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REVIEW

Revisiting the melanomagenic pathways and current therapeutic approaches

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Abstract

Melanoma is one of the most aggressive forms of skin cancer with a steady increase in global incidence and mortality rate over the past five decades. Paradoxically, both reduced and excessive sun exposure has been linked to increased risk of melanoma incidence and death. Although the histological classification of melanoma is useful in diagnosis, its molecular subtypes are often determined by somatic mutations, which could be UV-dependent or -independent. Multiple genes involved in cancer development are often mutated dysregulating molecular pathways with concomitant phenotypic heterogeneity. Hence, treating melanoma has been a challenge, with patients experiencing poor clinical outcomes to current therapeutic options. This presents an unmet need to understand the interaction of molecular networks underpinning melanogenesis. This review describes the crosstalk of signaling cascades in melanoma development and the putative druggable targets, with the view of elucidating newer and better therapeutic strategies for the disease.

Keywords Melanoma · Melanomagenesis · Molecular pathways · Diagnosis · Therapeutics

Introduction

The skin is one of the largest human organs with a huge surface area that interacts with the immediate environment. Consequently, the skin cells can undergo a malignant transformation in any part of the body [1]. This often involves aberrant melanocytes located within the epidermal basal layer. Of all the types of skin cancers known, melanoma is the least common but the most aggressive subtype. It is the 17th most common malignancy accounting for nearly 32,463.5 million newly diagnosed cases in the year 2020. With about 173,844 males and 150,791 females affected globally, this neoplasm has rapidly increased over the last 50 years and currently results in about 57,043 deaths annually [2, 3]. In the United States, melanoma alone is responsible for 8.7% of the deaths, as against 2.8% due to other malignancies across all races and ethnicity, with a mortality-associated productivity loss [4–6]. The determinants of melanoma development and prognosis frequently involve both genetic and environmental components [7]. For yet to be uncovered mechanisms, gender constitutes a major biological/genetic factor in melanoma morbidity and mortality [8]. Furthermore, a specific genetic condition that predisposes the population to this disease includes Xeroderma Pigmentosum and mutations in the BRCA2 and CDKN2A genes. Such susceptibilities confer a 1000-fold increased risk to melanoma [7]. The additional burden of this disease in fair-skinned- people with Fitzpatrick type I skin color have a higher risk than those with Fitzpatrick type VI, thus identifying the Ultraviolet B (UVB) exposure as a crucial yet modifiable environmental risk factor. Paradoxically, the geographical disparity is associated with reduced suboptimal sun UVB exposure that results in vitamin D deficiency or insufficiency with a compromised immune response for skin-associated sunburns [9] and indoor workers [10]. This has been termed as the intermittent UV exposure theory of melanomagenesis which propounds that "intermittent and intense sun exposure that places individuals at increased risk for melanoma", particularly in the presence of both low melanin content and deficient basal DNA repair capacity [3,

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11, 12]. Consequently, UV melanomagenesis could occur through (i) initiation of the normal melanocyte due to DNA Damage, (ii) promotion of the initiated melanocyte by solar nevogenesis, (iii) increased genomic instability by continued DNA damage, and (iv) specific immunosuppression by induction of suppressor cells [11, 12]. In this review, we briefly describe the clinical subtypes and diagnostic techniques in melanoma. We also give a detailed account of various druggable targets and therapeutic interventions available for melanoma.

Clinical subtypes of melanoma

The histo-genetic classification of melanoma given by Wallace Clark, (1967) remains the premise for the recent new-age classification made by WHO (World Health Organization). According to Clark, melanoma can be divided based on '*intra-epidermal component of tumor and peripheral to dermal invasive component*' [13, 14]. The three subtypes under this classification were (i) superficially spreading melanoma, (ii) lentigo maligna melanoma, and (iii) nodular melanoma. In this classification, the depth of invasion according to the level of skin layers involved is correlated with melanoma prognosis. In 1970, McGovern came up with slightly different terminology viz. melanoma arising in Hutchinson's melanotic freckle (Clark- lentigo maligna), melanoma arising from pre-malignant melanosis (Clark- superficial spreading melanoma), and nodular melanoma (Clark- nodular melanoma) [15]. In 1972, at the International Pigment Cell Conference and International Cancer Conference, Sydney, a consensus-based upon histological reports from patients, two forms of melanoma in situ were defined. The non-invasive form included: Hutchinson's melanotic freckle and superficial spreading melanoma; and invasive form included: melanoma, (i) with an adjacent intra-epidermal component of Hutchinson's melanotic freckle type, (ii) with an adjacent intra-epidermal component of superficial spreading type and (iii) without adjacent intra-epidermal component [16]. These initial classifications were modified by contributions from clinicians and scientists at the Sydney conference in 1982 (Fig. 1) [17]. Accordingly, the present types and subtypes are based on clinicopathological features. The updated World Health Organization (WHO) classification from 2018 is charted in Fig. 2 [13, 18, 19].

Diagnosis in melanoma

The complexity and heterogeneity of melanoma makes its diagnosis intricate. Therefore, the existing focus of research is on target identification and understanding the mechanisms of melanomagenesis to develop novel treatments. Prime risk factors in melanoma evolution include—family history of cancers, genetic defects, and environmental exposure specifically to UVB [20]. The risk of melanoma directly correlates to an increase in first-degree relatives with melanoma. The initial clinical diagnosis usually focuses on patient history, physical and histopathological examinations. A routine skin examination usually helps in discovering melanotic lesions

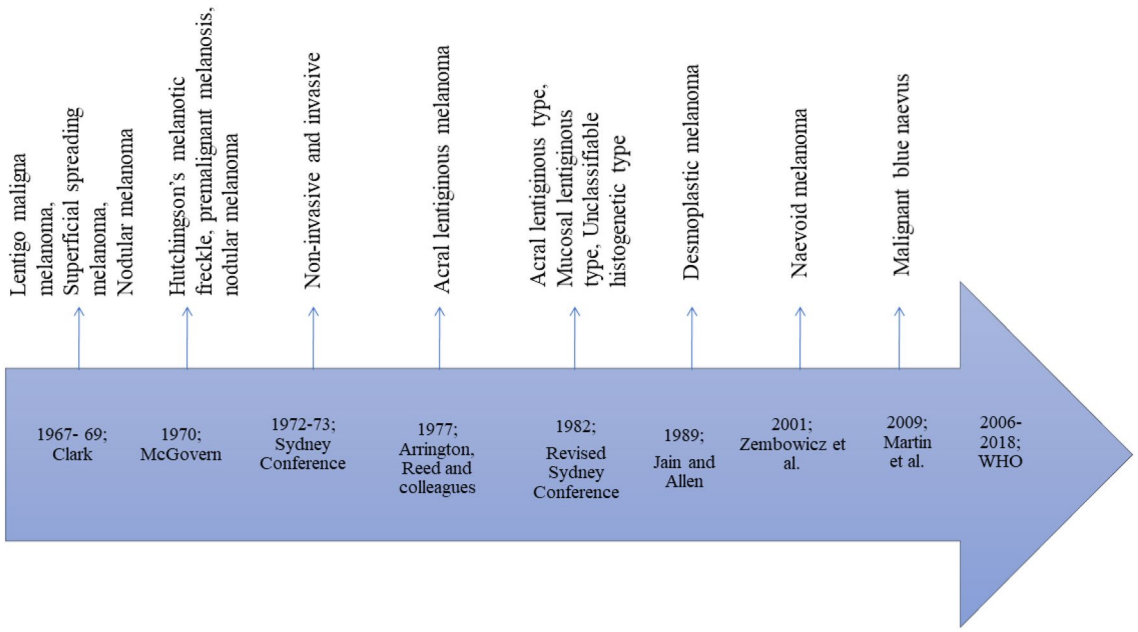

Fig. 1 Timeline of Development of Classical Subtypes of Melanoma

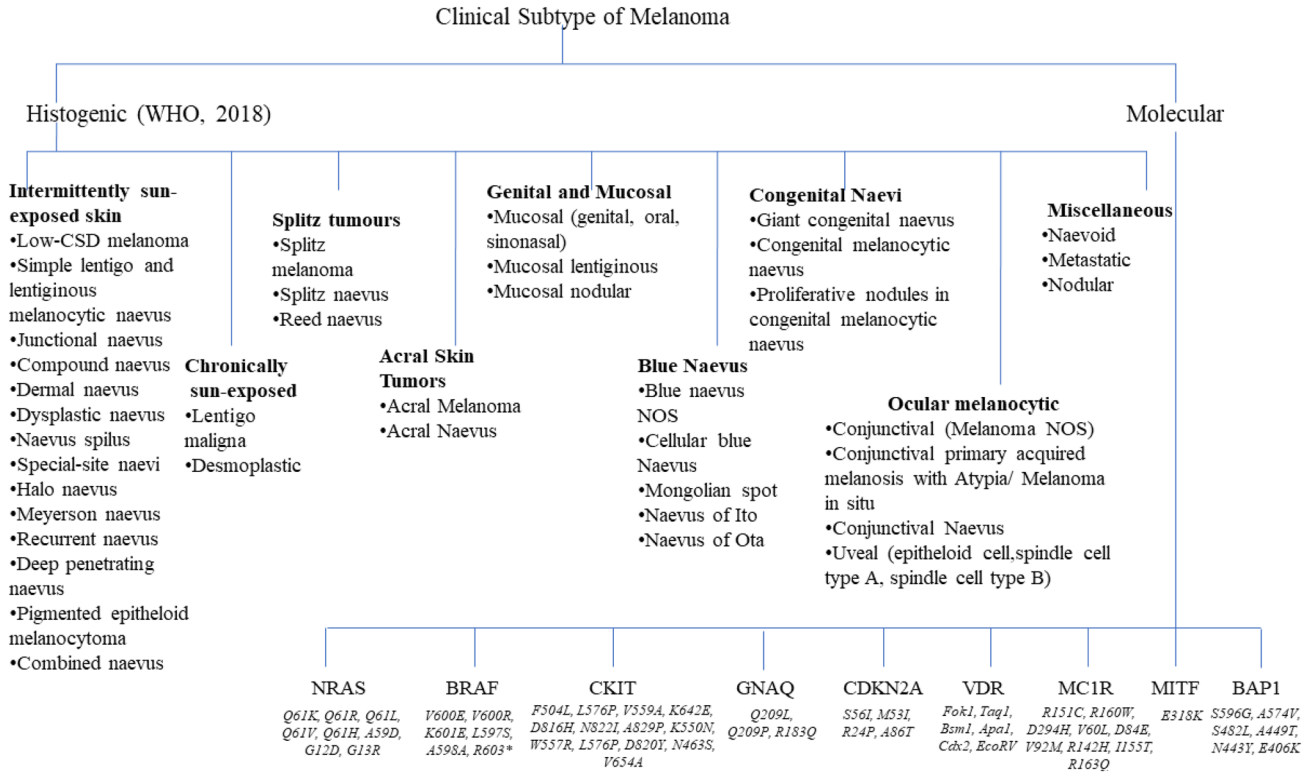

Fig. 2 Histogenic (WHO, 2018) and Molecular Classification of Melanoma

which are pigmented nodule that results from constant bleeding and itching in the early stages. Generally, melanomas may go undiagnosed since they are asymptomatic and might show up only in advanced stages. As mentioned earlier, there is a correlation between melanin pigment and developing melanoma, with less pigmented people being more prone to melanoma [20]. The clinicians usually detect sore, unhealed outgrowths or moles with changing color and size followed by additional symptoms viz. headaches, seizures, cough, hemoptysis, shortness of breath, dyspnoea, changes in vision or bowel habits, back pain, fevers, chills, night sweats, and visible weight loss [20, 21].

Classically the '**ABCDE**' acronym (1985), stands for **A**symmetric moles of variable size and shape; **B**orders of nevi, their **C**olour, and **D**iameter (greater than 6 mm) dictates the **E**volution of outgrowth and has been used for physical examination of melanoma [22]. Alongside the surrounding areas are examined for the presence of satellite lesions or in-transit metastatic foci further to which suspicious lesions are biopsied and used for histologic analysis [22]. The analysis is carried out considering the vertical depth of invasion, thickness, subtype, mitotic rate, margin status, presence or absence of ulcer, infiltration, cellular regression, and invasion [23]. An external biopsy is not only a diagnostic procedure but also approved as a part of a treatment regimen by the American Academy of Dermatology. The differential diagnosis (Supplementary Figure S1) enabled by the American Joint Committee on Cancer (AJCC) uses TNM (tumor, node, metastasis) staging system that allows physicians to categorize melanoma to plan a precise treatment regimen. The upgraded manual has improved tumor thickness assessment, removed mitotic activity as a cause to upstage a thin melanoma, expansion, and invasion into regional lymph nodes (N), and metastasis (M) categories depending on the location of secondary tumors. Higher variability in inter and intra-observations among pathologists has allowed melanoma diagnosis to move from '*subjective to objective observations*' which include prebiopsy non-invasive imaging techniques and post-biopsy immunohistochemistry (IHC), fluorescent in situ hybridization (FISH), comparative genome hybridization (CGH), or mass spectrometry (MS) techniques [24, 25]. The resemblance of this disease to carcinomas, sarcomas, lymphomas, neuroendocrine, and germ cell tumors, makes the histological diagnosis of melanoma very challenging.

Histopathologic and IHC have been a useful tool in the identification of various biomarkers (clinical, serological, prognostic, epigenetic) related to melanocytic differentiation and melanoma progression. Although it provides subjective scoring, the development of multiple biomarker-based diagnosis systems for optimal standardization procedures and strong interpretation criteria has made it successful in

melanoma diagnosis [26]. Apart from IHC, gene expression using RT-PCR assists in the diagnosis of melanocytic tumors [25].

Table 1 enlists various differentiation and progression markers that are highly specific for the diagnosis of poorly differentiated malignant tumors and staging of the primary melanocytic lesion, micro-invasive tumor cells, or depth of invasion [27]. In brevity, we describe some important markers here. The widely used proliferative marker Ki67, when over-expressed in melanoma, becomes a predictor of poor prognosis which is related to the tumor thickness. Other proliferative markers have a sensitivity ranging from 58 to 100% and are well characterized as per the mutational signature. It is known that in advanced melanoma these markers may not be of high prognostic value, but they are invariably involved in DNA ploidy especially markers like p53, Ki67, and PCNA. The fine balance of cell cycle regulators like p16INK4a, p21WAF1, GADD, and cell cycle progressors such as Cyclin A/B/D1/D3/E, CDK2, is lost in melanoma and has a critical role in the vertical and metastatic growth phase of the disease making them important cell proliferative markers. S100 is the most sensitive of the differential markers followed by (MART-1), tyrosinase, MITF which demonstrate ~ 97–100% specificity, and Melan-A, HMB-45 with the least specificity for melanoma of sentinel lymph node. Though the association between growth factor receptors like VEGF and melanoma progression or survival could not be established, in cutaneous melanoma, osteonectin can serve as a predictor of clinical outcome. Frequent mutations of BRAF and NRAS cause hyper activation of EGFR and FGFR growth receptors and increase the mutagenic effect on the melanogenic cells. Besides this, BRAF, RAS mutations along with mutations of PTEN are involved in activating cell proliferative pathways like AKT/PKB and downstream transcriptional factors ATF-1, AP-2, enhancing the transcriptional control of melanoma metastasis. AP-2α also regulates the expression of adhesion molecules and matrix metalloproteases indicating their role as markers in melanoma progression. Decreased disease-free survival was observed for MMP-1 and MMP-3 positive melanoma. The progressive markers like HLA-I, II, and CD26/40 are implicated in increasing proliferative lesions while apoptotic modulators like FASL increase the immune privilege of melanogenic cells [28]. Many CTAs have been found to express in metastatic melanomas which potentiates them to be diagnostics markers and therapeutic targets. *High throughput techniques have come to aid in melanoma diagnosis since it has helped in evaluating the frequency of specific mutations in disease subtypes and at various stages of the disease. It has also helped in discovering novel targets for diagnostic and therapeutic advancements.*

To move toward an accurate, sensitive, quantitative, and objective diagnosis, various imaging techniques are being used with improvisations. The digital imaging with computer-aided examination includes—MoleMax, SIAscope, SolarScan, MelaFind; laser-based confocal scanning laser microscopy (CSLM), optical coherence tomography (OCT), laser doppler perfusion imaging (LDPI), ultrasonography, MRI, PET, Nevisense, Molemate, dermoscopy and telederm-moscopy, total body photography, MSS (Multispectral imaging in the spatial domain), MSF (Multispectral imaging in the frequency domain), and Terahertz imaging (THz), that enhances the accuracy in diagnosis, the performance of clinicians and simultaneously reduces the mortality by facilitating earlier and easier detection of melanoma [26, 29]. Singh and Gupta (2018) have listed several methods, algorithms, and patterns for image pre-processing of melanoma lesions invented by scientists between 1999- 2016 [30]. A total of 26 Android/ IOS dermatology-based mobile apps have been developed that enables the high-risk group to monitor themselves [30]. These health apps have proven successful in raising public awareness to differentiate between sunburns, lumps, and cancerous lesions.

Table 1 List of differentiation and progression markers

Differentiation markers	Progression markers	
S100, gp100/HMB-45, tyrosinase, MART-1/Melan-A, HMW-MAA, tyrosinase, microphthalmia transcription factors (MITF), SOX10, MUM-1, MCR-1	Growth factor receptors	VEGF, VEGFR-3, bFGF, EGF/EGFR, Osteonectin, TGF-β, c-kit, transferrin receptor
	Signaling molecules	PTEN, AKT/ PKB
	Transcription factors	ATF-1, AP-2
	Proliferation molecules	Ki67/ MIB1, PCNA, Cyclin A/B/D1/D3/E, CDK2, $p16^{INK4a}$, $p21^{WAF1}$, $p27^{kip1}$, p53, HDM2, GADD
	Adhesion molecules	CD63, Fascin, CD171, VCAM-1, ALCAM, ICAM, CD44, ILK, integrins, N/E-cadherins, α/β-catenin
	Immunoregulators	HLA-I and II, CD26/40, FAS, FLIP, CTAs
	Proteases	MMP2/9, MTA-MMP, EMMPRIN, Cathepsin B/D/L, FAP
	Other proteins	APAF-1, metallothionein, ACS/TMS1, skeletrophin, MTAP, Versican, SKI, and h-CD/CNh1

The crosstalk of signaling cascades in melanomagenesis

The key signaling pathways involved in the onset of melanoma include- MAPK-ERK, PI3K-Akt, NFκB, JAK-STAT, Wnt-β catenin, Notch, TGF-β, and CDKN2A. Under normal conditions, these pathways are involved in general functions of cell cycle regulation, cell growth, survival, differentiation, proliferation, migration, and tissue homeostasis. Some of these pathways also help in specialized functions like stem cell maintenance, hypoxia response, embryogenesis, synthesis of the extracellular matrix, mammary gland development, lactation, adipogenesis, inflammation, and other immunological responses [31]. Supplementary Fig. 2 summarizes the progression of melanoma and the pathways involved corresponding to different stages. The crosstalk between various signaling cascades and important deviations during melanomagenesis are represented in Fig. 3.

The tumor suppressor gene **CDKN2A** encodes for two different proteins, p16 (INK4a) and p14 (ARF), both involved with Rb and p53 proteins in two separate pathways for cell cycle regulation. While the loss of function in p16 results in the upregulation of cyclin-CDK activity, functional loss of p14 facilitates the accumulation of Mdm2 and p53 degradation, both resulting in cell cycle progression despite DNA damage [32]. In 1992, locus 9p21 was first described to be involved in familial melanoma [33]. In 1994, eight p16 germline substitutions (splice donor site, nonsense, and missense) were reported by Hussussian et al. in approximately 72% of familial melanoma cases [33, 34]. Later, multiple studies explored the role of CDKN2A in melanoma susceptibility. About 20% of melanoma-prone families are found to have mutant CDKN2A, although the frequency of mutation varies anywhere between 5 and 72% depending upon the geographic location and ethnicity [35].

In healthy cells, the MAPK pathway transmits signals through Ras-GTP complex, the formation of which is initiated by binding of growth factors or mitogens with receptor tyrosine kinase (RTK). The Ras-GTP then activates the first effector kinases A-Raf, BRAF, and C-Raf via GTP hydrolysis by NF-1 like GTPase-activating proteins (GAPs). This, in turn, phosphorylates **MEK1/2** and **ERK1/2** followed by their translocation into the nucleus and regulation of gene expression by transcriptional factors like c-Fos, c-Myc, MITF, cyclin-D1, p90RSK, and c-Jun [36]. In melanoma, mutations in BRAF, RAS, or NF1 proteins cause constitutive activation of the MAPK pathway. Davies et al. (2002) reported major somatic missense substitution at 600th amino acid—V600E (Valine to Glutamic acid) of BRAF in approximately 66% of malignant melanoma cases [37]. In cells harboring $BRAF^{V600}$ mutations, MAPK pathway activation is constitutive and renders the cancer cells resistant to BRAF and MEK inhibitors [38].

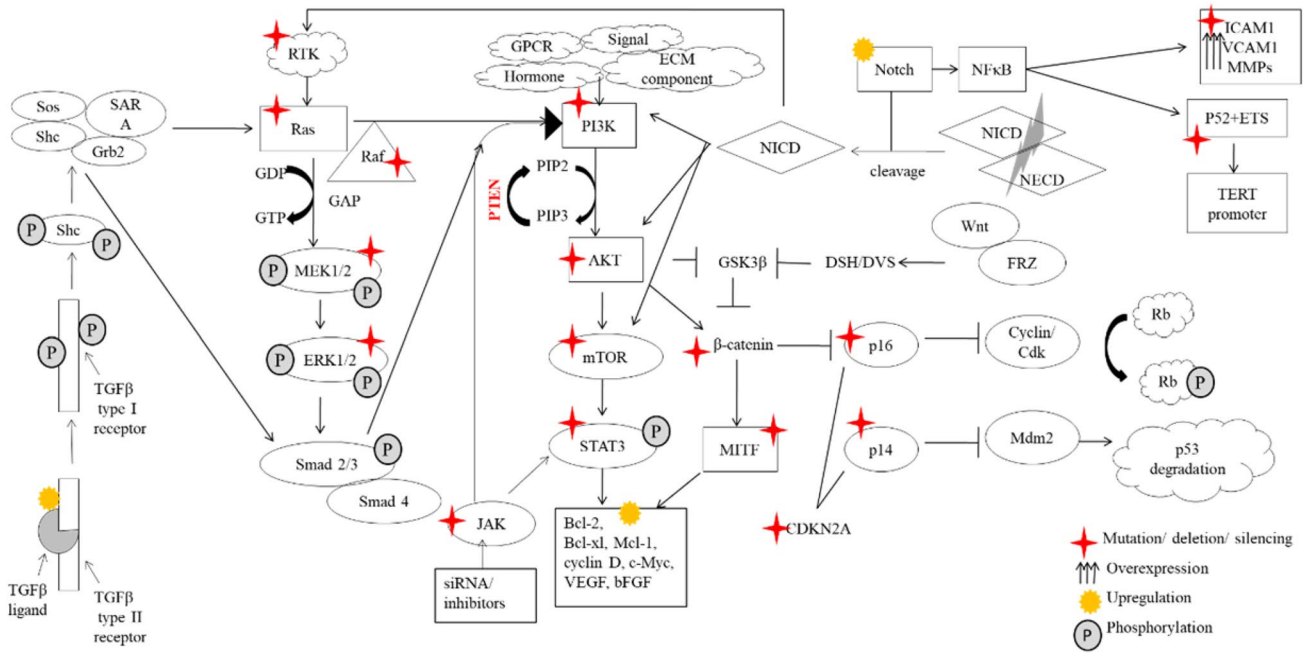

Fig. 3 Crosstalk among Various Signalling Pathways involved in Melanomagenesis; *NICD*- Notch intracellular domain, *NECD*- Notch extracellular domain, *FRZ*- Frizzled, *DSH/DSV*- Dishevelled, *GSK3β*- Glycogen synthase kinase 3 beta, *Shc*- Src homology domain 2 containing, *Grb2*- Growth factor binding protein 2, *SARA*- Smad anchor for receptor activation, *TERT*- telomerase reverse transcriptase

The TGFβ (Transforming growth factor β) is a cytokine involved in cellular proliferation whose functions depend on the type of receptor binding in a given cell type. Upon binding to the receptors, it phosphorylates Smad2/3 proteins (R-smads). R-smads in turn associate with Smad4 (Co-Smad) which as a complex translocates into the nucleus to regulate gene transcription by direct binding to the target gene promoter and/or through the interaction with transcriptional cofactors in a cell-type-specific manner. TGF-β receptors also influence other pathways that indirectly activate Smads, like activation of RAS via TβRI which forms a ShcA-Grb2–Sos complex. The type I TGF-β receptor can work through the PI3K-Akt pathway to activate Smads. These activations are essential for epithelial to mesenchymal transition (EMT) in cancer cells which is associated with metastasis [31, 39]. Mutational activation of Ras family proteins, PI3K, Akt, mTOR, and RTK can result in constitutive activation of PI3K-Akt cascade. Dysregulation of these pathways is associated with a poor prognosis of melanoma and non-melanoma skin cancers. In cutaneous melanoma, the resistance toward the growth inhibitory effect of TGF-β is observed, leading to tumorigenesis. As the disease progresses, the upregulation of TGF- β in melanoma cells causes inhibition of immune response supporting the tumor growth. Besides, TGF-β shows a direct effect on tumor cell invasion and motility and an indirect effect via alteration of stroma and extracellular matrix (ECM), which in turn supports angiogenesis and evasion of immune surveillance. As TGF-β interacts with multiple signaling cascades, modulating it can help define therapeutic targets [40].

In normal adult cells, where Wnt (Wingless and Int-1) is sparsely active. The WNT/β-catenin signal is involved in the formation and maintenance of cancer stem cells. The epithelial to mesenchymal switch in this pathway triggers melanoma progression to metastasis [41].

In mammals, the Janus Kinase (JAK: JAK1- 3, TYK2) and signal transducer and activator of transcription (STAT: STAT1- 4, STAT5A/B, STAT6) family contain various functional domains (SH2 domain being common) that are involved in multiple physiological and immune responses. The transcription activation of STAT3, which is downstream of BRAF has functional aberrations in cells with mutant BRAF [42]. Silencing of JAK2 using siRNA or inhibitors suppresses STAT3 phosphorylation, and in melanoma cell lines, this has been found to restore sensitivity to BRAF inhibitors [43].

NF-κB is a 'positive modulator' of immune response and inflammation. In this pathway, Akt directly binds with IKKα and IKKβ (IKappaKinase-α/β). In the non-canonical NF-κB pathway, the p52 subunit binds with ETS (E26 transformation-specific or E-twenty-six) transcriptional factor to interact with TERT promoter inducing its activation [31, 44] and imparting cell survival. In malignant melanoma, constitutive activation of IKK results in continuous degradation of IκB and sustained increase in NF-κB in the nucleus which might participate in melanoma invasion and metastasis by inducing expression of ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and MMPs (metalloproteinases) like adhesion molecules [45]. In melanoma, deregulations of upstream signaling pathways viz. RAS/RAF, PI3K/Akt, and NIK (NF-κB-inducing kinase) of NF-κB lead to its constitutive induction which in turn is associated with mutational activation of BRAF [46].

The fundamental role of Notch signaling is epidermal development and keratinocyte differentiation. The upregulation of Notch1, Notch2, and their ligands are well established in metastatic melanoma. The interaction of the Notch pathway with MAPK, PI3K-Akt, NF-κB, and p53 may contribute to the plural effect of melanoma [31, 42].

The microphthalmia-associated transcription factor (MITF) plays a relevant role in melanoma. Apart from skin pigmentation, MITF controls the proliferation and differentiation of melanocytes. A low or null MITF expression predisposes a cell to apoptosis, whereas an intermediate level promotes proliferation and cell survival. Overexpression of MITF induces cellular differentiation and, subsequently, exerts an anti-proliferative effect. In melanoma, constitutive activation of ERK is associated with a marked degradation of MITF, reported earlier in invasive melanomas, and is seen to be associated with a poor prognosis and clinical progression of the disease [32, 47].

Vitamin-D relation to melanoma

The photolysis of the B-ring in 7-DHC, induced by UVB radiation (28–320 nm), causes vitamin D to be produced primarily in sun-exposed skin. A cascade of chemicals are formed subsequently from the 7-DHC substrate—previtamin D3 to vitamin D3 which is then converted into 25-OHD and finally $1,25(OH)_2D_3$. The presence and function of the nuclear vitamin D receptor (VDR) are strongly linked to the effects of vitamin D. The structural analog $1,25(OH)_2D_3$ interacts with VDR and heterodimerizes with the retinoid X receptor to perform its biological activities. This complex further interacts with certain sequences of vitamin D responsive element (VDRE), located at the vitamin D responsive gene in the nucleus. The VDRE controls the expression of 900 genes involved in the cell cycle, differentiation, and apoptosis [48]. The levels of $1,25(OH)_2D_3$ depend upon the action of 1α-hydroxylase, an enzyme encoded by the CYP27B1 gene. A high amount of vitamin D metabolites are protective against some cancers while the deficiency leads to an increased risk of melanoma development as well as initiation and progression of respiratory infections, autoimmune diseases, neuromuscular

disorder, cardiovascular ailments, hypertension, and diabetes. Most importantly, the cutaneous malignant melanoma (CMM), squamous cell carcinoma (SCC), and basal cell carcinoma (BCC) express VDR. In primary melanomas and CMM, a relatively lower expression of VDR and CYP27B1 correlated to more aggressive forms of tumor accompanied by shorter overall survival suggesting the analog $1,25(OH)_2D_3$ to play a role in disease prognosis [48]. Independent research reports by Afzal et al. (2013) and van der Pols et al. (2013) showed an increased level of 25(OH)D to be associated with a higher incidence of CMM and BCC [49]. Despite harboring anticancer properties, the hypercalcemic effects limit the use of $1,25(OH)_2D_3$. This can be reverted by eliminating or lowering the cholesterol-type side chains. Moreover, the hunt for similar Vitamin D3 analogs might overcome this scenario.

Druggable targets in Melanoma

Melanoma is driven by the major hallmarks of cancer like every other type of malignancy [50]. In recent years there has been a huge surge of data output from the whole genome, exome, and proteome sequencing studies in cancer. This has obliterated the bottleneck of a lack of available targets. However, since these studies have established multiple possible targets, prioritizing the targets in the order of their mutational frequencies and their involvement in various signaling pathways remains the prime pursuit in the discovery and development of newer drugs in melanoma. Besides establishing novel targets to develop effective therapeutics, scientists have to gain insights into drug resistance to existing treatments and ways to overcome them. Malignant melanoma evolves as a spectrum of neoplasms that have diverse mutations that affect multiple biochemical pathways, have varied histopathological appearances, and altered clinical outcomes [51]. Best technologies are employed to drive novel drug discovery, but they still fall short in rendering progression-free survival in melanoma patients.

Surgical Interventions and Radiotherapy

In the treatment of cutaneous melanoma, the stage of the disease, its location, and the chances of recurrence are significantly important considerations though the research for target-specific drugs is ongoing. Stage 0 cutaneous melanoma usually deals with superficial development, also called 'melanoma in situ', and is surgically removed through wide-excision (5 mm around the cancer site) with none or additional follow-up treatments. Sometimes the more sensitive form of the surgery termed as Mohs surgery or application of imiquimod cream is also advised by medical professionals. Along with wide excision, depending on the involvement of the nodes, sentinel lymph node biopsy (SLNB) or complete lymph node dissection (CLND) are carried out. Surgical excisions of localized cutaneous melanoma remain the best option with an overall 5-year survival rate of 92% of patients [52]. A summary of the therapies discussed along with their potential advantages and disadvantages are mentioned in Table 2.

- *Radiation Therapy (RT)* may be used in certain cases of stage 0 as it also continues to be a lead therapeutic option in melanoma as in other cancers. Adjuvant RT following lymph node retrieval in node-positive melanoma patients is known to prevent recurrence though it is debatable among the scientific community. Stereotactic radiosurgery (SRS) and stereotactic body radiotherapy (SBRT) are considered advanced therapies in melanoma, which aid in local tumor control with minimum toxicity. However, RT is used as a primary treatment for non-operable tumors such as lentiginous, mucosal, and ocular melanomas [53]. Radiation therapy is also carried out concomitantly with the administration of chemo- or immune-therapeutics agents. With increasing stages of melanoma medical practitioners resort to more targeted treatments with or without surgical removal of the tumor.
- *Photodynamic Therapy* Photodynamic therapy (PDT), also termed blue light therapy, is a clinically approved, minimally invasive therapeutic procedure. Under the exposure of mild radiation, there is the induction of reactive oxygen species (ROS) post initial localization of photosensitizer (PS) in neoplastic cells and vasculature [54, 55]. The therapy induces response either by direct transfer of the energy from photosensitizer to oxygen molecules in the tissue and generation of superoxide radicals or by phototoxicity to cells by direct interaction with membranes and other molecules, and transfer of hydrogen atom (electron) to form hydroxyl radicals [56]. In any form, the phototherapy is toxic to the cancer cells and thus regresses the growing tumor. Dark toxicity produced by the photosensitizers to the non-exposed areas of the body has limited the use of PDT in melanoma. However, with localized control of exposure and overall low side effects as compared to radio- or chemotherapies, it is still a promising procedure [57]. PDT has the edge over certain earlier therapies since it can stimulate anti-tumor immune memory and thus can prevent recurrence to some extent [58]. The major drawback of this treatment comes from photosensitizers, which are organic dyes poorly soluble in water and prone to photobleaching needing repeated injections. After each injection, the efficacy to regress the tumor reduces with a simultaneous increase in dark toxicity [57, 59]. *For unresectable cutaneous melanoma chemotherapy and immunotherapy*

Table 2 Advantages and disadvantages of different therapeutic approaches

Therapy	Advantages	Disadvantages
Surgery	Easy excision of cancerous areas Quick and a standard procedure The 5-year survival rate of 92%	Only applicable to local melanoma lesions cannot be applied in metastatic cases
Radiation therapy	Primarily used in non-operable tumors like ocular, mucosal, and lentiginous melanomas Can be used along with other therapies	Hair loss at the site of radiation Fatigue Nausea Sunburn-like skin problems
Photodynamic therapy	Minimally invasive procedure Low side effects as compared to radiotherapy or chemotherapy Stimulates anti-tumor immune memory	Dark toxicity issues The dyes used in photodynamic therapy have poor water solubility and are prone to photobleaching needing repeated injections
Chemotherapy	Systemic treatment Can be used as monotherapy or in Combination therapy Tumor shrinkage ~ 12–15%	PFS is less than 2 months Median survival for DTIC and TMZ is 6.4 and 7.7 months only Phototoxic skin reactions and Development of lymphopenia
Electrochemotherapy	Higher intracellular concentration of cytostatic agents Decreased drug wash-out due to local contraction of arterioles Can be used in combination therapy	Contraindications including renal failure, pulmonary fibrosis, epilepsy, and cardiovascular complications were observed Side effects include local pain, swelling, and redness that may lead to ulcers and depigmentation
Biochemotherapy	Single or combination of chemotherapies with immunotherapy Early and advanced-stage metastatic melanoma can be treated Superior response with delayed progression at 6 months	Multiple cycles of treatment are required Relatively high toxic side effects No reduction in mortality rates at 12 months The overall quality of life declines
Targeted therapy	BRAF inhibitors like Vemurafenib and Dabrafenib are used in treating stage III and IV cutaneous melanoma carrying mutations in BRAF gene. Used as monotherapy or in combination PLX4032, a BRAF inhibitor is in phase III clinical trials Overall increase in patient survival compared to the DTIC A combination of BRAF inhibitors with various MEK inhibitors has shown enhanced frequency of tumor shrinking, delays in the growth of the tumors, and longer life Tyrosine kinase inhibitors, imatinib, and Nilotinib have shown promising effects in metastatic melanoma patients with KIT mutations CDK inhibitor, LEE011 has been implemented to overcome resistance in patients with upregulated CDK4 Simultaneous inhibition of CDK4/6 and BRAF kinases has shown better response against metastatic melanomas ERBB4 (HER4) inhibitor, lapatinib, a with dual-tyrosine kinase is used in melanoma treatments via induction of apoptosis Anti-VEGF therapy using Bevacizumab and a combination of paclitaxel or carboplatin in advanced mucosal melanoma showed a non-significant but improved response in overall survival The use of TMZ with bevacizumab achieved better efficacy and survival in melanoma patients without BRAF mutations as compared to those carrying BRAF mutations	The major drawback of BRAF inhibitors is that the cancer cells become resistant to the treatment within 8–12 months Toxic side effects Edema, nausea, fatigue, rash, leukopenia, and anemia Fatigue, skin rash, emesis, abnormal liver function tests, hyponatraemia, and hypokalemia Diarrhea and skin rash are common side effects Rash, fatigue, diarrhea, hand-foot skin reaction, exfoliative dermatitis are the common side effects

Table 2 (continued)

Therapy	Advantages	Disadvantages
Immunotherapy	Interleukin -2 (IL-2) therapy induces the growth of natural killer (NK) cells Effective in treating metastatic melanoma Peg-IFN treatment shows anti-proliferative effects on tumor cells Peg-IFN can be used as monotherapy or as a combination therapy Peginterferon-alfa-2b improved relapse-free survival (RFS) Inhibiting CTLA-4 enhances anti-tumor immunity of T-cells Ipilimumab is one of the commercially available CTLA-4 inhibitors CTLA-4 inhibitors work in combination therapy CTLA-4 inhibitors increase IFN-γ production by aiding tumor regression in murine models and patients Oncorine and T-VEC are commercially available oncolytic therapies for melanoma Replicative capacity within the tumor Induces tumor-specific immune response Simple mode of administration Ease of storage Economically feasible Bacillus Calmette–Guerin (BCG) and imiquimod are approved by FDA for monotherapy against melanoma. Increases cancer cell apoptosis, activates effector T-cells, induces innate and adaptive immune responses TLR agonists as adjuvant vaccines activate dendritic cells and T-cell response while suppressing the Treg cells A combination of radiation therapy, chemotherapy, monoclonal antibodies along with targeted therapies modulate TLR agonists and enhance anticancer effects Depletion of Treg cells via cyclophosphamide, OX40 Treg agonist or anti-PD 1 show a significant reduction in Tregs resulting in enhanced antitumor immunity and therapeutic outcome Anti-cancer agents like sunitinib, sorafenib, and imatinib also reduced the load of intra-tumoral Treg cells Adoptive cell therapy (ACT) is a durable therapy with a complete response and extended survival observed in melanoma patients Works through the elimination of Tregs	IL-2 has a short plasma half-life. Multiple administrations are required Severe toxicity issues Adverse events were reported Adverse events like Hepatitis, dermatitis, colitis, enterocolitis, and thyroiditis have been observed Headache, muscle aches, tiredness, nausea, fatigue, fever, and chills Safety concerns TLR also enhances metastasis through tumor-derived soluble mediators Tumor cells may also express TLRs which promote cell proliferation, resist apoptosis, and suppress anti-tumor immune responses Complete ablation of Treg population disturbs natural self-tolerance and leads to several chronic and destructive autoimmune disorders Depletion of Tregs in transplantation models resulted in increased severity of acute graft-versus-host disease (GVHD) Treg inhibition has tissue toxicity because other effector T cells share the same target protein or receptor Still awaits FDA approval

are the best lines of treatments, however, immunotherapy has been a more successful option in the management of melanoma.

- *Chemotherapy*: Chemotherapeutic agents are normally used as systemic treatment, single drug, or in combination with other drugs at a time in a set of multiple cycles. Chemotherapy is not the first line of treatment in melanoma since the immune checkpoint inhibitors had gained momentum and have been successful in controlling advanced melanoma. However, some drugs have still made it to the melanoma treatment regimen due to their potential to target and destroy cancer cells. Dacarbazine (DTIC) is the only approved drug for chemotherapy in melanoma with Temozolomide (TMZ) as the oral form of the same drug used in stage IV melanoma. Though these drugs are known to have low side effects and shrink the tumors by 12–15%, the overall progression-free survival (PFS) in patients with advanced melanoma is less than 2 months. The median survival for DTIC and TMZ is 6.4 and 7.7 months respectively, however, the feeling of general healthiness in patients is higher with TMZ treatment [60]. DTIC induces phototoxic skin reactions while the relative risk to develop lymphopenia by temozolomide is higher [61, 62]. Some of the other generic chemotherapeutic agents used in the treatment of melanoma are cisplatin, fotemustine, lomustine, the taxanes, and vinblastine [63].
- *Electrochemotherapy (ECT)*: The use of advanced chemotherapy in the form of electrochemotherapy (ECT) is a treatment option in metastatic melanoma. As the name

suggests, it uses electroporation to deliver chemotherapeutic drugs. Mild electric current is applied to the tissues to temporarily alter the permeability of the cell membrane, which in turn allows free influx of large drug molecules, solving the crucial problem of drug transportation through the cells. The toxic drugs directly target the cancer cells, making them susceptible to apoptosis [64, 65]. Various cytostatic agents such as bleomycin, cisplatin, carboplatin, mitomycin-c, and cyclophosphamide used with electroporation show different outcomes [66]. Intra-tumor administration of Bleomycin and cisplatin with ECT increased the toxicity to the cells by 1000 times and 80 times respectively. The overall action of ECT can be attributed to the increased intracellular concentration of cytostatic agents, decreased drug wash-out due to local contraction of arterioles, increased toxicity to endothelial cells associated with electrolyte influx, and disruption of transmembrane [65]. For more promising results, ECT administered in combination with the immunotherapeutic drug, ipilimumab, resulted in the regression of cutaneous and visceral metastasis for one year. The accompanying reduction of distant non-ECT treated tumors was ascribed to the depletion of certain subtypes of T-cells (regulatory T cells- Tregs) [67, 68]. This treatment does come with certain major contraindications including renal failure, allergy to bleomycin or cisplatin, pulmonary fibrosis (in the case of bleomycin), epilepsy, and cardiovascular complications. ECT demonstrates some minor side effects like local pain, swelling, and redness that may lead to ulcers and depigmentation [65, 69].

- *Biochemotherapy*: Similar to ECT this therapy is also a modified version of chemotherapy. It can be defined as a treatment regimen that includes a single or combination of chemotherapies with immunotherapy. To treat early and advanced stage metastatic melanoma chemotherapeutic agents are co-administered with immune checkpoint inhibitors (biochemotherapy). As compared to chemotherapy, biochemotherapy had a superior response with delayed progression at 6 months, but with no reduction in mortality rates at 12 months. Additionally, the overall quality of life declines with multiple cycles of this therapy since it has relatively high toxic side effects [70]. Clinical study in stage III melanoma (with node involvement) patients with the use of cisplatin, vinblastine, dacarbazine, interleukin-2, and low-dose interferon alfa-2b showed a significant median recurrence-free survival (RFS). The five-year RFS with biochemotherapy treatment was 48% though there was no overall survival benefit to the treated patients [71].
- *Targeted Therapies*: This therapy involves the targeting of specific molecules, including genes or proteins contributing towards the progression of the disease. With new-age sequencing technologies, the genetic landscape of melanoma has become increasingly complex as pointed out in the diagnostic section. Many heterogeneous somatic mutations are dispersed throughout the genes or entire genes, dysregulating the functions of important proteins and contributing to the genesis and progression of melanoma. Certain clinical variations like $BRAF^{V600}$ and NRASQ61 are frequent in melanoma and are considered as 'major hotspots' for devising drug targets. On the other hand, variations in ERBB4 and CKIT, though considered driver mutations, are termed as 'minor hotspots' due to low frequency in cutaneous melanoma.

Currently, BRAF (serine/threonine-protein kinase B-raf) kinase inhibitors (Vemurafenib, Dabrafenib) and MEK (Mitogen-activated protein kinase) inhibitor (Trametinib) are approved by the FDA for treating Stage III and IV non-removable melanomas carrying BRAF/MEK mutations [72]. Dysregulation of the MAPK pathway in cancers leads to increased cell proliferation, which in melanoma is mediated through mutant BRAF, a serine–threonine-specific protein kinase, belonging to the RAF family. BRAF acts downstream of RAS and upstream of MEK in the MAPK signaling pathways. Researchers have identified that about 66% of the cutaneous melanoma patients carry BRAF mutations, of which 90% have modifications at 600^{th} position from Valine to Glutamine (p.Val600Glu) [37]. This has led to the development of MAPK pathway inhibitors as effective therapy individually or in combination. Currently, PLX4032, a BRAF inhibitor specific for p.Val600Glu is in phase III clinical trials. It has shown an overall increased patient survival as compared to the DTIC. The major drawback of this drug is that the cancer cells become resistant to the treatment within 8–12 months [73]. A combination of BRAF inhibitors with various MEK inhibitors has shown enhanced frequency of tumor shrinking, delays in the growth of the tumors and longer life though with toxic side effects [74]. While most of the melanomas may have mutations in the BRAF gene, some patients show mutations in the NRAS gene. In minor percentages of mucosal or uveal melanomas, activating mutations exist in the CKIT gene dominantly in exons 11 or 13. Copy number variations in the KIT gene also make it a focus of targeted therapy in melanoma subtypes like lentigo malignant melanoma, mucosal melanoma, and acral lentiginous melanoma. Since the KIT receptor protein belongs to the tyrosine kinase family, the kinase inhibitor imatinib, dominantly used in the treatment of chronic myeloid leukemia (CML), has proved efficacious in patients with advanced melanoma harboring KIT mutations [75]. Clinical trials in melanoma patients having KIT mutations, particularly L576P in exon 11 treated with imatinib showed a positive response. Another kinase inhibitor Nilotinib, generally used

for imatinib-resistant CML patients, also showed promising results in metastatic melanoma with KIT mutations [76]. The cell cycle is well regulated by the interplay of cyclin-dependent kinases (CDKs), their inhibitors (CDKIs), RB, and p53 proteins. The upregulation of CDKs impacts RB-pathway which is dysregulated in more than 90% of melanomas, which in turn affects the downstream RAS/RAF/MEK/ERK pathway. The development of small-molecule CDK inhibitors against such early events of melanomagenesis is important. Broad range and selective CDKIs have been developed for the treatment of melanoma. Cyclin-dependent kinase (CDK) inhibitor like LEE011 has been implemented to overcome resistance in patients with upregulated CDK4. Simultaneous inhibition of CDK4/6 and BRAF kinases has shown better response against metastatic melanomas and hence clinical trials using a combination of LEE011 and novel BRAF inhibitor, encorafenib are being conducted [77]. Recently, ERBB4 (HER4) has been implemented as a driver mutation in melanoma. Mutant ERBB4 alters the normal signaling of the PI3K-AKT pathway. To inhibit the dysregulation of downstream signaling pathways, lapatinib, a small molecule with dual-tyrosine kinase inhibition is used in melanoma treatments. It is presumed that with lapatinib treatment the mutant ERBB4 signaling is inhibited, which in turn decreases Akt signaling, and induces apoptosis [78]. Melanomas are highly angiogenic, and hence anti-vascular endothelial growth factor (anti-VEGF) therapy remains an important treatment option. Bevacizumab (Avastin), a monoclonal antibody blocks the binding of VEGF to its receptor by interacting with cellular VEGF and is well studied in the treatment of metastatic melanoma. A combination of chemotherapeutic drugs like paclitaxel or carboplatin with bevacizumab in advanced mucosal melanoma showed a non-significant but improved response in overall survival. The use of TMZ with bevacizumab achieved better efficacy and survival in melanoma patients without BRAF mutations as compared to those carrying BRAF mutations [79]. Currently, Dasatinib, imatinib, and nilotinib are KIT inhibitors being tested for stage IV melanoma. Larotrectinib is a tumor-agonist targeted therapy used in cases of metastatic melanoma with NTRK (tropomyosin receptor kinase (Trk) receptor family) fusion [80].

- *Apoptotic Therapeutic Targets*: Evasion of apoptosis along with increased cell survival in tumors is now recognized as a major driver for resistance to frontline therapies in melanoma, which also presents the opportunities to discover novel targets. Apoptosis in melanoma is typically initiated through the intrinsic or extrinsic pathways [81]. The intrinsic pathway is regulated by the Bcl-2 family of proteins wherein BAX, a pro-apoptotic cytosolic monomeric protein oligomerizes with BAK in the mitochondria during apoptosis to stimulate the release of cytochrome c, resulting in a concomitant activation of the caspase cascade [82]. Oligomerization of BAX/BAK could be prevented by Bcl-2 to induce cellular immortality in melanoma cells. The expression of Bcl-2 in skin tumor cells as compared to adjacent normal areas is well documented and is a good target for biochemotherapy [83]. Consequently, the Bax/Bcl-2 ratio has been used as a biomarker for the development of anti-melanoma therapeutic candidates. These have included attenuated Salmonella engineered with plant-derived and synthetic apoptosis-inducing factors [84] like sanggenol L from *Morus alba* [85], cinobufagin from *Venenum bufonis* [86], himachalol from *Cedrus libani* [86], and Quinalizarin [87]. Oblimersen -an antisense oligonucleotide of Bcl-2 mRNA- in combination with dacarbazine, has been efficient in the treatment of metastatic melanoma [88, 89]. The extrinsic apoptotic pathway is driven by the binding of FasL and TNFα to the Fas receptor and TNF receptor, respectively. This binding consequently activates the recruitment of proteins such as the TNF receptor-associated death domain (TRADD), the Fas-associated death domain (FADD), and the proenzyme caspase 8 [81, 90]. Although this cocktail of targets has proven useful in the development of chemotherapies, there is still considerable resistance due to the reduced sensitivity of tumors to therapy-induced apoptosis [91]. To overcome this challenge, there have been major advances in identifying novel immunological targets that are not hampered by the lack of functional and/or overexpression of antiapoptotic genes [91].
- *Immunotherapy*: Immunotherapy is designed to boost the body's defense mechanism against cancer cells. In recent years, there have been major advances in the treatment of stage III and IV melanoma with immunotherapy. The development of immune checkpoint inhibitors has transformed the treatment and showed improved survival for patients with advanced melanoma [92], though certain subsets of patients do not respond to these drugs suggesting that newer biomarkers need to be identified to overcome drug resistance (Table 3).

(i) *Interleukin-2 (IL-2)*

Interleukins are a group of cytokines that boost the immune system and hence have been used in anti-cancer therapies. Interleukin -2 (IL-2), a lymphokine helps in the proliferation of responsive T cells and induces the growth of natural killer (NK) cells [93]. High-dose of interleukin 2 (IL-2) has been used as an initial line of immunotherapy in patients with advanced melanoma, and studies have shown positive response [94]. The short plasma half-life of IL-2, however, leads to multiple and repeated admin-

Table 3 Summarizes the various immunotherapeutic approaches available for the treatment of melanoma

Immunotherapeutic approach	Summary
Interleukin-2 (IL-2)	Effective in T-cell differentiation and proliferation also induces natural killer cell growth Nanoparticle-based delivery is possible
Peginterferon-alpha-2b (IFNα-2b)	Promising anti-cancer activity in melanoma Can be used as monotherapy or combination therapy Improved RFS in melanoma patients Currently used in combination with ipilimumab in BRAF mutant melanoma patients
Blockade of PD-1	Improved progression-free survival Used as monotherapy as well as in combination therapy Increase cytokines such as IL-2, TNF-α, and IFN-γ Available as Nivolumab and pembrolizumab
Blockade of CTLA-4	T-cell suppression and inhibition of IL-2 Enhances anti-tumor activity Median overall survival of 11.2 months in combination with dacarbazine Used as monotherapy as well as in combination therapy Showed 5-year recurrence-free and overall survival Boost IFN-γ production aiding tumor regression
Oncolytic virus	Stimulates host immune response Selectively attacks melanoma cells Can be used in combination therapy Oncorine and T-VEC are commercially available oncolytic viruses Safe to use, simple mode of administration, and economically feasible

istrations exhibiting severe toxicity and limiting its long-term use [95]. With the aid of recombinant adenoviruses expressing IL-2, this treatment can be administered as an effective gene therapy [96], but biosafety issues associated with the vector, obstruct its clinical application. Currently, direct intra-tumoral delivery of IL-2, using nanoparticles are being developed as safe, low-cost, and low immunogenic alternatives to efficiently treat metastatic melanoma [97].

(ii) *Peginterferon α-2b (Peg-IFN)*

Interferons belong to the family of cytokines and function as immunomodulatory proteins that have anti-proliferative effects on tumor cells. Type 1, interferon alfa (IFN-α), alone or in combination with other drugs has shown the most promising anti-cancer activity in melanoma Three types of IFN-α are commercially available: interferon alfa-2a, 2b, and peginterferon alfa-2b [98]. Peginterferon-alfa-2b was approved by FDA based on the studies conducted by the European Organization for Research and Treatment of Cancer (EORTC) which showed that as an adjuvant treatment for resected node-positive melanoma, this drug statistically improved the relapse-free survival (RFS) interval among the patients though there was no overall survival (OS) advantage [99]. IFN-α can thus be effective adjuvant regimens with other promising therapies which have shown overall survival benefit in stage IV melanoma management. Currently, trials using a combination of ipilimumab with high-dose IFN and interferons with targeted/immune checkpoint inhibitors for patients with BRAF mutations in melanoma are ongoing [100]. Adjuvant interferon treatments remain relevant to patients until novel therapies that have an overall survival advantage come along [101].

(iii) *Blockade of Cytotoxic T lymphocyte-associated Antigen 4 (CTLA-4)*

CTLA-4 belongs to the immunoglobulin (Ig) superfamily that shares homology with CD28 T cells, both of which are expressed on the surface of activated T-cells and regulatory T (Treg) cells. However, unlike CD28, CTLA-4 plays a vital role in shutting down T-cell-mediated immune responses through tight binding with ligands CD80 and Cd86 on Antigen Presenting Cells (APCs) [102]. CTLA-4 causes T-cell suppression and inhibition of IL-2 which leads to blockade of cell cycle progression following initial activation [103]. This in turn causes CTLA-4 mediated block in T-cell anti-tumor immune responses before these cells can identify and eradicate tumor cells. Hence, inhibiting CTLA-4 can abolish the inhibitory signals and can enhance the anti-tumor immunity of T-cell. Ipilimumab is an immunotherapy that targets CTLA4 and has demonstrated median overall survival of 11.2 months when combined with dacarbazine in previously untreated metastatic melanoma patients [104]. Animal studies have shown that CTLA-4 inhibitors work synergistically with radiation therapy, chemotherapy, molecularly targeted therapy, and tumor vaccination

to eradicate established tumors [105]. This may be due to the depletion of CTLA-4 which increases the ratio of effector T-cells to Foxp3 positive Treg cells combating the cancer progression. Ipilimumab is also used as adjuvant therapy for stage III melanoma patients with pathological involvement of regional lymph nodes. Patients who had surgical interventions along with CTLA-4 inhibitors showed 5-year recurrence-free, overall survival which can be due to expansion of tumor-suppressing T-cell clones, otherwise not detected until the administration of the therapy [106]. Additionally, CTLA-4 inhibitors have been shown to increase IFN-γ production by T-cells which aids tumor regression in murine models and patients [107]. Another CTLA-4-inhibiting antibody, tremelimumab was developed but failed to bring any change in patient survival and hence has been withdrawn (Fig. 4).

(iv) *Programmed Cell Death Protein 1(PD1)/PD-1 Ligand (PD-L1) Blockade*

Programmed cell death protein 1 (PD-1) like CTLA-4 belongs to the Ig superfamily but unlike CTLA-4 (which inhibits the T-cells in an early stage of activation), it inhibits activated T-cells in peripheral tissues at a later stage. Upregulation of PD-1 expression in cancer induces a state of T-cell exhaustion wherein activated T-cells lose their effector function [105]. The two known ligands of PD-1 are PD-L1 and PD-L2, which are both highly active in cancers. While cytokines increase the PD-L1 expression, PD-L2 is expressed on APCs and can be induced on tumor cells including 2% of melanoma cases [108, 109]. PD-1 has a role in melanomagenesis as proved by patient-derived xenograft models. This also emphasizes the need to develop PD-1 blockers that may contribute to anti-cancer treatment. Nivolumab and pembrolizumab are human monoclonal antibodies of the IgG4 isotype that competitively binds to the PD-1 receptor preventing its ligands from interaction and downstream effects [105]. Though there are currently no antibodies against PD-L1 ligand approved by FDA, many drugs are in pipeline. T-cell functions against tumor immunity are restored upon blocking of PD-1/PD-L1 signaling axis regressing the tumors in the preclinical studies. For patients with advanced-stage melanoma who failed to respond with ipilimumab or targeted therapies, nivolumab and pembrolizumab both separately showed improved progression-free survival [110, 111]. Though the clinical studies showing a change in the quantity of Tregs through PD1-PD-L1 inhibitors are currently limited, the hypothesis prevails that since PD-1 is expressed on Tregs, the blockade could be involved in the direct attenuation of Tregs. Studies reporting a change in the frequency of Treg cells in response to nivolumab or pembrolizumab are presently unavailable [112].

- *Combining PD-1 and CTLA 4 inhibitors:* CTLA 4 inhibitor ipilimumab was found to be more toxic as compared to PD-1 inhibitors, nivolumab, and pembrolizumab. However, a combination of CTLA 4 and PD-1 inhibitors is approved for the treatment of patients with advanced melanoma. A combination of these inhibitors in stage III and IV melanoma has

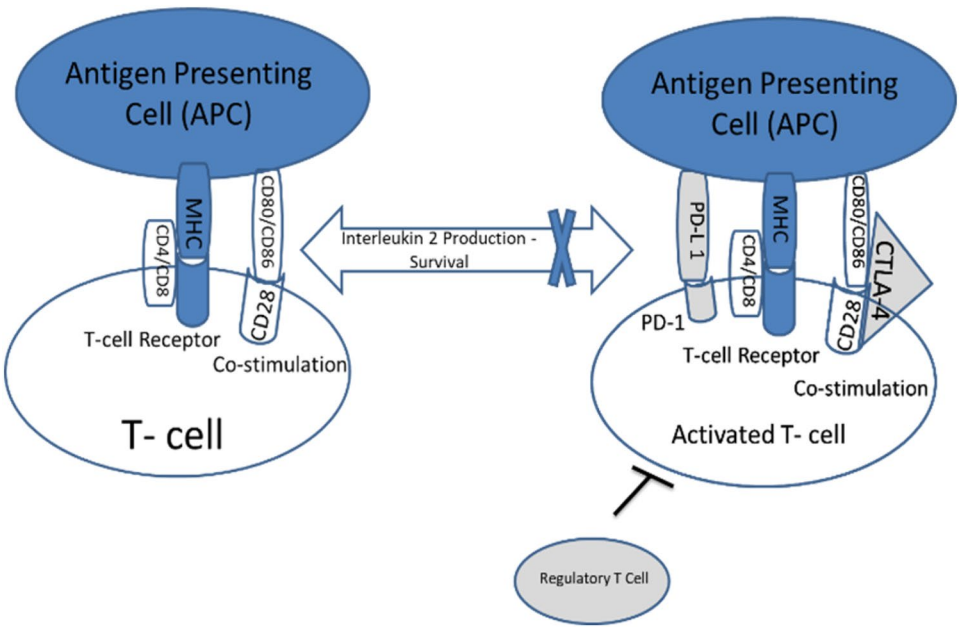

Fig. 4 Demonstrates Cancer Cells Evading Immunosurveillance through PD1/CLTA4

shown better output as compared to each drug individually, however with no overall survival benefit. Nonetheless, the patients presented with increased median progression-free survival [113]. Though the exact mechanism by which the combined treatment works is not completely understood, it can be predicted that CTLA 4 and PD-1 may attenuate Treg cells population at different phases (early and late) and may trigger an immune response against cancer cells. Besides PD-1 and CTLA 4 inhibitors, research involving blocking of other immune checkpoint receptors like LAG-3, and TIM-3 are being conducted and the outcome of their treatments is yet not known [114].

(v) Toll-like receptor (TLR) agonists

Toll-like receptors (TLR) are invariant receptors that aid in host defense via the recognition of pathogenic molecular signals. Humans have ten distinct TLR which activate innate and adaptive immune responses. TLR signaling activates type I interferons and proinflammatory cytokines. While the dendritic cell (DC) subset, TLR agonist, and signaling adaptors determine the cytokine induction patterns, the interferons aid in the immune response against cancer cells. The interferons act by facilitating antigen cross-presentation leading to increased cytotoxic CD8 + T cells, T-cell proliferation, DC maturation, and activation of NK cells. Thus, developing TLR agonist immuno-therapy warrants an anticancer treatment. Two TLR agonists, Bacillus Calmette–Guerin (BCG) and imiquimod are approved by FDA for monotherapy against cancer [115]. Locally applied TLR agonists, 5% imiquimoid (TLR7 agonist) for the treatment of cutaneous melanoma caused increased cancer cell apoptosis, effectively by inducing innate and adaptive immune response which altered the tumor-microenvironment [116]. TLR agonists as adjuvant vaccines activate dendritic cells and T-cell response while suppressing the Treg cells. Many infectious disease vaccines are used in TLR related therapeutics. Of these, BCG (which stimulates TLR2, TLR3, TLR4, and possibly TLR9) when administered as a monotherapy showed a better outcome in melanoma patients as compared to its use as adjuvant therapy with allogeneic melanoma vaccine [117]. While allogeneic vaccines did not help against melanoma, autologous whole-cell tumor lysate vaccines administered together with BCG augmented anti-tumor response and aided survival of patients with melanoma, colorectal cancers, and renal cell cancers. These outcomes can be attributed to activation of effector T-cells along with stimulation of innate immune cells [118, 119]. Other vaccine adjuvants like Polyriboinosinic-polyribocytidylic acid and its derivatives are being studied as potent activators of DC and cytokines against cancer cells. There are ongoing studies of patients being administered with peptide or DC vaccines in various advanced malignancies [119]. A combination of radiation therapy, chemotherapy, monoclonal antibodies along targeted therapies can modulate TLR agonists and enhance anticancer effects. However, it is important to consider the genetic polymorphisms of TLR and their response to cancer therapies. TLR has also been shown to enhance metastasis through tumor-derived soluble mediators. Additionally, as major drawback tumor cells may also express TLRs which can promote cell proliferation, resist apoptosis, and suppress anti-tumor immune responses [115].

(vi) Inhibition of Tregs (Regulatory T-cells)

The primary objective of cancer immunotherapy is to modulate anti-tumor T cells to reduce the burden of tumor cells that evade immunosurveillance. However, the presence of regulatory T (Treg) cells interferes with anti-tumor immunity. The counter-successful anticancer strategy can be to modulate the immune system by suppressing the effects of Tregs and enhancing the overall response against cancer cells. About 2–3% of CD4 T cells are Tregs with high expression of CD25 (IL-2Rα) and transcriptional factor FOXP3 (forkhead box protein P3) [120]. Tregs are immunosuppressive in nature and can inhibit the anti-tumor immune response in tumors thus leading to cancer progression and associated poor prognosis. Tregs may suppress the activation and proliferation of normal T cells by secreting cytokines in a contact-dependent and independent manner [121]. BRAF mutations have been shown to increase the intra-tumoral density of FOXP3 + Tregs by two folds in melanomas [122]. Tumor Treg cells have unique transcriptional signatures and express markers like CLTA 4 and PD-1 as compared to peripheral circulating cells. Treg assay in vitro showed that they compete with T cells for IL-2 and so decrease the availability of IL-2 that affect the T-cell functionality [123]. IL-2 inducing drugs (NKTR-214) preferentially bind to IL-2Rβ leading to a greater CD8 T cell to Treg cell ratio causing IL-2 mediated Treg cell suppression [124, 125]. Using low doses of cyclophosphamide alone and in combination with OX40 (secondary immune checkpoint) agonist or anti-PD 1 showed a significant reduction in Treg cells with a high proliferative rate and increase effector T cells to Treg ratio [126–128]. Besides these several anti-cancer agents like tyrosine kinase inhibitors—sunitinib, sorafenib, and imatinib also reduced the

load of intra-tumoral Treg cells. Administration of CLTA-4 inhibitors, in patients with advanced melanoma, showed reduced CLTA-4, FOXP3 positive Tregs thus regressing the tumors [123]. A recent approach involved epigenetic modification of Treg cancer cells to disrupt their lineage and functional stability. For example, molecular targeting of tumor-infiltrating Treg cells with upregulated, enhancer of zeste homolog 2 (EZH2) and epigenetic remodelling them to IFN-γ producing cells, led to an increase in anti-tumor immunity [128].

(vii) CAR-T Therapy in Melanoma

Chimeric Antigen Receptor (CAR-) T cell therapy involves genetic modification of autologous T-cells to express fusion proteins combining a single-chain fragment variable from a specific monoclonal antibody and one or more intracellular signaling T-cell receptor domains [129]. These cells have yielded successful outcomes in cases of haematological malignancies, highlighting its strong anti-cancer potential. However, in solid tumors, there are still certain issues that need to be resolved. Currently, scientists are working towards engineering CAR cells that can improve the therapeutic index within the tumor microenvironment (TME) in solid tumors [129]. One of the strategies for melanoma and other solid tumors is to combine immune checkpoint inhibitors with CAR-T to modify the TME and increase anti-tumor immune response [130].

(viii) Adoptive T-cell therapy

Adoptive cell therapy (ACT) is powerful immunotherapy that has shown durable and complete response with extended survival in melanoma patients. This therapy uses various types of immune cells that are amplified in the laboratory and are administered to kill cancer cells. These immune cells can be naturally derived from melanoma or the blood of the patients (tumor-infiltrating lymphocytes -TILS, endogenous T-cell therapy) or modified by introducing T-cell receptor genes that recognize the neoantigens on the cancer cells (CAR-T and TCR transduced T-cells). ACT-TIL therapy yet awaits FDA approval though clinical trials have shown that half of the melanoma patients benefit from this therapy with improved clinical responses [96, 131, 132]. The anti-tumoral activity of ACT is not fully elucidated but it may be working through the elimination of Tregs, exclusion of cytokine sinks, and eradication of host tumor immunosuppressive factors [133].

- *Oncolytic virus therapy*: The use of replication-competent viruses that can target the cancer cells while avoiding the normal cells is defined as oncolytic viro-therapy. The viruses used in the therapy are either in their native form or modified to selectively infect and kill malignant cells via cell lysis, thereby projecting anti-tumor responses. Oncorine (H101- recombinant adenovirus) and T-VEC (Talimogene laherparepvec- type I herpes simplex virus genetically modified) are commercially available treatments for melanoma. Measles virus, rhabdovirus, Newcastle disease virus (NDV), adenovirus, vaccinia virus, herpes virus, retrovirus, coxsackievirus, and reovirus are the ones under clinical development. T-VEC is made from a genetically modified herpes virus designed to replicate inside melanoma cells to kill them while simultaneously enhancing the immune response against cancer. T-VEC in combination with other related therapeutic agents has also gained momentum. However, the use of viral infections in the treatment of cancer is not yet a safe alternative due to their transmissible capabilities and escaping tumor selectivity. On a positive note, to date, no serious secondary infections to the health care specialists have been reported while administering the viral treatments. The advantages of oncolytic virus therapy are their replicative capacity within tumors, simple method of administration, and ease of storage, all of which suggest efficient, economically feasible options with minimum complications in the treatment of melanoma and other cancers [134–136] (Table 3). Oncolytic virus-immunotherapy is a novel approach that uses native or attenuated live viruses that selectively kill melanoma cells while concurrently inducing tumor-specific immune responses. Oncolytic virotherapy is armed with therapeutic genes and is able to enhance the interaction between fibroblasts, cytokine-induced killer cells, and cancer cells within the microenvironment, leading to enhanced tumor cell death [136]. Viruses like talimogene and laherparepvec are in the clinical development phase and have shown improvement in response to advanced melanoma [135]. Thus, manipulating the tumor microenvironment has become an important pursuit of melanoma virotherapy (Table 4).
- *gp100 peptide vaccine*: A 100 kDa artificial peptide vaccine constructed from melanoma antigen glycoprotein including the amino acids 280 to 288 and having potential anti-cancer activity is named as gp100 vaccine. In gp100 valine is substituted at 288thposition to increase the immunogenicity of this vaccine. This peptide-based vaccine stimulates the cytotoxic T-lymphocytes that recognize gp100 antigen-positive tumor cells and destroy them, thus causing tumor regression [147]. A study by Schwartzen-

Table 4 Immunological Targets of anti-melanoma Oncolytic Viruses

Targets	Oncolytic virus	Source
Bcl-2	apoptin protein derived from chicken anaemia virus	[137]
PD1/PD-L1 & CTLA4	Newcastle disease virus	Vijayakumar et al. (2019) [138]
retinoblastoma pathway	adenovirus ICOVIR5	Garcia et al. (2019) [139]
	Adapted ECHO-7 virus	Alberts et al. (2018) [140]
	Mumps Virus vaccine strain Leningrad-3 (MV L-3)	Ammour et al. (2018) [141]
P2Y2 and P2X7	Ad5/3-D24-GMCSF—serotype 5/3 chimeric oncolytic adenovirus that express GM-CSF (granulocyte macrophage colony-stimulating factor)	Hemminki et al. (2015) [142]
CD91, TLR2, TLR4, SREC1, and FEEL1	Parvovirus H-1 (H-1PV)	Goepfert et al. (2019) [143]
TLR2, TLR4, RAGE, and TIM3	Herpes Simplex Virus Type 1 Mutant HF10	Watanabe et al. (2008) [144]
	Measles Virus	Donnelly et al. (2013) [145]
	vaccinia virus	Greiner et al. (2006) [146]
	parvovirus H-1	Goepfert et al. (2019) [143]

truber et al. (2011) showed that the gp100 peptide vaccination increased the number of circulating T cells that were able to recognize and destroy melanoma cancer cells in vitro [148].

Conclusion

Understanding melanoma biology in terms of intra- and inter-population differences, as well as their phenotypic diversity, is crucial for the precise therapy of this elusive disease. This will expand on previously identified molecular pathways and aid in improving the disease's clinical outcome. Despite remarkable advances in melanoma pathogenesis, diagnosis, prognosis, and therapy, scientific approaches are required to address the underlying limitations. The tumor and patient heterogeneity influence the transcriptional plasticity, promoting resistance to the multimodal line of treatment including targeted therapies. The selection of 'dose escalation' to different cohorts of subjects often renders toxic responses. All of these negatively impact the clinical consequences subjugating the 'risk–benefit ratio'. Since the preclinical models are not well competent to mimic the human tumor microenvironment, hence the mechanisms of resistance can differ. This in turn poses difficulties during translation of the expected outcome in patients [149, 150]. The identification, analysis and appropriate monitoring of melanoma-related biomarkers though crucial but can also be compromised at various levels due to disparities in the viewpoint of academic researchers, pharmaceutical organizations, regulatory bodies, as well as funding agencies. With emerging opportunities and dramatic technological advancement, retaliating to such challenges might be beneficial for the melanoma community in designing and optimizing innovative strategies to establish stronger insights. Furthermore, future efforts should focus on using data on molecular melanomagenesis to design preventive interventions in risk populations and individuals.

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