**P16-specific DNA methylation by engineered zinc finger methyltransferase inactivates gene transcription and promotes cancer metastasis**

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**Abstract**

**Background:** P16 DNA methylation is well known to be the most frequent event in cancer development. It has been reported that genetic inactivation of P16 drives cancer growth and metastasis, however, whether P16 DNA methylation is truly a driver in cancer metastasis remains unknown.

**Results:** A P16-specific DNA methyltransferase (P16-dnmt) expression vector is designed using a P16 promoter-specific engineered zinc finger protein fused with the catalytic domain of dnmt3a. P16-dnmt transfection significantly decreases P16 promoter activity, induces complete methylation of P16 CpG islands, and inactivates P16 transcription in the HEK293T cell line. The P16-Dnmt coding fragment is integrated into an expression controllable vector and used to induce P16-specific DNA methylation in GES-1 and BGC823 cell lines. Transwell assays show enhanced migration and invasion of these cancer cells following P16-specific DNA methylation. Such effects are not observed in the P16 mutant A549 cell line. These results are confirmed using an experimental mouse pneumonic metastasis model. Moreover, enforced overexpression of P16 in these cells reverses the migration phenotype. Increased levels of RB phosphorylation and NFκB subunit P65 expression are also seen following P16-specific methylation and might further contribute to cancer metastasis.

**Conclusion:** P16 methylation could directly inactivate gene transcription and drive cancer metastasis.

**Keywords:** Cancer, Engineered methyltransferase, Metastasis, Methylation, P16

**Background**

P16 (CDKN2A or Ink4a) is one of the most frequently deleted genes in cancer genomes and has been studied extensively [1]. P16 germline mutation carriers have been shown to have a greatly increased predisposition to familial melanoma [2–4]. Recently, genetic inactivation of P16 has been proven to be a driver for cancer metastasis in mice [5].

While genetic alterations in P16 do occur, gene methylation is far more prevalent in human cancers [6–10]. Studies have shown that P16 DNA methylation is correlated with a decreased level of expression in tissues [6–10] and is linked to the development and metastasis of many cancers [11–15]. It is therefore highly likely that P16 DNA methylation may play an important role in cancer development.

It has been reported that artificial P16 DNA methylation induced through the insertion of alu motifs increased the susceptibility of mice to developing cancer [16]. However, whether P16 DNA methylation drives cancer metastasis has not been characterized. In the present study, a P16-specific DNA methyltransferase (P16-dnmt) was used to directly inactivate P16 transcription and the subsequent effects on proliferation, migration, and invasion of cancer cells were evaluated in vitro. These results were further confirmed in immuno-deficient mice. This study provides experimental evidence that
strongly implicates $P16$ DNA methylation as a driver in cancer metastasis.

**Results**

$P16$ DNA methylation directly inactivates gene transcription

In order to determine whether $P16$ DNA methylation directly inactivates gene transcription, a $P16$ promoter-specific DNA methyltransferase ($P16$-dnmt) was initially constructed using the pcDNA3.1_myc/His vector as described in the methods section (Fig. 1a). Western blot analysis confirmed that endogenous $P16$ was greatly reduced in HEK293T cells 48 h following transient transfection with the $P16$-dnmt vector (Fig. 1b). A dual-luciferase reporter assay further illustrated that $P16$ promoter activity was significantly inhibited in the $P16$-dnmt-transfected cells (Fig. 1c). Notably, methylation of CpG islands within both the $P16$ promoter and exon-1 regions was detected using denatured high performance liquid chromatography (DHPLC) and bisulfite-sequencing (Fig. 1d and e). An additional control lacking approximately 80 % of the DNA methyltransferase activity (R882H mutant) was constructed to evaluate the impact of steric hindrance from $P16$-Dnmt DNA binding on gene transcription. As expected, chromatin-immunoprecipitation (ChIP)-PCR analysis showed that the mutant still bound with the $P16$ promoter DNA fragment (Fig. 2a), but did not induce $P16$...
DNA methylation (Fig. 2b). Furthermore, its capacity to repress \(\text{P16}\) expression was sharply decreased in both HEK293T and BGC823 cells (Fig. 2c and d). These data suggest that \(\text{P16}\) DNA methylation is directly responsible for \(\text{P16}\) repression as opposed to steric hindrance. Taken together, these results indicate that \(\text{P16-dnmt}\) encodes an active methyltransferase for \(\text{P16}\) CpG islands, and \(\text{P16}\) DNA methylation is sufficient to inactivate endogenous \(\text{P16}\) expression.

In order to specifically methylate \(\text{P16}\) CpG islands, the \(\text{P16-dnmt}\) coding-sequence was then integrated into the pTRIPZ lentivirus vector carrying a ‘Tet-on’ switch to allow the gene expression to be controlled. Expression of \(\text{P16-Dnmt}\) protein was induced in GES-1 cells stably transfected with the \(\text{P16-dnmt}\) pTRIPZ vector after treatment with 0.25 \(\mu\)g/mL doxycycline for 3 days (61KD; Fig. 3a). Significant inhibition of endogenous \(\text{P16}\) expression was observed in Western blot and quantitative RT-PCR analysis when compared to GES-1 cells transfected with the \(\text{dnmt3a}\) and \(\text{7ZFP}\) control vectors (Fig. 3a and b). Confocal microscopy revealed that the average density of nucleic \(\text{P16}\) gradually decreased in the \(\text{P16-dnmt}\) expressing cells (Fig. 3c). In fact, after treatment with doxycycline for 3 and 7 days, \(\text{P16}\) expression levels were decreased by 21.4 % and 53.3 %, respectively (\(P < 0.001\)). Most importantly, intensive methylation of \(\text{P16}\) CpG islands was induced in the GES-1 cells stably transfected with \(\text{P16-dnmt}\) and treated with doxycycline, but not in cells transfected with the control vectors, nor in cells that did not receive doxycycline treatment (Fig. 3d). Similarly, \(\text{P16}\) DNA methylation and subsequent repression of \(\text{P16}\) expression was also induced by \(\text{P16-Dnmt}\) in the BGC823 cell line (Additional file 1: Figure S1).

ChIP-PCR analysis also showed that \(\text{P16-Dnmt}\) specifically bound the \(\text{P16}\) promoter, but not the \(\text{P14}\) promoter (Additional file 1: Figure S2). Similarly, ChIP-sequencing confirmed that the \(\text{P16-Dnmt}\) binding fragment was only detected in the promoter of \(\text{P16-Dnmt/Myc}\) antibody immunoprecipitated DNA from the \(\text{P16-dnmt}\)-expressing BGC823 cells, but not in the IgG control, nor the cells transfected with the control vector (Fig. 4a, red fragment; Additional file 2: File S1, Additional file 3: File S2, and Additional file 4: File S3). Although most of the \(\text{P16-Dnmt}\) binding fragments were found in intergenic and intron sequences (Additional file 1: Figure S3A), the main \(\text{P16-Dnmt}\) binding motif was found to closely match the antisense strand of the targeted fragment in the \(\text{P16}\) promoter with a similarity of 21/23 (91.3 %) base pairs (Additional file 1: Figure S3C, red-framed motif). Genome-wide methylation analysis of \(\text{P16-Dnmt}\) expressing BGC823 cells was performed using an Infinium Methylation 450 K array. The results illustrated that 647 of 481,615 informative CpG sites...
(0.13 %) were significantly hypermethylated (Δβ >0.50). Interestingly, 229 of these 647 CpG sites were located in intragenic CpG islands and shores corresponding to 203 genes (Additional file 5: File S4). The targeted P16 CpG island was included in the list of differentially hypermethylated sites (Fig. 4a, blue arrow). Furthermore, DNA methylation was not induced in the CpG islands of two control genes, P14 (located within the same CDKN2A locus as P16) and ZNF382 (located on a different chromosome) (Fig. 4b). These results suggest
that doxycycline-induced *P16-dnmt* expression could specifically methylate *P16* CpG islands.

**P16-specific DNA methylation promotes migration and invasion of cancer cells**

Various assays were then conducted to further characterize the biological behaviors of cancer cells following *P16*-specific inactivation by DNA methylation. Transwell assays revealed that the migration ability of GES-1 and BGC823 cells was significantly increased following *P16*-specific DNA methylation (Fig. 5a and b). Similarly, Matrigel assays showed that the invasion capacity of these cell lines was also significantly enhanced by *P16*-specific DNA methylation (Fig. 5c and d).

Four weeks after BGC823 cells stably transfected with *P16-dnmt* were injected into the tail vein of the NOD SCID mice, metastatic nodules were observed in the lung (Fig. 6a). The average lung weight, which correlates with the number of metastatic cells, in the *P16-dnmt* group was 152.5 % that of the empty vector control group (Mann–Whitney test, *P* < 0.001; Fig. 6b). The average proportion of metastatic nodule area to total lung area in the *P16-dnmt* group was also significantly higher than the control group (*P* < 0.004, Fig. 6c).

In addition, *P16*-specific DNA methylation was found to slightly, but significantly, inhibit proliferation of GES-1 cells, while the proliferation of BGC823 cells was not affected (Additional file 1: Figure S4). However, growth inhibition of the *P16-dnmt* transfected GES-1 cells was not observed in the SCID mice despite detection of methylated-*P16* alleles in the xenografts (Additional file 1: Figure S5).

In order to confirm whether the enhanced migration of cancer cells is *P16* DNA methylation-specific, a rescue
assay was carried out in the P16-dnmt expressing BGC823 cells through transient transfection of a P16 expression vector. Results of the Transwell assay demonstrated that enforced P16 overexpression significantly reversed the enhanced migration phenotype of these cells (Fig. 7a). Similar results were also observed in HONE-1 cells (Additional file 1: Figure S6). In contrast, downregulation of endogenous P16 expression through transient siRNA transfection significantly enhanced the migration of BGC823 and GES-1 cells (Fig. 7b). Furthermore, the migration capacity of A549 cells, which lack P16 alleles, was not changed following stable transfection of P16-dnmt and 7 days of doxycycline treatment (Fig. 7c). Taken together, these results imply that the enhanced migration and invasion phenotypes of cancer cells are P16-specific.

Fig. 5 Migration and invasion assays carried out with cell lines stably transfected with the P16-dnmt pTRIPZ vector in vitro. a, b Results of Transwell migration assays for GES-1 and BGC823 cell lines following 48-h and 36-h incubation, respectively; c, d Results of Matrigel invasion assays for GES-1 and BGC823 cell lines following 108-h and 96-h incubation, respectively; the average cell number and s.d. are displayed (Right). Dox (+), with 0.25 μg/mL doxycycline treatment; Dox (−), without doxycycline treatment. These experiments were independently repeated in triplicate.

Fig. 6 P16-specific methylation promotes experimental pneumonic metastasis of BGC823 cells. a Images of representative metastatic nodules in the lung of SCID mice (H&E staining). b The lung weights of mice in the P16-dnmt pTRIPZ and control groups at day 19. c The ratio of metastatic nodule area to lung area of mice in the P16-dnmt pTRIPZ and control groups.
**P16-specific DNA methylation promotes RB phosphorylation and upregulates NFκB subunit P65 expression**

To confirm that **P16** DNA methylation affects its downstream signal pathway, **P16-CDK4/6-RB**, the phosphorylation level of RB protein was analyzed using Western blot analysis. As expected, increased levels of phosphorylated RB were detected in the **P16-dnmt** transfected BGC823 and GES-1 cells treated with doxycycline when compared to those without doxycycline induction and those transfected with the control vector. Total RB protein levels were not changed (Fig. 8a and b). Furthermore, the expression level of nuclear factor NFκB subunit P65 was also increased in the **P16-dnmt** transfected cells.

**Discussion**

It is well known that methylation of CpG islands around transcription start sites is inversely correlated with the
expression level of genes in many cells and tissues. P16 DNA methylation may occur as a long-term mechanism to maintain gene suppression following transcriptional silence induced by repressive histone modifications [17]. Whether P16 promoter methylation alone is capable of silencing transcription has not been well studied. In the present study, we found that P16-Dnmt-induced methylation of P16 CpG islands could directly inactivate gene expression and promote metastasis of cancer cells.

Studies have shown that target-specific methylation/demethylation is associated with transcriptional inactivation/reactivation of several other human genes [18–20]. Zhang et al. reported that P16-specific artificial transcription factor (P16-ATF) could induce P16 DNA demethylation and re-activate its expression [21]; however, it is not known whether DNA demethylation is essential for re-activation of this gene. Yu et al. successfully established a p16 DNA methylation model in mice through the insertion of alu motifs into the mouse p16 promoter and found subsequent gene inactivation [16]; however, the possibility that the insertion of alu motifs directly contributed to transcriptional repression cannot be excluded. In order to increase the targeting specificity in the present study, we employed the pTRIPZ vector that allowed for controllable expression of P16-Dnmt. Our results showed that P16-Dnmt-induced DNA methylation was sufficient to silence transcription in two human gastric epithelial cell lines. This result is consistent with a recent report which demonstrated that engineered transcription activator-like effector (TALE)–Dnmts induced P16 DNA methylation, inactivated gene expression, and increased replication in human fibroblasts [22]. Additionally, steric hindrance from P16-Dnmt DNA binding does not appear to play a significant role in repressing gene transcription as was demonstrated using the P16-dnmt R882H mutant control. Taken together, the evidence suggests that P16 DNA methylation primarily accounts for the inactivation of P16 transcription.

Genome-wide CRISPR screens in mouse models have shown that genetic p16 inactivation may be a driver for tumor growth and metastasis [5]. Luo et al. have reported that the proportion of methylated P16 alleles is significantly associated with metastasis of gastric cancers [13]. Zhang et al. have also suggested that re-activation of methylated P16 by P16-ATF inhibits migration and invasion in AGS and H1299 cancer cell lines [21]. Here, we have provided evidence to demonstrate that P16-Dnmt mediated DNA methylation might promote metastasis of cancer cells in vitro and in vivo. Moreover, we found that such an effect was not observed in A549 cells lacking P16 alleles, and siRNA downregulation of P16 expression also promoted the migration of cell lines, and overexpression of P16 reversed the cell migration phenotype. These facts strongly implicate inactivation of P16 by DNA methylation as a possible promoter of migration/invasion and metastasis of cancer cells.

Inactivation of the P16 gene results in higher cyclin D-dependent protein kinase activity and thus induces aberrant phosphorylation of RB protein. Therefore normal cell cycle checkpoints are bypassed allowing accelerated
cell growth and increased genomic instability [23, 24]. We found that the induction of P16-specific DNA methylation could also increase the phosphorylation of RB.

NFκB subunit P65 is the master regulator in the senescence-associated secretory phenotype (SASP) [25]. In melanomas, the expression of P65 is increased while P16 expression is decreased [26]. P65 also regulates the transcription of a group of metastasis-related genes, including MMP-9/2 [27–29]. In this study, we also found that induction of P16 DNA methylation also increases the amount of P65 protein in cancer cells. Additional studies are required to determine other pathways involved in the P16 DNA methylation-related metastasis phenotype.

Conclusion

Engineered zinc finger protein-targeted P16 DNA methylation directly inactivates P16 expression and promotes invasion and metastasis of cancer cells.

Methods

Cell lines and cultures

HEK293T, BGC823, and GES-1 cell lines were kindly provided by Professor Yang Ke at Peking University Cancer Hospital and Institute. The A549 cell line lacking the P16 locus was kindly provided by Professor Zhigang Zhang at the same institute. The HONE-1 cell line was kindly provided by Professor Zhen Sun at Capital Medical University School of Stomatology, Beijing. All of these cell lines were tested and authenticated using the Goldeneye20A STR Identifiler PCR Amplification Kit (Beijing Jianlian Genes Technology Co., Ltd.) before being used in this study [30]. These cell lines were cultured in RPMI1640 medium supplemented with 10 % FBS and maintained at 37 °C in humidified air with 5 % CO2.

Construction of vectors and transfection

The P16-dnmt plasmid was constructed by fusing a SP1-like engineered seven-zinc finger protein (7ZFP) 6I capable of specifically binding the 21-bp fragment (5'-GAG GAA GGA AAC GGG CGG GGG-3', including a Sp1-binding site) within the human P16 promoter [21] with the catalytic domain (approximately 608–908aa) of mouse dnmt3a in the pFast Bac HT A-dnmt3a vector (kindly provided by Professor Keith Robertson at Georgia Regents University, USA) [31]. Point mutation R882H in the catalytic domain of Dnmt3a is the most frequent somatic mutation in acute myeloid leukemia [32]. The methyltransferase activity of R882H DNMT3A is reduced by approximately 80 % compared with the wide-type [33]. Thus, a P16-dnmt R882H mutant control was constructed as a negative control. The P16-dnmt coding sequence was integrated into a pcDNA3.1 vector and an expression controllable pTRIPZ vector carrying a ‘Tet-on’ switch (Open Biosystem, USA), respectively. Control vectors for the Dnmt3a catalytic domain or 7ZFP(6I) were also constructed. The purified P16-dnmt plasmid DNA was mixed with VSVG and Δ8.9 (Addgene, USA) to prepare lentivirus transfection particles. The P16 expression vector was constructed using wild-type P16 coding sequence cDNA and integrated into the pIRES2-EGFP vector. The cells (4.5 × 10⁶) were transiently transfected with the pIRES2-P16 expression vector; seeded into each well, and incubated for 43 h. P16 specific siRNAs (5'-CCGUA AAUGU CCAUU UAUAT T-3' and 5'-UAUAA AUGGA CAUUU ACGGT T-3') were synthesized (GenePharma) and used to transiently transfect cells at a final concentration of 1.0 μg/1 mL. The scramble siRNAs (5'-UUCUC CGAAC GUGUC ACGUT T-3' and 5'-ACGUG ACACG UUCCG AGAAT T-3') were used as negative control. The fresh lentivirus particles were used to transfect human cells.

Bisulfite-DHPLC, –sequencing, MethyLight, and methylation-specific PCR (MSP)

The 392-bp fragments isolated from the antisense-strand of P16 exon-1 in cultured cells were amplified with a CpG-free primer set and analyzed using DHPLC and clone sequencing as described previously [13, 34]; however, the PCR annealing temperature was fixed at 57.0 °C to avoid amplification bias between methylated and unmethylated P16 alleles. The 567-bp fragment in the antisense-strand of the P16 promoter was also amplified using a CpG-free primer set (forward, 5’-gaggt ttttg attta gtgaa tt-3’; reverse, 5’-acctcc atccc tcaaa tcta-3’) at an annealing temperature of 65 °C, analyzed at the partial denaturing temperature of 54 °C in DHPLC analysis, and confirmed using clone sequencing. Methylated and unmethylated P16 were also analyzed by 150/151-bp MSP [35].

The 272-bp P14 CpG island fragment was amplified using a CpG-free primer set (forward, 5’-gttgt ttatt tttgg tgtta-3’; reverse, 5’-acctct tccta cctcct tccca-3’) at the annealing temperature of 51.0 °C, and analyzed at the partial denaturing temperature of 57.7 °C in DHPLC analysis. The 437-bp ZNF382 CpG island fragment was amplified and analyzed by DHPLC as previously described [30].

Quantitative RT-PCR, Western blot, and confocal analysis of P16 expression

The P16 mRNA and protein level in cell lines were analyzed as described [21].
Chromatin immunoprecipitation (ChIP) assays
The 124-bp P16 and 61-bp P14 DNA fragments within CpG islands bound to P16-Dnmt were quantitated as described [21, 36]. Anti-Myc antibody was used to precipitate P16-Dnmt protein containing a Myc tag. The Myc-ChIPed-DNA samples were sequenced using the Illumina HiSeq2500 (Shanghai Biotechnology Co., China). The readouts were preprocessed using online fastx software (version 0.0.13; http://hannonlab.schul.edu/fastx_toolkit/index.html), mapped to the human genome hg19 using Bowties (version 0.12.8) [37], and enriched using MACS (version 1.4.2) [38]. The protein-binding motif was identified using MEME software [39]. The detected peaks/annotated information is presented as Additional file 2: File S1, Additional file 3: File S2, and Additional file 4: File S3.

Genome-wide analysis of DNA methylation
Illumina Infinium HD Methylation450K arrays were used to perform differential CpG methylation analyses on BGC823 cells stably transfected with the P16-dnmt and pTRIPZ control vectors following 14 days of doxycycline treatment according to the Assay Manual. Two parallel samples were tested for each group. DNA methylation levels for each CpG site were computed as the ratio of normalized methylated signal intensity to the sum of methylated and unmethylated signal intensities using GenomeStudio software. Using the control vector as a reference, Δβ was calculated to represent differential methylation for each CpG site in the P16-dnmt expressing cells. The differential methylation was considered to be significant when the Δβ value was >0.50. The raw methylation data are available as Additional file 6: File S5.

Dual-luciferase reporter assay
The P16 promoter (approximately −597 to +155 nt) was integrated into the pGL3-Basic vector and used for promoter activity analysis as previously described [21].

Transwell migration and Matrigel invasion tests
The Transwell migration and Matrigel invasion tests were performed using GES-1 and BGC823 cells suspended in 150 μL serum-free medium (2 × 10^5 cells/mL). The BGC823 cells were incubated for 36 h and 96 h at 37 °C in 5 % CO2 before quantifying their migration and invasion capacity, respectively. Similarly, the GES-1 cells were quantified following 48-h and 108-h incubation, respectively [21]. Wound healing status was dynamically recorded using the IncuCyte ZOOM™ live-cell imaging platform. Each trial consisted of three independent samples, and all the assays were repeated two to three times.

Xenografts and pneumonic metastases in SCID mice
GES-1 cells (1.4 × 10^6 cells in 200 μL Matrigel suspension) were stably transfected with the P16-Dnmt or control vector, induced with 0.25 μg/mL doxycycline for 7 days, and then injected subcutaneously into the lower limb of NOD SCID mice (female, 5 weeks old, weight 10–20 g, purchased from Beijing Huafukang Biotech). The mice were provided distilled, sterile water containing 2 μg/mL doxycycline. These mice were sacrificed 48 days after transplantation. The weight and volume of tumors were then analyzed.

For the pneumonic metastasis assay, BGC823 cells stably transfected with the P16-dnmt or control vector were also induced with 0.25 μg/mL doxycycline for 7 days, and then injected into the tail vein of the SCID mice (1.5 × 10^6 cells in 0.15 mL medium) (10 randomly allocated mice per group). The lung weight was detected at the 19th experimental day for each mouse [40]. The lung organs were fixed with Bouin solution, paraffin-embedded and cut into 5 μm slides along the maximum area, and examined microscopically following H&E staining. The lung area and total tumor nodule area were measured using INFINITY Analyze (Version 4.0, Luminera Sci). The nodule area to the lung area ratio was calculated for each mouse.

Statistical analysis
Results were displayed by constituent ratios of enumeration or ranked data. All P values were two-sided, and a difference with P <0.05 was considered statistically significant.

Ethical approval
This study was approved by the institute’s animal ethics committee (approval no. AE-2012-06).

Data and materials availability
The methylation array data have been deposited into the Gene Expression Omnibus under accession number GSE74233. The ChIP-sequencing raw data have been deposited into the bioproject database under accession number SRA306603.

Additional files

- Additional file 1: Figures S1 to S6. (DOC 3228 kb)
- Additional file 2: File S1 (annotated_tss_P16Dnmt-Myc.fq_macs_peaksFile). (XLSX 1943 kb)
- Additional file 3: File S2 (annotated_tss_P16Dnmt-IgG.fq_macs_peaks). (XLSX 139 kb)
- Additional file 4: File S3 (annotated_tss_vector-Myc.fq_macs_peaks). (XLSX 153 kb)
- Additional file 5: File S4 (P16-Dnmt induced methylation sites). (XLSX 4152 kb)
- Additional file 6: File S5 (450 K RawSignal). (CSV 46821 kb)
Abbreviations
7ZFP: seven zinc finger protein; DHPLC: denatured high performance liquid chromatography; Doc: doxycycline; P16-ATF: P16-specific transcription factor; P16-Dnmt: P16-specific methyltransferase.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
CC and YG carried out the cellular and molecular biological studies, participated in the design of the study and construction of various expression vectors, and drafted the manuscript. LG carried out the confocal immunoassays. JW edited the manuscript. ZL participated in methylation analysis by DHPLC. BZ and DD conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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References
1. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, D'Amico A, et al. The landscape of somatic copy-number alteration across human cancers. Nature. 2010;463:899–905.
2. Liu L, Lassam NJ, Slingerland JM, Bailey D, Cole D, Jenkins R, et al. Germline p16INK4A mutation and protein dysfunction in a family with inherited melanoma. Oncogene. 1995;11:605–12.
3. Hussussian CJ, Sterwein JP, Goldstein AL, Higgins PA, Ally DS, Sheahan MD, et al. Germline p16 mutations in familial melanoma. Nat Genet. 1994;8:15–21.
4. Kannevgesier C, Brookes S, del Arroyo AG, Pharm D, Bombled J, Barrois M, et al. Functional, structural, and genetic evaluation of 20 CDKN2A germ line mutations identified in melanoma-prone families or patients. Hum Mutat. 2009;30:64–74.
5. Chen S, Sanjana N, Zheng K, Shalem O, Lee K, Shi X, et al. Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. Cell. 2015;160:246–60.
6. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, et al. 5′p CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. Nat Med. 1995;1:686–92.
7. Serrano J, Goebel SU, Peghini PL, Lubensky IA, Gibril F, Jensen RT. Alterations in the p16INK4a/CDKN2A tumor suppressor gene in metastatic gliomas. J Clin Endocrinol Metabol. 2000;85:4146–56.
8. Herman JG, Merlo A, Mao L, Lapidos RG, Isa JP, Davidson NE, et al. Inactivation of the Cdkn2a/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res. 1995;55:4525–30.
9. Gonzalez-Zulueta M, Bender CM, Yang AS, Nguyen T, Beart RW, Van Tornout JM, et al. Methylation of the 5′p CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res. 1995;55:4331–5.
10. Kretsy LA, Mallely SR, Knobloch TJ, Song HJ, Lloyd M, Casto BC, et al. Alterations of p16(INK4a) and p14(ARF) in patients with severe oral epithelial dysplasia. Cancer Res. 2002;62:2925–30.
11. Sun Y, Deng DJ, You WC, Bai H, Zhang L, Zhou J, et al. Methylation of p16 CpG islands associated with malignant transformation of gastric dysplasia in a population-based study. Clin Cancer Res. 2004;10:5087–93.
12. Bellinsky SA, Liechty KC, Gentry FD, Wolf HI, Rogers, J, Yu K, et al. Promoter hypermethylation of multiple genes in sputum precedes lung cancer incidence in a high-risk cohort. Cancer Res. 2006;66:3338–44.
13. Luo DY, Zhang BZ, Lv LB, Xiang SY, Liu YH, Ji JF, et al. Methylation of CpG islands of p16 associated with progression of primary gastric carcinomas. Lab Invest. 2006;86:918–9.
14. Gao J, Zhou J, Gao Y, Gu L, Meng H, Liu H, et al. Methylation of p16 CpG island associated with malignant progression of oral epithelial dysplasia: a prospective cohort study. Clin Cancer Res. 2009;15:5178–83.
15. Liu HW, Liu XW, Dong GY, Zhou J, Luy, Gao Y, et al. P16 methylation as an early predictor for cancer development from oral epithelial dysplasia: a double-blind multicentre prospective study. EBioMedicine. 2015;2:342–6.
16. Yu DH, Waterland RA, Zhang P, Schady D, Chen MH, Guan Y, et al. Targeted p16(INK4a) epimutation causes tumorigenesis and reduces survival in mice. J Clin Invest. 2014;124:3708–12.
17. Hinshelwood RA, Melki JR, Huschtscha LI, Paul C, Song JZ, Strazzer C, et al. Ablation of novo methylation of the p16p16/INK4A CpG island is initiated post gene silencing in association with chromatin remodelling and mimics nucleosome positioning. Hum Mol Genet. 2009;18:3098–109.
18. Maeder ML, Angstmann UF, Richardson ME, Linder SJ, Casco VM, Tsai SQ, et al. Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. Nat Biotechnol. 2013;31:1137–42.
19. Chen H, Kazemier HG, de Groote ML, Ruiters MH, Xu GL, Rots MG. Induced DNA demethylation by targeting Ten- Eleven Translocation 2 to the human ICM-1 promoter. Nucleic Acids Res. 2011;42:1563–74.
20. Rivenbark AG, Stolzenburg S, Beltran AS, Yuan X, Rots MG, Stahl BD, et al. Epigenetic reprogramming of cancer cells via targeted DNA methylation. Epigenetics. 2012;7:350–60.
21. Zhang B, Xiang S, Zhong Q, Yin Y, Gu L, Deng D. The p16-specific reaction and inhibition of cell migration through demethylation of CpG islands by engineered transcription factors. Hum Gene Ther. 2012;23:1071–81.
22. Bernstein DL, Le Lay JE, Ruano EG, Kaestner KH. TALE-mediated epigenetic suppression of CDKN2A increases replication in human fibroblasts. J Clin Invest. 2015;125:1998–2006.
23. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature. 1993;366:704–7.
24. McDermott KM, Zhang J, Holst CR, Kozakiewicz BK, Singla V, Tlsty TD. p16(INK4a) prevents centrosome dysfunction and genomic instability in primary cells. PLoS Biol. 2006;4:es1.
25. Chen Y, Scuoppo C, Wang X, Fang X, Balgley B, Borden JE, et al. Control of the senescence-associated secretory phenotype by NF-κB promotes senescence and enhances chemoresistance. Genes Dev. 2011;25:2125–36.
26. Ghiorzo P, Mantelli M, Gariglio S, Gramigni C, Pastorino L, Baneli BS, et al. Inverse correlation between p16INK4a expression and NF-kappaB activation in melanoma progression. Hum Pathol. 2004;35:1029–37.
27. Song Z, Ni JS, Wu P, Bao YL, Li T, Li M, et al. Studies-specific protease 50 promotes cell invasion and metastasis by increasing NF-kappaB-dependent matrix metalloproteinase-9 expression. Cell Death Dis. 2015;6:1093.
28. Liu H, Han L, Chen HR, Meng F, Liu QH, Pan ZQ, et al. P16INK4A serves as a potential prognostic indicator for clear cell renal cell carcinoma and inhibits its invasion and metastasis by suppressing MMP-2 via NF-κB-dependent transcription. Oncotarget. 2015;6:21406–20.
29. Zhang Y, Huang H, Zhou H, Du T, Zeng L, Cao Y, et al. Activation of nuclear factor κB pathway and downstream targets survivin and livin by SHARPIN contributes to the progression and metastasis of prostate cancer. Cancer. 2014;120:3208–18.
30. Liu ZJ, Zhang J, Gao YH, Pei LR, Zhou J, Gu LK, et al. Large-scale characterization of DNA methylation changes in human gastric carcinomas with and without metastasis. Clin Cancer Res. 2014;20:9588–612.
31. Van Emburgh BO, Robertson KD. Modulation of Dnmt3b function in vitro by interactions with Dnmt3L. Dnmt3a and Dnmt3b splice variants. Nucleic Acids Res. 2011;39:5004–5002.
32. Xu J, Wang YY, Dai YJ, Zhang W, Zhang WN, Xiong SM, et al. DNMT3A Arg882 mutation drives chronic myelomonocytic leukemia through disturbing gene expression/DNA methylation in hematopoietic cells. Proc Natl Acad Sci U S A. 2014;111:2620–5.

33. Russler-Germain DA, Spencer DH, Young MA, Lampecht TL, Miller CL, Fulton R, et al. The R882H DNMT3A mutation associated with AML dominantly inhibits wild-type DNMT3A by blocking its ability to form active tetramers. Cancer Cell. 2014;25:442–54.

34. Deng DJ, Deng GR, Smith MF, Zhou J, Xin HJ, Powell SM, et al. Simultaneous detection of CpG methylation and single nucleotide polymorphism by denaturing high performance liquid chromatography. Nucleic Acids Res. 2002;30:13E.

35. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin S. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A. 1996;93:9821–6.

36. Li Q, Wang X, Lu Z, Zhang B, Guan Z, Liu Z, et al. Polycomb CBX7 directly controls trimethylation of histone H3 at lysine 9 at the p16 locus. PLoS One. 2010;5, e13732.

37. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10:R25.

38. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based Analysis of ChIP-Seq (MACS). Genome Biol. 2008;9:R137.

39. Machanick P, Bailey TL. MEME-ChIP: motif analysis of large DNA datasets. Bioinformatics. 2011;27:12:1696–7.

40. Kijima-Suda I, Miyamoto Y, Toyoshima S, Itoh M, Osawa T. Inhibition of experimental pulmonary metastasis of mouse colon adenocarcinoma 26 sublines by a sialic acid: nucleoside conjugate having sialyltransferase inhibiting activity. Cancer Res. 1986;46:858–62.