Review

Restoring the tumour suppressive function of p53 as a parallel strategy in melanoma therapy

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Abstract

The tumour suppressor p53 is a master sensor of stress and it controls the expression of hundreds to thousands of genes with diverse biological functions including cell cycle arrest, apoptosis, and senescence. Consequently p53 is the most mutated gene found in human cancer and p53 mutation rate varies from 5% to 95%. Importantly p53 activity is often inactivated in tumours expressing structurally wild type p53. Thus one of the major challenges in cancer research is to restore the tumour suppressive function of p53. Intensive studies in the past decade have demonstrated that in addition to mutation, p53 activities are largely regulated by cellular factors that control the expression level and/or transcriptional activities of p53. MDM2, MDM4, p14ARF and the ASPP family of proteins are among the most studied regulators of p53. With increased understanding of the complexity of p53 regulation, various p53 reactivating approaches are being developed. This review will focus on the recent understanding of p53 inactivation in melanoma and the approaches to reactivate p53 in preclinical studies. Recent success in the therapeutic targeting of the BRAFV600E oncogenic protein was accompanied with subsequent relapse caused by acquired drug resistance. Restoration of the tumour suppressive function of p53 presents a parallel cancer therapeutic opportunity alongside BRAFV600E inhibition. Thus targeted therapy and concurrent reactivation of p53 may be a fertile ground to achieve synergistic killing of the 50% of cancer cells that express structurally wild type p53.

1. Introduction: The tumour suppressor p53

Discovered thirty-five years ago, the tumour suppressor TP53 is the most mutated gene found in human cancer with the p53 gene being mutated in around 50% of human cancer cases. As a transcription factor, p53 is able to transactivate and transrepress numerous target genes. Hundreds of p53 target genes have been identified and characterized to date, and they are involved in diverse biological processes including cell cycle arrest, apoptosis, DNA repair, differentiation, angiogenesis and metabolism. Over 80% of p53 mutations occur in p53’s DNA-binding domain (DBD) and many of the cancer derived p53 mutations inactivate its transcriptional function [1]. The importance of the transcriptional activity of p53 in mediating its tumour suppressive function has been demonstrated experimentally through mutant p53 mouse models. The transgenic mouse carrying a transactivation-dead p53 with quadruple missense mutations at residues 25, 26, 53 and 54 in the transactivation domains of p53, p53^{25,26,53,54}, is tumour prone, analogous to loss of p53 [2]. Interestingly, the mouse expressing the transcriptionally impaired mutant p53^{25,26} is not susceptible to develop spontaneous tumours even though p53^{25,26} is defective in inducing G_{1}-arrest or apoptosis. However, p53^{25,26} is capable of inducing senescence. Consistent with this, a p53 knock-in mouse possessing triple mutations of p53 at three lysine residues (121, 164 and 165), p53^{3KR/3KR}, is also not tumour prone even though p53^{3KR/3KR} is largely defective in inducing p53 target genes involved in cell cycle arrest and apoptosis [3]. Moreover p53 in a transgenic mouse deleted for three of its most studied target genes, p21^{waf1}, PUMA and Noxa that are involved in cell cycle arrest and apoptosis, also maintains its tumour suppressive function [4]. All these recent studies illustrated that despite extensive studies of p53 target genes we know very little about the key p53 targets that mediate its tumour suppressive function. These findings reinforced the urgent need to understand the complex barcode of p53 function [5].

Nonetheless, the transcriptional activity of p53 is crucial for its tumour suppressive function. Importantly, the majority of p53 mutations detected in human cancers occur in the central DBD of p53, a region that is evolutionarily conserved and shares significant
sequence homology to its family members, p63 and p73. Mutations in p53 can enhance its ability to bind p63 and p73 [6–8]. In addition to DNA, increasing numbers of proteins have been identified to bind the DBDs of p53. The DNA tumour viral protein SV40 Large T antigen binds the DBD of p53 and inactivates its tumour suppressive function suggesting that mutations in the DBD of p53 may alter its ability to interact with proteins as well as DNA [9,10]. Consistent with this, a co-crystal structure of the DBD of p53 and the C-terminus of ASPP2, the first identified DBD interacting cellular protein of p53, illustrated that a number of p53 residues that only contact ASPP2 but not DNA are among the top 20 most mutated residues of p53 found in human cancers. Furthermore, the most frequently mutated p53 hotspots, p53R248Q and p53R273H, contact both DNA and ASPP2 [11,12]. A co-crystal structure between the DBA binding domain of p73 and the C-terminus of ASPP2 has also been recently solved [13]. ASPP2/p53 and ASPP2/p73 interactions are able to enhance the transcriptional activities of p53 or p73 on selected target genes [14,15]. It is therefore tempting to speculate that in addition to binding DNA, the ability of p53 to interact with its siblings and cellular interacting proteins may also contribute to its tumour suppressive function.

p53 is mutated in around half of human cancers. However, the mutation frequency of p53 among different cancer types varies dramatically ranging from less than 10% in prostate and leukaemia, to around 50–70% in human lung and colon cancer and up to 95% in human ovarian cancer (http://p53.iarc.fr/, http://cancergenome.nih.gov/). Those cancer types with high p53 mutation frequencies, such as lung, colon and ovarian, tend to have a high background mutation load [1]. One exception to this rule is human melanoma. Although its background mutation load is similar as that seen in lung and colon cancers, p53 is only mutated in 10–20% of human melanoma [16], in contrast to the high p53 mutation frequency (50–70%) observed in human lung and colon cancer. A key question is why p53 is rarely mutated in human melanomas. Answers to this question will enable us to identify ways to inhibit tumour growth by restoring the tumour suppressive function of p53 in tumours that express structurally wild type p53.

Metastatic melanoma is characteristically refractory to treatment and accounts for 80% of skin cancer deaths [17]. Around 20% and 50% of melanomas have activating mutations of N-RAS and BRAF respectively, with elevated RAS/RAF kinase activities promoting tumour growth [18]. Recent advances in BRAF inhibitors, such as vemurafenib, have underscored the importance of the BRAF oncogene in the development and maintenance of melanoma. Vemurafenib inhibits cell proliferation by selectively targeting BRAFV600E expressing melanoma [19]. Despite promising results in inhibiting melanoma growth in the initial stages of treatment, the majority of vemurafenib treated patients relapse within a few months. Mechanisms of acquired drug resistance have been attributed to an up-regulation of PDGFR or N-Ras mutation [20,21]. To overcome acquired drug resistance, co-targeting BRAFV600E and MEK has been suggested [22]. However, as BRAF and MEK are components of the same signalling pathway, it is difficult to predict whether targeting two nodes of the same pathway will achieve a sustained clinical response. An alternative strategy would be to target two independent pathways, with p53 as an attractive option since it functions largely independent of BRAF. BRAFV600E is present in around 50% of human melanoma; however, up to 84% of human melanoma express wild type p53 (Fig. 1, cBioPortal, TCGA skin cutaneous melanoma data) [23,24], indicating the opportunity to reactivate p53 in melanoma.

2. p53 inactivation in human melanoma

MDM2 and MDM4 are the most well-known p53 inhibitors. They both bind the N-terminal transcriptional domain of p53 and inhibit p53 transcriptional activity [25,26]. An additional function of MDM2 is to bind p53 and act as the E3 ubiquitin ligase to target p53 for proteasome mediated protein degradation [27]. MDM4 is highly homologous to MDM2 although lacks ubiquitin ligase activity. MDM4 inhibits p53 activity mainly through its ability to bind MDM2 [28]. The oncogenic properties of MDM2 and MDM4 are experimentally demonstrated using MDM2 and MDM4 transgenic mice. In human tumours, both MDM2 and MDM4 are frequently overexpressed due to gene amplification [29–32]. Additionally, inactivation of p53 in melanoma may also be achieved through a frequent deletion in CDKN2A [33], which inactivates both p14ARF and p16INK4A. p14ARF binds MDM2 and prevents MDM2 from targeting p53 for degradation [34], p14ARF also functions as a p53 activator by potentiating MDM2-MDM4 binding and thus promoting MDM4 degradation [35]. Consistent with this, mutation analysis of existing skin cutaneous melanoma sequencing data showed that p53 mutation or deletion (17%), MDM2 amplification (4%) and CDKN2A deletion or mutation (42%) occur in around 55% of melanoma cases. Importantly, MDM2 amplification and CDKN2A deletion mainly occur in wild type p53 expressing melanoma (Fig. 1). Amplification and mutation of MDM4 occurs in 6% of melanoma cases. Interestingly, MDM2 and MDM4 amplification are almost mutually exclusive and they predominantly occur in wild type p53 expressing cells. Most CDKN2A deletions occur in wild type p53 expressing melanomas that do not have MDM2 and MDM4 amplifications (Fig. 1). All these findings suggest that MDM2 and MDM4 overexpression or loss of p14ARF may contribute to the inactivation of wild type p53 function. The importance of p53 in suppressing melanoma growth is supported by the rapid proliferation of BRAFV600E containing melanocytes on loss of p53 function [36]. Also, p53 prevents progression of naevi to melanoma in a transgenic mouse model [37]. In agreement with this Avery-Kiejda et al. observed abnormal expression of p53 downstream target genes, in particular pro-apoptotic genes including Bax and PIG3 in melanoma [38]. It has now been established that despite containing structurally wild type p53, the tumour suppressive function of p53 in melanoma cells is largely lost. Since TP53, MDM2, MDM4, and CDKN2A alterations only occur in around 50% of wild type p53 expressing human melanoma (Fig. 1), one of the major questions in melanoma and p53 research

![Fig. 1](http://example.com/fig1.png) Genetic alteration of TP53, MDM2, MDM4 and CDKN2A in melanoma. Bar graph shows the mutation and copy number alteration profile of TP53, MDM2, MDM4 and CDKN2A across the set of melanoma patients recorded in TCGA.
is whether p53 activity is inhibited in addition to MDM2, MDM4 or p14ARF. Answers to these questions are crucial to restore the tumour suppressive function of p53.

3. Reactivating p53 by inhibiting MDM2 and MDM4 in human melanoma

Pharmacological targeting of MDM2 to prevent binding and subsequent p53 degradation with small molecules is a conceptually viable strategy to reactivate p53. The most well-studied small molecule inhibitor is Nutlin3, which specifically disrupts the interaction between MDM2 and p53 [39]. As a result, Nutlin3 can stabilize p53 in MDM2 overexpressing cells and consequently up-regulates the expression of p21, encoded by p53 target gene CDKN1A, to mediate cell cycle G1 phase arrest. The ability of Nutlin3 to suppress tumour growth has been demonstrated in various preclinical models and Nutlin3 is undergoing clinical trial evaluation [40]. However, despite promising effects of Nutlin3 on inducing p53 expression, Nutlin3 alone only caused modest reactivation of p53-mediated cell death in melanoma [41,42]. Nutlin3 induced p53 failed to induce caspase 3 and caspase 7 activity and to trigger p53 mediated apoptosis in melanoma cell lines [42]. These observations suggest that wild type p53 is negatively regulated by other inhibitors besides MDM2 in melanoma cells.

Recently, Gembaraska et al. demonstrated that MDM4 protein level is upregulated in around 60% of melanomas even though its copy number is amplified in less than 7% of melanoma (Fig. 1). Sah-p53-8, a MDM4 specific inhibitor, restored p53 function and increased the sensitivity of melanoma to the BRAFV600E inhibitor vemurafenib [32]. As MDM4 is poorly targeted by Nutlin3, therefore it may remain active upon Nutlin3 treatment. This provided an explanation to why Nutlin3 alone is inefficient in targeting melanoma cells. However, a large number of melanomas do not express high levels of MDM2 or MDM4, implying other cellular factors may also regulate p53 activity in melanoma.

4. Restoring p53 function by preventing iASPP and MDM2 from inhibiting p53

Apoptotic function of p53, which is an irreversible process, is tightly linked to its tumour suppression function on which the efficacy of many cancer therapies depends. As a therapeutic strategy, selective reactivation of p53’s apoptotic function is an attractive proposition. The evolutionarily conserved ASPP family of proteins was identified as regulators of p53 induced apoptosis. The ASPP family consists of three members, ASPP1, ASPP2 and iASPP. All ASPP family members interact with the DBD of p53. However, ASPP1 and ASPP2 activate whereas iASPP inhibits the apoptotic function of p53 and its family members p63 and p73 [15,43]. All three ASPP members have been implicated in human cancer. Firstly, ASPP deregulation has been widely observed in various cancer types in past decade [14,43–47]. ASPP deregulation can be achieved via various mechanisms. ASPP1 and ASPP2 are transcriptional targets of E2F1, a key transcription factor that promotes cell cycle G1 to S phase transition. In many cancers, ASPP1 and ASPP2 have been found to be down-regulated by promoter methylation [48,49]. For example, reduced ASPP1 expression by promoter methylation is associated with high relapse rate and mortality in acute lymphoblastic leukaemia [48]. Down-regulation of ASPP2 in diffuse large B-cell and follicular centre lymphoma is also associated with poor prognosis [47]. Secondly, ASPP deregulation also associates with cellular sensitivity and resistance of cancer cells to various treatments. A correlation between ASPP2 mRNA levels and cellular sensitivity to ultraviolet and X-ray radiation in various cancer cell lines has been described [50]. Overexpression of iASPP was shown to induce cellular resistance to apoptosis induced by chemotherapeutic drugs including cisplatin and paclitaxel [43,51]. In addition, the ASPP deregulation primarily occurs in wild type p53 expressing cells [14,43]. Interestingly, iASPP overexpression frequently occurs in cancers expressing a unique p53 polymorphism, p53Pro72 [52,53].

In addition to expression level, post-translational modifications can also contribute to deregulation of ASPP activity. Lu et al. recently reported that iASPP activity is regulated by phosphorylation [46]. Overexpressed cyclin B1/CDK1 in melanoma cells phosphorylates iASPP at Ser84 and Ser113, leading to the inhibition of iASPP self-dimerization via its N and C-terminus and promoting iASPP monomer nuclear entry. Interestingly iASPP does not contain an identifiable nuclear localization signal (NLS). However, it’s ankyrin repeats containing C-terminus possess nuclear localization capability [54]. Mechanistically two adjacent 13th hydrophobic residues in ankyrin repeats containing proteins form a code for RanGDP binding. The ankyrin repeats containing proteins enter the nucleus via RanGDP/NTF2 pathway independent of importin. This novel nuclear import pathway is named the RaDAR (RanGDP/Ankyrin Repeat) pathway. iASPP uses this RaDAR pathway to enter the nucleus [55]. Compared to unphosphorylated cytoplasmic iASPP, phosphorylated nuclear iASPP binds p53 better and is more potent in inhibiting p53’s apoptotic function [46].

Phosphorylated nuclear iASPP, rather than cytoplasmic iASPP, occurs in 91% of wild type p53 expressing melanoma cell lines [46]. High levels of phosphorylated nuclear iASPP are associated with poor survival in melanoma patients. To inhibit phosphorylated nuclear iASPP, a panel of G2/M kinase inhibitors was screened and a potent cyclinB/CDK1 kinase inhibitor JNJ-7706621 was identified as the most efficient inhibitor of iASPP phosphorylation at Ser84 and Ser113. Treatment with JNJ-7706621 specifically induces p53-dependent apoptosis in wild type p53 expressing melanoma cells but not in mutant p53 containing melanoma cells. Interestingly, phosphorylated nuclear iASPP tightly correlates with MDM2 over-expression in wild type p53 containing melanoma cells [46], highlighting the need to co-target MDM2 and iASPP to reactivate p53. Co-inhibition of phosphorylated nuclear iASPP and MDM2 by JNJ-7706621 and Nutlin3 synergistically induced apoptosis in wild type p53 expressing melanoma cells. Furthermore, they also exhibit a synthetic suppression of cell growth in vitro and in two xenograft models. As p14ARF modulates the p53 pathway via MDM2, a MDM2 inhibitor could also kill melanoma cells harbouring deleted CDKN2A. This finding provided a proof of principle strategy to synergistically restore the tumour suppressive function of p53 by preventing the inhibitory properties of iASPP and MDM2 (Fig. 2, left panel).

5. Preventing other mechanisms from inactivating p53

Apart from MDM2, MDM4, p14ARF and ASPPs, numerous p53 co-factors (directly binding p53) and regulators (modulating p53 activity) have been reported to be deregulated in melanoma, suggesting roles in inactivating melanoma p53. These include PIASy [56], Tip60 [56,57], Y box-binding protein 1 [58,59], p63 [60], and p73 [61]. Both p63 and p73 are rarely mutated in human cancer. In melanoma, both isoforms of p63 (TAp63 and DeltaNp63) negatively regulate apoptosis through ameliorating expression of Bcl-2 through transllocation to the mitochondria and preventing nuclear p53 stabilization in response to genotoxic stress [60]. In case of p73, DeltaNp73 is considered as an oncoprotein since the expression of DeltaNp73 is linked to progression and metastasis in melanoma [61,62]. In contrast, TAp73 plays a role of tumour suppressive role in melanoma. In wild type p53-expressing melanoma cells, camptothecin, a cytotoxic quinoline alkald which
p53-expressing melanoma cells. Notably, ASPP is also able to bind in independent of p53 as it can be detected even in mutant induced TAp73 mediated apoptosis and this property of TAp73 is methotrexate (MTX/TMECG) treatment and consequently MTX was found that both p53 and TAp73 can be upregulated upon therapy involving of TAp73 in inducing apoptosis [64]. It in inducing apoptosis [63]. Recently, a study confirmed the p73-dependent apoptosis whereas in mutant p53-expressing melanoma cells, p73 and caspase-2 are predominantly involved in inducing apoptosis [63]. Recently, a study confirmed the involvement of TAp73 in inducing apoptosis in melanoma [64]. It was found that both p53 and TAp73 can be upregulated upon methotrexate (MTX/TMECG) treatment and consequently TAp73 induced TAp73 mediated apoptosis and this property of TAp73 is independent of p53 as it can be detected even in mutant p53-expressing melanoma cells. Notably, ASPP is also able to bind and regulate the p53 family members, p63 and p73 [15,65,66]. Thus, phosphorylated nuclear iASPP may also inhibit p63 and p73 and decide melanoma cell fate.

6. Reactivating p53 as a parallel strategy in future cancer therapy

Inactivation of the tumour suppressor p53 pathway is a common event in human cancer. p53 mutation occurs in around 50% of human tumours, but of the remaining 50% that express structurally wild type p53, many show impaired p53 function. The tumour suppressive function of p53 in human melanoma cells is notoriously unresponsive. The ability to restore the apoptotic function of p53 in melanoma cells in preclinical studies suggests that restoration of p53 can serve as a parallel strategy in the treatment of melanoma. Restoring wild type p53 function and inhibiting BRAFV600E represents a new means of enhanced cell killing in human melanoma and this was supported by recent studies (Fig. 2) [32,42,46]. In agreement with this, a combined treatment with inhibitors of BRAFV600E (veumafenib), iASPP (JNJ-7706621) and MDM2 (Nutlin3) suppressed the growth of wild type p53 and BRAFV600E expressing melanoma cell lines in vitro and xenograft tumours in vivo by at least 80% (Fig. 2, right panel). In contrast, single-agent treatment with vemafenib, JNJ-7706621 and Nutlin3 resulting in growth inhibition of 40%, 20% and 10%, respectively [46]. An optimized derivative of JNJ-7706621 together with a more potent Nutlin3 analog such as RG7112 may enhance the apoptotic function of p53 [67]. This initial study provided a proof of principle that the tumour suppressive function of p53 can be reactivated effectively by inhibiting both MDM2 and phosphorylated nuclear iASPP.

With recent understanding on the complexity of p53 inactivation and the preclinical successes in p53 reactivation in melanoma, p53-based therapy may become feasible in treating melanoma patients by restoring p53 function. Targeting iASPP in melanoma therapy represents a promising opportunity, although its regulation of other binding partners such as p63 and p73 in melanoma requires further investigation. Given these efforts, we would anticipate that modulating iASPP function may prove to be an effective strategy to restore p53 function in tumours such as melanoma that express structurally wild type p53. Restoration of the tumour suppressive function of p53 presents an opportunity for a parallel therapeutic strategy with BRAF600E inhibition to achieve maximal efficacy to kill melanoma cells. Strategies that target both p53-dependent and -independent pathways are likely to achieve synergistic killing in the 50% of cancers that express wild type p53.

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References


