Provisional chapter

The Potential Importance of K Type Human Endogenous Retroviral Elements in Melanoma Biology

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Additional information is available at the end of the chapter

1. Introduction

Human endogenous retroviruses (HERVs) are thought to be germline-integrated genetic remnants of exogenous retroviral infections and comprise approximately 8% of the human genome [1, 2]. Similar to exogenous retroviruses such as human immunodeficiency virus (HIV) and human T cell leukemia virus (HTLV), a complete HERV sequence is composed of GAG, POL, and ENV genes flanked by two long terminal repeats (LTRs). Although most HERVs are degenerated with disruptive open reading frames, a few proviruses have retained intact genes, and the corresponding proteins can thus be expressed [1, 3]. HERVs have been implicated in the etiology of cancer, chronic inflammation, and other diseases [2], and emerging data support a role of HERV-K in melanomagenesis. For example, HERV-K is activated in melanomas but not in melanocytes [4, 5], and inhibition of HERV-K by RNA interference (RNAi) suppresses the *in vivo* growth of melanoma cells [6-9]. We have demonstrated recently that expression of K-type human endogenous retrovirus (HERV-K) correlates with ERK activation and p16 loss in human melanoma specimens, and that inhibition of MEK and CDK4 in combination, suppresses HERV-K expression [10, 11]. Importantly, if HERV-K drives melanomagenesis downstream of the BRAF-MEK-ERK and p16/CDK4 pathways, when HERV-K is already turned on, cells may be resistant to therapies targeting BRAF-MEK-ERK and p16-CDK4. Triple therapies, such as simultaneous targeting of HERV-K, BRAF/MEK and CDK4, may be necessary to produce more effective and long-lasting therapeutic effects. This strategy is analogous to HIV "cocktail" therapy that disrupts human immunodeficiency virus (HIV) at different stages of viral replication and has brought many acquired immune deficiency syndrome (AIDS) patients from near death to fairly normal and productive lives.

2. The K type Human Endogenous Retroviral Element (HERV-K)

HERVs are thought to be germline-integrated genetic remnants of exogenous retroviral infections and comprise approximately 8% of the human genome [1,2]. They are transmitted vertically by Mendelian inheritance [12]. HERVs can be classified into over 20 families based on transfer RNA (tRNA) specificity of the primer binding site used to initiate reverse transcription; thus, HERV-K would use lysine and HERV-W tryptophan if they were replicating viruses [13]. Through millions of years of evolution, HERVs have become indispensible parts of the human genome. For example, syncytin-1, encoded by the envelope (ENV) gene of HERV-W, mediates intercellular fusion of trophoblast cells to form syncytiotrophoblast as well as preventing maternal immune attack against the developing embryo, thereby facilitating implantation of the embryo [1,2]. Similar to exogenous retroviruses such as human immunodeficiency virus (HIV) and human T cell leukemia virus (HTLV), a complete HERV sequence is composed of group-specific antigen (GAG), polymerase (POL), and ENV genes flanked by two long terminal repeats (LTRs) (Fig. 1). Although most HERVs are degenerated with disruptive open reading frames, a few proviruses have retained intact genes, and the corresponding proteins can thus be expressed [1, 3]. Increased HERV expression has been found under pathological conditions, particularly in cancer and inflammatory disease [12, 14]. Unlike most HERVs that harbor defective mutations, the HML-2 group of HERV-Ks has open reading frames that code for functional viral proteins, which may form noninfectious particles [15, 16]. HERV-K proteins and virus-like particles have been demonstrated in human cell lines of teratocarcinoma [17], breast cancer [18], placenta [19], and melanoma [5].

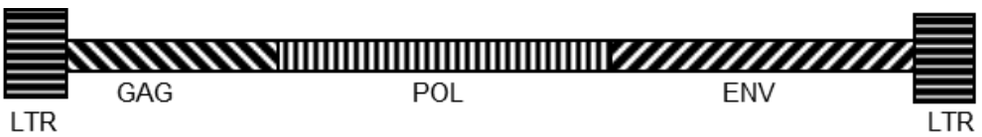

Figure 1. HERV-K genome organization. An intact HERV-K genome is approximately 9700 nucleotides long, like other retrovirus. HERV-K has several genes, mutated so unable to encode functional viral proteins to produce infectious virus. However, pieces of protein domains can be produced by HERV-K sequences. In wild-type retroviral sequences, the group-specific antigen (GAG) codes for the Gag polyprotein. Gag protein provides the basic physical infrastructure of retrovirus; Polymerase (POL) codes for viral enzyme reverse transcriptase, integrase, and protease; envelope (ENV) codes for proteins embedded in the viral envelope which enable the virus to attach to and fuse with target cells. Flanking functional genes are long terminal repeat (LTR) regions that play important roles in initiating viral DNA synthesis and its integration as well as regulating transcription of the viral genes.

HERVs have been implicated in the etiology of cancer, chronic inflammation, and other diseases [2], and emerging data support a role of HERV-K in melanomagenesis. For example, HERV-K is activated in melanomas but not in melanocytes [4, 5], and inhibition of HERV-K by RNAi suppresses the *in vivo* growth of melanoma cells [6-9]. Putative pathogenic mechanisms for HERV-K include mutagenesis by retro-transposition [8, 20], regulation of gene transcription downstream of the insertion sites [21], HERV-K mediated intercellular fusion [Huang et al., in preparation], subversion of immune surveillance by viral proteins [6], as well as direct influences on cell proliferation, differentiation, and anchorage-dependent growth [7, 9].

Like functional retrovirus, HERV-K transcripts can retro-transpose and insert back to the host genome, depending on the insertion sites, may lead to mutagenesis. The enzymes encoded by HERV-K may also induce retro-transposition of Long-interspersed nuclear element-1 (LINE-1) elements, as in the case of HIV-1 [22]. We designed experiment to determine HERV-K genomic integration sequences by HERV-K anchored polymerase chain reaction (PCR). The PCR amplicons can be sequenced after subcloning of individual sequences or next generation sequencing (NGS).

We designed and performed preliminary experiments as shown in Fig. 2. DNA was extracted from human cells using QIAamp® DNA Mini kit (Qiagen, Valencia, CA) following manufacturer's procedures. The optical density (OD) values of DNA were measured before samples were stored at -80°C for further use. Primers used (based on HERV-K108, accession # AF164614) were: 5'3': GCG GTC CCA AAA GGG TCA GTN NNN NNN NNN; 5'ERV (nucleotide 2156-): TTT GCC AGA ATC TCC CAA TC; 3'ERV (nucleotide 4063-): TTG AGC CTT CGT TCT CAC CT; 5'Tag: GCG GTC CCA AAA GGG TCA GT. To perform PCR, about 75ng DNA from human cells was amplified with 0.3 μM 5'3' and 5'ERV or 3'ERV primers, 0.4 mM dNTPs, 1X Pfx Buffer, 1mM MgSO4, and 1.25 unit Platinum® Pfx DNA Polymerase (Invitrogen, Carlsbad, CA). The PCR reaction was carried out in a 9700 thermocycler (Applied Biosystem, Carlsbad, CA). Initial denaturing was at 94°C for 5 minutes, then 94°C for 15 seconds, 36°C for 30 seconds, and 68°C for 4 minutes, for 35 cycles, with an extension at 68°C for 10 minutes after the last cycle. Then 10 μl of the PCR products were used for the 2nd PCR using 5'Tag and 5'ERV or 3'ERV primers with the same amplification conditions except 55°C for annealing. The 2nd PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). The resulting templates can be sequenced after subcloning or directly by next generation sequencing.

In a recent study, Macfarian etc shown that during embryogenesis, transcripts of embryonic and extraembryonic tissues are initiated from long terminal repeats derived from endogenous retroviruses [21]. It is conceivable that HERV-K LTR may also regulate gene transcription during melanomagenesis, a hypothesis that can be tested in future studies. The experimental strategy used in Fig. 2 may also apply to determine transcripts downstream of HERV-K sequences.

As with some other viruses, syncytium formation is a characteristic of retroviral infection. As shown in Fig. 3B, pleomorphic cells with features of nuclear atypia, multinuclear/nuclear syncytia are characteristic of malignant cells. We hypothesize that HERV-K mediated intercellular fusion contributes to the formation of pleomorphic cells in tumor specimens.

We performed experiments to identify fusogenic domains and epitopes in HERV-K ENV protein, and to generate HERV-K ENV monoclonal antibodies and evaluate for ability to block ENV mediated intercellular fusion. The fusogenic activity of syncytin has been located to its N-terminus [23]. To map fusogenic domains of HERV-K ENV protein, we will generate a series of C-terminal truncations of HERV-K ENV by PCR using HERV-K108 sequence as template. PCR amplicons will be cloned into pcDNA 3.3-TOPO TA Mammalian Expression Vector using TA Cloning Kit (Invitrogen, Grand Island, NY). The ENV expression constructs will be transfected into melanoma cells and examined for activities to rescue fusogencity inhibited by HERV-K inhibition.

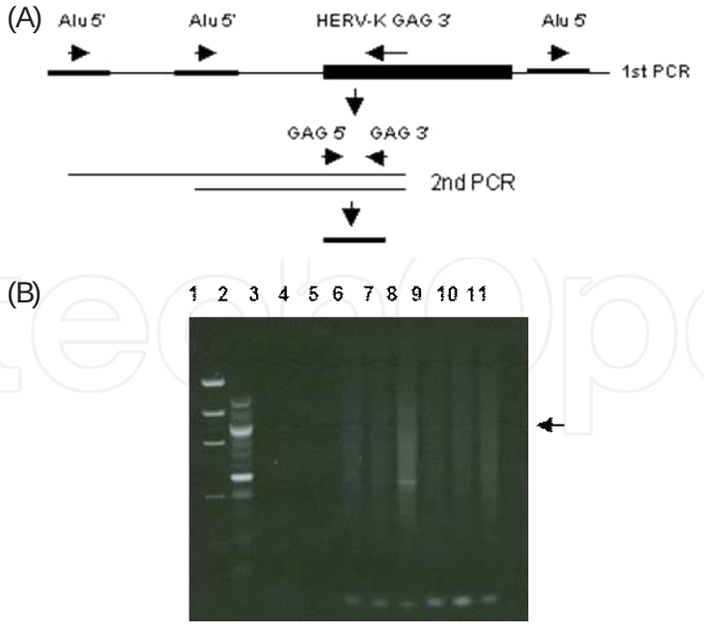

Figure 2. Experimental design to detect HERV-K genome insertion sites. A. HERV-K and Alu elements are bolded. Alu is a short stretch of approximately 300 bp originally characterized by the action of the Alu (*Arthrobacter luteus*) restriction endonuclease. There are over one million Alu elements interspersed throughout the human genome, and it is estimated that about 10.7% of the human genome consists of Alu sequences. PCR is designed to amplify HERV-K and flanking Alu sequneces. Amplicon sequences flanking HERV-K can be determined by subcloning or NGS. B. Gel electrophoresis of 1st PCR products. 1 and 2, molecular weight markers; 3-11 1st round PCR amplicons of genomic DNA extracted from different clones of cultured A375 melanoma cells. Sample 8 may have a unique insertion site captured by the PCR assay (arrow).

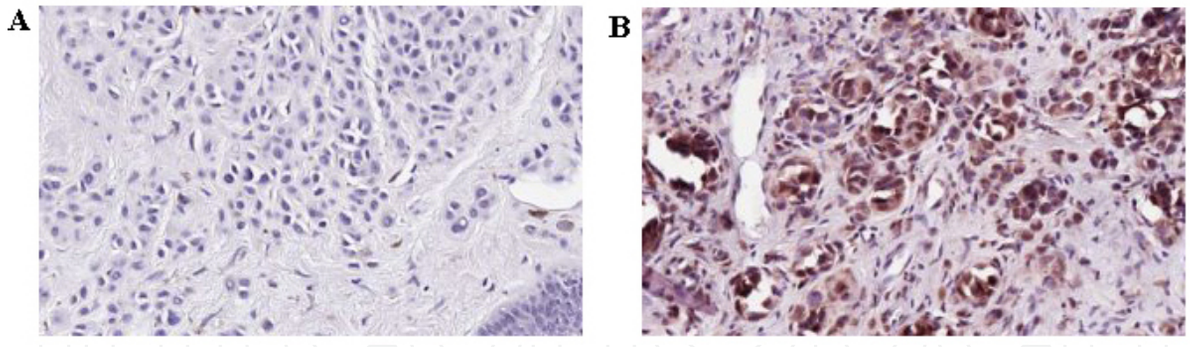

Figure 3. HERV-K may contribute to the generation of pleomorphic tumor cells. Formalin-fixed, paraffin embedded microscopic sections of nevus (A) and melanoma (B) were analyzed to examine cell morphology. Pleomorphic cells with features of nuclear atypia, multinuclear/nuclear syncytia were easily detected in melanoma (B) but not in nevus (A) cells. In an unpublished study, we have shown that such pleomorphic cells in melanomas express HERV-K (Huang et al., submitted). Magnification: x 200.

3. Possible regulation of HERV-K by BRAF-MEK and p16-CDK4

We noticed that the growth characteristics of melanoma cells that can be modified by HERV-K activation (e.g., changes in cell shape, loss of melanin, anchorage-independent growth) [9] overlap with those that can be blocked by suppression of BRAF-MEK-ERK signaling pathway,

especially with simultaneous restoration of p16 or inhibition of CDK4 [23-26]. This observation, together with the knowledge that aberrations in BRAF-MEK-ERK and p16-CDK4 pathways are early events and often co-exist during melanomagenesis, and the evidence that RAF-MEK-ERK signaling pathway is required for the completion of HIV-1 reverse transcription [27], prompted us to hypothesize that HERV-K is regulated by BRAF-MEK-ERK and p16-CDK4 pathways. We have reported that expression of HERV-K GAG and ENV proteins correlates with ERK activation and p16 loss in a panel of melanocytic specimens, and that inhibition of MEK and CDK4, especially in combination, suppresses HERV-K protein expression in cultured melanoma cells [11].

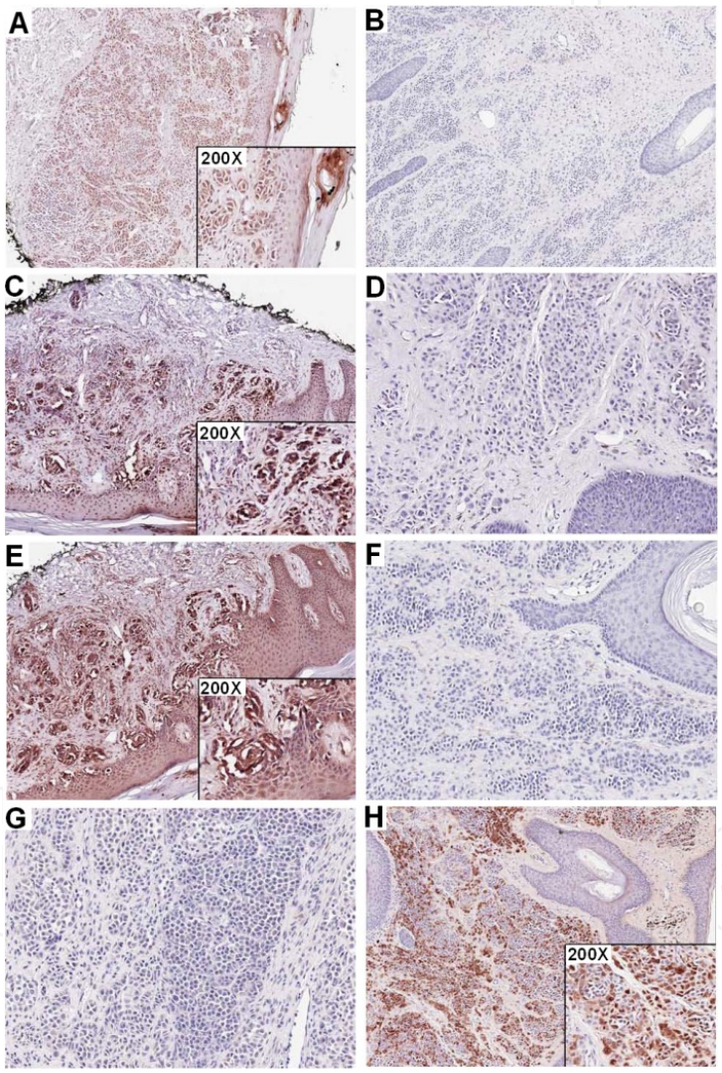

Figure 4. Expression of HERV-K GAG and ENV, p-ERK and p16 in neval and melanoma specimens [8]. Formalin-fixed and paraffin-embedded routine pathology specimens of melanoma (A, C, E, G) and nevus (B, D, F, H) were analyzed by immunohistochemical staining using HERV-K GAG (A, B), ENV (C, D), p-ERK (E, F), and p16 (G, H) specific antibodies. Shown is a representative staining pattern. HERV-K GAG and ENV were mainly detected in melanoma cells (A and C), but rarely expressed in neval cells (B and D). p-ERK was mainly detected in melanoma cells (E) but rarely found in neval cells (F). p16 was rarely expressed in melanoma (G) but prominently expressed in neval cells (H). Magnification: ×100 and ×200 (insets).

Seventy two formalin-fixed, paraffin-embedded pathological specimens were available from the archives at the University of Texas Medical Branch (UTMB). The samples included 38 nevi (11 junctional, 3 compound, and 24 dermal) and 34 melanomas (10 Lentigo maligna, 10 superficial spreading, 7 nodular, and 7 metastatic). The study was approved by the UTMB institutional review board (IRB) for the protection of human subjects. Immunohistochemical staining was modified from the protocol of All-in-One Kit for Immunohistochemical Staining for Tissues with Antibodies (Invitrogen, Carlsbad, CA). Four-micron-thick sections of paraffin blocks were dewaxed in three changes of xylene and rehydrated through a graded series of alcohol concentrations into water. Sections were washed with PBS Tween (PBST) (Sigma, St Louis) three times, each for 5 minutes, then blocked with horse serum for 30 minutes at room temperature. For antigen retrieval, sections were heat-treated in a microwave oven for 20 minutes in 0.01 M citrate buffer (pH 6.0, 100 mM stock), and cooled for 20 minutes in a beaker. For immunostaining the slides were incubated at 4 ℃ overnight with primary antibodies including phospho-p44/42 ERK (Thr202/Tyr204,Cat. #9101, Cell Signaling Technology, Danvers, MA), p16 (Cat. #MS-887 NeoMarkers, Fremont, CA), HERV-K GAG and ENV (Cat. #s 1841-5 and 1811-5, respectively, Austral Biologicals, San Ramon, CA) following the manufacturer's instructions. The slides were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by signal development, counterstaining and mounting using All-in-One Kit for Immunohistochemical Staining for Tissues with Antibodies (Invitrogen, Carlsbad, CA). As described previously [28], blood vessel were stained distinctly for p-ERK and used as an internal control for p-ERK antibody. Epidermal keratinocytes and adnexal glands were internal controls for p16 [28]. Melanoma cell line A375 was reported positive for HERV-K using the same HERV-K antibodies [9], and was used as positive control for HERV-K GAG and ENV antibodies, as described [11].

As shown in Fig. 3 [11], we examined the expression of HERV-K GAG and ENV proteins, the active form of ERK (phospho-ERK, p-ERK), and p16 in a panel human melanocytic specimens including 38 benign nevi and 34 melanomas. Both HERV-K GAG and ENV proteins were largely cytoplasmic, but occasional nuclear staining was observed, whereas p-ERK was typically co-expressed in cytoplasm and nucleus. It has been reported that wild-type *INK4A* is expressed in both nucleus and cytoplasm, whereas mutant p16, if expressed, is either nuclear or cytoplasmic [29]. HERV-K GAG cytoplasmic staining was over 10-fold more frequent in melanoma than in nevus (38% of melanomas vs. 3% of nevi, $p < 0.001$) (Table 1). Similarly, HERV-K ENV immunoreactivity was detected in the cytoplasm in 44% of melanomas and 11% of neval specimens (a 4-fold difference, $p = 0.003$). The nuclear staining of GAG and ENV were infrequently detected in both nevi and melanomas, and the differences did not reach statistical significance (Table 1). p-ERK staining was 5-fold more often positive in melanomas than in nevi (68% and 13%, respectively, $p < 0.001$). p16 staining, both in the cytoplasm and nucleus, was more frequently observed in nevi than in melanomas (cytoplasmic, 79% vs. 50%, $p = 0.014$; nuclear, 79% vs. 15%, $p < 0.001$) (Table 1). Figure 3 demonstrates representative staining pattern of HERV-K GAG, HERV-K ENV, p-ERK and p16 in melanoma and neval specimens.

Antigen	Positivity (%)		*p value*
	Nevi [n=38]	Melanoma [n=34]	
HERV-K GAG, cytoplasmic	3	38	$p < 0.001$**
HERV-K GAG, nuclear	0	6	$p = 0.493$
HERV-K ENV, cytoplasmic	11	44	$p = 0.003$**
HERV-K ENV, nuclear	8	12	$p = 0.7$
p-ERK, cytoplasmic and nuclear	13	68	$p < 0.001$**
p16, cytoplasmic	79	50	$p = 0.014$*
p16, nuclear	79	15	$p < 0.001$**

Protein immunoreactivity, cytoplasmic or nuclear, was dichotomized as negative/decreased (<30% of cells staining positively) and positive (>30% of cells staining positively).

** Difference is significant at the ≤0.01 level (2-tailed)

* Difference is significant at the ≤.05 level (2-tailed).

Table 1. Expression of HERV-K GAG and ENV, p-ERK and p16 in neval and melanoma specimens [8]

Further analysis showed that the expression of HERV-K GAG in the cytoplasm of melanoma was positively correlated with p-ERK ($p = 0.005$), and negatively correlated with p16 cytoplasmic expression ($p = 0.012$) (Table 2). The expression of HERV-K ENV in the cytoplasm of melanomas was positively correlated with p-ERK ($p < 0.001$), and negatively correlated with p16 nuclear expression ($p = 0.046$) (Table 2).

	HERV-K GAG cytoplasmic	HERV-K GAG nuclear	HERV-K ENV cytoplasmic	HERV-K ENV nuclear
p-ERK, cytoplasmic and nuclear	$p = 0.005$**	$p = 0.093$	$p < 0.001$**	$p = 0.2$
p16, cytoplasmic	$p = 0.012$*	$p = 0.058$	$p = 0.114$	$p = 0.608$
p16, nuclear	$p = 0.123$	$p = 0.378$	$p = 0.046$*	$p = 0.251$

Note: ** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Table 2. Associations of the expression of p-ERK, p16, and HERV-K GAG and HERV-K ENV[8]

As described [11], human melanoma cell lines 624Mel, A101D, A375, and OM431 were kindly provided by Dr. Stuart Aaronson (Mount Sinai School of Medicine, New York, NY). Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO) and 50 units/mL penicillin-streptomycin (Invitrogen, Carlsbad, CA) in a humidified incubator at 37 °C with 5% CO_2. Total RNA was extracted from cultured melanoma cells using an RNA

extraction kit ToTALLY RNA (Ambion, Austin, TX) according to the manufacturer's instructions, and purified with RNase free DNase Set (Qiagen, Valencia, CA). RNA concentration was assessed spectrophotometrically at 260/280 nm. Two μg of total RNA was reverse transcribed and amplified by One-Step RT-PCR (Qiagen, Valencia, CA) using 50 pmol of primer pairs specific for HERV-K POL, ENV genes and GAPDH for control as described [9]. Briefly, samples were reverse transcribed at 50 C for 30 min, then heat-activated by incubation at 95 C for 15 min. Samples were subjected to 30 cycles of PCR amplification as described [9] in MasterCycler® Personal (Eppendorf, Hamburg, Germany). Each cycle consisted of 1 min at 95 C, 1 min at 56 C and 1 min at 72 C. After the final cycle, samples were incubated at 72 °C for 10 min. PCR products were inspected by separation through 1% agarose gels. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified PCR products were directly sequenced on both strands using a 3100 Capillary Array Sequencer and Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA). NCBI BLAST search of the human genome was performed to identify the amplified sequences [11].

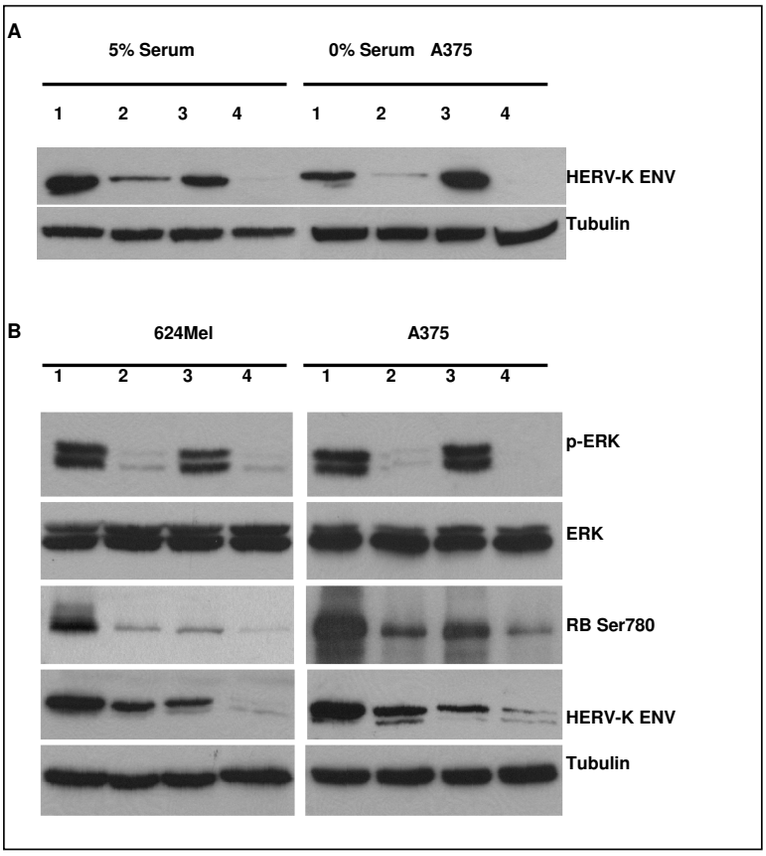

Figure 5. MEK and CDK4 inhibitors suppress HERV-K ENV protein expression [8]. A. 624Mel (1), A101D (2), A375 (3), and OM431 (4) melanoma cells were cultured in DMEM with 5% serum or serum starved overnight, and cell lysates collected for Western blotting. B. 624Mel and A375 melanoma cells were treated with solvent vehicle control (1), 25 μg MEK inhibitor PD98059 (2), 1 μg CDK4 inhibitor 219476 (3), and 25 μg PD98059 plus 1 μg 219476 (4) for 48 h under serum starvation, and cell lysates collected for Western blotting. Western blotting was performed using 50 μg total cell extracts and commercially available HERV-K ENV antibody that recognized a 37 Kd spliced transmembrane domain of ENV protein. Tubulin was used as loading control.

Western blots were performed as described [24, 11]. Briefly, harvested cells were lysed in Lysis Solution (Cell Signaling, Danvers, MA) supplemented with Complete Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics Corporation, Indianapolis, IN). Protein concentration of lysates was determined using the Quick Start Bradford 1 x Dye Reagent (Bio-Rad, Hercules, CA). Lysates were separated in 10% SDS-polyacrylamide gel, electrophoretically transferred to Immobilon-P membrane (Millipore Corp, Billerica, MA), and probed with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The following antibodies were used: phospho-p44/42 ERK (Thr202/Tyr204) and p44/42 ERK (Cell Signaling, Danvers, MA), PhosphoPlus(R) RB (Ser780, Ser795, Ser807/811) Antibody Kit (Cell Signaling, Boston, MA), HERV-K GAG and ENV (Cat. #s1841-5 and 1811-5, respectively, Austral Biologicals, San Ramon, CA), and HRP-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA). Immunoreactive bands were visualized with SuperSignal chemiluminescence substrate (Pierce, Rockford, IL). The blots were exposed to blue sensitive blue x-ray film (Phenix Research, Candler, NC).

MEK inhibitor PD98059 (Calbiochem, San Diego, CA) was dissolved in DMSO as a 50 mM stock solution, aliquoted and stored in –20 °C. CDK4 inhibitor 219476 (Cat. #219476, Calbiochem, San Diego, CA) was dissolved in DMSO as a 2 mM stock solution and stored at 4 °C. 1×10^6 melanoma cells were seeded in a cell culture dish in DMEM containing 10% FBS and antibiotics. On the second day, cells were treated with PD98059 and 219476 in DMEM without serum for 48 h and then harvested. Statistical analyses were performed by using SPSS 13.0 software (SPSS Inc., Chicago, IL). Fisher exact test was used to detect the differences in immunostaining between melanomas and nevi. Pearson Correlation was used to test the correlations of immunostaining (HERV-K GAG, HERV-K ENV, p-ERK, and p16).

The expression of HERV-K has been reported in several melanoma cell lines [4, 5, 9, 30]. We examined HERV-K in four melanoma cell lines, 624Mel, A375, A101D, and OM431 that have constitutive activation of p-ERK, and loss of wild-type p16 with corresponding hyperphosphorylation of RB protein [24, 25, 26]. As previously reported [11], HERV-K expression was detected using a specific HERV-K ENV antibody as described [5, 9], that recognizes a 37 Kd spliced transmembrane domain of ENV protein [4, 5, 30]. HERV-K ENV protein was prominently expressed in 624Mel and A375 cells, weakly positive in A101D cells, but barely detectable in OM431 cells (Fig. 4A). We extracted total cellular RNA from all the four cell lines and performed conventional RT-PCR using specific HERV-K POL and ENV primers as described [9]. Direct sequencing of RT-PCR amplicons and NCBI BLAST analysis showed that the expressed sequences share 96%-98% overall homology with Group N HERV-K [31] (data not shown), as reported in other melanoma cells [32].

We have established experimental conditions (including time course and dose-response curve) of treating several melanoma cells with PD98059 and 219476, to respectively inhibit MEK and CDK4, alone and in combination [26]. 624Mel and A375 lines were used to test whether HERV-K EVE expression was suppressed by the MEK and CDK4 inhibitors. As expected, treatment with PD98059 inhibited ERK phosphorylation in both 624Mel and A375 cells (Fig. 4B, lane 2). It is worth noting that we consistently observed somewhat further reduced levels of p-ERK by PD98059 when combined with CDK4 inhibition (Fig. 4B, p-ERK, lane 4 vs. lane 2; also in Li et

al., 2009, Fig. 1a, lane 4 vs. lane 2); which may contribute to the observed functional interaction between BRAF-MEK-ERK and p16-CDK4-RB pathways [25, 26]. Phosphorylation of serine 780, a CDK4 target in the RB protein, was reduced by PD98059 and 219476, especially in combination in both cell lines (Fig. 4B). Similarly, HERV-K ENV expression was inhibited by either PD98059 or 219476, especially when used in combination (Fig. 4B). The results were consistent with findings of the association between HERV-K expression and p-ERK and p16 in melanocytic specimens (Table 2).

Multiple endogenous and exogenous factors have been linked to the activation of HERVs including hormones, cytokines, and cytotoxic chemicals [33]. To our knowledge, a direct association between ERK and p16-CDK4 pathways and HERV expression has only been reported by our group [11]. It has been shown that HERV-K sequences, as other host genes, are regulated by DNA methylation in the promoter/enhancer sequences located in the 5'-LTR regions [34]. Since RB protein, a downstream mediator of BRAF-MEK-ERK and p16-CDK4 signaling pathways, is a key regulator of DNA methylation [35], it is conceivable that the observed association between HERV-K, p-ERK and p16-CDK4 may act through RB, a notion that will surely prompt further investigation. It is worth noting that the four melanoma cell lines examined, 624Mel, A375, A101D and OM431, all have *BRAF* T1799A mutation, constitutive activation of ERK, loss of wild-type p16, and over-expression of phospho-RB protein [24]. However, only 624Mel and A375 cells express high levels of HERV-K ENV protein (Fig. 4A), suggesting that HERV-K ENV protein is regulated by mechanisms in addition to ERK and p16-CDK4 pathways.

HERV-K may prove to be a key mediator of BRAF-MEK-ERK and p16-CDK4-RB pathways during melanoma pathogenesis. Activation of BRAF/ERK was recently shown to drive chromosome abnormality and aneuploidy in melanocytes [36]. It is conceivable that the effect may be mediated, at least partly, through HERV-K sequences that are capable of jumping around by retro-transposition leading to mutagenesis and chromosomal abnormalities [8, 20]; HERV-K proteins have been shown to suppress the host immune system [6]. Activation of HERV-K by BRAF-MEK-ERK and CDK4 signaling may facilitate tumor progression especially in the blood/lymph stream when circulating tumor cells are under attack by the immune system, thus providing a critical survival/escape mechanism for invasion and metastasis [12, 37]. Alternatively, it is possible that the observed growth promotion and anti-apoptotic effects of activated ERK and CDK4 [25, 26] can be mediated, at least in part, by HERV-K since HERV-K has been shown to directly affect melanoma cell proliferation, differentiation, and anchorage related survival [7, 9].

4. Combined inhibition of CDK4 potentiate the effect of MEKi

Constitutive dysregulation of MEK-ERK and p16-CDK4-RB pathways both occur at high frequencies in melanoma. We found that simultaneous inhibition of both MEK and CDK4 generated more potent and dramatic lethal effects than individual inhibition in melanoma cells [10, 25, 38]. We also found that the expression of K- type human endogenous retrovirus (HERV-

K) correlates with ERK activation and p16 loss in melanoma cells and that inhibition of MEK and CDK4 can block HERV-K expression [11]. As shown in Fig. 5, if HERV-K drives tumor progression downstream of RAF-MEK-ERK and p16/CDK4, when HERV-K is already turned on, cells may escape the inhibitory effects of therapies targeting RAF-MEK-ERK and p16/CDK4. Given that HERV may drive malignant growth downstream of MEK and CDK-4, we hypothesized that cells with activated HERV may not respond to the therapeutic effects of MEK and CDK4 blockers and that triple therapy, such as targeting HERV-K, MEK and CDK4, may be necessary to produce more effective and long-lasting therapeutic effects than single or, as we have proposed previously, double inhibition of MEK-ERK and CDK4 [25, 26]. This strategy is analogous to HIV "cocktail" therapy that disrupts HIV at different steps of replication and brought many AIDS patients from death to fairly normal and productive lives [39].

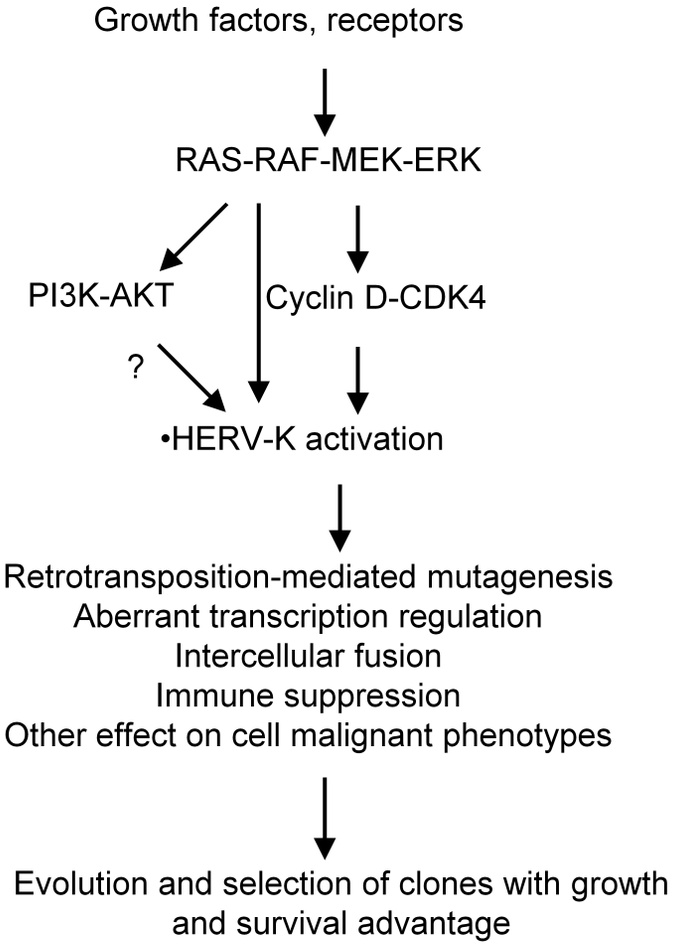

Figure 6. Potential regulation and activity of BRAF-MEK-ERK, CDK4, and HERV-K in carcinogenesis and tumor progression. Constitutive activation of RAS-RAF-MEK-ERK and cyclin D-CDK4 are common paradigm of cancer biology. We have reported that that simultaneous inhibition of both BRAF-MEK and CDK4 generated more potent and dramatic lethal effects than individual inhibition in melanoma cells [10, 25, 38]. We have also shown that the expression of HERV-K correlates with ERK activation and p16 loss in melanoma cells and that inhibition of MEK and CDK4 can block HERV-K expression [11]. When HERV-K is already turned on by MEK, CDK4 or alternative pathways, cells may escape the inhibitory effects of therapies targeting RAF-MEK-ERK and p16/CDK4. Triple inhibition of BRAF-MEK, CDK4, and HERV-K may stop the evolution and selection of cell clones with growth and survival advantage.

Group	Treatment
1	solvent control
2	solvent control + BRAFi/MEKi
3	solvent control + CDK4i
4	solvent control + BRAFi/MEKi + CDK4i
5	HERV-Ki + solvent control
6	HERV-Ki + BRAFi/MEKi
7	HERV-Ki + CDK4i
8	HERV-Ki + BRAFi/MEKi + CDK4i

The triple inhibition experiment (Table 3) can be performed using cultured melanoma cells or in mouse melanoma xenograft, as described [10, 24, 25, 38]. Inhibition of BRAF (BRAFi), MEK (MEKi), CDK4 (CDK4i), and HERV-K (HERV-Ki) can be achieved using either RNA interference (RNAi) or small molecular inhibitors that are in active clinical development (http://clinicaltrials.gov/).

Table 3. Triple Therapy Treatment Groups

We designed experiments to examine the effects of combined inhibition of HERV-K, BRAF/MEK, and CDK4 in melanoma cells (Table 3). Human melanoma cell lines MeWo, A101D and A375 that harbor wild-type, heterozygote, and loff of heterozygosity (LOH) *BRAF* T1799A alleles, respectively [24, 40] can be used. These cell lines express HERV-K transcripts and/or proteins, have constitutive activation of MEK-ERK signaling pathway, and high levels of phospho-RB (p-RB) [24, 25, 3840, 41] (and data not shown). Stable suppression of HERV-K, BRAF, or CDK4 can be achieved by stable expression of HERV-K short hairpin RNA (shRNA) [7, 9], *BRAF* shRNA, or *INK4A* cDNA [24], or using small molecular inhibitors of BRAF inhibitor (BRAFi), including Vemurafenib (PLX4032, RO5185426, Hoffmann-La Roche) that has been U.S. Food and Drug Administration (FDA) approved for treatment of melanoma, Dabrafenib (GSK2118436, GlaxoSmithKline), PLX3603 (RO5212054) (Hoffmann-La Roche); MEK inhibitor (MEKi), including PD-325901 (Pfizer), GSK1120212 (GlaxoSmithKline), MSC1936369B (EMD Serono), ARRY-438162 (MEK162) (Array BioPharma), AZD6244 (Astra-Zeneca), and BAY86-9766 (Bayer); CDK4 inhibitor (CDK4i), including PD-0332991 (Pfizer), LY2835219 (Eli Lilly and Company), LEE011 (Novartis Pharmaceuticals). All are in active clinical development (http://clinicaltrials.gov/).

We performed experiments to simultaneously inhibit MEK and CDK4 using known pharmacological inhibitors PD98059 and 219476, respectively, along with using azidothymidine (AZT) to target HERV-K reverse transcription activity as described [42]. AZT (also called ZDV) is a nucleoside analog reverse-transcriptase inhibitor (NRTI), a type of antiretroviral drug used for the successful treatment of HIV infection. It is a therapeutic analog of thymidine. Optimal results were obtained when cultured A101D and 624Mel cells were treated for a period of 48 hours (Fig. 6). A decrease in the number of viable cells was observed for both A101D and 624Mel cells. The data from this study provided preliminary data of the effect AZT has on melanoma cells in combination with inhibitors of MEK and CDK4, in support of our hypothesis

and our rationale for using AZT as a complementary treatment. Several follow-up studies will be performed. The effects of the chemical treatment on melanoma cells, e.g., cell proliferation, apoptosis, and cell cycle progression will be measured using established methods [10, 38]. Molecular mediators of the effects (phospho-ERK, phospho-RB, HERV proteins) will be measured using commercial available antibodies by Western blotting. Future studies are also necessary to further investigate the regulation and activities of HERV-K in melanoma pathogenesis.

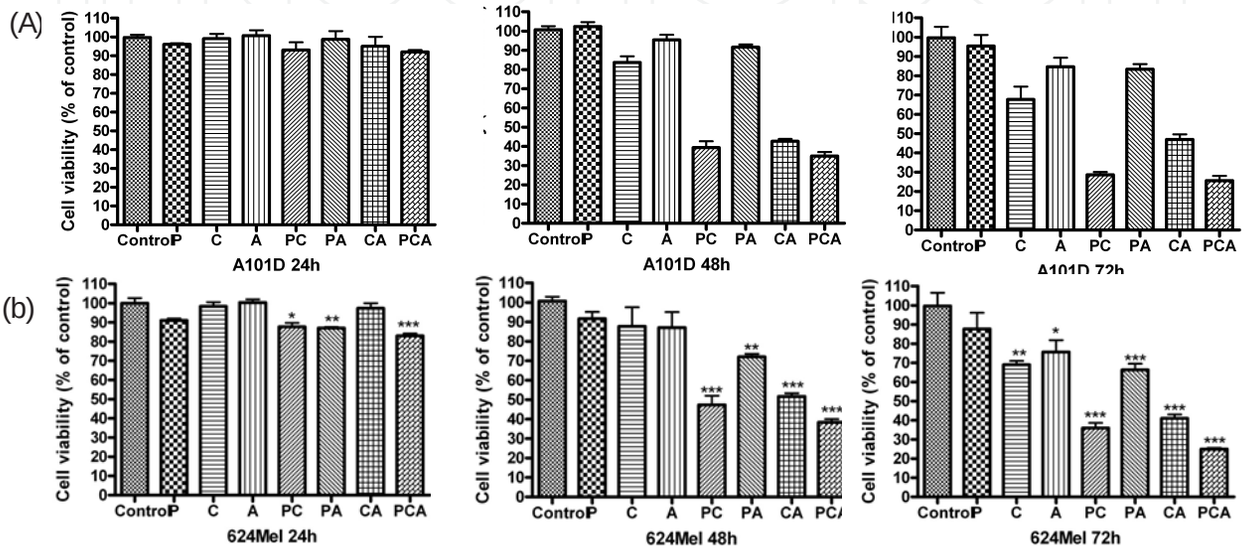

Figure 7. Combined use of MEK, CDK4, and HERV-K inhibitors enhances the therapeutic effect in cultured melanoma cells. A101D (A) and 624Mel (B) human melanoma cell lines were grown in culture as described [10, 38]. CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI) was used to measure total viable cells following manufacture's instructions. 5,000 melanoma cells were seeded in each well of 96-well plates, and were grown for 24 hrs in media with 10% Fetal Bovine Serum (FBS). Afterward, media were aspirated and various combinations (Table 3) of treatment chemicals were added to each well in media without serum. Based on dose-response analyses, the final concentrations used were: PD90895 (P) at 25 μM; 219476 (C) at 1 μM; AZT (A) at 1000 μM. The plates were incubated for a period of 24 hrs, 48hrs, and 72 hrs, respectively. Subsequently, MTS solution was added to each well according to the manufacturers protocol. The plates were then incubated for 2-4 hrs, afterward absorbance was measured at 490nm. Data were analyzed and compared for treatment efficiency. Triple inhibition (PCA) generated more inhibitory effects in control solvent, single, or double combination.

We have learnt the significance of "contaminating DNA" in the measurement of HERV-K expression from our recent investigation of the correlation between HERV-K expression and HIV-1 viral load in plasma specimens [Esqueda et al., in press]. We performed RNA extraction using HIV-1 ViroSeq RNA preparation method (Abbott Molecular, Des Plaines, IL). According to the manufacturer this RNA preparation kit extracts total nucleic acid including RNA plus DNA (Dr. Gavin Cloherty, personal communication). According to Qiagen, "The QIAamp Viral RNA Mini Kit is not designed to separate viral RNA from cellular DNA, and both will be purified in parallel if present in the sample" (page 9, QIAamp Viral RNA Mini Handbook, 04/2010). We used DNase I digestion to effectively remove contaminating cellular DNA in the RNA samples. As demonstrated in Fig. 7, without DNase I digestion, HERV-K RT-PCR was

uniformly positive in all five HIV-1 positive plasma specimens, whereas only samples #3 and #5 were positive after DNase I digestion. The results emphasize that optimized methodology in the laboratory is essential to the accurate assessment of HERV-K activation.

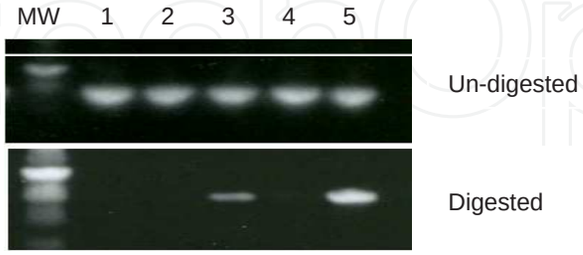

Figure 8. DNase I digestion affects HERV-K RT-PCR results. Viral RNA was extracted from five HIV-1 positive specimens using HIV-1 ViroSeq kit. Without DNase I digestion, HERV-K RT-PCR was uniformly positive in all the 5 cases, whereas only samples #3 and #5 remained positive after DNase I digestion. MW, molecular weight marker.

5. Conclusion

Constitutive dysregulation of MEK-ERK and p16-CDK4-RB pathways both occur at high frequencies in melanoma. We found that simultaneous inhibition of both MEK and CDK4 generated more potent and dramatic lethal effects than individual inhibition did, in melanoma cells. It has been shown recently that the K-type human endogenous retroviral sequence (HERV-K) is expressed in melanoma cells but not in melanocytes. HERVs are germline-integrated genetic remnants of retroviral infections that are transmitted vertically by Mendelian inheritance. It is estimated that 8% of the human genome consists of retroviral elements including HERVs (4). HERVs can be classified to over 20 families based on tRNA specificity of the primer binding site used to initiate reverse transcription. HERV sequences are similar to the HIV sequence. 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. We have shown that the expression of HERV-K correlates with ERK activation and p16 loss in melanoma cells and that inhibition of MEK and CDK4 can block HERV-K expression. Given that HERV may drive malignant growth downstream of MEK and CDK-4, we hypothesized that cells with activated HERV may not respond to the therapeutic effects of MEK and CDK4 blockers and that triple inhibition of MEK, CDK4, and HERV should be more effective than double treatment, a hypothesis supported by our preliminary data. Additional studies are necessary to further examine the regulatory relationship between aberrant MEK-ERK and p16-CDK4-RB pathways and HERV-K activation, the roles and mechanisms of HERV-K in carcinogenesis and clonal evolution of malignant cells. The potential therapeutic efficacy of triple inhibition of BRAF-MEK, CDK4, and HERV-K need to be further explored in our fight against melanoma and other malignancies.

Nomenclature

AIDS: acquired immune deficiency syndrome

Alu: *Arthrobacter luteus*

BRAF: v-raf murine sarcoma viral oncogene homolog B1

BRAFi: BRAF inhibitor

CDK4: cyclin-dependent kinase 4

CDK4i: CDK4 inhibitor

DMEM: Dulbecco's modified Eagle medium

DNA: deoxyribonucleic acid

ENV: envelop

ERK: extracellular-signal-regulated kinase

FBS: fetal bovine serum

FDA: Food and Drug Administration

FGF: fibroblast growth factor

FISH: fluorescent *in situ* hybridization

GAG: group-specific antigen

HERV: human endogenous retroviruse

HERV-K: K-type human endogenous retrovirus

HIV: human immunodeficiency virus

HTLV: human T cell leukemia virus

IHC: immunohistochemical staining

INK4A: inhibitor of cyclin-dependent kinase 4A; part of cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*), also known as multiple tumor suppressor 1 (*MTS1*)

LINE-1: long-interspersed nuclear element-1

LOH: loss of heterozygosity

LTR: long terminal repeatRs

MEK: mitogen-activated protein kinase/ERK kinase

MEKi: MEK inhibitor

NGS: next generation sequencing

OD: optical density

PAGE: polyacrylamide gel electrophoresis

PARP: poly (ADP-ribose) polymerase

PBS: phosphate buffered saline

PCR: polymerase chain reaction

p-ERK: phopho-ERK

PI3K: phosphatidylinositol 3-kinase

POL: polymerase

RAF: v-raf murine sarcoma viral oncogene homolog. Human has three RAF: CRAF, BRAF, and ARAF

RAS: rat sarcoma viral oncogene homolog. Human has three RAS: HRAS, NRAS, and KRAS (KRAS4A and KRAS4B proteins arise from alternative splicing)

RB: retinoblastoma proteins including pRB, p107, and p103

RNA: ribonucleic acid

RNAi: RNA interference

RT-PCR: reverse transcription polymerase chain reaction

SDS: sodium dodecyl sulfate

shRNA: short hairpin RNA

tRNA: transfer RNA

Acknowledgements

We thank Dr. Stuart Aaronson for human melanoma cell lines. This work was supported by Bill Walter III Melanoma Research Fund, Harry J. Lloyd Charitable Trust, and Cancer and Leukemia Group B Foundation (to J.D.).

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