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Implications of Genetic and Epigenetic Alterations of *CDKN2A* ($p16^{INK4a}$) in Cancer

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ABSTRACT

Aberrant gene silencing is highly associated with altered cell cycle regulation during carcinogenesis. In particular, silencing of the *CDKN2A* tumor suppressor gene, which encodes the $p16^{INK4a}$ protein, has a causal link with several different types of cancers. The $p16^{INK4a}$ protein plays an executional role in cell cycle and senescence through the regulation of the cyclin-dependent kinase (CDK) 4/6 and cyclin D complexes. Several genetic and epigenetic aberrations of *CDKN2A* lead to enhanced tumorigenesis and metastasis with recurrence of cancer and poor prognosis. In these cases, the restoration of genetic and epigenetic reactivation of *CDKN2A* is a practical approach for the prevention and therapy of cancer. This review highlights the genetic status of *CDKN2A* as a prognostic and predictive biomarker in various cancers.

Contents

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1. Introduction

Cancer comprises a collection of complex genetic and epigenetic diseases that arise through multistep processes (Tallen and Riabowol, 2014). Tumor cells acquire common properties, including unlimited proliferation potential, self-sufficiency in growth signaling, neovascularization for nutrient and oxygen supply, and resistance to anti-proliferative and apoptotic stimuli (Hanahan and Weinberg, 2011). In resting cells, the cell cycle is strictly managed by a set of regulatory proteins that control the various cell cycle checkpoints (Collado et al., 2007; Collins et al., 1997). This cell cycle machinery is often deregulated in cancer as a consequence of the silencing of various tumor suppressor genes (Collins et al., 1997; Tallen and Riabowol, 2014). In fact, loss of tumor suppressor genes and their encoded proteins through deletion, inactivating mutations, epigenetic silencing or post-translational modification results in tumorigenesis. The progression of the mammalian cell cycle from G1 to mitosis is regulated by various cyclin proteins and their catalytic subunits referred to as cyclin-dependent kinases (CDKs) (Nabel, 2002). A family of cyclin–CDK inhibitor proteins (CDIs), which bind and inactivate the CDKs, has been isolated. This family includes the $p16^{INK4a}$, $p21^{CIP1}$, $p27^{KIP1}$, and associated proteins $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$ and $p57^{KIP2}$ (Nabel, 2002). These proteins potentially act as tumor suppressors and their inactivation corresponds with human carcinogenesis.

One of the tumor suppressor proteins that is inactivated in cancer is the $p16^{INK4a}$ protein, which is encoded by the *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*) or *multiple tumor suppressor 1* (*MTS1*) gene (Fig. 1) (Witcher and Emerson, 2009). The *CDKN2A* gene is located within the frequently deleted chromosomal region 9 of p21 (Gil and Peters, 2006). This gene (8.5 kb full length) contains two introns and three exons and encodes the $p16^{INK4a}$ protein. The $p16^{INK4a}$ protein is a protein consisting of 156 amino acids with a molecular weight of 16 kDa and is a negative regulator of the cell cycle (Serrano et al., 1993). In addition to $p16^{INK4a}$, *CDKN2A* encodes a completely unrelated tumor suppressor protein, alternate open reading frame (ARF or $p19^{Arf}$ in mice), which interacts with the p53 regulatory protein, mouse double minute 2 homolog (MDM2) (Pomerantz et al., 1998). The simple tandem arrangement is complicated by the presence of an additional exon 1β, which is transcribed from its own promoter. The resulting RNA incorporates exons 2 and 3, but specifies a distinct protein because the exons are translated by an alternative reading frame. Thus, while exons 2 and 3 are shared by the two mRNAs, they encode different protein products, $p16^{INK4a}$ and ARF (Quelle et al., 1995). The specific binding of the $p16^{INK4a}$ protein to CDK4 or CDK6 induces an allosteric conformational change in these proteins and inhibits the formation of the complex between CDK4 or 6 and cyclin D (Serrano et al., 1993). The lack of this complex formation maintains the retinoblastoma protein (Rb) in its hypo-phosphorylated and growth-suppressive states. This leads to the induction of G1 phase cell cycle arrest through the formation of the Rb/E2Fs-repressive complex (Fig. 1) (Weinberg, 1995). The loss of $p16^{INK4a}$ is increasingly common with advancing stages of various neoplasms, suggesting that $p16^{INK4a}$ inactivation may contribute to cancer progression. The frequent inactivation of $p16^{INK4a}$ induced by homozygous deletion or promoter hyper-methylation and point mutation has been observed in various cancers (Table 1).

Epigenetic alterations are suggested to regulate gene expression without affecting the base sequence. These modifications include genomic DNA-methylation, histone modifications, chromatin remodeling and miRNA/non-coding RNA-induced regulation of gene expression (Hauptman and Glavac, 2013; Portela and Esteller, 2010; Sarkar et al., 2015). The *polycomb group* (*PcG*) genes, first identified in *Drosophila melanogaster*, encode the highly conserved PcG proteins, which form two large macromolecular complexes classified as polycomb repressive complex-1 (PRC1) and -2 (PRC2). The PRC1 complex consists of B lymphoma Mo-MLV insertion region 1 homolog (BMI1), mPh1/2, Pc/chromobox (CBX) and RING1A/B, and sustains silencing of chromatin. The mammalian PRC1 might harbor specificity against selecting one of the multiple Pc/CBX homologs. The PRC2 complex is composed of

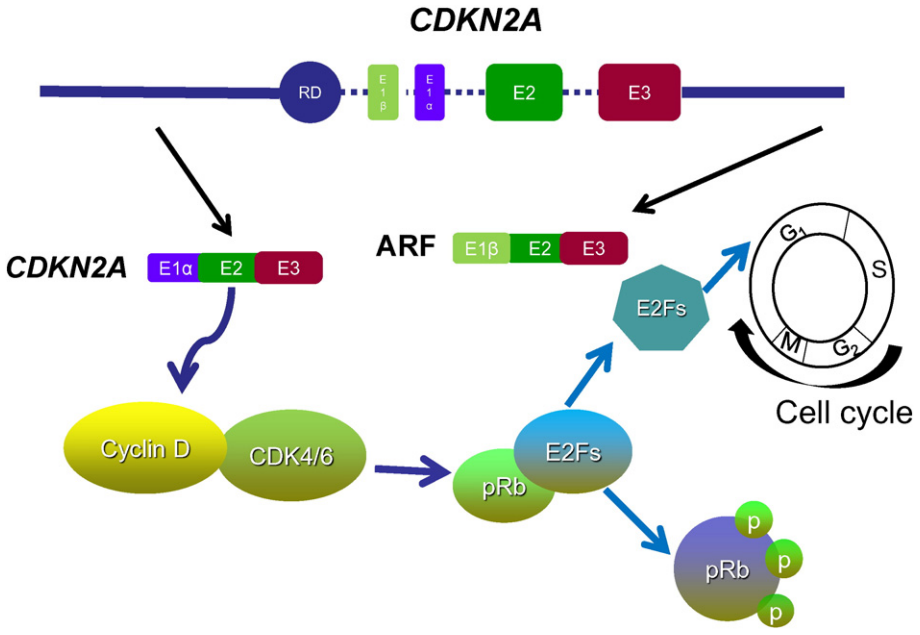

Fig. 1. Schematic structure of the *INK4a/ARF* locus and the role of $p16^{INK4a}$ in cells. *CDKN2A* is produced by alternative splicing of E1, E2 and E3. The $p16^{INK4a}$ protein binds to the cyclin D and CDK4/6 complexes and inhibits the activation of the transcription factor, E2F1, which induces proteins to move from the G1 phase to S phase in the cell cycle.

Table 1
Changes of *CDKN2A* in various cancers.

Cancer types	Status of alteration	Frequency (%)	Reference
Gastric lymphoma	Promoter hyper-methylation	29.7% (11/37)	Huang et al. (2007)
Burkitt's lymphoma	Promoter hyper-methylation	72.5% (37/51)	Robaina et al. (2015)
Skin cancer	Promoter hyper-methylation, histone modification	50–70%, 80–90%	Chen et al. (2012a)
Melanoma	Promoter hyper-methylation	25.9% (15/58)	Kostaki et al. (2014)
	Histone modification		Sarkar et al. (2015)
	Promoter hyper-methylation, non-synonymous mutation	25% (15/59), 16% (9/56)	Jonsson et al. (2010)
Head and neck squamous cell carcinoma	Mutation/promoter hyper-methylation	57% (138/243)	Chung et al. (2015)
	Promoter hyper-methylation	54.5% (6/11 cell line) and 66.7% (20/30)	El-Naggar et al. (1997)
Oral cancer	Promoter hyper-methylation	60% (6/10)	Asokan et al. (2014)
Pancreatic adenocarcinoma	Mutation	11.8% (2/17)	Salo-Mullen et al. (2015)
	Promoter hyper-methylation	24.6% (14/57)	Jiao et al. (2007)
Non-small cell Lung cancer (NSCLC)	Homozygous deletion (HD)/mutation/promoter hyper-methylation	53%/13%/33% in cell lines	Tam et al. (2013)
	Mutation/frameshift/HD	19% (12/63)	Marchetti et al. (1997)
	Loss of heterozygocity (LOH)/mutation	13% (5/38)/6% (4/67)	Kuwabara et al. (2011)
Esophageal squamous cell carcinoma	Promoter hyper-methylation	81.7% (210/257)	Chen et al. (2012b)
	Deletion	20% (22/106)	Qureshi et al. (2012)
Gastric cancer	Hyper-methylation	81.6% (40/49, EBVaGC), 33.3% (15/45, EBVnGC)	He et al. (2015)
Colorectal cancer	Promoter hyper-methylation	11.9% (34/285)	Kim et al. (2010)
Epithelial ovarian caricnoma	Promoter hyper-methylation	17.1% (87/497)	Rajendran et al. (2015)
Prostate cancer	Promoter hyper-methylation	43% (58/134)	Bhagat et al. (2014)
	Expression of ANRIL, CBX7, and EZH2		Yap et al. (2010)
	Promoter hyper-methylation	47.6% (10/21)	Ameri et al. (2011)

embryonic ectoderm development (EED), suppressor of zeste (SUZ)12 and enhancer of zeste homolog (EZH)1/2, and initiates repression of target genes by di- and trimethylation of lysine 27 of histone H3 (H3K27me2 and H3K27me3) (Bracken et al., 2007; Cao et al., 2002). Thus, activation of PRC1 and PRC2 induces gene silencing through histone modification ultimately affecting the chromatin structures. Long non-coding RNAs are transcripts longer than 200 nucleotides and lack protein-coding capacity. These RNAs might play a major role in programming the epigenome by cooperating and coordinating with the PcG complexes to impose chromatin states in a dynamic manner (Aguilo et al., 2011). The purpose of this mini-review is to shed light on the molecular mechanisms of genetic and epigenetic changes in $p16^{INK4a}$ and the implications in carcinogenesis.

2. Genetic alterations of *CDKN2A* in various cancers

The changes in *CDKN2A*/$p16^{INK4a}$ status are highly variable depending on the type of cancer. The following section describes the mechanisms of $p16^{INK4a}$ inactivation in various cancers (Table 1; Fig. 2).

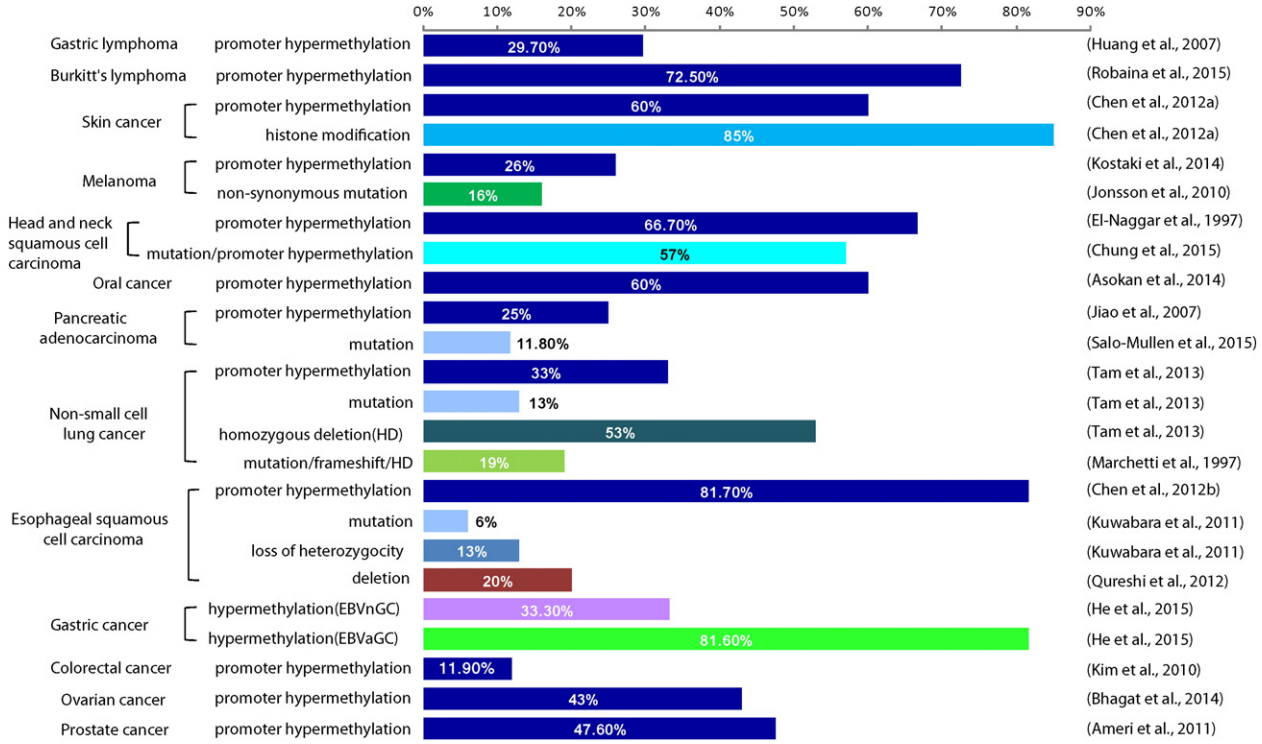

Fig. 2. Frequency of *CDKN2A* alterations in various cancer types. Based on the types of aberrant *CDKN2A*, the bars represent the percentage of changes reported.

2.1. Lymphoma

Several studies have demonstrated that promoter hyper-methylation leads to the loss of $p16^{INK4a}$ expression in lymphoma. Gastric lymphoma is an extra-nodal non-Hodgkin's lymphoma and is associated with 2–8% of all gastric cancers (Alevizos et al., 2012). Huang et al. reported that 26.5% (13 of 49) of primary gastric lymphomas exhibited hyper-methylation of the *CDKN2A* promoter, suggesting that expression of $p16^{INK4a}$ is a clinical risk factor for gastric lymphoma (Huang et al., 2007). Burkitt's lymphoma is a common subtype of B-cell non-Hodgkin's lymphoma in children and adolescents (Molyneux et al., 2012). A recent analysis of 51 Burkitt's lymphoma tumor samples revealed that methylation of the *CDKN2A* promoter occurred in 72.5% of the samples and nuclear expression of the $p16^{INK4a}$ protein remained undetectable in about 41% of the samples (Robaina et al., 2015). In this study, *CDKN2A* promoter methylation was detected in 32 patient samples (80%) at stage III/IV of the cancer (Robaina et al., 2015).

2.2. Skin cancer and melanoma

Solar ultraviolet (UV) radiation is the most common risk factor for the initiation and promotion of melanoma and non-melanoma skin carcinogenesis (de Gruijl, 1999). The *CDKN2A* gene is a melanoma susceptibility gene and its mutations are present in 20 to 40% of familial and 2 to 3% of sporadic melanomas (Kostaki et al., 2014). The inactivation of *CDKN2A* in skin cancer involves histone modifications as well as DNA methylation. Chronic exposure of HaCaT skin keratinocytes to UVA radiation has been reported to cause 80 to 90% histone methylation (H3K4m3) and 50 to 70% DNA methylation in the *CDKN2A* promoter region (Chen et al., 2012a). Deletion of $p16^{INK4a}$ has also been detected in 50% of melanomas and its inactivation by point mutations occurs in about 9% of cases, which correlates with an increased risk of metastases and disease progression. Methylation of the *CDKN2A* gene promoter occurs in about 5 to 19% of sporadic melanomas, whereas promoter methylation of the gene was found in 27 to 33% of melanoma metastases (Kostaki et al., 2014). In cutaneous melanoma metastases, the *CDKN2A* promoter is hyper-methylated in about 25% (15/59) of cases and non-synonymous mutation of the gene was observed in about 16% (9/56) of cases examined (Jonsson et al., 2010).

ANRIL (antisense noncoding RNA in the INK4 locus) mediates a cis-acting silencing mechanism. ANRIL binds with PRC1 at the RNA-binding domains of CBX7 and methylates the *CDKN2A* promoter region at H3K27, which inhibits *CDKN2A* gene expression (Sarkar et al., 2015; Yap et al., 2010; Sato et al., 2010).

2.3. Head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous disease that arises in the areas of the nasal and oral cavities, pharynx and larynx. Although HNSCC can be caused by infection with human papilloma virus (HPV) (Bishop et al., 2013), approximately 90% of HPV-negative HNSCC tumors exhibit low expression of *CDKN2A*. This is largely due to mutations, loss of heterozygosity, and DNA hyper-methylation of the gene (Veganzones et al., 2015). In one study, 57% of HPV-negative HNSCC from the TCGA (The Cancer Genome Atlas) dataset was due to mutation or loss of the *CDKN2A* gene (Chung et al., 2015). In primary HNSCC with deficient expression of *CDKN2A*, 66.7% (20/30) of the tumors exhibited DNA methylation of the *CDKN2A* promoter. The number of methylations was found to be 4 methylations at exon 1, 7 at exon 2 and 9 each at exons 1 and 2 (El-Naggar et al., 1997).

Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. More than 90% of all oral neoplasms are oral squamous cell carcinomas (OSCCs) (Markopoulos, 2012). Genetic alterations in OSCC might activate mutations or methylation of cell cycle regulatory genes, thereby promoting cell survival and proliferation (Al-Kaabi et al., 2014). In OSCC patients, hyper-methylation of the *CDKN2A* promoter was observed 60% of the time and oral leukoplakia patients also exhibited 60% hyper-methylation in the *CDKN2A* promoter (Asokan et al., 2014).

2.4. Pancreatic cancer

Because of their severe metastatic potential, pancreatic tumors are the most aggressive types of cancer. Expression of *CDKN2A* has been reported to be aberrant in ~95% of pancreatic adenocarcinomas, and approximately 15% of those (El-Naggar et al., 1997) and 24.6% in another study (Jiao et al., 2007) were attributed to promoter hyper-methylation. The *CDKN2A* gene is abnormally methylated in 27% of pancreatic cancer cell lines (Moore et al., 2001). In addition, the mutation of *CDKN2A* occurred 11.8% of the time in hereditary pancreatic cancer patients (Salo-Mullen et al., 2015).

2.5. Lung cancer

Lung cancer is the leading cause of cancer death and most patients with lung cancer suffer from non-small cell lung cancer (NSCLC) (Siegel et al., 2015). Alterations in *CDKN2A* were examined in 63 NSCLCs samples of which 30 samples were primary resected NSCLCs with metastatic involvement of thoracic lymph nodes and 33 NSCLCs were without lymph node metastases (Marchetti et al., 1997). The analysis revealed that 6 NSCLC tumor samples had somatic aberrations of the *CDKN2A* gene, including 4 mutations, 1 frame-shift and 1 homozygous deletion (Marchetti et al., 1997). These alterations of *CDKN2A* were significantly associated with lymph node metastasis of NSCLC. Further analysis of 40 NSCLC adenocarcinoma cell lines revealed that *CDKN2A* was inactivated in 75% (30/40) of cases including 16 homozygous deletions, 10 methylations and 4 mutations (Tam et al., 2013). In another study, alteration of *CDKN2A* in NSCLC adenocarcinoma tissue samples was noted as 38% (17/45) and included 10 homozygous deletions, 4 methylations and 3 mutations (Tam et al., 2013).

2.6. Esophageal cancer

Esophageal cancer is the 6th leading cause of cancer death worldwide (Zhang, 2013). Esophageal squamous cell carcinoma (ESCC) is more common in the developing world and arises from the epithelial cells. The promoter region of *CDKN2A* was highly methylated in tissue samples from Chinese subjects (81.7% or 210/257) (Chen et al., 2012b). Among 38 Japanese ESCC samples examined, 13% showed a loss of heterozygosity of the *CDKN2A* gene and 67 tumors showed 6% with *CDKN2A* mutations (Kuwabara et al., 2011). A novel 7 base pair (TCCAGCC) deletion in 22 of 106 (~20%) esophageal tumor samples was observed in exon 2 of *CDKN2A* (Qureshi et al., 2012). An *in silico* study revealed that the deletion of these 7 base pairs resulted in premature termination and caused instability of the $p16^{INK4a}$/CDK6 complex (Qureshi et al., 2012).

2.7. Gastric cancer

Gastric (stomach) cancer develops in the lining of the stomach. In a meta-analysis of Chinese gastric cancer patients, hyper-methylation of the *CDKN2A* promoter was recorded as 43.3% of the median average (28.3–64.4%), and was significantly correlated with *CDKN2A* promoter methylation and carcinogenesis (Peng et al., 2014; Wang et al., 2014). Infection with the Epstein-Barr virus (EBV) is one risk factor for gastric cancer. EBV-associated gastric carcinoma (EBVaGC) accounts for approximately 8–10% of all gastric carcinomas (Liang et al., 2014). Hyper-methylation of the *CDKN2A* promoter was detected in 81.6% and 33.3% of the EBVaGC and EBVnGC (EBV-negative gastric carcinomas), respectively (He et al., 2015). These findings suggest that a high level of *CDKN2A* promoter methylation is a risk factor for developing EBV-associated gastric cancers. Another important etiologic factor for gastric carcinogenesis is *Helicobacter pylori* (*H. pylori*) infection. Matsusaka et al. demonstrated that *H. Pylori* infection promotes *CDKN2A* DNA methylation

from 21.3% to 45.0%, which correlates with poor tumor differentiation, increased lymph node metastasis, and lower survival rates of gastric cancer patients (Matsusaka et al., 2014; Qu et al., 2013).

2.8. Colorectal cancer

The development of colorectal cancer involves genetic and epigenetic changes of several tumor suppressor genes, including *CDKN2A* (Chan et al., 2002). Similar to other cancers, the *CDKN2A* gene is silenced through promoter hyper-methylation during the course of colorectal cancer pathogenesis (Kim et al., 2010). Kim et al. reported that promoter methylation of *CDKN2A* occurred in 11.9% of 285 sporadic colorectal cancer patients. Among these *CDKN2A* hyper-methylated cancer patients, 10.6% of 264 patients exhibited microsatellites with low frequency, whereas 28.6% of 21 patients showed microsatellites with high frequency (Kim et al., 2010). In another study, *CDKN2A* promoter methylation occurred in 17.1% of 497 patients and its regulation was highly associated with poor survival in stage II and III colorectal cancer patients treated with adjuvant fluoropyrimidine (Lee et al., 2015). The prognostic value of concurrent methylation of the *CDKN2A* promoter was associated with gender variation and was significant only in male patients (Lee et al., 2015).

2.9. Ovarian cancer

Ovarian cancer is the most common form of gynecological cancer and a leading cause of cancer-related mortality among women (Jayson et al., 2014). This cancer is diagnosed usually at an advanced stage and has a high rate of recurrence (Hennessy et al., 2009). In a recent study, Bhagat et al. demonstrated that the frequency of *CDKN2A* promoter methylation was 43% in 134 invasive cancer cases including 22% of 23 low malignancy patient tumors and 42% of 26 benign cases (Bhagat et al., 2014). Down-regulation of *CDKN2A* mRNA expression and hyper-methylation of the *CDKN2A* promoter are significantly associated.

2.10. Prostate cancer

Prostate cancer presents mostly as adenocarcinomas and is the second leading cause of cancer death in men (Siegel et al., 2015). Silencing of the *CDKN2A* gene plays a critical role in prostate cancer progression. Methylation of the *CDKN2A* promoter has been reported to occur in 47.6% of 21 patients with poor prognosis (Ameri et al., 2011). Epigenetically, ANRIL and H3K2me are involved and alternatively bind with CBX7 of PRC1, thereby repressing *CDKN2A* gene transcription (Yap et al., 2010).

2.11. Renal cell carcinoma

Accumulation of various genetic aberrations, including the *CDKN2A* gene, underlies the development of renal cell carcinomas (Dulaimi et al., 2004). Evaluation of the methylation status of the promoter CpG island of *CDKN2A* and immunohistochemical detection of the $p16^{INK4a}$-encoded protein in 57 patients with renal carcinomas revealed that the *CDKN2A* gene was hyper-methylated in 22.9% of patients and none of them expressed the $p16^{INK4a}$ protein product (Vidaurreta et al., 2008). The lack of $p16^{INK4a}$ protein expression was detected in 52.9% of the tumors, suggesting the involvement of another genetic alteration or post-transcriptional modification of the protein (Vidaurreta et al., 2008).

3. Molecular switches associated with $p16^{INK4a}$ aberrations

The inactivation of *CDKN2A* in cancer is neither an isolated event nor does it appear to have a direct link with carcinogen exposure (Table 2). Analysis of the current literature reveals that promoter methylation of *CDKN2A* is not associated with HPV infection, which is a cause of head and neck squamous cell carcinomas (Chung et al., 2015). However, the epigenetic changes in *CDKN2A* are associated with simultaneous genetic or epigenetic alterations in other cancer-related oncogenes or tumor suppressor genes (Veganzones et al., 2015; Chung et al., 2015; Kostaki et al., 2014; Tam et al., 2013; Wilson et al., 2010; Jonsson et al., 2010). Thus, the study of the concordance between $p16^{INK4a}$ inactivation with other tumor-related genetic/epigenetic lesions could provide new avenues for developing anticancer therapies. Tam et al. have demonstrated that mutations of the *epidermal growth factor receptor* (*EGFR*) and *KRAS* are major oncogenic drivers in the pathogenesis of lung adenocarcinomas. The mutation of *EGFR* is reportedly associated with *CDKN2A* homozygous deletion or mutation, whereas *KRAS* mutation is related to hyper-methylation of the *CDKN2A* promoter (Tam et al., 2013). However, changes in *STK11* (also known as *LKB1*), a gene frequently mutated in lung adenocarcinomas, have no impact on *CDKN2A* inactivation mechanisms (Tam et al., 2013). Veganzones and colleagues investigated the frequency of simultaneous methylation of *CDKN2A* and *hMLH1*, a gene that encodes a DNA repair enzyme, in colorectal cancer patients with microsatellite instability (MSI) (Veganzones et al., 2015). The outcome of the analysis of 51 samples of MSI-positive sporadic colorectal cancer was expressed by a new variable referred to as combined methylation of *CDKN2A* and *hMLH1* (*CMETH2*). Results indicated that 17 of 51 patients (33.3%) tested positive for *CMETH2* expression, which was associated with poorly differentiated tumors in proximal locations. The clinicpathological implication of *CMETH2* was reflected in the overall survival of patients with distal tumors (Veganzones et al., 2015).

Histone modification, especially histone 3-lysine-9 methylation (H3K9) is a common mechanism of transcriptional repression of gene expression (Yoruker et al., 2012). SETDB1, which belongs to the family of SET domain histone methyltransferases including Suv39H, EZH2 and G9a, contains a highly conserved motif of 150-amino acids and is involved in the modulations of chromatin structure (Yang et al., 2002). The SETDB1 protein structure contains a CpG DNA methyl-binding domain, which regulates H3K9 methylation activity (Ceol et al., 2011). The CpG island methylation of the *CDKN2A* gene promoter and expression of SETDB1 in sporadic cutaneous melanomas are highly correlated with histologic prognostic parameters (Kostaki et al., 2014).

Table 2
Genes and factors correlated with changes in *CDKN2A*.

Genes/Factors	Cancer types	Note	Reference
HPV	Head and neck squamous cell carcinoma	*CDKN2A* alteration in non-HPV-cancer	Chung et al. (2015)
EGFR	Lung adenocarcinoma	*CDKN2A* homozygous deletion or mutation is correlated with EGFR mutation	Tam et al. (2013)
KRAS		Hypermethylation of *CDKN2A* promoter is correlated with KRAS mutation	
hMLH1 (human mutL homolog 1)	Colorectal cancer (microsatellite instability)	Hypermethylation of both *CDKN2A* and *hMLH1* is 33.3% (17/51)	Veganzones et al. (2015)
SETDB1	(Sporadic cutaneous) Melanoma	Cytoplasmic SETDB1 expression correlates with higher frequency of *CDKN2A* methylation and $p16^{INK4A}$ expression	Kostaki et al. (2014)
NRAS	Cutaneous melanoma	NRAS-mutated tumors have *CDKN2A* promoter methylation (52%, 12/23)	Jonsson et al. (2010)
SNF5	–	Inactivation of H3K27 tri-methylation of *CDKN2A* by EZH2 in deficient SNF5	Wilson et al. (2010)

Methylation of the *CDKN2A* promoter is associated with 52% (12/23) of *NRAS*-mutated cutaneous melanomas compared with *BRAF*-mutated (7%, 2/27) tumors (Jonsson et al., 2010). EZH2, a histone methyltransferase and a catalytic subunit of the PRC2 polycomb repressor, mediates gene silencing by tri-methylating H3K27 of various target gene promoters (Bracken et al., 2007; Cao et al., 2002). SNF5, one of the core subunits of Brahma-associated factor (BAF) chromatin remodeler complexes, down-regulates EZH2 transcription (Wilson et al., 2010). Thus, the loss of SNF5 function leads to increased EZH2 expression, thereby repressing the expression of *CDKN2A* through the tri-methylation of H3K27 in the *CDKN2A* promoter region (Wilson et al., 2010).

4. Epigenetic induction of *CDKN2A*

Reactivation of silenced *CDKN2A* or the inhibition of epigenetic repression of the gene could be a rational strategy for the prevention or treatment of various cancers. While epigenetic silencing of *CDKN2A* is caused by DNA hyper-methylation and histone modification (Bracken et al., 2007; Cao et al., 2002; Collado et al., 2007), restoration of $p16^{INK4a}$ transcriptional activation can be achieved by a set of intracellular regulatory genes as well as a series of small molecule epigenetic modifiers (Table 3, Figs. 3, 4) (Crea et al., 2009; Feng et al., 2009; Hassler et al., 2012; Kollmann et al., 2011; Li et al., 2013a; Majid et al., 2008; Nandakumar et al., 2011; Valdez et al., 2015; Zhang and Tong, 2014).

4.1. Induction of CDKN2A by regulatory genes

The FOXA1 (Forkhead box A1) protein is a transcription factor that regulates the expression of $p16^{INK4a}$ (Li et al., 2013a; Zhang and Tong, 2014). In hormone-dependent cancers, such as breast and prostate cancers, the expression of FOXA1 is positively correlated with the expression of $p16^{INK4a}$, but inhibits the expression of EZH2, which is a component of PRC2 and an epigenetic repressor of *CDKN2A* (Nanni et al., 2006). FOXA1 directly binds to the C-terminal histone-binding motif and inhibits EZH2 methyltransferase activity, thereby inducing the expression of $p16^{INK4a}$ and suppressing cancer cell growth (Zhang and Tong, 2014).

ZBP-89, a four-zinc finger transcription factor, plays a role in the regulation of target genes by binding to the GC-rich DNA elements (Wu et al., 2007). ZBP-89 directly recruits histone deacetylase-3 (HDAC3) to the *CDKN2A* promoter and represses the expression *CDKN2A* by histone hypo-acetylation (Feng et al., 2009). Therefore knockdown of ZBP-89 (ZBP-89i) by a specific small interfering RNA vector induced the expression of $p16^{INK4a}$ and cell senescence in NCI-H460 human lung cancer cells by promoting histone acetylation (Feng et al., 2009).

Jmjd3 (jumonji domin containing 3) is a histone lysine demethylase, which especially works to remove the tri-methylation of histone H3 at lysine 27. Jmjd3 induces expression of $p16^{INK4a}$ and senescence of Schwann cells under the conditions of nerve regeneration and tumorigenic stimulation (Gomez-Sanchez et al., 2013). The UHRF1 (ubiquitin-like plant homeobox domain (PHD) and RING finger containing 1) protein interacts with DNA methyltransferase 1 (DNMT1) and recognizes hemi-methylated CpG dinucleotides through its SRA (SET- and RING-associated domain) region (Bostick et al., 2007). The aromatic cage mutant of tandem tudor domain (TTD, 124–285 aa) within UHRF1 cannot bind to H2K9me3 and therefore induces the expression of $p16^{INK4a}$ (Nady et al., 2011).

The c-Jun protein is a component of activator protein (AP)-1 and a common regulator of cell cycle components and was reported to have a direct role in regulating gene transcription of *e.g.*, p53 and cyclin D1. Although JunB, as a tumor enhancer, reportedly inhibits promoter methylation of *Cdk6* in BCR-ABL-induced leukemia (Ott et al., 2007), binding of c-Jun to the promoter region shows a protective function and prevents methylation and silencing of these genes. Because the promoter region of the *CDKN2A* gene includes binding sites for AP-1, c-Jun can inhibit methylation of the *CDKN2A* promoter (Kollmann et al., 2011)

4.2. Induction of $p16^{INK4a}$ by reagents

A number of natural and synthetic small molecule modifiers of epigenetic regulation of gene expression have been identified (Fig. 4). Many of these small molecules are capable of restoring the expression of $p16^{INK4a}$ in various cancers. Genistein, an isoflavone present in soybean, is a cancer chemopreventive agent that inhibits cell proliferation and induces apoptosis (Gullett et al., 2010). Li et al. reported that genistein represses early breast tumorigenesis through epigenetic regulation of *CDKN2A* by impacting histone modifications as well as by recruiting the BMI1-c-MYC complex to the regulatory region in the *CDKN2A* promoter (Li et al., 2013b). In prostate cancer, treatment with genistein increased acetylated histones 3 and 4 of the *CDKN2A* transcription start sites (Majid et al., 2008). Thus, soybean products containing genistein might be useful in preventing breast and prostate cancer through transcriptional activation of *CDKN2A* by modulating its epigenetic silencing (Li et al., 2013b; Majid et al., 2008). Sulforaphane, a major component

Table 3
Epigenetic induction of $p16^{INKa}$.

	Inducer	Mechanisms	Cancer types	References
Genes	FOXA1	EZH2 inhibition	Breast cancer and prostate cancer	Zhang and Tong (2014), Li et al. (2013a)
	Si-ZBP-89	HDAC3 inhibition	NCI-460 human lung cancer cells	Feng et al. (2009)
	Jmjd3	Histone demethylation	Neurofibroma Schwann cells	(Gomez-Sanchez et al. (2013)
	Mutant UHRF1	H3K9me3 inhibition		Nady et al. (2011)
	c-JUN	Protect DNA methylation		Kollmann et al. (2011)
Compounds	Genistein	Induction of histone acetyl transferase (HAT)	Prostate cancer	Majid et al. (2008)
		Modification of histone and Inhibition of binding BMI1 and c-MYC to *CDKN2A* promoter	Breast cancer	Li et al. (2013b)
	Sulforaphane (SFN)	HDAC3 inhibition by induction of Nrf2	Colon cancer	Rajendran et al. (2015)
	EGCG	DNA demethylation and histone acetylation	Skin cancer cell	Nandakumar et al. (2011)
	TSA	HBP1 transcription factor acetylation		Wang et al.1 (2012)
	EGCG and TSA	DNA demethylation	Lymphoma	Wu et al. (2013)
	TSA and 5-aza-2′-deoxycytidine	DNA demethylation	Epstein–Barr virus-associated gastric cancer cells	He et al. (2015)
	5-aza-2′-deoxycytidine	DNA demethylation	Large cell lymphoma	Hassler et al. (2012)
	5-aza-2′-deoxycytidine and irinotecan	DNA demethylation	Colorectal cancer cells	Crea et al., (2009)
	Cladribine, clofarabine	DNA demethylation	Acute myeloid leukemia	Valdez et al. (2015)
	Doxorubicin, FUMI (5-fluorouracil + mitomycin C)	DNA demethylation	Breast cancer	Klajic et al. (2014)

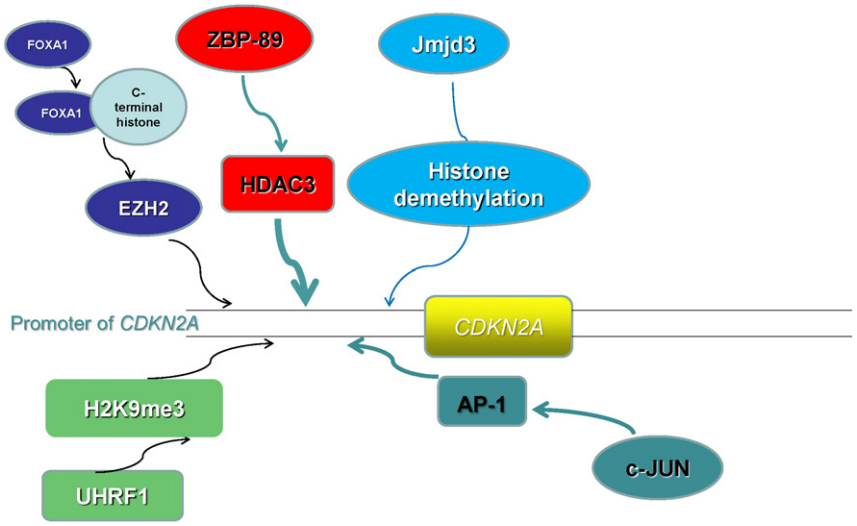

Fig. 3. The epigenetic induction of $p16^{INK4a}$ by regulatory genes. FOXA1, Si-ZBP-89, Jmjd3, Mutant UHRF1 and c-JUN induce $p16^{INK4a}$ protein expression by re-activation of the *CDKN2A* promoter.

of broccoli and other cruciferous vegetables, is an inducer of nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2), which regulates the transcriptional activation of a battery of genes encoding various phase 2 detoxification enzymes (Fahey et al., 1997; Kensler et al., 2013). Rajendran et al. reported that *Nrf2* wildtype mice showed a higher incidence of colon tumors than *Nrf2* heterozygous ($Nrf2^{+/-}$) mice when treated with 1,2-dimethylhydrazine. Tumors from wildtype mice exhibited higher HDAC3 levels globally and deregulated $p16^{INK4a}$ levels locally. Treatment with sulforaphane markedly reduced the tumor burden in wildtype mice but not in $Nrf2^{+/-}$ mice. Sulforaphane, an inhibitor of HDAC3, a repressor of $p16^{INK4a}$, induced $p16^{INK4a}$ expression and reduced the tumor burden in this model (Rajendran et al., 2015).

Epigallocatechin-3-gallate (EGCG), a main polyphenol component of green tea, stimulated expression of $p16^{INK4a}$ by inhibiting DNA methylation and increasing histone acetylation in the *CDKN2A* promoter of human skin cancer cells (Nandakumar et al., 2011). Trichostatin A (TSA) is an HDAC4 inhibitor that elevated the acetylation of HBP1 (K419), a member homologous to the sequence-specific high mobility group (HMG) family of transcription factors, and increased the expression of $p16^{INK4a}$, thereby inducing apoptosis and differentiation (Wang et al., 2012). The combination treatment with inhibitors of DNMTs and HDACs might be a representative therapeutic strategy in cancer. Treatment of CA46 lymphoma cells with EGCG (24 μg/ml) or the combination treatment of EGCG (6 μg/ml) and TSA (15 ng/ml) resulted in lower

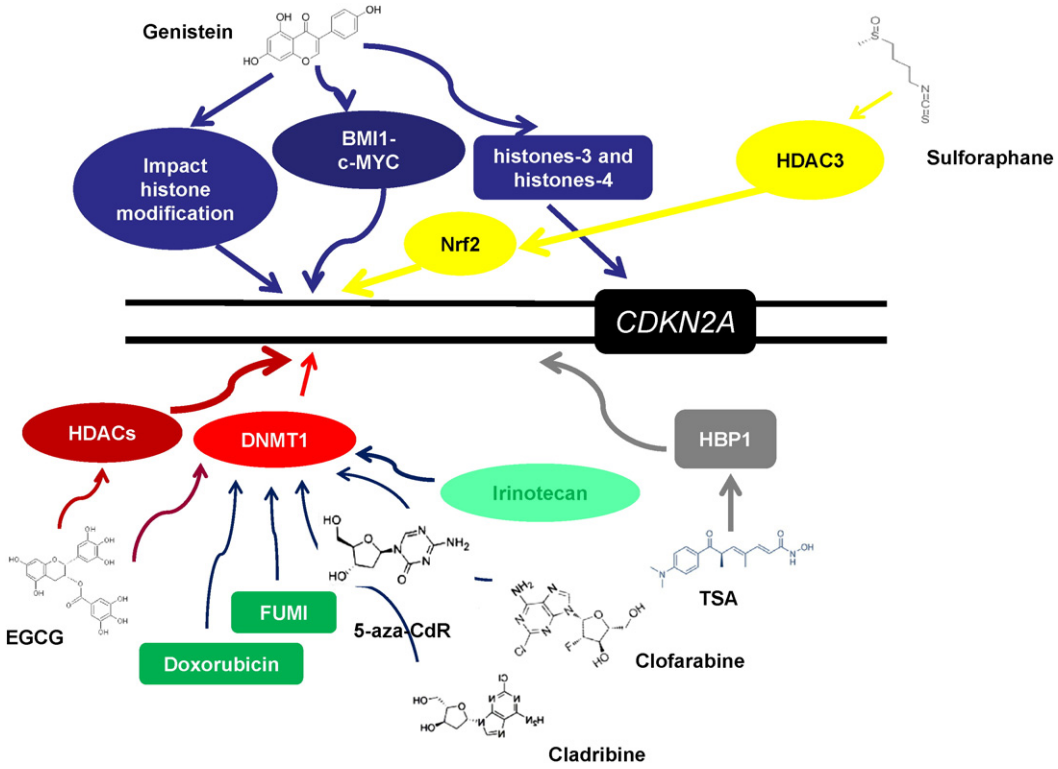

Fig. 4. The epigenetic induction of $p16^{INK4a}$ by reagents. Phytochemicals such as genistein, SFN and EGCG and synthetic chemicals, TSA, 5-aza-2′-deoxycytidine, irinotecan, cladribine, clofarabine, doxorubicin, FUMI (5-fluorouracil + mitomycin C) and combinations reactivate $p16^{INK4a}$ protein expression by prohibiting alterations in the *CDKN2A* promoter.

proliferative indices when compared to the other groups. Co-treatment with EGCG and TSA decreased the DNA methylation of *CDKN2A*, which coincided with increased *CDKN2A* mRNA and protein expression. Thus, EGCG and TSA synergistically induced *CDKN2A* expression by reducing promoter methylation, which might decrease CA46 lymphoma cell proliferation (Wu et al., 2013).

5-Aza-2′-dexoycytidine (5-aza-CdR, decitabine, Dacogen®) is a DNMT inhibitor, which has been approved by the Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome, a hematological malignancy (Scandura et al., 2011). Treatment of anaplastic large cell lymphoma cells with 5-aza-CdR resulted in the inhibition of proliferation through G1 phase cell cycle arrest *in vitro* and *in vivo* through re-expression of $p16^{INK4a}$ (Hassler et al., 2012). Furthermore, combination treatment with TSA and 5-aza-CdR exerted synergic cell growth inhibition and apoptosis in EBV-associated gastric carcinoma through the induction of *CDKN2A* by inhibiting methylation (He et al., 2015). On the other hand, co-treatment with 5-aza-CdR and irinotecan, a topoisomerase-1 inhibitor, caused a more sensitive cytotoxic effect in *p53*-mutated colon cancer cells, such as HT-29, SW620, and WiDr cells (Crea et al., 2009). The synergistic effect of 5-aza-CdR and irinotecan was significantly associated with topoisomerase I up-regulation by 5-aza-CdR, and combined to induce *CDKN2A* expression through promoter demethylation as well as Sp1 up-regulation. Expression of $p16^{INK4a}$ enhanced cell cycle arrest after irinotecan treatment (Crea et al., 2009).

Cladribine and clofarabine are second-generation analogues of 2′-deoxyadenosine. Fludarabine (Fludara) and Busulfan (Myeran) and are cell cycle non-specific alkylating antineoplastic agents. Although both cladribine and clofarabine induced $p16^{INK4a}$ expression, cladribine was effective at a lower concentration compared to clofarabine (Valdez et al., 2015). The combination of cladribine or clofarabine with fludarabin and busulfan caused synergic cytotoxicity in acute myeloid leukemia. Moreover, the addition of panobinostat, an HDAC inhibitor, and 5-aza-CdR with these combinations also enhanced cytotoxicity. Inclusion of panobiostat and 5-aza-CdR increased histone modifications and DNA demethylation, and increased the levels of the $p16^{INK4a}$, $p15^{INK4b}$ and $p21^{Waf1/Cip1}$ proteins (Valdez et al., 2015).

Treatment with doxorubicin and FUMI (5-fluorouracil and mitomycin C) in breast cancer resulted in demethylation of the *CDKN2A* promoter by 19.3% and 9.1%, respectively (Klajic et al., 2014). The *CDKN2A* gene was differentially methylated before/after treatment with doxorubicin and before/after treatment between responders. Lower levels of methylation were observed after treatment in the responder groups. Also, *CDKN2A* methylation levels among the non-responders were significantly changed (Klajic et al., 2014).

5. Conclusion

A high frequency of genetic and epigenetic alterations (*e.g.*, promoter hyper-methylation, homozygous deletion or mutation) in the *CDKN2A* gene has been observed in human cancer cell lines derived from various tumor types. However, a significantly lower frequency of *CDKN2A* abnormalities has been reported in fresh, non-cultured primary tumors and cell lines. Therefore, regulation of *CDKN2A* abnormalities will have benefits for the cancer prevention and/or therapy.

Outstanding questions

Besides genetic and epigenetic aberrations, the regulation of gene expression is also associated with the status of many other proteins, such as p53, BRAF, ataxia talengectia mutated (ATM), phosphatase and tensin homolog deleted at chromosome 10 (PTEN), KRAS and phosphatidylinositol 3-kinase (PI3-K) as well as the components of the *CDKN2A* promoter, such as the PRC1 and PRC2 complexes and long non-coding RNAs. HPV, a small DNA virus (major types HPV-16 and HPV-18) expresses E6 and E7 oncoproteins and is linked causally to most cervical cancers that show a high expression of $p16^{INK4a}$ (Kobalka et al., 2015; Munger et al., 2013; Pauck et al., 2014; Nehls et al., 2008). The E6 and E7 oncoproteins directly block p53 and pRb tumor suppressors resulting in the accumulation of $p16^{INK4a}$, which is upstream of p53 and pRb (Dyson et al., 1989; Scheffner et al., 1993). HPV-infection-associated cervical cancer also mediates the high expression of $p16^{INK4a}$ by epigenetic modulation of the *CDKN2A* upstream promoter (Nehls et al., 2008; McLaughlin-Drubin et al., 2013). Thus in HPV-infection-associated cancer, we should consider the $p16^{INK4a}$ as a biomarker in an opposite manner than what is generally expected. Although smoking and/or drinking is a risk factor for lung, head and neck, and pancreatic cancers (Gillison et al., 2012; Jiao et al., 2007; Tam et al., 2013), meaningful correlations of $p16^{INK4a}$ expression with smoking or drinking are not yet supported by strong evidence (Jiao et al., 2007; Gillison et al., 2012; Tam et al., 2013). Because $p16^{INK4a}$ is a critical regulator of cell proliferation and is inactivated during the course of tumorigenesis, methods for restoration of $p16^{INK4a}$ function could provide good therapeutic strategies. To achieve this goal, the focus has been directed toward understanding the molecular mechanisms, especially genetic and epigenetic changes that lead to $p16^{INK4a}$ inactivation in cancer, and developing small molecule modifiers of these mechanistic switches. For example, ZBP-89 can induce the expression of $p16^{INK4a}$ (Zhang et al., 2010). Likewise, FOXA1 and jmjd3 enhance *CDKN2A* expression by direct transcriptional activation and promoter di-methylation, respectively. Thus, discovery of small molecule activators of ZBP-89, jmjd3 or FOXA1 might be a future research goal for developing novel anticancer therapies. On the other hand, factors that repress *CDKN2A* gene expression, such as SETDB1, EZH2, or PcB proteins, could be potential therapeutic targets to induce $p16^{INK4a}$ functionality and tumor suppression. Although several natural and synthetic compounds, such as genistein, sulforaphane, EGCG and 5-aza-CdR have been shown to intervene against the epigenetic silencing of *CDKN2A*, mechanism-based development of $p16^{INK4a}$ activators warrants further studies.

Search strategy and selection criteria

This review was prepared by searching in PubMed with querying key words: $p16^{INK4a}$, epigenetics, alteration, gene alteration, gene silencing, cancer, promoter alteration and induction of $p16^{INK4a}$. The search was conducted for research articles including clinical reports up to 28 December 2015 and we especially summarized research articles in each cancer type.

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No potential conflicts of interest are disclosed.

Authors' contribution

RZ contributed to the literature search and collection of articles, assisted with designing the figures and writing; BYC contributed to the design and organization of the manuscript; MHL did most of the original writing; AMB did all the editing and confirmation of references and accurate information; ZD supervised the studies and allocated the funding.

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