Effect of methylation-associated silencing of the death-associated protein kinase gene on nasopharyngeal carcinoma

Wei-Jia Kong, Song Zhang, Chang-Kai Guo, Yan-Jun Wang, Xiong Chen, Su-Lin Zhang, Dan Zhang, Zheng Liu and Wen Kong

Death-associated protein kinase (DAPK) is a Ca^{2+}/calmodulin-regulated serine/threonine kinase and a positive mediator of apoptosis. Loss of expression of the DAPK gene by aberrant promoter methylation may play an important role in cancer development and progression. The aim of this study was to investigate the frequency of gene promoter methylation of DAPK in nasopharyngeal carcinoma (NPC) and the effect of 5-Aza-2′-deoxycytidine (5-Aza-CdR), a demethylating agent, on CNE cells, a human nasopharyngeal carcinoma cell line, and on xenografts of CNE cells. Methylation-specific PCR and RT-PCR were used to determine the promoter methylation status and mRNA expression of the DAPK gene in NPC. Furthermore, CNE cells were treated *in vitro* and *in vivo* with 5-Aza-CdR to explore the effect of demethylating agents on DAPK mRNA expression and tumor growth. Hypermethylation of the DAPK gene promoter was found in 35 (76.1%) of 46 NPC samples. There was no significant difference in the promoter hypermethylation rate among samples from patients with different TNM stages. No promoter hypermethylation of the DAPK gene was found in all six chronic inflammatory nasopharyngeal tissue specimens. DAPK mRNA expression was not detected in NPC tumor specimens with promoter hypermethylation. However, DAPK mRNA expression was observed in unmethylated NPC tumors and in the chronic inflammatory nasopharyngeal tissue specimens. Promoter hypermethylation of the DAPK gene was found and no DAPK mRNA expression was detected in CNE cells. DAPK mRNA expression in CNE cells and xenografts could be restored by treatment with 5-Aza-CdR. The CNE cell xenografts of nude mice treated with 5-Aza-CdR were obviously smaller in tumor volume than those of nude mice treated with PBS. These results demonstrate that loss of DAPK expression could be associated with promoter region methylation in NPC. 5-Aza-CdR may slow the growth of CNE cells *in vitro* and *in vivo* by reactivating the DAPK gene silenced by *de novo* methylation. *Anti-Cancer Drugs* 17:251–259 © 2006 Lippincott Williams & Wilkins.

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Introduction

Nasopharyngeal carcinoma (NPC) is a common tumor in the head and neck with a high incidence in Southeast Asia. Previous studies demonstrated that the development of NPC might be due to a complex interaction of genetic factors, dietary exposure to chemical carcinogen and Epstein–Barr virus (EBV) infection. The tumorigenesis of NPC is thought to be a multistep process, and involves multiple genetic and epigenetic changes. There is growing evidence demonstrating that alteration in the distribution of 5-methylcytosine is an important factor in multistep carcinogenesis [1] and hypermethylation of CpG islands in the promoter regions of growth controlling genes might play a special role in carcinogenesis. Hypermethylation of normally unmethylated CpG islands is associated with transcriptional inactivation of defined tumor-suppressor genes in some human cancer [2,3]. Hypermethylation of CpG islands located in the promoter regions of tumor-suppressor genes is now firmly established as an important mechanism for gene inactivation [1,4].

Programmed cell death (apoptosis) is an important regulatory mechanism that eliminates cells during development and maintenance of tissue homeostasis. Several apoptotic genes have been identified over recent years. Death-associated protein kinase (DAPK) is a novel 160-kDa Ca^{2+}/calmodulin-dependent enzyme with serine/threonine kinase activity [5]. It is an essential mediator involved in interferon (IFN)-γ-induced programmed cell

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death. Overexpression of DAPK induces programmed cell death, whereas a catalytically inactive mutant of the DAPK gene protects cells from IFN-γ-induced apoptosis. DAPK is also involved in tumor necrosis factor (TNF)-α- and Fas-induced apoptosis [6]. It was found that DAPK mRNA and protein expression is frequently lost in various human cancer cell lines [7]. Analysis of the methylation status of the DAPK gene promoter in DNA extracted from fresh tumor samples showed a high incidence of hypermethylation in stage I non-small cell lung cancer and B cell malignancies [8,9]. Our recent studies demonstrate that loss of DAPK expression could be associated with aberrant promoter region methylation in laryngeal squamous cell cancer. 5-Aza-2′-deoxycytidine (5-Aza-CdR), a demethylating agent, may slow the growth of Hep-2 cells *in vitro* and *in vivo* by reactivating the DAPK gene silenced by *de novo* methylation [10].

NPC differs from laryngeal carcinoma not only in terms of anatomical origin, but also in the hypothesis of carcinogenesis, and mechanism of tumor invasion and metastasis. Tumorigenesis of laryngeal carcinoma may be partially associated with human papilloma viral infection (HPV) [11], but that of NPC is associated with EBV [12]. NPC usually metastasizes to lymphoid nodes of the neck in the early stage. Moreover, the main therapeutic treatment of NPC is radiotherapy combined with chemotherapy. In contrast, laryngectomy combined with radiotherapy is the essential treatment for laryngeal carcinoma. Kwong *et al.* [13] found that the incidence of promoter hypermethylation of DAPK gene is 76% in NPC; however, the status of DAPK expression in NPC tissue was not investigated. At present, it is not clear whether promoter hypermethylation could lead to transcriptional inactivation of the DAPK gene in NPC. In addition, it was not known whether promoter region methylation of the DAPK gene was associated with tumorigenesis of NPC. Moreover, the potential therapeutic role of 5-Aza-CdR in NPC has not been investigated.

In the present study, the methylation status of the DAPK gene and the expression of DAPK mRNA in NPC and CNE cells were investigated. Furthermore, the CNE cells and xenografts were treated with 5-Aza-CdR. We found that the incidence of promoter hypermethylation of the DAPK gene is 76.1% in NPC and there was no expression of DAPK mRNA in those methylated tumor tissues. In CNE cells, the DAPK gene promoter was methylated and there was no expression of DAPK mRNA. In contrast, DAPK mRNA was re-expressed in CNE cells *in vitro* and in xenografts *in vivo* after treatment with 5-Aza-CdR. Moreover, 5-Aza-CdR can slow down the growth of CNE cells *in vitro* and xenografts *in vivo*. These results demonstrate that transcriptional inactivation of the DAPK gene is associated with its promoter hypermethylation and with the occurrence of NPC.

Material and methods

Tissue samples

Forty-six cases of primary tumors (NPC) were included. Thirty-five paraffin-embedded tumors and 11 tumor biopsies were obtained from NPC patients with consent before treatment at the Department of Otolaryngology, Union Hospital of Tongji Medical College. Six fresh nasopharyngeal tissue specimens were also collected from patients with chronic nasopharyngitis with consent as normal controls before treatment. All specimens were subjected to histological diagnosis by a pathologist. The male:female ratio of the NPC patients was 5.3:1. The age range was 32–68 years (mean 51 years). On the basis of TNM stage classification (UICC 2002), six patients had stage I disease (13.04%), 11 patients had stage II disease (23.91%), 10 patients had stage III disease (21.74%), nine patients had stage IVA disease (19.57%) and 10 patients had stage IVB disease (21.74%).

Cell culture

The CNE cell line, a NPC cell line [14], was obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. It was established by the Tumor Institute, Chinese Academy of Medical Sciences, from tissues of a highly differentiated nasopharyngeal squamous cell carcinoma in 1975 [15]. Neither Herpes-like virus particles nor EBV capsid antigens were found in the CNE cell line. CNE cells were maintained in RPMI 1640 with 10% FBS. Twenty-four hours after being plated in six-well plates, CNE cells were treated with 0, 5×10^{-8}, 10^{-7}, 5×10^{-7} and 10^{-6} mol/l 5-Aza-CdR (Sigma, St Louis, Missouri, USA). The medium was changed to normal medium (without 5-Aza-CdR) 24 h after 5-Aza-CdR treatment and subsequently every 3 days. Total RNA was isolated 9 days after treatment. The experiment was repeated 3 times.

Nude mice

CNE cells were harvested from culture bottles and suspended in PBS. Cells (10^7) were injected s.c. into the right flank of 12 female BALB/c-*nu*/*nu* nude mice (4 weeks old). After 20 days when the mean tumor diameter was at least 0.5 cm, the mice were randomized into two groups. Each nude mouse in the experimental group was given three i.p. injections per week of 1 μg/g of 5-Aza-CdR (0.1 ml/injection) for the duration of the 4-week period. An equal amount of PBS (0.1 ml/injection) was injected into each nude mouse of the control group under the same conditions. For the duration of the 4 weeks of drug administration, tumor volumes were estimated twice weekly by caliper measurements assuming spherical geometry (volume = $d^3 \times \pi/6$). Tumors were then isolated from nude mice, and total RNA and DNA of tumor tissues was extracted.

The Animal Care and Use Committee of TongJi Medical College approved this experimentation on animals.

DNA preparation

Genomic DNA was isolated from about 20 mg NPC tissues or 10^7 CNE cells using 1% SDS and 0.4 mg/ml proteinase K at 55°C for 3 h. DNA was purified by phenol/chloroform extraction and ethanol precipitated.

Methylation-specific PCR (MSP)

DNA methylation status in CpG islands of the DAPK gene was determined by chemical modification with the CpGenome DNA Modification kit (Intergen, New York, New York, USA) according to the manufacturer's protocol and MSP procedure [16] described in a previous study [10]. MSP distinguishes the methylation status of a given region based on sequence changes produced by sodium bisulfite treatment. Primer sequences for the methylated (M) sequence were: 5′-GGA TAG TCG GAT CGA GTT AAC GTC-3′ (sense) and 5′-CCC TCC CAA ACG CCG A-3′ (antisense); primer sequences for the unmethylated (U) sequences were: 5′-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3′ (sense) and 5′-CAA ATC CCT CCC AAA CAC CAA-3′ (antisense). The 5′ position of the sense U and M primers corresponds to base pairs 2 and 5 of the GenBank sequence (no. X76104), respectively. Both antisense primers originate from base pair 87 of this sequence. The U primers amplify a 106-bp PCR product only from a promoter unmethylated gene, but not from a promoter methylated gene. The M primers amplify a 98-bp PCR product from a promoter methylated gene, but not from a promoter unmethylated gene. Specimens with purely unmethylated promoters will have positive PCR products when using U primers, but not M primers. A specimen that contains purely methylated promoters will have positive PCR products by using M primers, but not U primers. A specimen that contains heterogeneous tissues of both methylated and unmethylated promoters will have positive PCR products by using both U and M primers. Amplification was carried out in a PTC200 temperature cycler for 40 cycles. The annealing temperature for both the unmethylated and methylated reactions was 60°C. Human placental DNA was treated *in vitro* with excess SssI methyltransferase (New England Biolabs, Boston, Massachusetts, USA) to generate completely methylated DNA at all CpGs and used as the positive control for methylated alleles of the DAPK gene. DNA from normal lymphocytes was used as a positive control for unmethylated alleles. A vacuity control containing all PCR components except sample DNA was also included in all PCRs. The PCR products (10 µl) were then electrophoresed on a 3% agarose gel (containing ethidium bromide) and visualized under UV illumination.

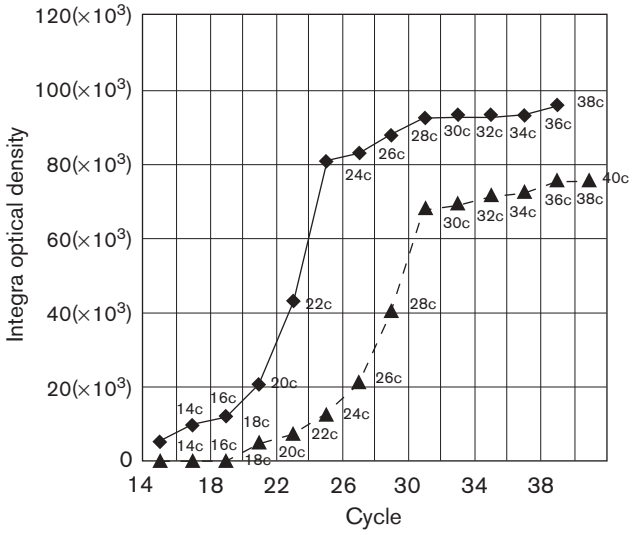
PCR product sequencing

Both unmethylated (U) and methylated (M) products of the MSP were excised from the agarose gel and purified by a Gene Clean DNA purification kit (BIO101, Vista, California, USA). The PCR products (M or U) were transformed into DH5α (*Escherichia coli*) after ligation with pMD18-T vector and a positive colony was cultured. Plasmid DNA was extracted by using the alkali lysis method. Recombinant plasmid DNA sequence was analyzed by a 377 ABI Prism automatic sequencer (Perkin-Elmer, Foster City, California, USA) after identification by restriction analysis.

RT-PCR

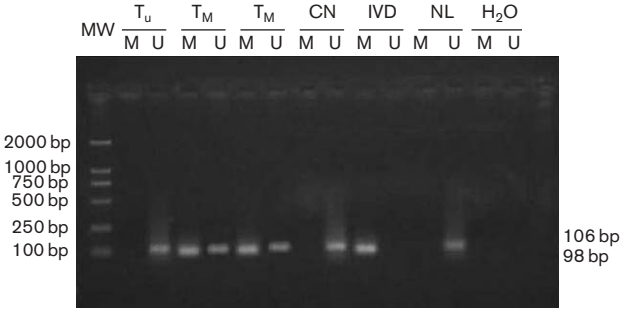
RNA cannot be extracted from paraffin-embedded tumor tissues because it has been degraded by RNase. Therefore, RNA can be extracted only in tumor biopsies and in fresh nasopharyngeal tissues with chronic inflammation. Total RNA (about 50 mg tissue or 10^7 cells) was extracted by using Trizol reagent (Sigma) according to the manufacturer's protocol. RNA was then dissolved in RNase-free water and quantified by a UV spectrometer. Two micrograms of total RNA was reverse transcribed with oligo(dT), deoxynucleoside triphosphates (dNTPs) and MMLV reverse transcriptase (Promega, Madison, Wisconsin, USA) in a 25-µl reaction mixture according to the manufacturer's protocol. PCR primer sets were designed with the aid of computer software (Primer Premier 5.0) according to the published sequences of DAPK and β-actin mRNA (GenBank) as follows: primer sequence for DAPK: 5′-GCC TGG AGA CGG AGA AGA T-3′ (sense) and 5′-AAG TCC CGT GGC TGG TAG A-3′ (antisense); for β-actin: 5′-GTG CGT GAC ATT AAG GAG-3′ (sense) and 5′-CTA AGT CAT AGT CCG CCT-3′ (antisense). To ensure credibility in comparison of DAPK and β-actin, PCR amplification was conducted by using the 'primer dropping' method [17], and each reaction was stopped during exponential growth (before the plateau of PCR). Preliminary experiments were conducted to determine the optimal number of PCR cycles for DAPK and the internal standard β-actin. Preliminary experiments indicated that nasopharyngeal tissues with chronic inflammation have a higher expression of DAPK mRNA. Therefore, the cDNA from nasopharyngeal tissues with chronic inflammation was used to determine the PCR reaction plateau of DAPK. The PCR reaction plateau of β-actin was also defined by using cDNA from nasopharyngeal tissues with chronic inflammation as a template. In detail, primers for DAPK and β-actin were used in separate PCR reactions with equal amounts of cDNA. The original PCR volume was 100 µl. From cycle 18 (DAPK) or cycle 14 (β-actin), 5 µl was removed from the original reaction tube every 2 cycles during the PCR process. The PCR reaction was stopped at cycle 38 (β-actin) or cycle 40 (DAPK). PCR products (5 µl) were electrophoresed on a 1.5% agarose gel and imaged by the Grab-it system. Then, the optical density of each strip was analyzed by Gelworks 1D software. The results indicate that the plateau of DAPK begins from cycle 30 and the plateau of β-actin begins from cycle 24 (Fig. 1). These cycle numbers were therefore used in all subsequent PCR procedures when

Fig. 1



Relationship between PCR cycle number and yield. There was an approximate exponential relationship of the PCR cycles and the PCR product below 30 cycles for DAPK (triangles) or below 24 cycles for β-actin (diamonds). The quantity of PCR products was no longer in exponential growth after 30 cycles for DAPK or after 24 cycles for β-actin.

Fig. 2



Methylation analysis of the DAPK gene in nasopharyngeal cancer and nasopharyngeal tissues with chronic inflammation. T_U, unmethylated tumor tissues; T_M, methylated tumor tissues; CN, nasopharyngeal tissues with chronic inflammation; *in vitro* methylated DNA (IVD) and normal peripheral blood lymphocytes (NL) were used as positive controls for methylated and unmethylated DNA, respectively. H_2O, vacuity control; lane M, amplified product (98 bp) with primers recognizing methylated sequences; lane U, amplified product (106 bp) with primers recognizing unmethylated sequences. A specimen from chronic nasopharyngitis and unmethylated tumor tissues that contains purely unmethylated promoter only has PCR products by using the U primers, but not the M primers. A specimen from methylated tumor tissues could amplify PCR products by using both U and M primers sequences because it contains heterogeneous tissues.

two primer pairs were included in the same reaction tube (multiplex PCR). To achieve the intended number of cycles, each primer pair was added individually during the PCR procedure at its predetermined time point (primer dropping). The final multiplex PCR reaction volume was 25 µl, including 2.5 µl cDNA, 1.5 mmol/l Mg^{2+}, 0.4 mmol/l each dNTP, 0.3 µmol/l each primer and 1 U Taq DNA polymerase. After initial melting at 95°C for 5 min, thermal cycling was carried out for 45 s at 95°C, 45 s at 54°C and 45 s at 72°C; DAPK primer was added first and β-actin primer was added after 6 cycles. The multiplex PCR reaction was ended after a total of 28 cycles, with a final extension at 72°C for 5 min. Ten microliters of PCR products was electrophoresed on a 1.5% agarose gel (containing ethidium bromide), visualized under UV illumination and imaged. The integra optical density of each strip was analyzed. DAPK gene expression was normalized to β-actin expression.

Cell cycle analysis

CNE cells were plated and treated with 5×10^{-7} mol/l 5-Aza-CdR or with DMSO only (without 5-Aza-CdR). CNE cells were harvested two passages after drug treatment to ensure complete recovery from the immediate toxic effect of 5-Aza-CdR. Cells were fixed with 75% ethanol, treated with RNasin (20 µg/ml) and stained with propidium iodide (PI; 50 µg/ml). The rate of cell apoptosis and DNA content at each cycle stage was determined via flow cytometry.

Statistical analysis

Statistical analysis was performed using SPSS 10.0 for Windows. ANOVA was used to assess the association between methylation and clinicopathological parameters. The *t*-test was used to assess the difference in tumor sizes and weights of mice between two groups of nude mice.

Results

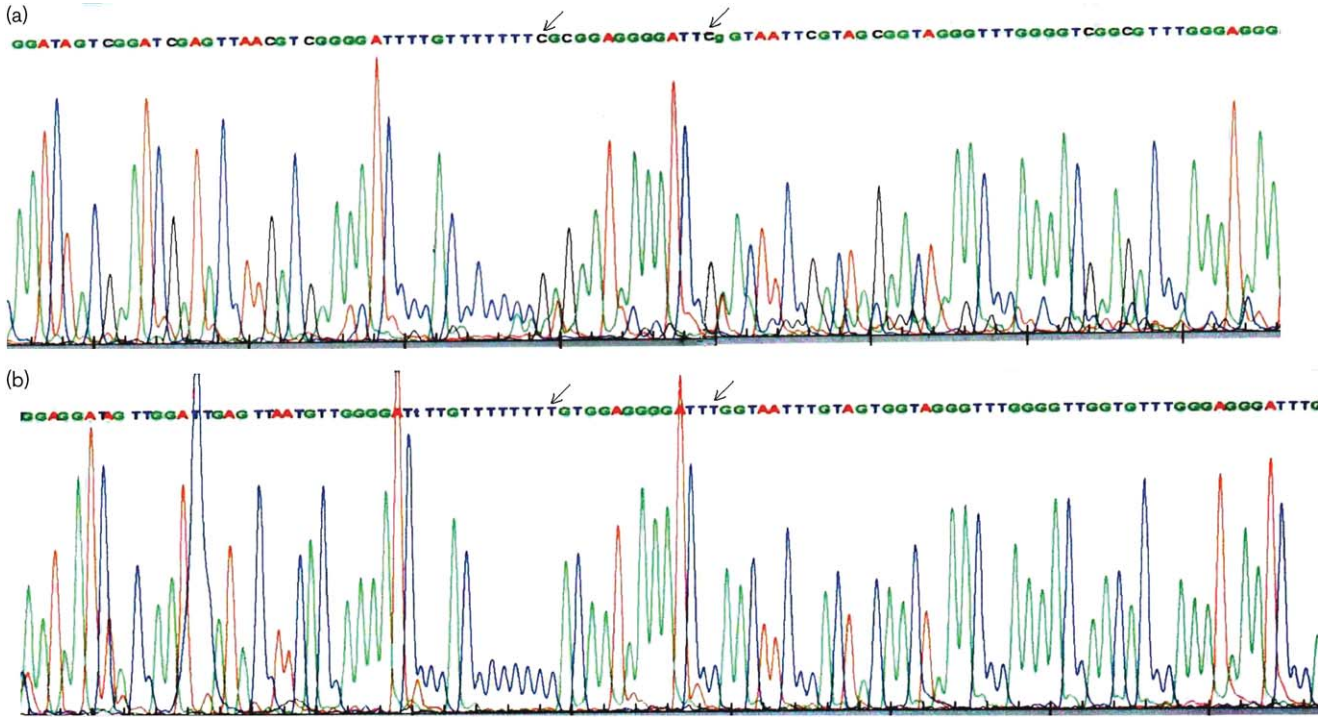
Methylation status of DAPK in tissues

Methylation of the DAPK gene promoter was found in 35 of 46 (76.1%) nasopharyngeal carcinoma tissues. None of six nasopharyngeal tissues with chronic inflammation showed any methylation of the DAPK gene promoter (Fig. 2). ANOVA showed that there was no significant difference in methylation rate of the DAPK gene promoter among the samples from patients with different TNM stages ($F = 0.155$ $P = 0.695$).

PCR product sequence analysis

In the MSP product from the M primers, only the cytosine residues in CG dinucleotides were found to remain as cytosine, which indicated the presence of methylated cytosine in these CG dinucleotides. In the MSP product from U primers, all cytosine nucleotides including those within CG dinucleotides were found to be changed to thymine, due to the conversion of all cytosine nucleotides to uracil by sodium bisulfide modification of the specimen DNA, which indicated there was no methylation of the DAPK gene (Fig. 3).

Fig. 3



MSP product sequence analysis. (a) MSP product amplified by M primers; only the cytosine residues in CG dinucleotides were found to remain as cytosine (arrows). (b) MSP product amplified by U primers; all cytosine nucleotides including those within CG dinucleotides were found to be changed to thymine (arrows).

DAPK mRNA expressions in various tissues

No expression of DAPK mRNA was detected in any tumor tissues with the promoter methylated. Expression of DAPK mRNA was detected in all tumor tissues with the promoter unmethylated and nasopharyngeal tissues with chronic inflammation (Fig. 4). These results suggest that the presence of hypermethylation of the CpG islands correlates with the loss of expression of DAPK mRNA.

Effects of 5-Aza-CdR on DAPK mRNA expression and on the methylation of the DAPK gene in the CNE cell line

The products of the MSP reaction showed that the DAPK gene promoter was methylated in CNE cells. No DAPK mRNA expression was found in CNE cells by the RT-PCR assay. Treating the cells with 5-Aza-CdR at concentrations greater than 10^{-7} mol/l could induce DAPK mRNA re-expression in a dose-dependent manner (Fig. 5). Furthermore, both methylated and unmethylated products were found in 5-Aza-CdR-treated CNE cells by the MSP assay (Fig. 6). It showed that the methylated DAPK gene was partly demethylated in the 5-Aza-CdR-treated CNE cells.

Effect of 5-Aza-CdR on the cell cycle

CNE cells treated with 5×10^{-7} M 5-Aza-CdR or DMSO only (without 5-Aza-CdR) were analyzed by flow cytometry to identify any changes in their cell cycle profiles. In comparison to the CNE cells which were treated with DMSO, it was found that after treatment with 5-Aza-CdR the proportion of apoptotic cells was increased from 8.71 to 29.92% and the proportion of G_0/G_1 stage cells was increased from 36.61 to 40.03%, while the proportion of G_2/M stage cells was reduced from 36.66 to 8.83% (Fig. 7).

Effects of 5-Aza-CdR on the growth of tumors in xenografts of CNE cells *in vivo*

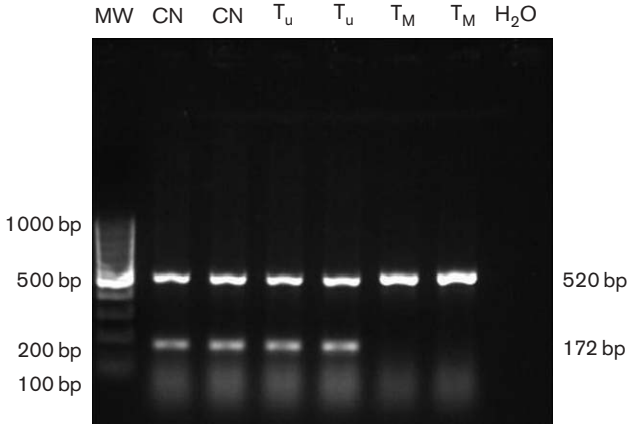
Tumor volumes of xenografts of CNE cells in nude mice were estimated by caliper measurements twice weekly during the duration of 5-Aza-CdR or PBS treatment. Tumor growth curves were drawn from week 1 to week 4 (Fig. 8). Tumors in the experimental group treated with 5-Aza-CdR averaged $195.32 \pm 27.57\,mm^3$ in volume, whereas tumors in the control group treated with PBS averaged $343.67 \pm 23.08\,mm^3$ in volume at the end of week 4. Significant differences were identified between the two groups by the t-test ($t = 10.11$, $P < 0.01$). Weights of nude mice were measured before they were sacrificed. The average weight was 24.995 ± 0.837 g in the control group treated with PBS and 24.947 ± 0.996 g in the experimental group treated with 5-Aza-CdR. No significant differences were identified between the two

groups by the *t*-test ($t = 0.011$, $P > 0.05$). These results indicate that 5-Aza-CdR was able to slow the growth of tumors *in vivo* and 5-Aza-CdR at the concentration of 1 μg/g is non-toxic to nude mice.

Effects of 5-Aza-CdR on DAPK mRNA in xenografts of CNE cells *in vivo*

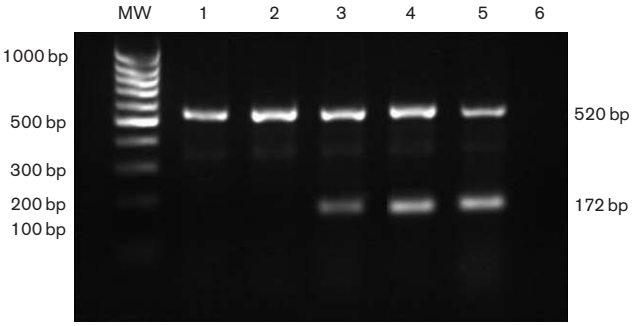
The possibility of re-expression of DAPK mRNA in xenografts of CNE cells *in vivo* by the application of 5-Aza-CdR at non-toxic concentrations (1 μg/g) was investigated. The nude mice were sacrificed 4 weeks after treatment with either 5-Aza-CdR or PBS and total RNA was extracted from the tumor tissues in xenografts of CNE cells. Re-expression of DAPK mRNA was detected via RT-PCR in all six tumors from the mice treated with 5-Aza-CdR, whereas no DAPK mRNA expression was found in tumor tissues from the mice treated with PBS only (Fig. 9).

Fig. 4



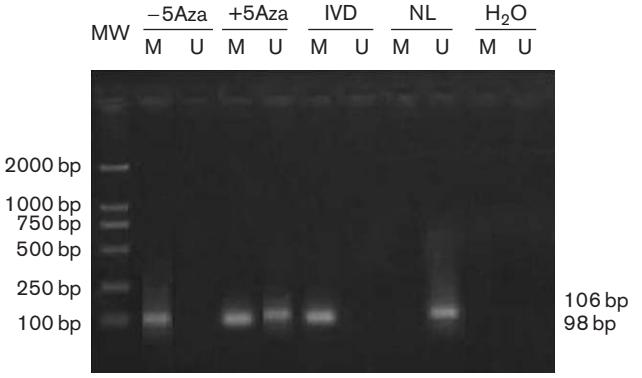
DAPK mRNA expression in various tissues. CN, nasopharyngeal tissues with chronic inflammation; T_U, unmethylated tumor tissues; T_M, methylated tumor tissues; H_2O, vacuity control. β-actin: 520 bp, DAPK: 172 bp. Expression of DAPK mRNA was detected in promoter unmethylated tumor tissues and nasopharyngeal tissues with chronic inflammation. No expression of DAPK mRNA was detected in promoter methylated tumor tissues.

Fig. 5



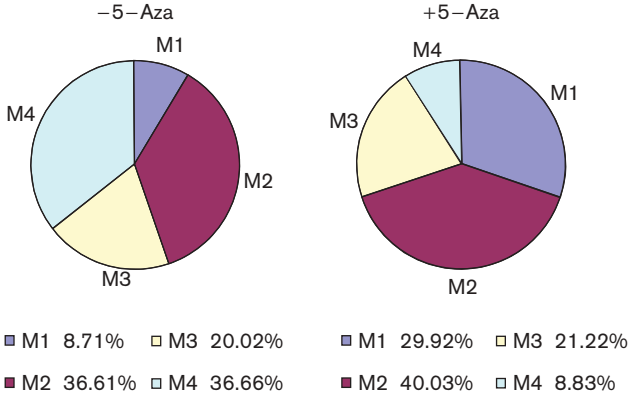
Effects of 5-Aza-CdR on DAPK mRNA expression *in vitro*. RNA was isolated from CNE cells after treatment with 5-Aza-CdR. DAPK mRNA was measured via RT-PCR. The expression of β-actin was measured as an internal control in the same reaction system (as described in Materials and methods). Lanes 1–6 for cells treated with 0, 5×10^{-8}, 10^{-7}, 5×10^{-7} and 10^{-6} mol/l 5-Aza-CdR, and H_2O vacuity control, respectively.

Fig. 6



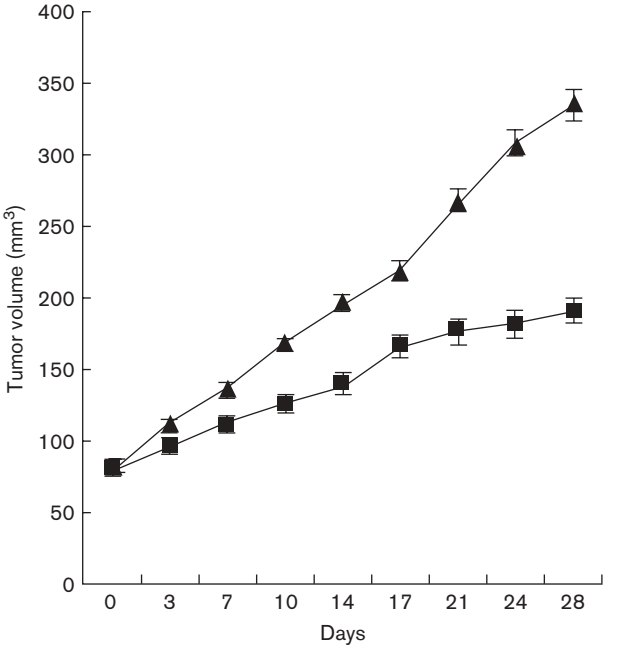
Effects of 5-Aza-CdR on methylation of the DAPK gene. −5Aza, without 5-Aza-CdR; +5Aza, 5×10^{-7} mol/l 5-Aza-CdR; *in vitro* methylated DNA (IVD) and DNA of normal peripheral blood lymphocytes (NL) were used as positive controls for methylated and unmethylated DNA, respectively. H_2O, vacuity control; lane M, amplified product (98 bp) with primers recognizing methylated sequences; lane U, amplified product (106 bp) with primers recognizing unmethylated sequences. A specimen from CNE cells treated without 5-Aza-CdR has PCR products only by using the M primers, but not the U primers. A specimen from CNE cells treated with 5-Aza-CdR could amplify PCR products by using both U and M primer sequences.

Fig. 7



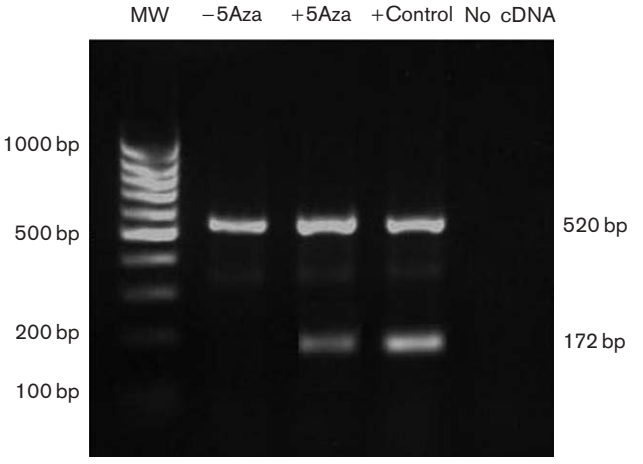
Effect of 5-Aza-CdR on cell cycle. −5Aza, cells were treated only with DMSO (without 5-Aza-CdR); +5Aza, cells were treated with 5×10^{-7} mol/l 5-Aza-CdR; M1, proportion of apoptosis in total cells; M2, M3 and M4, proportion of G_0/G_1, S and G_2/M stage cells, respectively.

Fig. 8



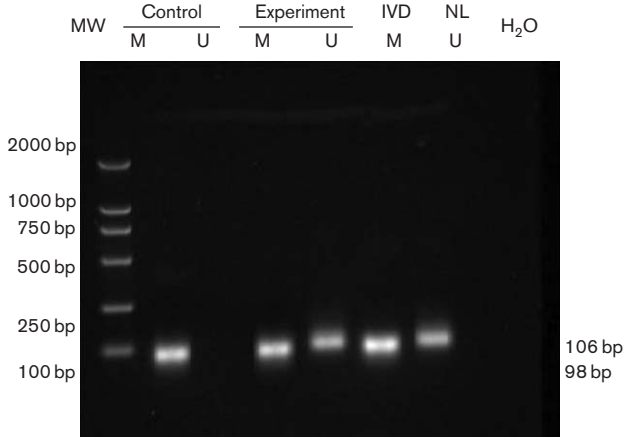
Growth curve of tumor in xenografts of CNE cells *in vivo.* Control (triangles), volume of tumors from nude mice treated with PBS; experiment (squares), volume of tumors from nude mice treated with 5-Aza-CdR (1 μg/g). The volumes of tumors from nude mice of the experiment group are smaller than those from mice of the control group.

Fig. 9



Effects of 5-Aza-CdR on DAPK expression *in vivo.* −5Aza, without 5-Aza-CdR; +5Aza, 5×10^{-7} mol/l 5-Aza-CdR; +control, DNA from nasopharyngeal tissues with chronic inflammation. β-actin: 520 bp, DAPK: 172 bp. No expression of DAPK mRNA was detected in tumors from nude mice treated with PBS. Expression of DAPK mRNA was detected in tumors from nude mice treated with 5-Aza-CdR.

Fig. 10



Effects of 5-Aza-CdR on methylation of the DAPK gene *in vivo*. Control, tumor tissues from nude mice treated with PBS; experiment, tumor tissues from nude mice treated with 5×10^{-7} M 5-Aza-CdR. *In vitro* methylated DNA (IVD) and normal peripheral blood lymphocytes (NL) were used as positive controls for methylated and unmethylated DNA, respectively. H_2O, vacuity control; lane M, amplified product (98 bp) with primers recognizing methylated sequences; lane U, amplified product (106 bp) with primers recognizing unmethylated sequences. A specimen from nude mice treated with PBS has PCR products only by using the M primers, but not the U primers. A specimen from nude mice treated by 5-Aza-CdR could amplify PCR products by using both U and M primer sequences.

Effects of 5-Aza-CdR on methylation of the DAPK gene in xenografts of CNE cells *in vivo*

Nude mice were sacrificed 4 weeks after treatment with either 5-Aza-CdR or PBS and DNA was extracted from tumor tissues in xenografts of CNE cells. MSP was used to examine the change of DAPK gene methylation. Only the products of methylation were detected in all six tumors from nude mice treated with PBS. In contrast, however, both products of methylation and unmethylation were detected in all six tumors from nude mice treated with 5-Aza-CdR (Fig. 10). This demonstrated that the DAPK gene was partly demethylated by treatment with 5-Aza-CdR in xenografts of CNE cells.

Discussion

DAPK is a pro-apoptotic serine/threonine kinase that participates in a wide spectrum of apoptotic signals, including IFN-γ, TNF-α, activated c-Myc, transforming growth factor-β and detachment from the extracellular matrix [5,18–21]. The death-promoting effects of DAPK depend on its intact catalytic activity, the correct intracellular localization and the presence of the death domain. It was found that DAPK mRNA and protein expression is frequently lost in various human cancer cell lines and some human tumor tissues. Analysis of the methylation status of the DAPK gene promoter region showed a high incidence of hypermethylation in these cell lines and tumor tissues [7,8]. Our present studies show that hypermethylation of DAPK gene promoters was found in 35 of 46 (76.1%) NPC tissues and the CNE cell line. Hypermethylation was not detected in naso-

pharyngeal tissues with chronic inflammation. No expression of DAPK mRNA was detected in all promoter hypermethylated cancer tissues and the CNE cell line. These results indicate that DAPK gene promoter hypermethylation may contribute to the transcriptional loss of DAPK mRNA in NPC tissues. Sanchez-Cespedes *et al.* [22] reported that DAPK gene promoter hypermethylation was detected in three of 17 (18%) cases of serum DNA from head and neck cancer patients whose tumor tissues had DAPK gene promoter methylation. Our recent studies showed that promoter hypermethylation of the DAPK gene was found in six of 39 tissues adjacent to tumors from laryngeal carcinoma patients whose tumor tissues had DAPK gene promoter hypermethylation [10]. These results demonstrate that DAPK gene promoter hypermethylation is possibly an early molecular event in cell carcinogenesis.

DNA methylation alterations are now widely recognized as a contributing factor in human tumorigenesis [23]. Many studies have demonstrated that CpG islands of multiple cancer-related genes are frequently methylated in a variety of human cancers, but are usually unmethylated in the corresponding normal tissues. When these CpG islands are methylated in cells, expression of the corresponding gene is silenced; the epigenetic inactivation of these genes may affect many molecular pathways, such as the cell cycle (P^{16} and P^{15}) [24,25], DNA repair (hMLH1, MGMT, and BRCA1) [26–28], apoptosis (DAPK) [6], metabolic enzymes (GSTP1) [29] and APC/β-catenin route (APC) [30]. Therefore, it is possible to find simultaneous inactivation of several pathways by aberrant methylation compromising all of the described functions in any given tumor. Transcriptional silencing of those tumor-suppressor genes by hypermethylation of CpG islands in the promoter region may result in loss of cell growth control and then occurrence of cell carcinomatous change. It is reported that DNA methylation is a reversible biological modification. Transcriptional silencing of a gene resulting from *de novo* methylation could be partially relieved by demethylation of the promoter region [31]. Several preclinical and clinical trials have been developed to use DNA methylation inhibitors, such as 5-Aza-CdR, in attempts to reactivate silenced genes in human cancers [32,33]. 5-Aza-CdR is an inhibitor of DNA methyltransferase. It is substituted for cytosine during replication and is recognized by DNA methyltransferase. Therefore, attempted transfer of methyl groups to 5-Aza-CdR covalently traps the enzyme to newly synthesized DNA. 5-Aza-CdR inhibits DNA methylation and is often used *in vitro* to induce the re-expression of genes putatively silenced by promoter methylation. Some studies have demonstrated that methylation-regulated genes are released from silencing after treatment of tumor cells with 5-Aza-CdR *in vitro*. The re-expression of these genes after 5-Aza-CdR treatment correlated with cell growth inhibition, re-establishment of cell–cell adhesion and renewed ability for mismatch repair [34–36]. Our data demonstrate that DAPK gene promoter methylation was detected in the CNE cell line and no DAPK mRNA expression was found in CNE cells by RT-PCR. DAPK mRNA re-expression was detected after CNE cells were treated with increasing concentrations of 5-Aza-CdR. The rate of cell apoptosis was increased after treatment with 5-Aza-CdR. There was an increase in stage G_0/G_1 and a reduction in stage G_2/M after treatment with 5-Aza-CdR in the CNE cell line. These findings indicate that DAPK mRNA re-expression could promote cell apoptosis and inhibit CNE cell growth *in vitro*. In addition, our data demonstrate that the DAPK gene was reactivated and tumor growth in nude mice was inhibited by treatment with 5-Aza-CdR *in vitro*. Bender *et al.* [31] reported that after treatment with 5-Aza-CdR, cell growth was suppressed in seven tumor cell lines (except fibroblast strains), whereas treatment of T24 cells with an equitoxic dose of Ara-C did not increase the proportion of cells in the G_0/G_1 stage. These results demonstrate that the cytotoxic effects of 5-Aza-CdR did not suppress cell growth, although 5-Aza-CdR may restore growth control of tumor cells by reactivating tumor-suppressor genes silenced by *de novo* methylation *in vitro* or *in vivo*.

To date, 5-Aza-CdR has been used in chemotherapeutic clinical trials for leukemia and myelodysplastic syndromes [37]. However, there are few reports concerning the application of 5-Aza-CdR in the treatment of solid tumors. Laird *et al.* [38] reported that administration of 5-Aza-CdR to Min mice resulted in more than 98% inhibition of ApcMin-induced intestinal neoplasia. Lantry *et al.* [39] reported that 5-Aza-CdR is chemopreventive in a mouse lung tumor model when administered prior to chemical initiation and throughout the promotion phase of mouse lung tumorigenesis. In the present study, we used 5-Aza-CdR *in vivo* at non-toxic concentrations (1 μg/g) to induce re-expression of DAPK in xenografts of the CNE cell line and slow the growth of the tumor *in vivo*. The present results indicate that methylation inhibition is a potential approach in the early intervention, prevention and treatment of tumors. Further studies are needed to explore the possible application of demethylating agents, such as 5-Aza-CdR, in the treatment of solid tumors in the clinic.

References

1 Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alternations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998; **72**:141–196.

2 Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, *et al.* Silencing of the VHL tumor-suppressor gene by methylation in renal carcinoma. *Proc Natl Acad Sci USA* 1994; **91**:9700–9704.

3 Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, *et al.* 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med* 1995; **1**: 686–692.

4 Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999; **21**:163–167.

5 Cohen O, Feinstein E, Kimchi A. DAP-kinase is a Ca^{2+}/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. *EMBO J* 1997; **16**:998–1008.
6 Cohen O, Inbal B, Kissil JL, Raveh T, Berissi H, Spivak-Kroizaman T, *et al.* DAP-kinase participates in TNF-α and Fas-induced apoptosis and its function requires the death domain. *J Cell Biol* 1999; **146**:141–148.
7 Kissil JL, Feinstein E, Cohen O, Jones PA, Tsai YC, Knowles MA, *et al.* DAP-kinase loss of expression in various carcinoma and B-cell lymphoma cell lines: possible implications for role as tumor suppressor gene. *Oncogene* 1997; **15**:403–407.
8 Katzenellenbogen RA, Baylin SB, Herman JG. Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. *Blood* 1999; **93**:4347–4353.
9 Tang X, Khuri FR, Lee JJ, Kemp BL, Liu D, Hong WK, *et al.* Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. *J Natl Cancer Inst* 2000; **92**: 1511–1516.
10 Kong WJ, Zhang S, Guo C, Zhang S, Wang Y, Zhang D, *et al.* Methylation-associated silencing of death-associated protein kinase gene in laryngeal squamous cell cancer. *Laryngoscope* 2005; **115**:1395–1401.
11 Rees L, Birchall M, Bailey M, Thomas S. A systematic review of case-control studies of human papillomavirus infection in laryngeal squamous cell carcinoma. *Clin Otolaryngol Allied Sci* 2004; **29**:301–306.
12 Wei WI, Sham JS. Nasopharyngeal carcinoma. *Lancet* 2005; **365**: 2041–2054.
13 Kwong J, Lo KW, To KF, Teo Peter ML, Johnson PJ, Huang DP, *et al.* Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. *Clin Cancer Res* 2002; **8**:131–137.
14 Hu LH, Zou HB, Gong JX, Li HB, Yang LX, Cheng W, *et al.* Synthesis and biological evaluation of a natural ester sintenin and its synthetic analogues. *J Nat Prod* 2005; **68**:342–348.
15 Chinese Academy of Medical Sciences, ZhongShan Medical College. Establishment of epithelium cell line and fusiform cell line of human nasopharyngeal carcinoma. *Science in China* 1978; **1**:113–118.
16 Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**:9821–9826.
17 Wong H, Anderson WD, Cheng T, Riabowol KT. Monitoring mRNA expression by polymerase chain reaction: the 'primer-dropping' method. *Anal Biochem* 1994; **223**:251–258.
18 Deiss LP, Feinstein E, Berissi H, Cohen O, Kimchi A, *et al.* Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev* 1995; **9**:15–30.
19 Inbal B, Cohen O, Polak-Charcon S, Kopolovic J, Vadai E, Eisenbach L, *et al.* DAP kinase links the control of apoptosis to metastasis. *Nature* 1997; **390**:180–184.
20 Raveh T, Droguett G, Horwitz MS, DePinho RA, Kimchi A. DAP kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. *Nat Cell Biol* 2001; **3**:1–7.
21 Jang CW, Chen CH, Chen CC, Chen JY, Su YH, Chen RH, *et al.* TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol* 2002; **4**:51–58.
22 Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, *et al.* Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res* 2000; **60**:892–895.
23 Karpf AR, Jones DA. Reactivating the expression of methylation silenced genes in human cancer. *Oncogene* 2002; **21**:5496–5503.
24 Herman JG, Jen J, Merlo A, Baylin SB, *et al.* Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. *Cancer Res* 1996; **56**:722–727.
25 Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, *et al.* Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res* 1995; **55**:4525–4530.
26 Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, *et al.* Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res* 1997; **57**:808–811.
27 Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 1999; **59**:793–797.
28 Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, *et al.* Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J Natl Cancer Inst* 2000; **92**:564–569.
29 Esteller M, Corn PG, Urena JM, Gabrielson E, Baylin SB, Herman JG, *et al.* Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. *Cancer Res* 1998; **58**:4515–4518.
30 Hiltunen MO, Alhonen L, Koistinaho J, Myohanen S, Paakkonen M, Marin S, *et al.* Hypermethylation of the APC (adenomatous polyposis coli) gene promoter region in human colorectal carcinoma. *Int J Cancer* 1997; **70**:644–648.
31 Bender CM, Pao MM, Jones PA. Inhibition of DNA methylation by 5-Aza-2-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res* 1998; **58**:95–101.
32 Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M, *et al.* Low-dose 5-Aza-2-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. *J Clin Oncol* 2000; **18**:956–962.
33 Lubbert M. DNA methylation inhibitors in the treatment of leukemias, myelodysplastic syndromes and hemoglobinopathies: clinical results and possible mechanisms of action. *Curr Top Microbiol Immunol* 2000; **249**:135–164.
34 Yoshiura K, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T, Hirohashi S, *et al.* Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc Natl Acad Sci USA* 1995; **92**:7416–7419.
35 Bender CM, Zingg JM, Jones PA, *et al.* DNA methylation as a target for drug design. *Pharm Res* 1998; **15**:175–187.
36 Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, *et al.* Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 1998; **95**:6870–6875.
37 Pinto A, Zagonel V. 5-Aza-2′-deoxycytidine (Decitabine) and 5-Aza- cytidine in the treatment of acute myeloid leukemias and myelodysplastic syndromes: past, present, and future trends. *Leukemia* 1993; **7**:51–60.
38 Laird PW, Jackson-Grusby L, Fazeli A, Dickinson SL, Jung WE, Li E, *et al.* Suppression of intestinal neoplasia by DNA hypomethylation. *Cell* 1995; **81**:197–205.
39 Lantry LE, Zhang ZQ, Crist KA, Wang Y, Kelloff GJ, Lubet RA, *et al.* 5-Aza-2′-deoxycytidine is chemopreventive in a -(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced primary mouse lung tumor model. *Carcinogenesis* 1999; **20**:343–346.