mice xenografts. Based on these results, a phase 1B trial of daily oral digoxin plus trametinib for 20 BRAF wild-type melanomas patients was completed at the University of Texas Southwestern. The most common adverse events were rash, diarrhoea, nausea, and fatigue. The disease control rate including partial responses or stable disease was 13/20 or 65% of patients, including 5/6 or 83% for patients with NRAS mutant melanomas and 8/14 or 57% for NRAS wild-type melanomas. Response

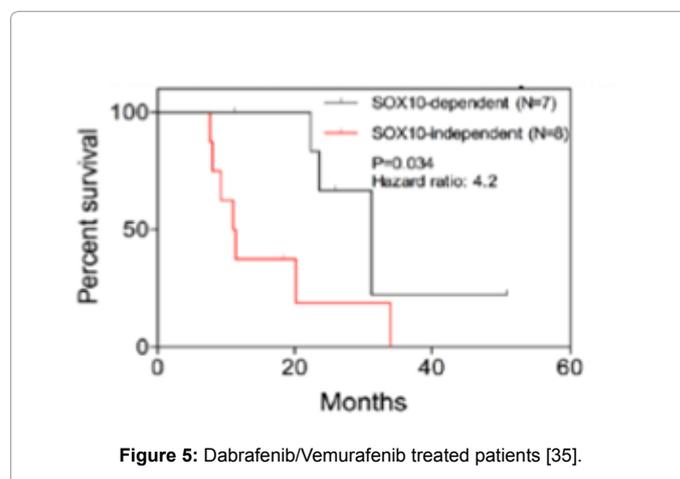
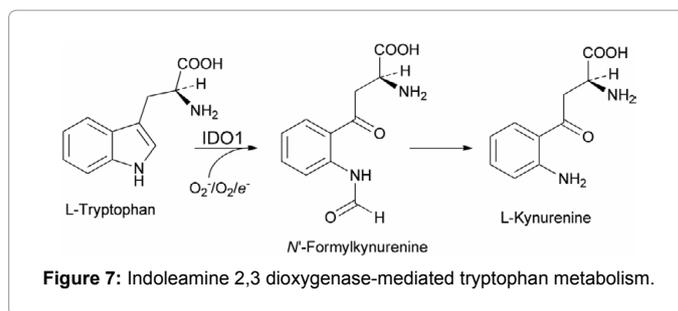


Figure 5: Dabrafenib/Vemurafenib treated patients [35].

durations were 2, 4, 6, and 8 months for patients with partial responses, and 2, 2, 2, 4, 5, 6, 7, 8+ and 10 months for patients with stable disease. Xenografts from four patients recapitulated the treatment responses observed in patients. This compares favourably with the single agent trametinib expected PR rate of 10% and PR+SD rate of 36% [39]. More patient accruals and follow-ups are needed to assess therapeutic benefit. In the subset of NRAS mutant patients, digoxin plus trametinib yielded an 83% disease control rate-DCR versus an expected rate of 58% DCR for MEK inhibitor alone [150].

While immunotherapy provides long-term disease control in many patients, up to half of patients fail to achieve clinical benefit. Immune checkpoint resistant melanomas lack intra-tumoral activated cytotoxic T lymphocytes [122]. A single modality-stereotactic ablative radiation-SAbR therapy provides tumor antigens and damage-associated molecular pattern agonists to dendritic cells [151,152]. The stimulated and primed antigen-presenting cells induce tumor-specific cytotoxic T cells that migrate to the melanoma metastases and alter the tumor microenvironment [153,154]. Evidence for radiation-elicited innate immunity and enhanced immunotherapy efficacy has been shown in mouse models [155,156]. Further, clinical reports document cases of SAbR synergy with immune checkpoint inhibition [157-160]. In an ongoing study at the University of Texas Southwestern, metastatic melanoma patients are treated concurrently in week one with SAbR+ipilimumab+nivolumab followed by ipilimumab+nivolumab alone. Tumor biopsies are done pre-treatment and after SAbR to measure T cell infiltration and cGAMP levels from the cGAS-STING pathway (Figure 6) [161]. Responses will be assessed by RECISTv1.1. An increase in response rate and PFS from the historical rates of 58% and 11.5 months is anticipated [117]. Irradiated tumors may show an increase in cGAMP levels and type I interferon gene signature post-radiation secondary to cytosolic DNA and increased interferon- γ producing tumor cytotoxic T lymphocytes secondary to macrophage/dendritic cell chemokine secretion.

Even in the presence of CTLs permeating the melanomas and immune checkpoint blockade with anti-CTLA4 and/or anti-PD1/PD-L1, a quarter of patients show tumor progression. Tumor cells produce indoleamine 2,3-dioxygenase-IDO [162]. IDO in the tumor microenvironment converts tryptophan to L-kynurenine (Figure 7). Tumor cytotoxic T cells lacking the essential amino acid tryptophan activate GCN2 leading to cell cycle arrest and functional energy [163]. Kynurenine binds aryl hydrocarbon receptors and drives *de*

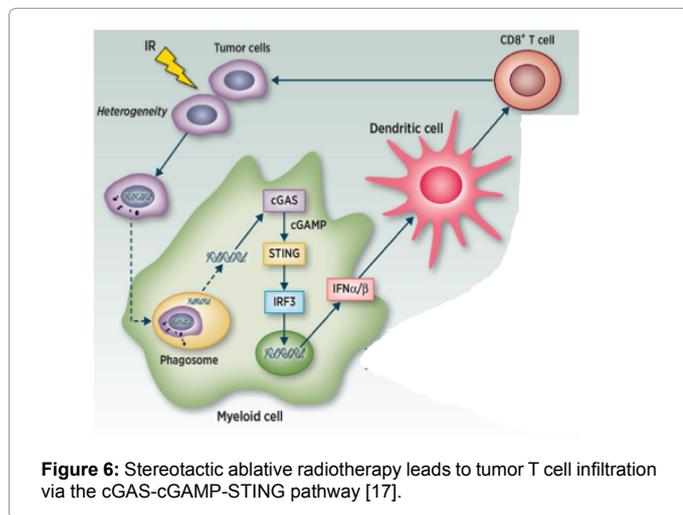


novo differentiation of inhibitory Foxp3+T regulatory cells-Tregs and myeloid derived suppressor cells-MDSCs. These cells, in turn, release cytokines that block recruitment of further cytotoxic T lymphocytes to the tumor. Note placental IDO blocks maternal T cell rejection of the fetus. The combination of an IDO inhibitor with immune checkpoint blockers including anti-CTLA-4 and anti-PD-1 yield additive to synergistic tumor growth inhibition in melanoma tumor models [164]. An Incyte-sponsored phase 1B study of the IDO inhibitor-epacadostat with the anti-PD-1 antibody-pembrolizumab in patients with metastatic melanoma is ongoing.

Thus, current research is identifying patient subgroups for novel surgery, novel pathway inhibitors and new combinations and approaches for immunotherapy. The melanoma field is leading the way both in basic and clinical oncology science.

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