INK4a/ARF

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Contributor: Helen Rizos

Genetic alterations in the INK4a/ARF (or CDKN2A) locus have been reported in many cancer types, including melanoma, head and neck squamous cell carcinomas, lung, breast and pancreatic cancers.

The CDKN2A locus encodes two critical tumor suppressor proteins, the cyclin-dependent kinase inhibitor p16INK4a and the p53 regulator p14ARF. The majority of CDKN2A alterations in melanoma selectively target p16INK4a or affect the coding sequence of both p16INK4a and p14ARF. There is also a subset of less common somatic and germline INK4a/ARF alterations that affect p14ARF, while not altering the syntenic p16INK4a coding regions.

This review describes the frequency and types of somatic and germline alterations affecting the CDKN2A locus and their functional consequences in melanoma development. The clinical implications of CDKN2A inactivating alterations and their influence on treatment response and resistance are also described.

Keywords: Melanoma, INK4a/ARF, CDKN2A, p16INK4a, p14ARF

1. Introduction

Melanoma is an aggressive and highly metastatic form of skin cancer that causes nearly 60,000 deaths globally each year[1]. Melanoma originates from neural-crest-derived pigment-producing cells known as melanocytes. These specialized, dendritic cells are predominantly located in the basal layer of the epidermis, and are also found in the vascular uvea of the eye and in mucosal membranes[2]. The transformation of melanocytes into melanoma, termed melanomagenesis, involves the sequential selection of genetic and epigenetic alterations that promote proliferation, invasion, and immune escape[3][4].

Cutaneous melanoma has the highest median coding mutation rate of any cancer type (14.4 coding mutations per megabase) and this reflects the high proportion of ultraviolet (UV)-induced C>T substitutions that occur at pyrimidines[5][6]. The high mutation burden in melanoma is also associated with a high rate of silent passenger mutations and this is consistent with the 2:1 ratio of non-synonymous to synonymous mutations in melanoma [7]. The systematic evaluation of over 500 melanoma genomes in the last decade has identified a series of frequently and significantly altered oncogenes and tumor suppressor genes, including BRAF, NRAS, RAC1, NF1, CDKN2A, PTEN, and ARID2[7][8][9].

The CDKN2A locus is located on chromosome band 9p21 and is also referred to as the INK4a/ARF locus. This is one of the most commonly altered sequences in cancer and is mutated, deleted, or methylated in 40–70% of sporadic melanomas[8]. Germline alterations in the CDKN2A locus have also been identified in approximately 40% of high-risk melanoma families with three or more melanoma cases[10]. The frequent alteration of CDKN2A reflects its capacity to encode two distinct tumor suppressor proteins, $p16^{INK4a}$ and $p14^{ARF}$, which are translated in alternate reading frames, from alternatively spliced transcripts with independent start sites and unique first exons (exon 1α for $p16^{INK4a}$ and exon 1ß for $p14^{ARF}$)[11]. $p16^{INK4a}$ forms binary complexes with the cyclin-dependent kinases 4 and 6 (CDK4/6) to inhibit cyclin D-CDK4/6-mediated phosphorylation of the retinoblastoma protein and, thus, prevents G1 to S phase cell cycle transition[12][13][14]. The functions of $p14^{ARF}$ are more complex, but it plays a central role in the stabilization and activation of p53 via the inhibition of the major p53 negative regulator MDM2 [15][16][17][18]. Despite sharing largely overlapping DNA sequences, the functional impact of CDKN2A alterations is complex and can be difficult to predict.

2. Clinical Implications of CDKN2A: Impact on Response and Resistance to Current Treatments in Melanoma

The restoration of $p14^{ARF}$ and/or $p16^{INK4a}$ functions has not yet been possible, and most therapeutic strategies involve modulating downstream cell cycle regulators or pathways to overcome the loss of CDKN2A-encoded functions.

2.1. MDM2 Inhibitors

To overcome $p14^{ARF}$ loss, small molecule inhibitors targeting MDM2 activity or the MDM2–p53 interaction have been developed. These inhibitors have shown promising anti-tumor effects in the preclinical setting. The small molecules nutlins (nutlin-1, nutlin-2, and nutlin-3) sterically disrupt the interaction between MDM2 and p53, resulting in p53 accumulation and activation[19]. Nutlin-3 in particular has been shown to inhibit melanoma growth and induce apoptosis in patient-derived xenograft models[20]. Similarly, the MDM2 inhibitor KRT-232 inhibited tumor growth in xenografts derived from 15 melanoma patients, when used alone or in combination with BRAF and/or MEK inhibitors[21]. Importantly, although MDM2 inhibitors will only benefit melanoma patients with p53 wild-type tumors, melanoma often retains expression of wild-type p53[20].

2.2. CDK Inhibitors

To circumvent $p16^{INK4a}$ loss, CDK inhibitors have been developed and tested with variable success. The first-generation CDK inhibitor, flavopiridol, has broad range activity against CDK1, CDK2, CDK4, and CDK7 and induced cell cycle arrest in preclinical melanoma models but failed to generate any significant clinical activity in a phase II trial of metastatic melanoma patients (NCT00005971[22]). Second generation CDK-specific inhibitors such as ribociclib (LEE011) and abemaciclib selectively target CDK4 and CDK6, and these have shown more promising results, especially when combined with MAPK inhibitors. For instance, ribociclib in combination with binimetinib (MEK inhibitor) enhanced tumor regression in $NRAS^{Q61K}$-mutant melanoma xenograft models compared to treatment with either ribociclib or binimetinib alone[23]. Ribociclib also demonstrated synergistic effects in combination with encorafenib (BRAF inhibitor) in $BRAF^{V600E}$-mutant melanoma models. Likewise, treatment with the selective CDK4/6 inhibitor abemaciclib inhibited tumor growth and delayed tumor recurrences in melanoma xenograft mouse models. Importantly, abemaciclib caused tumor regression in vemurafenib (BRAF inhibitor)-resistant tumors, suggesting that CDK4/6 inhibitors may be a viable therapeutic option for melanoma patients who progressed on BRAF/MEK inhibitors[24]. The combination of ribociclib and MDM2 inhibition also enhanced tumor regression and overcame resistance to CDK4/6 inhibitors in a melanoma xenograft model[25].

Selective CDK4/6 inhibitors have since been evaluated in clinical trials. The combination of CDK4 and MEK inhibitors (ribociclib and binimetinib) was tested in a phase Ib/II trial with advanced NRAS-mutant melanoma, and 60–70% of patients experienced clinical benefit (RECISR CR, PR, and SD)[26][27]. The combination of ribociclib and the BRAF inhibitor encorafenib was also evaluated in 18 patients with advanced BRAF-mutant melanoma, with more than half of patients showing clinical benefit (PR/SD)[28]. A triple combination of ribociclib, binimetinib, and encorafenib was evaluated in a phase Ib/II study of 21 patients with BRAF-mutant melanoma, and although increased toxicity was observed with this combination, clinical response was noted in over half of the patients[29].

Loss of CDKN2A has been shown to predict response to CDK4/6 inhibitors in melanoma, glioblastoma, ovarian, and rhabdoid tumor cells[30][31][32]. The activity of CDK4/6 inhibitors was also restricted to melanoma cells that retained expression of the retinoblastoma protein (the downstream effector of CDK4 and CDK6). The presence of an activating Arg24Cys CDK4 mutation, which abolishes the ability of CDK4 to bind to $p16^{INK4}$[33] was also associated with melanoma cell sensitivity to CDK4/6 inhibition[34]. These data suggest that activation of the CDK4/6 pathway via loss of $p16^{INK4a}$ or CDK4 activation and the retention of the retinoblastoma protein are key determinants of sensitivity to CDK4/6 inhibition.

2.3. Epigenetic Modulators

Considering that CDKN2A methylation can lead to $p14^{ARF}$ and $p16^{INK4a}$ loss, epigenetic reactivation of CDKN2A has also been attempted with inhibitors of DNA methyltransferase (DNMT), histone deacetylase (HDAC), histone methyltransferase, and histone acetyltransferase. These inhibitors have been shown to induce $p14^{ARF}$ and $p16^{INK4a}$ expression in cancer cell lines and preclinical models (reviewed in reference[35]). In melanoma, treatment of melanoma cell lines with 5-aza-2-deoxycytidine, a DNMT inhibitor, and vorinostat, an HDAC inhibitor, restored $p14^{ARF}$ and $p16^{INK4a}$ function, and this led to reduced cell proliferation, migration, and invasion[36]. However, given that these epigenetic modulators have promiscuous effects, it is difficult to attribute the consequent melanoma control on modulation of $p14^{ARF}$ and $p16^{INK4a}$ function alone.

2.4. BRAF/MEK Inhibitors

The $BRAF^{V600}$ inhibitors dabrafenib and vemurafenib, in combination with MEK inhibitors trametinib or selumetinib, have now become standard of care in $BRAF^{V600}$-mutant melanoma. The combination of dabrafenib and trametinib produced response rates of 64%, and progression-free survival and overall survival rates of 13% and 28% at 5 years, respectively,

in patients with advanced $BRAF^{V600}$-mutant melanoma[37], superior to single-agent treatment. However, despite improved response and progression-free survival rates, melanoma patients treated with these selective kinase inhibitors quickly develop resistance and progress within one year.

Despite the high frequency of CDKN2A alterations in melanomas, the impact of CDKN2A mutations on patient responses to BRAF/MEK inhibitors is not well established. For instance, in melanoma cell studies, the presence of $p16^{INK4a}$-resistant CDK4 mutations (including the melanoma-associated germline CDK4 Arg24Cys mutation) did not alter cell sensitivity to BRAF inhibitors. Conversely, the overexpression of cyclin D1 was associated with BRAF inhibitor resistance and resistance was enhanced when cyclin D1 overexpression was combined with the CDK4 Arg24Cys mutation[38]. Recurrent CDKN2A loss has been implicated in BRAF inhibitor resistance[39][40], although CDKN2A alterations have been found to be pre-existing in responding patients, and CDKN2A alterations commonly co-occur with other mechanisms of BRAF inhibitor resistance (i.e., PTEN loss, N-RAS mutations)[41]. Finally, although reduced CDKN2A copy number at baseline has been associated with poor BRAF inhibitor responses in melanoma[42], the genetic loss of CDKN2A is also a poor prognostic marker in melanoma[43].

It is important to mention that 15–40% of mucosal and acral melanomas show activating mutations or amplification of the receptor tyrosine kinase KIT, and the kinase inhibitor imatinib has shown efficacy in KIT-mutant melanoma with an overall response rate of 54%[44]. Imatinib is also used commonly in the treatment of BCR–ABL chronic myelogenous leukemia but has not been as successful in BCR–ABL positive acute lymphoblastic leukemia showing deletion in the CDKN2A gene, suggesting that expression of $p14^{ARF}$ and/or $p16^{INK4a}$ may sensitize cancer cells to imatinib treatment[45]. Thus, it is tempting to speculate that CDKN2A inactivation in melanoma may analogously diminish sensitivity to imatinib in melanoma.

2.5. Immune Checkpoint Inhibitors

The immune checkpoint inhibitors targeting the inhibitory receptors cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) have significantly improved survival of patients with advanced and high-risk stage III melanoma. The CTLA-4 inhibitor ipilimumab generates a response rate of around 20% in melanoma patients, and a small proportion of patients remain disease-free past 10 years[46]. Response rates are higher with PD-1 inhibitors (up to 45%) and the combination of CTLA-4 and PD-1 inhibitors further enhances the response rate to 60% [47][48][49][50].

Immune checkpoint inhibitors show most activity in immunogenic cancers, and in tumors showing IFNγ transcriptome signatures and evidence of infiltrating T cells[51]. In this context, knockout of the CDKN2A gene in mice resulted in increased inflammatory cytokine expression in the skin following chronic UVB irradiation. Additionally, more myeloid cells were identified in the CDKN2A knockout mice[52]. Interestingly, chromosomal 9p losses encompassing CDKN2A can also affect the JAK2 gene (JAK2 is located on chromosome band 9p24.1[53]). JAK2 is a critical transcription factor in IFNγ signaling, and the loss of JAK2 is associated with PD-1 inhibitor resistance[54]. Indeed, 75% of melanoma tumors carry concurrent loss of the JAK2 and CDKN2A alleles[55]. Hence, loss of CDKN2A may increase inflammatory responses, which may augment response to immune checkpoint blockade, but also confer susceptibility to immunotherapy resistance through IFNγ suppression. Given the complexity of the immune response and the heterogeneity of immune cell subsets, it is unclear if and how $p14^{ARF}$ and/or $p16^{INK4a}$ regulate melanoma response to immunotherapy.

CDKN2A mutations were not significantly associated with clinical outcomes such as median time to progression, overall survival, and disease control rate in a cohort of 102 cutaneous melanoma patients treated with immune checkpoint inhibitors. However, this study did report a trend towards improved time to progression and disease control rate in patients with CDKN2A mutations[56]. Similarly, melanoma patients with CDKN2A germline mutations also showed improved response to immune checkpoint blockade; approximately 58% of carriers responded to therapy, with 32% showing complete response[57], suggesting that CDKN2A mutation may be associated with better immunotherapy response rates. Although the mechanism for improved immunotherapy responsiveness in CDKN2A mutation carriers remains unclear, melanomas with somatic CDKN2A mutations have an increased mutational burden, and this may result in more neoantigens and stronger immune responses[57].

2.6. Chemotherapy

Chemotherapy is still used as salvage treatment for melanoma patients, especially those with BRAF wild-type disease, and in patients who have failed molecular targeted and/or immunotherapy[58]. Chemotherapy agents such as dacarbazine and temozolomide show low response rates of 12–13% and median overall survival of only 6–8 months[59], and although partial response rates for these agents can reach 15–28%, less than 2% of patients will have durable responses (reviewed in[60]).

Expression of $p14^{ARF}$ has been shown to enhance chemosensitivity. For example, $p14^{ARF}$ accumulation induced potent cell cycle arrest in a p53-dependent manner. On its own, $p14^{ARF}$ did not induce apoptosis, but rather sensitized cells to apoptosis in the presence of camptothecin and adriamycin, inhibitors of topoisomerase I and II, in osteosarcoma, colorectal, melanoma, and fibroblast cell lines[61]. This effect is also observed in osteosarcoma cell lines in response to cisplatin-induced apoptosis, however, effects were independent of p53[62], suggesting a distinct regulatory mechanism that may be treatment dependent.

Similar to $p14^{ARF}$, ectopic expression of $p16^{INK4a}$ in glioma cell lines also sensitized cells to the chemotherapy drug vincristine[63]. In melanoma cells, CDKN2A expression was associated with better response to chemotherapy in the form of melphalan or actinomycin-D, and enforced accumulation of $p16^{INK4a}$ induced cell death by augmenting response to these cytotoxic drugs[64].

3. Conclusions

The CDKN2A locus is the most common melanoma-dominant predisposition gene and somatic alterations encompassing this genetic sequence occur early in the development of melanoma. Many CDKN2A genetic and epigenetic changes impact both the $p16^{INK4a}$ and $p14^{ARF}$ protein products encoded by this locus, and although early studies confirmed the major contribution of $p16^{INK4a}$ in CDKN2A-associated melanoma, there is now significant evidence that $p14^{ARF}$ plays an important and additional role in melanomagenesis. CDKN2A loss is associated with histological features predictive of poor prognosis in melanoma and also correlates with diminished patient response to treatment, with loss of CDKN2A associated with poor response to BRAF/MEK inhibitors and chemotherapy but potentially improved responses to immune checkpoint inhibitors. The loss of the CDKN2A sequence also co-operates with the BRAF and NRAS oncogenes to promote melanoma development. Thus, there is renewed interest in restoring the functional loss of $p16^{INK4a}$ and $p14^{ARF}$ in melanoma, and the frequent loss of this locus in melanoma may provide unique therapeutic opportunities, as the downstream targets retinoblastoma protein and p53 are often retained. In recent work, the combination of CDK4 and MDM2 inhibitors demonstrated significant preclinical activity, and this combination was effective in melanoma models with genetic loss of CDKN2A. It remains to be determined whether combination therapies that functionally restore CDKN2A will be effective as salvage therapies. The CDKN2A locus may ultimately help stratify patients for optimal treatment and provide therapeutic options for patients who fail standard of care MAPK inhibitor-based and/or PD1-inhibitor-based therapies.

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