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Aberrant expression of p16^{INK4a} in human cancers – a new biomarker?

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Abstract

The *ARF* and *INK4a* genes are located in the same *CDKN2a* locus, both showing its tumor suppressive activity. ARF has been shown to detect potentially harmful oncogenic signals, making incipient cancer cells undergo senescence or apoptosis. INK4a, on the other hand, responds to signals from aging in a variety of tissues including islets of Langerhans, neuronal cells, and cancer stem cells in general. It also detects oncogenic signals from incipient cancer cells to induce them senescent to prevent neoplastic transformation. Both of these genes are inactivated by gene deletion, promoter methylation, frame shift, and aberrant splicing although mutations that change the coding region affect only the latter. Recent studies indicated that Polycomb genes *EZH2* and *BMI1* repressed p16^{INK4a} expression in primary cells, but not in cells deficient for pRB protein function. It was also reported that that p14^{ARF} inhibits the stability of the p16^{INK4a} protein in human cancer cell lines and mouse embryonic fibroblasts through its interaction with regenerating islet-derived protein 3γ. Overexpression of INK4a is associated with better prognosis of cancer when it is associated with human papilloma virus infection. However, it has a worse prognostic value in other tumors since it is an indicator of pRB loss. The p16^{INK4a} tumor suppressive protein can thus be used as a biomarker to detect early stage cancer cells as well as advanced tumor cells with pRB inactivation since it is not expressed in normal cells.

Introduction

Since the discovery as a product of the alternate reading frame of the mouse *Arf/Ink4a* locus signals, the Arf tumor suppressor has been identified as a key sensor of hyperproliferative stimuli such as those originating from oncoproteins to prevent early stage cancer cells undergo neoplastic transformation by inducing senescence or apoptosis [1,2]. p19^{Arf} and p16^{Ink4a} are transcribed from separate and unique first exons 1β and 1α which splice into two shared exons 2 and 3 (Figure 1). These two genes are different tumor suppressors since *p19^{Arf}* uses only exons 1 and 2 while *p16^{Ink4a}* uses all of the exons 1-3 for production of the protein [3,4]. This locus has a very unique genomic structure not found in other mammalian genes due to the unprecedented splicing utilized by *Arf* which causes an alternate reading frame in the coding region of exon two. Of note this *ARF-INK4a* (*CDKN2a*) locus is located 11.5 kbp apart from the genomic locus for *CDKN2b* that encodes for p15^{INK4b} in humans (Figure 1). The transcriptional regulation for the *ARF-INK4a* locus has been described [5,6]. The aberrant transcripts from *CDKN2a* locus have also been reviewed [7]. Both p14^{ARF} and p16^{INK4a} function as tumor suppressors [8-11] despite the lack of amino acid sequence similarity. Consistent with the findings in mice, frequent mutation, promoter methylation, or deletion of the *ARF/INK4a* locus in human cancers has been reported [5,12,13] second only to p53 in frequency.

ARF is a highly basic (the predicted pI is 11), insoluble protein which exhibits little structure apart from a pair of α helices at its amino terminus [14]. Ectopic Arf is capable of arresting immortal mouse cell lines such as NIH 3T3 as well as transformed human cells [3,4,15], a classic and requisite property of tumor suppressors. Arf sequesters MDM2 in the nucleolus, preventing p53 degradation [1,2]. Additionally, it inhibits transcription factor E2F activity. These actions lead to cell cycle arrest at G1 and G2 [4]. Importantly, Arf has both p53-dependent and independent functions [1,2,16,17].

Expression of p16^{INK4a} functions to limit cell-cycle progression and to promote cellular senescence in response to multiple stressors, including oncogene activation, telomere erosion, reactive oxygen species, and stalled replication forks [18-22] (Figure 1). Expression of p16^{INK4a} in healthy cells is low, but once induced, p16^{INK4a} binds and inhibits cyclin-dependent kinase 4/6 (CDK4/6) activity, thereby promoting a retinoblastoma (RB) - dependent cell-cycle arrest. This tumor suppressive mechanism is believed to limit the growth of early stage neoplasms, and accordingly, the p16^{INK4a}- CDK4/6-RB signaling is disrupted in most, if not all, human cancers, with inactivation of p16^{INK4a} being the most common lesion of this pathway [12] (Table 1). Although induction of p16^{INK4a} in response to oncogenic stimuli results in a beneficial, anti-cancer mechanism, expression of this tumor suppressor also accelerates mammalian cell aging [19-22]. Both senescent cells and levels of p16^{INK4a} progressively accumulate with age [23,24] and are associated with a decline in the replicative capacity of many tissue types [25,26]. p16^{Ink4a} overexpression has been reported in human cancer and senescent fibroblasts in response to oxidative stress, DNA damage, and changes in chromatin structure [27]. Although gene knock-out mice for *p15^{Ink4b}* does not show striking phenotypes, it acts as a tumor suppressor when the *Arf-Ink4a* locus is simultaneously inactivated [28] (Figure 1). Hybrid proteins that encode for p15^{Ink4b} and p16^{Ink4a} have been reported, indicating the synergy of co-deletion for tumor suppressor genes [29].

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Table 1. Mechanisms of gene inactivation of the *ARF-INK4a* locus. The p16^{INK4a} gene/protein could be either underexpressed or overexpressed in human cancer, which will cause inactivation. p16^{INK4a} upregulation detects early stage cancer cells, and thus is associated with better prognosis; however, it can also reflect the RB inactivation in tumors, and is associated with worse prognosis

Gene	Mechanism of inactivation	Human cancers affected	Impact on prognosis	References
INK4a	gene deletion			5, 12, 13, 18-22
	point mutation	25-70% of all human cancers		
	promoter hypermethylation	head and neck		
	frameshift	esophagus		
	splicing errors	biliary tract		
		liver		
		lung (LOH 36%, methylation 53%)		38
		bladder		
		breast (LOH 9%)	LOH does not have impact	33, 34, 88
		leukemia		
		lymphoma		
		glioblastoma		
		pancreatic carcinoma (98%)		
		malignant melanoma		
INK4a	overexpression	HPV (+) cervical, head & neck tumors	better prognosis	79-83
		colon	worse prognosis	32; 86; 92
		breast, 20%	worse prognosis	33, 34, 88, 93-95
		gall bladder		85
	Inverse relationship with RB loss	breast, lung, endometrium	possibly worse prognosis	86-91

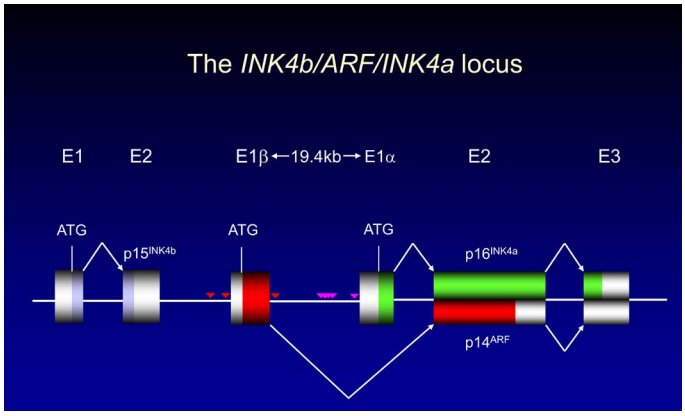

Figure 1. The structure for the human *p15^{INK4b}-p14^{ARF}-p16^{INK4a}* locus

The genomic structure is well-conserved between human and mice, and thus gene knockout studies have been extensively conducted in mice. The distance between exon 1β and exon 1α is 19.4 kbp in humans and 12.4 kbp in mice. The exon 1α is 3.8 kbp upstream of exon 2 in humans; 5.2 kbp in mice. The *ARF-INK4a* (*CDKN2a*) locus is located 11.5 kbp apart from the genomic locus for *CDKN2b* that encodes for p15^{INK4b} in humans. All of *p15^{Ink4b}*, *p19^{Arf}*, and *p16^{Ink4a}* genes act as tumor suppressors as reported by Krimpenfort, *et al.* [10, 28]. The reverse triangles indicate the position of Dmp1-binding sites (mouse), which is different on the *Arf* genomic locus (red: -1060, -183, and +290 from 5' end of *Arf* cDNA) from that on *Ink4a* (pink: -3482, -3390, -3292, -3171, -162 from 5' end of *Ink4a* cDNA). The DMP1 consensus is located -2.3 kb and -0.31 kb of *ARF* and -4.04 kb and -1.40 kb of *INK4a* in humans. Both of these are Dmp1 target genes [7, 96-98] although the mode of regulation is different [36].

This review is focused on the mechanism of regulation of INK4a, and its expression in human cancers. Special interests have been put on its value for early stage tumor detection for a potential biomarker since it is not expressed in normal tissues.

Transcriptional regulation of p16^{INK4a}

Transcriptional regulation plays a major role in p16^{INK4a} regulation as the half-life is long (8 hrs) [30]. A progressive increase of p16^{INK4a} expression has been described in passaged MEFs [8,31], transformation from normal tissue to pre-neoplastic lesions, and from pre-neoplastic lesions to carcinomas [32-34]. Human cells lacking functional pRB contain high levels of p16^{INK4a} mRNA and protein, suggesting a negative feedback loop by which pRB regulates p16^{INK4a} expression in late G1. Hara, *et al.* conducted nuclear run-on assays and promoter characterization of *p16^{INK4a}* in human fibroblasts [35]. They showed that *p16^{INK4a}* transcription was affected by the status of *RB* and defined the *p16^{INK4a}* promoter that was required for this response. *p16^{INK4a}* RNA was extremely stable, and the levels did not change during the cell cycle consistent with that fact that it lacked E2F or p53 consensus sequences [35]. Primary human fibroblasts expressed very low levels of p16^{INK4a}, but the mRNA and protein accumulated in late passage, senescent cells [35]. The overexpression of p16^{INK4a} in RB-negative cells is caused by 1) loss of repression by RB, and 2) an increase in the number of population doublings. The mouse *p16^{Ink4a}* promoter has the Dmp1-binding site in its proximal region, which can be transactivated by Dmp1 in response to cyclin D1 overexpression since cyclin D1 does not have the transactivation domain [36]. The mouse *p16^{Ink4a}* promoter is repressed by E2F1-3, indicating that p16^{Ink4a} is not overexpressed in *RB*-deficient cells due to E2F activation. This is consistent with that fact that the Dmp1 upregulates both *p19^{Arf}* and *p16^{Ink4a}*, and the *p16^{Ink4a}* promoter is repressed by E2Fs [37-41] (Inoue, *et al.* unpublished data).

Ohtani, *et al.* demonstrated a role for the Ets1 and Ets2 transcription factors based on their ability to activate the *p16^{INK4a}* promoter through the consensus sequences, and their patterns of expression in human diploid fibroblasts [42]. The induction of p16^{INK4a} by Ets2, which was abundant in young human diploid fibroblasts, was potentiated by signaling through the Ras-Raf-MEK kinase cascade, and inhibited by a direct interaction with the helix-loop-helix protein Id1 [42]. In senescent cells, where the ETS2 levels and MEK signaling decline, they saw a marked increase in p16^{INK4a} expression consistent with the reciprocal reduction of ID1 and accumulation of ETS1 [42]. These results indicated the opposing effects of Ets and Id proteins on p16^{INK4a} expression during cellular senescence.

Type 1 diabetes is associated with loss of functional pancreatic β-cells, and restoration of β-cells is a major goal for regenerative therapies [43]. The regenerative capacity of β-cells declines rapidly with age due to accumulation of p16^{INK4a}, resulting in limited capacity for adult endocrine pancreas regeneration [24]. Dhawan, *et al.* showed that TGFβ signaling via Smad3 integrated with the trithorax complex

to activate and maintain *Ink4a* expression to prevent β-cell replication [43]. Importantly, inhibition of TGFβ signaling resulted in repression of the *ARF/INK4a* locus, resulting in increased β-cell replication in adult mice. These data revealed a novel role for TGF-β signaling in the regulation of the *ARF/INK4a* locus. In mice homozygous for a hypomorphic allele of the *α-klotho* ageing-suppressor gene, accelerated ageing phenotypes were rescued by *p16^{Ink4a}* deletion, suggesting its dependency [44]. Indeed, p16^{Ink4a} repressed *α-klotho* promoter activity by blocking the functions of E2Fs, indicating that p16^{Ink4a} plays a role in downregulating *α-klotho* expression during ageing [44].

PRC (polycomb repressor complex) mediated inhibition of p16^{INK4a} expression

Both the expression of p16^{INK4a} and p14^{ARF} are regulated by promoter hypermethylation through proteins of the polycomb repressor complex (PRC1) and PRC2 complexes [45,46] (Figure 2). The polycomb group (PcG) of transcriptional repressor proteins was originally characterized in drosophila for maintenance proteins of pluripotency [47]. PRC2 functions as an initiator of transcription repression and PRC1 functions as a repressor maintenance complex. Thus the methylation mediated by PRC2 is a prerequisite for the binding of PRC1 to the chromatin [45,46,48]. The PRC1 complex is comprised of Polycomb (CXB2, 4, 6-8), Bmi1, HPH (HPH1-3), and RING (RING1 and 2) proteins. The primary mechanism of transcriptional repression by the PRC1 involves mono-ubiquitination of histone H2A K119 by histone H2A ubiquitin ligase [48] (Figure 2). The PRC2 complex is composed of three core proteins, EZH2 which mediates tri-methylation of histone H3K27, EED, SUZ12, and RbAp46 [49] (Figure 2). Histone methyltransferase EZH2 plays a critical role in epigenetic regulation as a bridge between histone methylation/deacetylation and DNA methylation. EZH2 is frequently overexpressed and considered to be an oncogene in cancers [49]. The SET domain of EZH2 possesses methyltransferase activity specific for histone H3K27 [50,51]. The role of deregulated PRC2 in tumor suppressor gene expression, DNA damage response, and the fidelity of DNA replication has been suggested [52], resulting in long-term reversible suppression p14^{ARF} and p16^{INK4a}.

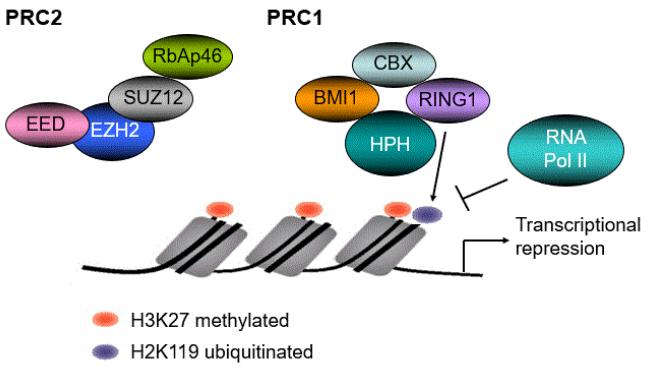

Figure 2. PRC1 and PRC2 repress transcription from the *ARF-INK4a* locus

Both the expression of *p14^{ARF}* and *p16^{INK4a}* are regulated by promoter hypermethylation through proteins of the polycomb repressor complex (PRC1) and PRC2 complexes [45-48]. The polycomb group of transcriptional repressor proteins was originally characterized in drosophila for maintenance proteins of pluripotency [47]. PRC1 functions as a repressor of the maintenance complex while PRC2 functions as an initiator of transcriptional repression. Thus the methylation mediated by PRC2 is a prerequisite for the binding of PRC1 to the chromatin [48]. The PRC1 complex is comprised of Polycomb (CXB2, 4, 6-8), Bmi1, HPH (HPH1-3), and RING (RING1 and 2) proteins. The most important mechanism for transcriptional repression by the PRC1 complex involves mono-ubiquitination of histone H2A K119 by histone H2A ubiquitin ligase (48). The PRC2 complex is composed of three core proteins, EZH2 which mediates tri-methylation of histone H3K27, EED, SUZ12, and RbAp46 [49].

The *ARF/INK4a* tumor suppressor locus, which is a key executor of cellular senescence, is regulated by members of the PcG of transcriptional repressors (Figure 2). Barradas, *et al.* [53] showed that signaling from oncogenic RAS overrides PcG-mediated repression of *INK4a* by activating the H3K27 demethylase JMJD3, and down-regulating the methyltransferase EZH2. In human fibroblasts, JMJD3 activated p16^{INK4a}, but not p14^{ARF}, and caused p16^{INK4a}-dependent cell cycle arrest. In MEFs, Jmjd3 activated both Ink4a and Arf and causes a p53-dependent arrest, echoing the effects of Ras in this system. Their findings directly implicate JMJD3 in the regulation of *ARF/INK4a* during oncogene-induced senescence (OIS), suggesting that JMJD3 has the capacity to act as a tumor suppressor [53]. Similar findings were also reported from two other groups, indicating the importance of EZH2 - mediated repression of the *ARF/INK4a* locus in cellular senescence [54,55].

Mechanisms of upregulation of *p16^{Ink4a}* in *Rb*-deficient cells

The p16^{Ink4a} protein is always upregulated in *Rb*-deficient cells [35,56]. So, what are the molecular mechanisms? It was believed that dysregulated activities of E2F proteins were responsible for overexpression of p16^{Ink4a} in *Rb*-deficient cells, but it is the *p19^{Arf}* promoter that is transactivated with E2F1-3 overexpression, not *p16^{Ink4a}*. Indeed E2F1-3 repressed the murine *p16^{Ink4a}* promoter and E2F4, even combined with nuclear localization signal, had no effect (Inoue, *et al.* unpublished data). This indicates that it is not E2Fs that is responsible for overexpression of p16^{Ink4a} in *Rb*-deficient cells.

Genetic studies have demonstrated that Bmi1 promotes cell proliferation and stem cell self-renewal with a correlative decrease of p16^{Ink4a} expression. Kotake, *et al.* demonstrate that Polycomb genes *EZH2* and *BMI1* repressed p16^{INK4a} expression in human and mouse primary cells, but not in cells deficient for pRB protein function [57]. The *p16^{INK4a}* locus was H3K27-methylated and bound by BMI1, RING2, and SUZ12 [48]. Inactivation of pRB family proteins abolished H3K27 methylation and disrupted BMI1, RING2, and SUZ12 binding to the *INK4a-ARF* locus, associated with a substantial increase of p16^{INK4a}. qRT-PCR analysis showed that among the four *INK4* and *ARF* genes, only *p16^{INK4a}* mRNA was significantly increased in WI38 cells when *BMI1* was silenced [57]. The level of *p18^{INK4c}* and *p14^{ARF}* mRNA was slightly decreased by *BMI1* silencing. *BMI1* knockdown resulted in slower cell growth associated with p16^{INK4a} increase. Together, these results demonstrate that *p16^{INK4a}* seems to be a specific target of *BMI1* function in human primary cells, confirming the difference between mouse and human cells for the relative importance for p19^{Arf} and p16^{Ink4a} [8-13]. Their results raised a model in which pRB recruit PRC2 to trimethylate p16^{INK4a}, priming the BMI1-containing PRC1L ubiquitin ligase complex to silence p16^{INK4a}, explaining why p16^{INK4a} levels are high in *RB*-deficient cells [57].

Regulation of p16^{INK4a} at the protein level

Kobayashi, *et al.* [30] found that that p14^{ARF} regulates the stability of the p16^{INK4a} protein in human cancer cell lines, as well as in MEFs. In particular, ARF promoted rapid degradation of p16^{INK4a} protein (the half-life became 3.5 hrs from 8 hrs), which was mediated by the proteasome and, more specifically, by interaction of ARF with one of its subunits, regenerating islet-derived protein 3γ (REGγ). Furthermore, this ARF-dependent destabilization of p16^{INK4a} was abrogated by

knock-down of REGγ or by pharmacological blockade of its nuclear export. Thus, their findings uncovered a novel crosstalk of two key tumor suppressors mediated by a REGγ-dependent mechanism [30].

p16^{INK4a} overexpression in pre-malignant lesions

Several pieces of evidence suggested that the ability to bypass senescence is the main molecular mechanism in the progression of pre-malignant to fully malignant cells [58,59]. This hypothesis is based on the concept of OIS, which was established after demonstration of p53- and p16^{Ink4a}-mediated senescent-like growth arrest in response to expression of oncogenic Ras in normal primary cells [18,58-60], which has been considered as highly possible mechanism to prevent proliferation of incipient cancer cells. Consistently, senescent cells have been reported in a number of different benign lesions, including nevi and neurofibromas [61,62], but not in cancer. Since p16^{Ink4a} is involved in OIS, the overexpression has been found in benign and pre-malignant lesions with senescent cells where the *ARF/INK4a* locus plays its role [61-63]. Indeed, human nevus lesions remain in a growth-arrested state, and rarely progress to malignant melanomas [64,65]. A mutation in the downstream effector of Ras, BRAF (BRAF^{V600E}) is often found in malignant melanomas, the expression of which in human melanocytes induces cell cycle arrest, followed by p16^{INK4a} induction [61-65]. A similar finding has been reported in schwannomas/neurofibromas [64]. Schwannomas express high levels of p16^{INK4a}, and show senescence-associated β-galactosidase activity, BRAF mutations, and very low proliferative activity [66]. Conversely, the malignant counterparts for these tumors are negative for p16^{INK4a}, and loss of p16^{INK4a} has thus been implicated in the development of both types of malignant neoplasms [64-68]. In other words, benign tumors overexpress p16^{INK4a}, which seems to inhibit cell proliferation in response to oncogenic stimuli, protecting cells from malignant transformation. p16^{INK4a} methylation could thus have a diagnostic value; it can be used in the differential diagnosis from pre-malignant and malignant lesions [69]. In fact, p16^{INK4a} methylation is found of 53.2 % of non-small cell lung cancer while p14^{ARF} methylation is found 6.5 % [38], the frequency 8 times higher than that in ARF.

OIS is emerging as a potent cancer-protective response to oncogenic events, serving to eliminate early neoplastic cells from the neoplastic tissue. Kuilman, *et al.* [70] reported a unique role of interleukin-6 (IL-6) in OIS of cancer cells. They found that OIS was linked specifically to the activation of an inflammatory transcriptome. Induced genes included IL-6, which upon secretion by senescent cells acted mitogenically in a paracrine fashion [70]. IL-6 was also required for the execution of OIS, but in a cell-autonomous fashion. Its depletion caused the inflammatory network to collapse and abolished senescence entry and maintenance. They also demonstrated that C/EBPβ cooperated with IL-6 to amplify the activation of the inflammatory network, including IL-8. In human colon adenomas, IL-8 specifically colocalized with p16^{INK4a}-positive epithelium [70]. They proposed a model in which interleukins connect senescence with an inflammatory phenotype and cancer. The role of IL-6 in OIS has been confirmed by later studies [71,72], indicating the unique role of this cytokine in cancer prevention.

Concerning the role of p16^{INK4a} in OIS, Burd, *et al.* has made a very intriguing report [73]. They have made a luciferase knock-in mouse (*p16^{LUC}*), which faithfully reports expression of p16^{INK4a}. Lifelong assessment of luminescence in *p16$^{+/LUC}$* mice revealed an exponential increase with aging, which was highly variable in a cohort of contemporaneously housed, syngeneic mice. However, the expression of p16^{INK4a} with aging did not predict cancer development, suggesting that the accumulation of senescent cells is not a principal determinant of cancer-related death. Importantly, in 14 of 14 (e.g. C3(1)TAg cells, mammary tumor: *MMTV-HER2/neu*, *K14-CRE; p53$^{Lox/Lox}$*, pancreatic cancer: *Pdx-CRE; LSL-Kras^{G12D}; p53$^{Lox/Lox}$*, B cell lymphoma: *Eμ-Myc*), and endometrial cancer (*Sprr2f-CRE; Lkb1$^{Lox/Lox}$*) tested tumor models, the expression of p16^{LUC} was focally activated by early neoplastic events, enabling visualization of tumors with sensitivity exceeding other imaging modalities [73]. Activation of p16^{Ink4a} was noted in the emerging neoplasm and surrounding stromal cells. They concluded that p16^{Ink4a} activation is a characteristic of all emerging cancers, making the *p16^{LUC}* allele a sensitive, unbiased reporter of neoplastic transformation. It seems p16^{Ink4a} is a gateway to detect oncogenic signals to force incipient cancer cells into senescence and/or apoptosis from this study; however, Zindy, *et al.* had reported that p19^{Arf} was the major player that played its role *in vivo* and *in vitro* in response to c-Myc overexpression since p16^{Ink4a} was not induced in response to c-Myc [74,75]. Our data indicate that the *p16^{Ink4a}* promoter is repressed in response to E2F1-3 although the *p19^{Arf}* promoter was strongly activated by these E2Fs [6,76]. Moreover, our HER2 study showed that p16^{Ink4a} was induced 2-16 folds (median 3 folds) while p19^{Arf} was induced 7-39 folds (median 12 folds) in wild type *Dmp1 ND* tumors [40] indicating that p19^{Arf} induction was the predominant player for quenching the oncogenic stress in tumors driven by c-Myc, E2Fs, and HER2 in the murine system.

It should be noted that the ARF protein is always overexpressed whenever the mRNA is high. However, that is not the case in p16^{INK4a}. Kobayashi, *et al.* reported that the p16^{INK4a} protein is unstable due to the increased turnover in ARF overexpressing cells, suggesting the crosstalk between ARF and p16^{INK4a}, and suggested possible discrepancy between p16^{INK4a} mRNA and protein [30]. Hence, analysis of p16^{INK4a} protein levels is necessary to establish its role in tumor suppression in those overexpressing ARF. The impact of aging should also be considered in the assessment of p16^{Ink4a} in aged mice [19-22]. As a matter of fact, p16^{Ink4a} remains high in MEFs that are passaged for more than 12 days (passage 4) regardless of the protocol [8,31]; it remains consistently elevated in later passages regardless of the mechanism of immortalization indicating the Cdk4 inhibition is not enough to inhibit cell proliferation in immortalized MEFs. Whether ARF or INK4a is major player in silencing oncogenic signals remains a big question that should be resolved in human systems.

p16^{INK4a} overexpression in HPV-infected human cancers and their prognostic values

The RB protein is inactivated by interaction with the high-risk HPV oncoprotein E7 [77,78], and oncoprotein E6 induces degradation of the tumor suppressor p53. RB inactivation releases p16^{INK4a} from its negative feedback control, causing a paradoxical increase in the levels of this protein, which attempts to inhibit uncontrolled cellular replication. As a consequence, p16^{INK4a} is overexpressed in HPV-expressing tumors such as cervical cancer and head and neck tumors [79-81] (Table 1).

Previous reports suggested that p16^{INK4a} immunostaining allows precise identification of even small cervical intraepithelial neoplasia or cervical cancer lesions in biopsies. The prognostic value of overexpressed p16^{INK4a} in cervical cancer has been evaluated for several years with controversial results. Lin, *et al.* performed meta-analyses of studies assessing the clinical and prognostic significance of overexpression of p16^{INK4a} in cervical cancer to evaluate the prognostic value of overexpressed p16^{INK4a} in cervical cancer (15 publications;

1,633 cases) [79]. Analysis showed that p16^{INK4a} overexpression was not significantly associated with tumor TNM staging, tumor grade, tumor size, or vascular invasion. However, overexpression of p16^{INK4a} was highly correlated with no lymph node metastasis, increased overall survival (p = 0.002) and increased disease-free survival (p = 0.001). They showed that overexpression of p16^{INK4a} in cervical cancer was connected with better prognosis of cervical cancer: increased overall and disease-free survival, consistent with previously published studies [80]. Coordes, *et al.* conducted a meta-analysis (18 articles, 4,424 patients) between HPV/p16 and clinical outcomes in head and neck squamous cell carcinoma (HNSCC; ref. 81). The meta-analysis showed a significantly improved 5-year overall survival (OS), 5-year disease-free survival and their corresponding hazard ratio for HPV(+)/p16(+) HNSCC in comparison to other groups. It is generally accepted that p16^{INK4a} expression is frequently associated with HPV infection and a better prognostic factor in HNSCC [82,83] since they respond to chemo/radiotherapy.

p16^{INK4a} overexpression in malignant tumors without HPV infection

Aberrant expression of p16^{INK4a}, either high or low, is found frequently in human cancers. In colon cancer, a very low p16^{INK4a} immunostaining in normal mucosa with a progressively higher expression in aberrant crypt foci, non-serrated adenomas, further increase in primary carcinomas and metastatic tumors have been reported [32] (Table 1). A similar pattern was reported in skin cancer where p16^{INK4a} expression increases from relatively low levels in pre-malignant lesions to high levels in *in situ* and invasive squamous cell carcinomas [84]. In breast cancer, p16^{INK4a} was negative or low in normal ductal epithelium, but a progressive increase was reported in benign lesions and carcinoma [33,34]. Increased nuclear p16^{INK4a} protein expression compared with normal epithelium has also been reported in pre-neoplastic and tumor tissues of the gallbladder [85]. Therefore, p16^{INK4a} induction as a protective mechanism for tumor development must be present in humans in relation to senescence, and inactivation of the p16^{INK4a} - RB pathway should be present in cases where a progressive increase of p16^{INK4a} is seen from pre-malignant lesions (RB wild type) to malignant neoplasia (RB loss). In this sense, inactivation of RB results in increased p16^{INK4a} expression in tumor cells due to corrupted feedback loop [86] as reported in an early study. Indeed, *RB*-loss is a frequent event in many neoplasms and it is associated with uncontrolled cell proliferation. Loss of heterozygosity (LOH) of *RB* was found in 39 % of breast cancers [87]. LOH of the *ARF/INK4a* locus was found in 20 % of breast cancer without association of the prognosis [88]. In the former samples, high p16^{INK4a} protein expression was observed with *RB* LOH cases. An inverse relationship between p16^{INK4a} and RB expression has been reported in breast cancer [87], lung cancer [89,90], and endometrial cancers [91] (Table 1). In short, the negative feed-back loop between RB and p16^{INK4a} could explain overexpression of the p16^{INK4a} in malignant tumors showing uncontrolled cell proliferation. The molecular mechanisms that explain p16^{INK4a} overexpression in *RB*-deficient cells are explained before this section.

Prognostic value of p16^{INK4a} in human cancer

In colon cancer, p16^{INK4a} overexpression was associated with clinical features of a worse prognosis, such as sex, distal location, tumor grade and stage [92] (Table 1). In breast cancer, p16^{INK4a} overexpression was detected in about 20 % of tumors and was significantly associated with unfavorable prognostic indicators, such as high grade and negative estrogen receptor status [93]. Garcia et al. showed that, simultaneous overexpression of p73 and p16^{INK4a} was correlated with the lymph node metastasis, positivity for p53, vascular invasion, and negative progesterone receptors [94]. Kerlikowske, *et al.* described the association between p16^{INK4a} overexpression in breast ductal carcinoma *in situ* and the risk of subsequent DCIS or invasive cancer [95]. In short, p16^{INK4a} overexpression, together with a high Ki67 and COX-2 expression, was associated with progression to an invasive breast cancer, whereas p16^{INK4a} overexpression with high Ki67 but without COX-2 expression was associated with DCIS. These results raise the idea that more molecular events than p16^{INK4a} overexpression are needed in malignant transformation of tumors, and that p16^{INK4a} overexpression is generally a bad prognostic marker in HPV (-) cells because it leads to RB inactivation.

Research in progress and future directions

We have reviewed p16^{INK4} alterations in cancer. Both *ARF* and *INK4a* genes are inactivated in human cancer by gene deletion, promoter methylation, gene frame shift, and splicing errors although mutations mainly affect only the latter [12,13]. It has also been reported that many human cancers overexpress p16^{INK4a} [84-86,92-94]. The mechanism of p16^{INK4a} overexpression in *RB*-null cells is related to aberrant PRC activity [57] rather than hyperstimulated E2Fs. Tumors that overexpress p16^{INK4a} should have better prognosis than the others in early stage since they stop cell proliferation due to inhibition of the cell cycle by activating RB. Conversely, if p16^{INK4a} is overexpressed in tumor cells as a result of *RB*-loss, such patients should have worse prognosis as shown in published studies. The exception for this rule is overexpression of p16^{INK4a} in human cervical and neck cancers with HPV; the prognosis of such patients is better than the others since they respond to therapies. In summary, we have to be cautious in using p16^{INK4a} as a for cancer biomarker since it can be associated with either favorable (early stage or HPV - positive tumors) or worse prognosis (HPV - negative tumors).

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Conflicts of interest

The authors declare no conflicts of interest.

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