Introduction

Malignant melanomas are the most lethal skin malignancy, notorious for aggressive growth and resistance to therapy. While the responses to selective BRAF and MEK inhibitors (BRAFi, MEKi), alone and in combination, in BRAF V600-mutant melanoma is encouraging, virtually all patients rapidly develop secondary resistance. We have shown that constitutive deregulation of both BRAF-MEK-ERK and p16INK4A-CDK4/6-RB pathways occur at high frequencies in melanomas, and that suppression of BRAF/MEK, or restoration of p16INK4A expression/inhibition of CDK4/6 can block the growth melanoma cells, and simultaneous correction of both BRAF-MEK and p16INK4A-CDK4/6 compounds this effect and also triggers significant apoptosis in melanoma cells. Our data suggests that BRAF-MEK-ERK and p16INK4A-CDK4/6-RB pathways may act additively or synergistically in the malignant growth of melanoma cells, and could be jointly targeted for treatment of melanoma. We also reported that the expression of K-type human endogenous retrovirus (HERV-K) correlates with ERK activation and p16INK4A loss in melanoma cells, and can be inhibited by MEK and CDK4/6 inhibitors, especially in combination. Given that HERV-K may destabilize the genome and act downstream of BRAF-MEK and CDK4/6, we hypothesize that cells with activated HERV-K may escape the therapeutic effects of BRAF-MEK and CDK4/6 blockers, and that triple inhibition of BRAF-MEK, CDK4/6, and HERV-K should be an effective therapy for melanomas.

Keywords: BRAF mutation; NRAS mutation; CDKN2A/p16INK4A lesion; HERV-K activation; combination therapy

Research Article

Triple Inhibition of BRAF/MEK, CDK4/6, and HERV-K for Melanoma Treatment

Gregorio Garza, Leon Li Tung, Jianli Dong

1 División de Ciencias de la Salud, Universidad de Monterrey, Nuevo León, México
2 Internal Medicine, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX, USA
3 Department of Pathology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX, USA
jidong@utmb.edu

Abstract: Malignant melanomas are the most lethal skin malignancy, notorious for aggressive growth and resistance to therapy. While the response to selective BRAF and MEK inhibitors (BRAFi, MEKi), alone and in combination, in BRAF V600-mutant melanoma is encouraging, virtually all patients rapidly develop secondary resistance. We have shown that constitutive deregulation of both BRAF-MEK-ERK and p16INK4A-CDK4/6-RB pathways occur at high frequencies in melanomas, and that suppression of BRAF/MEK, or restoration of p16INK4A expression/inhibition of CDK4/6 can block the growth melanoma cells, and simultaneous correction of both BRAF-MEK and p16INK4A-CDK4/6 compounds this effect and also triggers significant apoptosis in melanoma cells. Our data suggests that BRAF-MEK-ERK and p16INK4A-CDK4/6-RB pathways may act additively or synergistically in the malignant growth of melanoma cells, and could be jointly targeted for treatment of melanoma. We also reported that the expression of K-type human endogenous retrovirus (HERV-K) correlates with ERK activation and p16INK4A loss in melanoma cells, and can be inhibited by MEK and CDK4/6 inhibitors, especially in combination. Given that HERV-K may destabilize the genome and act downstream of BRAF-MEK and CDK4/6, we hypothesize that cells with activated HERV-K may escape the therapeutic effects of BRAF-MEK and CDK4/6 blockers, and that triple inhibition of BRAF-MEK, CDK4/6, and HERV-K should be an effective therapy for melanomas.

Keywords: BRAF mutation; NRAS mutation; CDKN2A/p16INK4A lesion; HERV-K activation; combination therapy
BRAF is a component of the RAS-RAF-mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling pathway, and p16INK4A (encoded by CDKN2A) is part of the p16INK4A-cyclin D-cyclin-dependent kinases (CDK) 4/6-retinoblastoma (RB) pathway. Constitutive deregulation of the BRAF-MEK-ERK and p16INK4A-CDK4/6-RB pathways occur at high frequencies in melanomas. We have shown that correction of either BRAF-MEK or p16INK4A-CDK4/6 abnormalities suppresses the in vitro and in vivo growth of melanoma cells, and that simultaneous inhibition of both BRAF-MEK and p16INK4A-CDK4/6 lesions, compounds this effect and also triggers significant apoptosis. Our data suggests that BRAF-MEK-ERK and p16INK4A-CDK4/6-RB pathways may act additively or synergistically in the malignant growth of melanoma cells, and could be jointly targeted for treatment of melanoma. We have shown that the expression of HERV-K correlated with ERK activation and p16INK4A loss in melanoma cells, and that HERV-K expression can be inhibited by MEK and CDK4/6 inhibitors, especially in combination. Since HERV-K can be activated and may drive malignant growth of melanoma downstream of BRAF-MEK and CDK4/6 pathways, cells with activated HERV-K may escape the therapeutic effects of MEK and CDK4/6 blockers leading to acquired treatment resistance. We propose that triple inhibition of BRAF-MEK, CDK4/6, and HERV-K could be an effective combo therapy for melanoma.

**BRAF/NRAS Activating Mutations in Melanoma**

In a systematic genome-wide screening for gene mutations, Davies et al. identified BRAF mutations at high frequencies, ranging from 59 to 80% in human melanoma samples; which included tumor cell lines, short-term cultures, and tumor tissues. A T1799A transversion in exon 15, resulting in a V600E missense mutation, accounts for approximately 90% of mutations detected in melanoma samples. In addition to melanomas, BRAF mutations have been identified in several other tumor types including thyroid, ovarian, colorectal, and lung tissues. The pathway conveys extra- and intracellular signals to nuclear transcription factors that regulate gene expression in response to such signals. BRAF is one of three members of the RAF family, which include serine/threonine kinases that transduce regulatory signals from RAS through MEK, to ERK. The ERK signaling pathway plays essential roles in cell proliferation, differentiation, and survival. Constitutive activation of the ERK pathway is believed to be essential in melanoma development. Pharmacological inhibition of the ERK pathway inhibited melanoma metastases in mice. We and others have detected BRAF mutations in over half of benign melanocytic nevi. In comparison, there are a very large number of melanocytic nevi in the general population compared with the relatively low incidence of melanomas. It is known clinically that nevi very often regress over time; thus, BRAF mutations alone are insufficient to cause malignant transformation in nevus cells.

About a third of all human cancers harbor mutations in one of the K-, N-, or H-RAS genes that encode an abnormal RAS protein, locked in a constitutively activated state, driving malignant transformation and tumor growth. NRAS-activating lesions are found in melanomas, but do not generally overlap with BRAF mutations in the same lesion. NRAS codons 12, 13, and 61 mutations are oncogenic lesions causing constitutive activation of MEK-ERK signaling pathway. NRAS mutations have been identified as one of the mechanisms to turn on phosphoinositide 3-kinase pathway leading to enhanced survival and resistance to BRAF inhibitors in melanoma cells.

**BRAF/MEK Inhibitors in Melanoma Treatment**

The high frequency of BRAF hot spot T1799A lesions provides the opportunity to examine the effects of specifically blocking this mutant allele. We used RNAi to specifically inhibit the expression of the T1799A mutant BRAF (mBRAF) and observed inhibited endogenous ERK signaling in melanoma cells that are positive for the BRAF mutation. Importantly, mBRAF RNAi also significantly inhibited the growth of these cells in tissue culture as measured by cell counting, and colony formation assay, and tumor growth in nude mice xenograft.
Triple Inhibition of BRAF/MEK, CDK4/6, and HERV-K for Melanoma Treatment

Interestingly, melanoma cells expressing mBRAF RNAi not only grow slower, but are also darker in color (shown in cell pellets, colonies, and xenografts), and produce more mature melanosomes 2. Since melanosome maturation and melanin production are signatures signs of melanocyte differentiation, the induced melanogenesis by BRAF inhibition may represent a reversion of melanoma cells to a more differentiated state. De-differentiation is characteristic of tumors cells 24. In many cell types, it is caused by constitutive activation of the RAS/RAF/MEK/ERK signaling 25,26. Suppression of mutant BRAF causes inhibition of the ERK signaling, which may explain the observed differentiation phenotype 27,28.

Consistent with the finding that inhibition of BRAF in melanoma cells not only induces growth inhibition, but also triggers cellular differentiation, our gene expression microarray analyses using Affymetrix human genome U133 GeneChip show that several genes involved in cell cycle control, cell growth, and differentiation are potential targets for mutant BRAF (Table 1). For the microarray expression analyses, 624Mel control and mBRAF RNAi expressing cells were cultured as previously described 1-3. RNA extraction, labeling, and hybridizations were performed at the Microarray Core Facility at Mount Sinai School of Medicine in New York City according to the manufacturer’s protocols. The microarray expression data were divided into control and mBRAF RNAi groups and the ratio of mBRAF RNAi over control were calculated (Table 1).

Table 1. Examples of putative mutant BRAF target genes identified by micro-array

<table>
<thead>
<tr>
<th>Gene class</th>
<th>Genes</th>
<th>Ratio of mBRAF RNAi/control</th>
<th>Fold ↑</th>
<th>Fold ↓</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>CCND1</td>
<td>425.9/686.7</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCNA1</td>
<td>16.3/35</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDK3</td>
<td>7.7/35.2</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>KITLG</td>
<td>0.5/1.6</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FGF17</td>
<td>5.2/30.1</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP1</td>
<td>1.2/12.3</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td>CEBPD</td>
<td>132.8/59</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MEOX2</td>
<td>16.1/2.2</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LHX2</td>
<td>23.4/5</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HOXD1</td>
<td>129.2/64.9</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HOXD3</td>
<td>53.3/32.8</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

CCND1: cyclin D1, a major downstream target of mitogenic signals 29.

CCNA1: cyclin A1, a CDK2 interacting cyclin that promotes cell cycle progression and a proliferation marker 30.

CDK3: cyclin dependent kinase 3, a CDK regulates the G1-phase of the cell cycle 31.

KITLG: KIT ligand, known to regulate developmental and functional processes of melanocytes 32.

FGF17: Fibroblast growth factor 17, involved in cell proliferation 33.

MMP1: matrix metallopeptidase 1, the matrix metalloproteinase (MMP) family degrades the extracellular matrix. MMP1 is up-regulated by the ERK signaling pathway in melanoma cells 34.

CEBPD: CCAAT/enhancer binding protein delta, transcription factor CCAAT/enhancer binding protein delta (also known as CEBPD, CRP3, CELF, NF-IL6beta) is implicated in diverse cellular functions, such as the acute phase response, adipocyte differentiation, and chromosomal stability 35.

MEOX2: mesenchyme homeobox 2, growth arrest specific mesenchyme homeo box 2, involved in patterning and differentiation 36. B. S. Skuntz, S. Harrigan,
The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites.**

**LHX2**: LIM homeobox 2, a LIM homeodomain protein involved in development.**

**HOXD1** and **HOXD3**: homeobox D1 and homeobox D3, genes involved in development and cancer.

The discovery that melanoma cells harboring BRAF V600 mutations are addicted to mutationally deregulated BRAF led to the exciting development and approval of PLX 4032 and other BRAF inhibitors for clinical use. Although inhibitors of mutant BRAF have generated great excitement because approximately 80% of BRAF mutant metastatic melanomas regressed in response to the treatment which was considered rare victory in the fight against melanomas, attention was quickly drawn to the fact that regressed tumors may resurge more aggressively within 1 year after the start of therapy, indicating that more research is needed to conquer melanoma.

**CDKN2A/p16INK4A Lesions in Melanoma**

Multiple genetic and environmental factors have been linked to the development of melanomas. For example, ultraviolet exposure, as well as defects in the cyclin dependent kinase inhibitor 2A (CDKN2A, encodes p16INK4A and p14ARF) locus are predisposing factors. Mutational spectra have shown that over 60% of CDKN2A gene mutations harbor UVB signature changes (A:T>T:A transversions). We have shown that loss of expression of CDKN2A (by Western blotting) co-exists with the hot-spot T1799A BRAF mutation in several melanoma cell lines, and that CDKN2A and BRAF lesions functionally interact to promote the proliferation and survival of cultured melanoma cells. BRAF mutations occur in over half of benign nevi, where BRAF is shown acting upstream of CDKN2A, leading to p16INK4A over-expression and senescence. The proportion of CDKN2A mutations in melanoma specimens co-existing with T1799A BRAF that also harbor UVB signature is unknown. Lesions in the p16INK4A-CDK4/6-RB pathway commonly co-exist and interact functionally with abnormal MEK-ERK pathway in melanoma cells. Similarly, the majority of melanomas have a deregulated p16INK4A-CDK4/6-RB pathway in which there is a loss of normal p16INK4A, resulting in disinhibition of CDK4/6. The mechanism(s) of CDKN2A and BRAF genetic interaction in the regulation of cell proliferation and survival is largely unclear. The observed induction of apoptosis and suppression of proliferation by simultaneous expressions of mutant BRAF siRNA and wild-type CDKN2A cDNA could result from double hitting of the "linear" BRAF-MEK-ERK-cyclin D:CDK4/6-RB pathways (additive effect). Alternatively, since BRAF and p16INK4A have non-overlapping activities in melanoma cells, their interaction can be non-linear and synergistic.

**Loss of CDKN2A/p16INK4A in Melanoma**

Genetic and epigenetic changes of CDKN2A have been identified in 30-70% of melanomas. Bi-allelic deletion of CDKN2A (p16INK4A null) occurs in 10-27% of melanomas. Other possible changes include mono-allelic deletion, point mutation, or promoter hypermethylation; thus resulting in various levels of p16INK4A expression/activity. p16INK4A shares coding sequences with ARF, however, the proteins are translated in different reading frames. ARF up-regulates p53 by interfering with p53 negative regulators, MDM2; while p16INK4A binds to and inhibits CDK4 and CDK6 and promotes cell-cycle arrest via the RB tumor suppressor pathway. Although deletions and mutations may affect both p16INK4A and ARF, several studies have identified mutations in melanoma specimens affecting only p16INK4A and lack of ARF involvement in human melanoma specimens. Mutational inactivation of RB tumor suppressor is rare in melanomas.
To examine reconstitution of wild-type CDKN2A in melanoma cells, the biological effect would be best measured in a cell that lacks or has abnormal endogenous CDKN2A. We examined CDKN2A in several melanoma cell lines. 624Mel cells were found to have an 18 bp deletion (GGAGGCGGGGGCGCTGC) in exon 1α. The deleted sequence encodes an evolutionarily conserved six amino acids (LEAGAL) in the p16INK4A N-terminal domain. The sequence shows loss of heterozygosity (LOH), suggesting that either the wild-type copy of the gene is deleted or that this is a homozygous deletion. Since matched constitutional DNA sample from the patient are not available, we cannot determine the cause of the LOH. Castellano et al. reported promoter hypermethylation in melanoma cells. Such data suggests that p16INK4A function is compromised in these melanoma cells. Florenes et al. reported deletion of p16INK4A in WM35 cells. Consistent with this report, we were unable to detect p16INK4A by Western blotting or CDKN2A mRNA, using reverse transcriptase PCR at high cycle numbers (>40 cycles) with appropriate positive and negative controls in WM35 cells. PCR failed to amplify CDKN2A exons 1α and 2, however exon 3 was amplified and showed a normal sequence.

It is believed that the acquisition of loss-of-function p16INK4A lesions allows melanoma cells to bypass senescence/growth arrest during melanoma development. To examine the effects of p16INK4A reconstitution, we subcloned the wild-type CDKN2AcDNA into a pBabe-neo retroviral vector. We transduced 624Mel and WM35 melanoma cells with either vector control or pBabe-neo-CDKN2A retroviruses. Melanoma cells expressing exogenous CDKN2A cDNA grew slower than control cells consistent with the tumor suppressor role of p16INK4A.

CDK4/6 inhibitors and companion diagnostic assays

Several CDK inhibitors have been developed including palbociclib (Ibrance, Pfizer), ribociclib (Kisqali, Novartis), and abemaciclib (Verzenio, Eli Lilly). Recently, the U.S. Food and Drug Administration (FDA) has approved the above three CDK4/6 inhibitors for patients with metastatic breast cancer, demonstrating that these drugs can be tolerated by cancer patients. There are ongoing investigation of CDK4/6 inhibitor-based therapies in melanoma. Our investigations have provided critical proof-of-concept preclinical studies to combine CDK4/6 with BRAF/MEK inhibitors to improve melanoma treatment. Given the increasing understanding and implementation of genetic and genomic medicine to improve cancer treatment efficacy and specificity, and the use of gene-signature, instead/in addition to tumor-or-origin as the basis of cancer treatment, for example the recent FDA approval of cancer treatment for any solid tumor with a specific genetic feature, and the ongoing clinical investigation of NCI-MATCH trial (https://www.cancer.gov/about-cancer/treatment/clinical-trials/nci-supported/nci-match), it is important to develop molecular tests that predict the efficacy of targeted melanoma therapy including CDK4/6 inhibitors. CDKN2A/p16INK4A assays should be validated for samples processed both by formalin-fixation with paraffin-embedding (FFPE) and frozen with optimal cutting temperature (OCT) compounds. Table 2 includes a list of the status of CDKN2A and p16INK4A that can be examined using molecular assays. Basically, wild-type CDKN2A without promoter hypermethylation should correlate with normal level of p16INK4A, mono-allelic deletion, various mutations and promoter hypermethylation with decreased levels of p16INK4A, whereas bi-allelic deletion should cause loss of p16INK4A expression.

<table>
<thead>
<tr>
<th>CDKN2A status</th>
<th>p16INK4A protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Normal</td>
</tr>
<tr>
<td>Various mutations</td>
<td>Various changes</td>
</tr>
<tr>
<td>Bi-allelic deletion</td>
<td>Protein null</td>
</tr>
<tr>
<td>Promoter hypermethylation</td>
<td>Lower levels of p16INK4A</td>
</tr>
</tbody>
</table>
HERV-K Activation in Melanoma

Endogenous retroviruses (ERVs) are sequences in the genome thought to be derived from ancient viral infections of germline cells in humans, mammals and other vertebrates; as such their proviruses are passed on to the next generation and now remain in the genome. Most retroviruses (such as HIV-1) infect somatic cells, but in very rare cases, it is thought that exogenous retroviruses have infected germline cells allowing integrated retroviral genetic sequences to be passed on to subsequent progeny, thereby becoming ‘endogenous’. Endogenous retroviruses have persisted in the genome of their hosts for thousands of years. Some human ERVs have been implicated in certain autoimmune diseases and cancers, such as rheumatoid arthritis, teratocarcinoma, melanoma and breast cancer. There are many thousands of endogenous retroviruses within human DNA, with human ERVs (HERVs) comprising nearly 8% of the human genome. HERVs can be classified to over 20 families based on tRNA specificity of the primer binding site used to initiate reverse transcription.

We reported that K type HERVs (HERV-K) are expressed and correlate with status of MEK-ERK and p16INK4A-CDK4/6 pathways in melanoma cells. It is interesting that we observed HERV-K expression in polyploid melanoma cells. We detected polyploid cells in melanomas but not in benign nevi and all such cells expressed HERV-K envelop protein ENV. Increased DNA content can be caused by mitosis error or intercellular fusion and is associated with enhanced malignant behavior.

Potential roles of HERV-K in melanoma pathobiology

HERV-K activation was observed to be correlated with changes in growth characteristics of melanoma cells (e.g., changes in cell shape, loss of melanin, anchorage-independent growth). These cellular changes share features that can be blocked by suppression of BRAF-MEK-ERK, especially in the presence of CDKN2A cDNA or CDK4/6 inhibitor. Of note, RAF signaling can aid in the replication and pathogenesis of the HIV and other retroviruses. HERV-K RT can reverse transcribe HERV-K RNA back to DNA, which may transpose into host genomic DNA, causing destabilization of the genome. We proposed the below model of regulatory interactions between BRAF-MEK-ERK, p16INK4A, and HERV-K in benign nevi and melanoma cells (Figure 1).

A. Nevus

Mutant BRAF

Wild-type p16INK4A

MEK-ERK activation

B. Melanoma

Mutant BRAF

Loss of wild-type p16INK4A

HERV-K activation

MEK-ERK activation

Fig1. A proposed model of interactions between BRAF-MEK-ERK, p16INK4A, and HERV-K in nevi and melanomas. A. In benign nevi, CDKN2A is wild-type and turned on by gain-of-function V600E BRAF; over-expression of wild-type p16INK4A inhibits activation of the BRAF-MEK-ERK signaling pathway; HERV-K is not induced. B. In melanomas, CDKN2A/p16INK4A is damaged by UVB or other factors leading to its loss of expression (nonsense mutation or promoter hypermethylation) or loss of activity (missense mutation). BRAF-MEK-ERK signaling is not in check by p16INK4A; HERV-K is activated to further drive malignant progression.

We have been intrigued by the findings that specific inhibition of HERV-K using RNAi can block intercellular fusion-mediated colony formation of melanoma cells, and that melanoma cell intercellular fusion can be inhibited by HERV-K ENV antibodies. We believe that efficient neutralizing HERV-K ENV antibodies can block intercellular fusion to stop the subsequent genetic changes that may lead to the evolution of tumor clones and emergence of more aggressive ones leading to tumor progression, metastasis, and treatment resistance.
Triple Inhibition of BRAF/MEK, CDK4/6, and HERV-K for Melanoma Treatment

HERV-K research and clinical applications need accurate analysis of HERV-K DNA, RNA, and proteins; which is challenged by the repetitive and homologous sequences of HERV-K elements. With new development to handle long-range sequencing and bioinformatics tools to correctly align homologous genomic sequences, we can better understand HERV-K sequence polymorphisms and copy number variations in normal and cancer samples, which should facilitate the investigation of HERV-K in human health and diseases.

**COMBINED INHIBITION OF BRAF/MEK, CDK4/6, AND HERV-K IN MELANOMA TREATMENT**

Although BRAF and MEK inhibitors have been shown to be amazingly effective in treating metastatic melanoma. Unfortunately, the effect was not curative, and tumor cells returned back after 1 year or so. These results show that further improvements must be made in the treatment of this disease. The best way to reach a cure, we believe, relies on rational combinations of BRAF/MEK inhibitors with other agents. Such combined treatment approaches have resulted in therapeutic “cocktails” that are effective in human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS). We propose that triple inhibition of BRAF/MEK, CDK4/6, and HERV-K can be an effective combined therapy for melanoma.

Creating identity crisis and cell death for melanoma treatment

We delineated independent regulation of proliferation and differentiation by BRAF and CDKN2A lesions in melanoma cells. Oncogenic BRAF can upregulate cyclin D through ERK pathway resulting in the activation of CDK4/6, and p16INK4A binds to and inactivates these CDKs, and activated CDKs phosphorylate and inactivate RB proteins resulting in the liberation of E2F transcription factors and cell cycle progression, therefore, both lesions lead to uncontrolled cellular proliferation consistent with their roles in the RB pathway. Note unlike melanoma cells with mutant BRAF inhibition, CDKN2A reconstituted cells are lighter in color (Figure 2). Since melanogenesis is a marker of melanocyte differentiation, the observed suppression of melanogenesis by p16INK4A and enhanced melanogenesis by BRAF inhibition, while unexpected, would suggest that proliferation and differentiation are regulated differently by BRAF and CDKN2A lesions in melanoma cells. Therefore, proliferation and differentiation are therefore separately regulated by BRAF and CDKN2A lesions in melanoma cells. It is believed that differentiation and malignancy are inversely correlated and cancer is a disease of cell differentiation, therefore, our findings have potential clinical significance.

**Fig2. Expression of CDKN2A and mutant BRAF RNAi induce opposite color change in 624Mel melanoma cells.**

Parental 624Mel cells (control) and 624Mel cells expressing CDKN2A or mutant BRAF RNAi (BRAF RNAi) were grown in regular media as described previously. Shown are regular pictures of cell pellets from similar numbers of the corresponding cells. CDKN2A and mBRAF inhibition induced lighter and darker colors, respectively.

It is well known that malignant cells, including those of melanomas, can maintain their normal differentiation program and retain sensitivity to differentiation modulators. “Differentiation therapy” that induces malignant reversion from malignant to benign phenotypes has been most successful for acute promyelocytic leukemia (APL). Such treatment has changed APL from a previously dismal outcome into one of the most treatable forms of leukemia. The understanding of mBRAF in the regulation of differentiation process of melanoma cells may provide not only insights into the roles of the mutation in melanoma biology, but also may provide strategies by targeting mBRAF in the “differentiation therapy” of melanomas.
**Triple Inhibition of BRAF/MEK, CDK4/6, and HERV-K for Melanoma Treatment**

We found that combined suppression of both BRAF-MEK and p16INK4A lesions not only further retarded cell growth, but also were synergistic in inducing apoptosis. During normal development, differentiation stimuli trigger the activation of microphthalmia-associated transcription factor (MITF) to promote cell-cycle arrest and initiate the melanogenesis process. Melanogenesis is a multi-step biochemical process resulting in the formation of melanin in pigment cells. There are three melanogenic factors, tyrosinase, tyrosinase-related protein-1 (TRP1), and dopachromatautomerase (DCT)/tyrosinase-related protein-2 (TRP2) that participate in the melanogenic pathway and are important melanocyte differentiation markers. Eberle et al. compared the expression of tyrosinase, TRP1, and DCT/TRP2 in cultured normal human melanocytes and melanoma cell lines by Northern blotting and reverse transcriptase-PCR (RT-PCR). They found that the three genes were expressed in the normal human melanocytes, but the expression was repressed in nearly all the 14 melanoma cell lines examined and was completely absent in 4 of the 14 lines. Hofbauer et al. compared the expression of tyrosinase, TRP1, and DCT/TRP2 in cultured normal human melanocytes and melanoma cell lines by Northern blotting and reverse transcriptase-PCR (RT-PCR). They found that the three genes were expressed in the normal human melanocytes, but the expression was repressed in nearly all the 14 melanoma cell lines examined and was completely absent in 4 of the 14 lines. Hofbauer et al. also found that tyrosinase expression level correlated inversely with clinical stage. Such data suggest that the normal melanogenesis mechanism is inhibited in melanoma cells. MITF and related melanogenesis factors are potential biomarkers for melanoma prognosis and treatment.

Inhibiting BRAF also up-regulates MITF in melanogenesis, a process somewhat inhibited by p16INK4A restoration. During cellular growth and differentiation, cells have specific spatial and temporal windows that are susceptible to exogenous influences of differentiation. Apoptosis occurs in cells with interrupted patterning programs. We propose that the differentiation process induced by BRAF-MEK inhibition is interrupted by wild-type p16INK4A and CDK4/6 inhibition, which create cell identity crisis and apoptosis (Figure 3).

**Fig 3. Creating identity crisis for cancer treatment.** A. Differentiation therapy. Tumor cells are induced to more mature and less malignant state. B. Differentiation confusion therapy. Cells have conflict between differentiation and de-differentiation. The confused cells undergo apoptosis in response to the identity crisis.
Melanoma is one of the most immunogenic tumors thought to due to its high mutational burden (caused by UV-damage) and MITF-induced molecular profile that make melanomas particularly susceptible to immunotherapy\textsuperscript{89,90}. Durable remission of metastatic melanoma is now achievable in some patients who receive to immunotherapy. However, it is unclear why some patients respond better than others. There are ongoing research to identify predictive biomarkers to immunotherapy and ways to enhance immunogenicity for better clinical efficacy\textsuperscript{89,90}. Given that HERV and MITF may modulate host immune reactivity\textsuperscript{73,89,90}, it will be interesting to examine whether activation of HERV-K and MITF correlates with response of melanoma cells to immunotherapy, and if so, develop corresponding predictive biomarkers for melanoma treatment.

**Blocking engine (BRAF-MEK), fixing brake (p16INK4A), stabilizing genome (HERV-K) for melanoma treatment**

Constitutive activation of BRAF-MEK-ERK (engine on) and loss of p16INK4A (brake off) are common paradigm in cancer biology. We have demonstrated that combinatorial inhibition of BRAF-MEK and p16INK4A lesions blocks proliferation and enhanced apoptosis in melanoma cells\textsuperscript{4,5}, the expression of HERV-K correlates with MEK-ERK activation and loss of p16INK4A in melanoma specimens and can be suppressed by MEK and CDK4/6 inhibitors\textsuperscript{5}, and combined use of MEK, CDK4/6, and HERV-K blockers enhance the inhibitory effect in cultured melanoma cells\textsuperscript{91}. Tumor development and acquired treatment resistance involve molecular and cellular changes leading to the evolution and selection of aggressive clones; for example, changes in survived cells under BRAF/MEK inhibition leading acquired treatment resistance. HERV-K encodes unique retroviral proteins, including reverse transcriptase (RT) and ENV that may be co-opted by melanoma cells leading to tumor progression and treatment resistance. It has been shown that constitutive BRAF-MEK signaling upregulation can drive aneuploidy in melanocytic cells\textsuperscript{92}, an effect is mediated, at least in part, by HERV-K retrotransposition-mediated mutagenesis and destabilized genome. We have shown that HERV-K ENV has fusogenic activity that can mediate intercellular fusion of melanoma cells\textsuperscript{4,5}). ENV may anchor tumor cells to target sites through intercellular fusions (melanoma cell as seed, ENV as root, target site as soil). Blocking HERV-K expression may stabilize the chromosome, inhibit cell fusion, and prevent evolution and the selection of more aggressive melanoma cells. Importantly, activation of HERV-K without normal cell cycle checkpoint control (due to abnormal p16INK4A-CDK4/6) may lead to acquired treatment resistance under BRAF/MEK treatment through HERV-K mediated intercellular fusion and retrotransposition. We believe that adding CDK4/6 and HERV-K inhibitors should block alternative and feedback survival routes and enhance tumor suppression under BRAF/MEK treatment. We contemplate that simultaneous inhibition of BRAF-MEK, CDK4/6, and HERV-K can broaden the pharmacodynamic profile, achieve target inhibition at lower bio-available drug concentrations, and generate additive/synergistic and long-lasting therapeutic effects.

Several CDK inhibitors have been developed, and Novartis CDK4/6 inhibitor LEE011 (ribociclib) has received FDA approval for treating advanced breast cancer\textsuperscript{66}. A wide variety of anti-retroviral drugs are available to treat HIV retrovirus\textsuperscript{93}. HERV-K elements can be inhibited by some of the anti-retroviral drugs, for example reverse transcriptase inhibitors\textsuperscript{91,94}. These inhibitors may be used in conjunction with BRAF-MEK and CDK4/6 inhibitors. While BRAF/MEK, CDK4/6, and retroviral drugs have been approved for clinical use, it is important to carry out clinical trials combining these agents to achieve more efficacious and long lasting effect so metastatic melanoma patients can live longer. Research and development of predictive biomarkers that can identify tumors with BRAF/MEK, CDK4/6, and HERV-K lesions should facilitate the selection of patients more likely to benefit from the combination therapy.

**Conclusion**

Constitutive deregulation of BRAF-MEK-ERK and p16INK4A-CDK4/6-RB pathways both occur at high frequencies in melanomas. We have shown that suppression of either BRAF-MEK or CDK4 inhibits cell growth, and that simultaneous inhibition of both BRAF-MEK and CDK4 compounds this effect and also triggers
significant apoptosis in melanoma cells. The data suggest that BRAF-MEK-ERK and p16INK4A-CDK4/6-RB pathways may act additively or synergistically in the malignant growth of melanoma cells, and could be jointly targeted for treatment of melanoma. We have also reported that the expression of HERV-K correlates with ERK activation and p16INK4A loss in melanoma cells and can be inhibited by MEK and CDK4/6 inhibitors, especially when in combination. Given that HERV-K may drive malignant growth downstream of BRAF-MEK and CDK4/6 and can also be activated by other factors that may be independent of BRAF-MEK and CDK4/6, we contemplated that cells with an activated HERV-K may escape the therapeutic effects of MEK and CDK4/6 inhibitors, and that triple inhibition of BRAF-MEK, CDK4/6, and HERV-K, would be more effective melanoma treatment.

Author Contribution

J.D. conceived and designed the experiments described in the review; G.G. and L.L.T. participated in the preparation of the review.

REFERENCES


**Triple Inhibition of BRAF/MEK, CDK4/6, and HERV-K for Melanoma Treatment**


Triple Inhibition of BRAF/MEK, CDK4/6, and HERV-K for Melanoma Treatment


