Induction of Epigenetic Alteration by CPUK02, An Ent-kaurenoid Derivative of Stevioside

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Abstract

**Background:** Dietary polyphenols, such as those found in green tea and red wine, are linked to antitumor activity. They are known to influence many signaling pathways epigenetically within the human body. In this regard, CPUK02 (15-Oxosteviol benzyl ester) is a new ent-kaurenoid derivative of stevioside and exhibits strong anti-cancer activity in vitro and in vivo. Nowadays, the role of epigenetics in cancer has been the subject of intensive study and DNA methylation targeting represents a relevant strategy for cancer treatment. There are no reports regarding the effects of CPUK02 on epigenetic alterations in colorectal cancer cell line. This study was an attempt to compare CPUK02 with 5-AZA as DNMT inhibitor agent and evaluate whether it can induce its anti-cancer effects via altering the level of DNMT3b mRNA, MGMT and SFRP2 methylation pattern in HCT 116 cell line.

**Methods:** To evaluate DNMT3b expression, DNMT3B mRNA levels in HCT116 CRC cell line were quantified by real-time reverse-transcriptase Polymerase Chain Reaction (PCR) assay after 24 hr of incubation time with CPUK02 and 5-AZA. In addition, the methylation patterns of 2 CpG islands in this cell line were examined by methylation-specific PCR methods.

**Results:** CPUK02 surprisingly, decreased the DNMT3b mRNA level. The average expression levels of DNMT3b in HCT116 treated with CPUK02 and 5-AZA relative to the GAPDH expression level in control were 0.16 and 0.5%, respectively. Furthermore, CPUK02 could decrease the methylated allele of MGMT and SFRP2 genes in HCT 116 after 24 hr.

**Conclusion:** In this study, positive correlation was found between mRNA expression of DNMT3b and gene promoter hypermethylation after treatment with CPUK02 and 5-AZA. Our data confirmed that CPUK02 like 5-AZA exhibits demethylating properties.

**Keywords:** 5-AZA, Colorectal neoplasm, DNMT, Epigenetic, Methylation

Introduction

Colorectal Cancer (CRC) is a multistep process with accumulation of genetic and epigenetic errors which causes a normal cell transformation to an invasive tumor cell 1. Three distinct pathways including chromosomal instability, microsatellite instability, and CpG island gene methylation pathways have been recognized in colon cancer initiation and progression. Alterations in DNA methylation patterns are the best understood epigenetic cause of the disease which occurs usually via either hypomethylation of global DNA or hypermethylation of tumor suppressor genes 2. DNA methylation is performed by at least two DNA methyltransferases (DNMT; 3a and 3b) and maintained by DNMT1 3. Overexpression of DNMTs has been also detected in a variety of malignancies, including lung, prostate, and colorectal tumors 4.

DNA hypermethylation is also associated with gene silencing and is often observed in CpG islands of cancer-related genes. Transcriptional silencing by the hypermethylation of CpG islands is an early event in tumor progression. In this regard, researchers suggest that hypermethylation of O6-methylguanine-DNA methyltransferase (O6-MGMT) is a pacemaker for certain gene mutations such as K-ras 3. On the other hand, aberrant methylation in genes related to WNT signaling...
pathway has crucial roles for cancer progression especially in CRC. Among specific genes in WNT signaling, SFRP2 methylation is considered as a marker with high sensitivity and specificity in serum and stool for colorectal adenomas and CRC screening. 5-Aza-CR and 5-Aza-CdR potentially inhibit the DNA methyltransferases (DNMTs) \(^{10,11}\). Nowadays, therapeutic targeting via the DNA methylation machinery has been the subject of intensive study. \(^14\)

Recently, natural compounds, such as curcumin, Epigallocatechin Gallate (EGCG) and resveratrol, have been considered to increase sensitivity of cancer cells to conventional agents and induce tumor growth inhibition via epigenetic mechanisms. \(^15\) One of the plants that has recently attracted the attention of researchers is stevia rebaudiana bertoni and its glycoside compounds. Among stevia derivatives, isosteviol and derivative compounds inhibit human cancer cell growth. \(^16\) Furthermore, only isosteviol potently suppresses both mammalian DNA polymerases and human DNA topoisomerase. \(^17,18\) Paul et al also reported that stevioside is a potent inducer of apoptosis via intracellular ROS generation in MCF7 breast cancer cell line. \(^19\) In this regard, the Chinese researcher made a new ent-kaurenoi d derivative of stevioside known as CPUK02. \(^20\) A large number of ent-kaurene diterpenoids isolated from different natural plants have been reported to display anticancer activities. \(^21\) The anticancer activity of CPUK02 was evaluated in vitro and in vivo models. CPUK02 strongly inhibits cell proliferation and induces apoptosis in several human cancer cell lines. Furthermore, CPUK02 demonstrates its anti-tumor effects much better than fluorouracil (5-FU) in mouse xenograft cancer model. \(^22\)

Lack of information about the effects of CPUK02 on epigenetic alteration in colorectal cancer mandates more research in this field. Therefore, the aim of this study was to further elucidate the anti-cancer effects of CPUK02 via epigenetic alteration. In this regard, the level of DNMT3b mRNA expression, MGMT and SFRP2 methylation pattern as representative of epigenetic changes in HCT 116 cell line were assessed.

**Materials and Methods**

The human colon cancer cell line, HCT 116 was obtained from the National cell bank of Iran (Pasteur Institute, IRAN). Cell culture medium, penicillin, streptomycin medium supplement, glutamine and fetal bovine serum were obtained from Gibco Life Technologies (UK). Tripure Isolation Reagent was purchased from Roche Applied Sciences (USA). DNA Synthesis Kit was purchased from Fermentas, EU. SYBR green DNA PCR Master Mix was purchased from the Applied Biosystem (ABI) Company, (Foster City, CA USA). CPUK02 was kindly provided as a gift from Drug Research Institute, China Pharmaceutical University.

**Cell line**

The human colon cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified 5% (v/v) CO\(_2\) incubator.

**Treatment with CPUK02**

Cells were seeded for 12 hr and then washed with PBS and medium was replaced with serum-free RPMI medium. Finally, non-growing, confluent cells were incubated with CPUK02 and 5-aza dissolved in DMSO to a final concentration of 4.5 μM and 1 μM, respectively for 24 hr. Optimum concentrations were determined according to MTT assay. At the end of the incubation, cells were harvested for RNA and DNA isolation.

**Methylation specific-PCR analysis**

DNA was extracted from cells according to the standard phenol/chloroform method. \(^23\) The status of promoter methylation of the MGMT-B, and SFRP2 genes was determined by Methylation-Specific PCR (MSP-PCR). The sequences of primers and annealing temperatures used for amplification of the promoter regions of genes are listed in table 1.

The genes promoter methylation status was determined by chemical treatment of DNA samples with sodium bisulfite and subsequent MS-PCR as previously described. \(^24\) In every reaction, DNA from peripheral blood lymphocytes was considered as a negative control. PCR products were analyzed by electrophoresis on 2% agarose gel.

**RNA extraction and semi-quantitative PCR**

The total RNA was extracted from the treated cells using the TRIZOL Reagent Kit, according to the manufacturer’s instructions. The RNA concentration was quantified by measuring the absorbance at 260 nm in a spectrophotometer. Ratios of absorption (260/280 nm) of all samples were between 1.8 and 2.0. RNA samples were subjected to electrophoresis through a 1.4% agarose formaldehyde gel to verify their integrity. The total RNA samples were stored at -80°C until analysis. The expression of DNMT3b mRNA was determined by semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR). Two μg of total RNA were reverse transcribed by incubation at 37°C for 1 hr in a 25 μl reaction mixture consisting of 100 U M reverse transcriptase, 8 U RNase inhibitor, 0.5 μg of oligo (dT), 50 mmol/L Tris-HCl (pH=8.3), 3 mmol/L MgCl\(_2\), 75 mmol/L KCl, 10 mmol/L DTT, and 0.8 mmol/L each dNTP. The reaction was terminated by heating at 95°C for 5 min and quickly cooling on ice. Two μl of the RT reaction mixture were used for PCR in a final volume of 25 μl, containing 2.5 μl of 10×PCR buffer, 2 μl of each 2.5 mmol/L dNTPs mixture, 1 unit of Taq DNA polymerase, and 10 pmol/L of each forward and reverse primer. The conditions of PCR amplification were as follows: one cycle at 95°C for 5 min, 33 cycles
at 95°C for 30 s, 60°C for 40 s and 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The PCR-amplified fragments were run alongside molecular weight markers on 2% agarose gels stained with gel red. The experiments were repeated twice. The primers and PCR conditions for DNMT3b, GAPDH are listed in Table 2. 232 and 113 bp amplicons were expected upon performing PCR for GAPDH and DNMT3b, respectively.

### Quantitative real-time PCR

Real-time PCR was carried out using the ABI real time PCR 7500 system. The PCR reaction mixture contained 2 μl of cDNA (tenfold diluted), 0.5 μl of 5 mmol/L solutions of each of the forward and reverse primers and 10 μl of SYBR green DNA PCR Master Mix in a total volume of 20 μl. All incubations included an initial denaturation step at 95°C for 10 min and 30 cycles (15 sec at 95°C and 30 sec at 60°C) subsequently. A melting curve analysis was achieved by performing 70 cycles of 10 s with a temperature increment of 0.5°C/cycle starting from 60°C. Efficiency of amplification was measured by the slope of a standard curve. Data were analyzed by using the 7500 Software v2.0.1. The relative expression level (fold changes) of DNMT3b gene was calculated by the 2-ΔΔCT formula. GAPDH was also considered as the internal control. Expression levels of target gene were normalized using the GAPDH as the housekeeping control gene.

### Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post-hoc Dunnett multiple comparison tests using SPSS [Version 16; SPSS, Chicago, IL, USA]. The values of p<0.05 were considered statistically significant.

### Results

#### Methylation of SFRP2 and MGMT genes

The methylated and unmethylated sense/antisense primers for MGMT produced a 127 bp fragments. However, the methylated and unmethylated sense/antisense primers for SFRP2 produced 138 and 145 bp fragments, respectively. In this study, it was observed that CPUK02 has a positive effect on SFRP2 and MGMT methylation pattern in HCT 116 cells. CPUK-02 and 5-aza had a similar effect on the methylation of MGMT gene promoter. As shown in figure 1B, both of them moderately decreased methylated allele. However, according to our results (Figure 1A), 5-aza completely inhibited SFRP2 methylation but CPUK02 moderately did.

#### DNMT3b expression analysis

The presence of appropriate bands for DNMT3b

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Table 1. Primer sequence of SFRP2 and MGMT genes

| Gene  | Primer sequence (5'-3') | Annealing temperature, °C | Product size (bp) |
|-------|-------------------------|---------------------------|------------------|
| MGMT  | MF: GGTCGTTTGTACGTTCGCC MR: TAACCCCTGCACCAGATCAA UF: GTAGGGTTGTGTAATGTGT | 59 | M: 127; U: 127 |
| SFRP2 | MF: TGGGTTTTTTTATTTCTGATGTTTCC MR: CACTAATACCCCGGGCTGCGCG UF: GTTTTGTGGTTTTTTATTTTATTGTAGTTTGT | 59 | M: 138; U: 145 |

Table 2. Primer sequence of DNMT3b and GAPDH genes

| Gene  | Primer sequence (5'-3') | Annealing temperature, °C | Product size (bp) |
|-------|-------------------------|---------------------------|------------------|
| DNMT3b | F: GGCAAGTTCTCCGGAAGGCTCT R: TGGTACATGGCTTTTCGATAGGA | 60 | 113 |
| GAPDH | F: CGACCACCTTTGGAACGCTCA R: AAGGGGTCTACATGGCCAACCTA | 60 | 228 |

Figure 1. Representative example of MSP-PCR for promotor methylation analysis of genes SFRP2 A) and MGMT, B) and in HCT 116 cells. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated genes; the presence of a product in those lanes marked M indicates the presence of methylated genes in control, cells treated with DMSO as vehicle as well as 5-AZA and CPUK02. Unmethylated lymphocytes (lymphocytes) DNA was used as the negative control.
CPUK02 as Inducer of Epigenetic Alteration

113 bp) and GAPDH (228 bp) amplicons was confirmed by semi RT-PCR. Real time PCR results in figure 2 showed that CPUK02 and 5-aza were able to decrease mRNA level of DNMT3b gene in comparison with control group in HCT 116 cells. The average expression levels of DNMT3b in HCT116 treated with CPUK02 and 5-AZA relative to the GAPDH expression level in control were 0.16 and 0.5%, respectively.

**Discussion**

For the first time, the epigenetic role of CPUK02 in HCT 116 cells was shown. CPUK02 as an ent-kaurenoi d compound exhibited strong effect upon DNMT3b mRNA level and methylation status of two important genes; MGMT and SFRP2 in CRC.

Researchers showed that CPUK02 can induce the apoptosis in different cancer cell lines via p53 22. However, there is no data to address the effects of CPUK02 on epigenetic alteration in colorectal cancer. Pierre-Olivier and colleague have found that DNMTs are integral part of the p53 protein network. On the other hand, the levels of DNMTs, especially DNMT3a and DNMT3b, are often increased in various cancer tissues and cell lines such as HCT 116 cells 23. Therefore, this phenomenon may increase hypermethylation of tumor suppressor genes in a variety of malignancies. Although cytosine promoter methylation is accomplished by five mammalian DNMTs (DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L), but in contrast to DNMT1, DNMT3a and DNMT3b are able to do de novo DNA methylation during normal development. DNMT1 is also required for maintenance of methylation patterns 26.

Regarding the importance of DNMT3b, several studies demonstrated that DNMT3b is overexpressed at higher frequency than DNMT3a and DNMT1 in CRC and breast cancer 27-29. In addition, DNMT3b overexpression is associated with CIMP-high phenotype in colorectal cancer 30. Furthermore, direct interaction between Dnmt3a and/or Dnmt3b and transcription factors provides a molecular mechanism which leads to DNA hypermethylation 31.

In this regard, Zhang et al showed that DNMT1, DNMT3b protein level decreased in HCT116 cells treated with DNA-damaging drug doxorubicin as the apoptosis-inducible agent 32. Furthermore, Qiang Li et al showed that 5-aza-2-deoxycytidine inhibited both DNMT1 and DNMT3B protein expression in breast, colon, and other types of cancer cells 33. Our data showed that CPUK02 was also able to decrease mRNA level of DNMT3b gene in HCT 116 cells. So, CPUK02 mimics the role of 5-AZA to decrease mRNA level of DNMT3b. However, considering CPUK02 as an epigenetic drug, further investigation is needed.

In the next part of our study, the effect of CPUK02 on the methylation status of two key genes in colorectal cancer initiation and progression was evaluated. SFRP2 as a negative regulator of Wnt signaling has important implications in tumorigenesis, and its down regulation has been correlated with CRC 34-36. It has been also postulated that lack of MGMT expression increases the spontaneous G:C:A:T mutation rate in tumors in vivo. In addition, MGMT methylation is also a central member in cancer development from normal adenoma to carcinoma 37.

According to the importance of SFRP2 and MGMT role in cancer progression, promoter methylation of these two genes was investigated in HCT 116 treated with CPUK02. It was revealed that CPUK02 and 5-AZA had a similar effect on methylation pattern of MGMT gene. Both of them induced moderate demethylation of MGMT gene. In case of SFRP2, it was shown that 5-aza completely restored unmethylated allele but CPUK02 moderately did.

In this case, the ER-negative breast cells were also treated with demethylating agent 5-aza and CPUK02. As shown in supplement figure, our results showed that CPUK02 like 5-AZA may effectively restore ER aberrant hypermethylation. Thus, inactivation of ER gene has a close relationship with the abnormal methylation of ER gene promoter which can be restored by 5-AZA 38 and probably CPUK02.

However, 5-aza and CPUK02 were not able to completely restore the unmethylated pattern of MGMT.
gene in CRC cell line. In this regard, Wodarz et al revealed that methylation rate of MGMT gene in HCT-116 cell line is slower than methylation rate of SFRP2 gene. Also, they showed that MGMT promoter methylation in HCT 116 cell line started with a delay. Given this finding, it can be concluded that methylation rate of MGMT gene will be slower than SFRP2. Therefore, increasing the incubation time during treatment from 24 hr to 48 might help the unmethylated allele of MGMT to appear. This experiment was performed on HCT 116 cells for longer time and the same results were obtained which are seen in ER-negative breast cells.

Recent researchers showed that epigenetic pharmaceuticals could be a replacement or adjuvant therapy for currently accepted treatment methods such as radiation and chemotherapy, or could sensitize cells to the current treatments. In this regard, although 5-Aza is the most potent demethylating agent in clinical usage, general side effects led to efforts for finding new DNA methylation inhibitors with greater potency and low cytotoxicity.

Recently, natural compounds, such as curcumin, EGCG, and resveratrol, have been shown to increase sensitivity of cancer cells to conventional agents via alteration in epigenetic mechanisms, which may lead to tumor growth inhibition. It has been shown that the epigenetic control of the proto-oncogene regions and the tumor suppressor sequences by conformational changes in histones directly affects the formation and progression of cancer. CPUK02 as a new semi natural compound which exhibits strong anti-cancer effect in in vitro and in vivo models might also manipulate epigenetic pathways and our data confirmed that CPUK02 like 5-AZA exhibits demethylating properties.

Conclusion
According to our data, CPUK02 is a promising molecule with epigenetic/anti-cancer properties and it might be a front-runner candidate for new pharmaceutical targets. However, little information exists about performance of CPUK02 and further investigations are needed to introduce it as a new epigenetic drug.

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Conflict of Interest
Authors declare they have no financial disclosure.

References
1. American Cancer Society [Internet]. USA: ACS. Colorectal Cancer Overview. 2011. Available from: http://www.cancer.org/acs/groups/cid/documents/webcontent/003047-pdf.pdf.
2. Søreide K, Janssen E, Søiland H, Körner H, Baak J. Microsatellite instability in colorectal cancer. Br J Surg 2006; 93(4):395-406.
3. Robertson KD. DNA methylation and chromatin-unraveling the tangled web. Oncogene 2002;21(35):5361-5379.
4. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA 1999;96(15):8681-8686.
5. Mokarram P, Zamani M, Kavousipour S, Naghibalhos-sani F, Iraje C, Moradi Sarabi M, et al. Different patterns of DNA methylation of the two distinct O6-methylguanine-DNA methyltransferase (O6-MGMT) promoter regions in colorectal cancer. Mol Biol Rep 2013;40(5):3851-3857.
6. Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. Nat Rev Cancer 2013;13(1):11-26.
7. Chang E, Park DI, Kim YJ, Kim BK, Park JH, Kim HJ, et al. Detection of colorectal neoplasm using promoter methylation of ITGA4, SFRP2, and p16 in stool samples: a preliminary report in Korean patients. Hepatogastroenterology 2009;57(101):720-727.
8. Zhang X, Song YF, Lu HN, Wang DP, Zhang XS, Huang SL, et al. Combined detection of plasma GATA5 and SFRP2 methylation is a valid noninvasive biomarker for colorectal cancer and adenomas. World J Gastroenterol 2015;21(9):2629-2637.
9. Lu H, Huang S, Zhang X, Wang D, Zhang X, Yuan X, et al. DNA methylation analysis of SFRP2, GATA4/5, NR-RG4 and VIM for the detection of colorectal cancer in fecal DNA. Oncol Lett 2014;8(4):1751-1756.
10. Constantinides PG, Taylor SM, Jones PA. Phenotypic conversion of cultured mouse embryo cells byaza pyrimidine nucleosides. Dev Biol 1978;66(1):57-71.
11. Gabbara S, Bhagwat AS. The mechanism of inhibition of DNA (cytosine-5)-methyltransferases by 5-azacytosine is likely to involve methyl transfer to the inhibitor. Biochem J 1995;307(Pt 1):87-92.
12. Momparler RL, Derse D. Kinetics of phosphorylation of 5-aza-2′-deoxycytidine by deoxycytidine kinase. Biochem Pharmacol 1979;28(8):1443-1444.
13. Santi DV, Norment A, Garrett CE. Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. Proc Natl Acad Sci USA 1984;81(22):6993-6997.
14. Issa JP, Kantarjian HM. Targeting DNA methylation. Clin Cancer Res 2009;15(12):3938-3946.
15. Brown VA, Patel KR, Viskaduraki M, Crowell JA, Perloff M, Booth TD, et al. Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: safety, pharmacokinetics, and effect on the insulin-like growth factor axis. Cancer Res 2010;70(22):9003-9011.
16. Boonkaweewan C, Burudom A. Anti-inflammatory and immunomodulatory activities of stevioside and steviol on colonic epithelial cells. J Sci Food Agric 2013;93(15):3820-3825.
17. Goyal SK, Samshier, Goyal RK. Stevia (Stevia rebaudiana) a bio-sweetener: a review. Int J Food Sci Nutr 2010;61(1):1-10.

18. Koyama E, Sakai N, Ohori Y, Kitazawa K, Izawa O, Kakegawa K, et al. Absorption and metabolism of glycosidic sweeteners of stevia mixture and their aglycone, steviol, in rats and humans. Food Chem Toxicol 2003;41(6):875-883.

19. Paul S, Sengupta S, Bandyopadhyay TK, Bhattacharyya A. Stevioloside induced ROS-mediated apoptosis through mitochondrial pathway in human breast cancer cell line MCF-7. Nutr Cancer 2012;64(7):1087-1094.

20. Li J, Zhang D, Wu X. Synthesis and biological evaluation of novel exo-methylene cyclopentanone tetracyclic diterpenes as antitumor agents. Bioorg Med Chem lett 2011;21(1):130-132.

21. Nagashima F, Kondoh M, Fujii M, Takaoka S, Watanabe Y, Asakawa Y. Novel cytoxic kaurane-type diterpenoids from the New Zealand liverwort Jungermannia Y, Asakawa Y. Novel cytotoxic kaurane-type diterpenoids as antitumor agents. J Nat Prod 2005;61(19):4531-4544.

22. Li Y, Wang K, Zhang DY, Hua WY, Wu XM. A new ent-kaurenoid derivative CPUK02 notably inhibits tumor growth through induction of apoptosis. Chin J Nat Med 2012;10(6):429-435.

23. Sambrook J, Russell DW. Purification of nucleic acids by extraction with phenol: chloroform. CSH Protoc 2006;2006(1). pii: pdb. prot4455.

24. Mokarram P, Zare N, Jafari H, Pour SK, Barazesh M, Naini MA. The methylation status of MGMT and SFRP-2 genes in Iranian patients with polyps. Thtira 2015;4(2):e28601.

25. Estève PO, Chin HG, Pradhan S. Human maintenance DNA (cytosine-5)-methyltransferase and p53 module expression of p53-repressed promoters. Proc Natl Acad Sci USA 2005;102(4):1000-1005.

26. Soejima K, Fang W, Rollins BJ. DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. Oncogene 2003;22(30):4723-4733.

27. Girault I, Tozlu S, Liderace R, Bièche I. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res 2003;9(12):4415-4422.

28. Roll JD, Rivenark AG, Jones WD, Coleman WB. DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. Mol Cancer 2008;7:15.

29. Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Szemegi J, Gonzales FA, et al. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res 1999;27(11):2291-2298.

30. Nosho K, Shima K, Irahara N, Kure S, Baba Y, Kirkner GJ, et al. DNMT3B expression might contribute to CpG island methylator phenotype in colorectal cancer. Clin Cancer Res 2009;15(11):3663-3671.

31. Hervouet E, Vallette FM, Cartron PF. Dnmt3/transcription factor interactions as crucial players in targeted DNA methylation. Epigenetics 2009;4(7):487-499.

32. Zhang Y, Gao Y, Zhang G, Huang S, Dong Z, Kong C, et al. DNMT3a plays a role in switches between doxorubicin-induced senescence and apoptosis of color-ectal cancer cells. Int J Cancer 2011;128(3):551-561.

33. Li Q, Bartlett DL, Gorry MC, O'Malley ME, Guo ZS. Three epigenetic drugs up-regulate homeobox gene Rhox5 in cancer cells through overlapping and distinct molecular mechanisms. Mol Pharmacol 2009;76(5):1072-1081.

34. Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet 2004;36(4):417-422.

35. Chung MT, Lai HC, Sytwu HK, Yan MD, Shih YL, Chang CC, et al. SFRP1 and SFRP2 suppress the transformation and invasion abilities of cervical cancer cells through Wnt signaling pathway. Gynecol Oncol 2009;112(3):646-653.

36. Ying Y, Tao Q. Epigenetic disruption of the WNT/β-catenin signaling pathway in human cancers. Epigenetics 2009;4(5):307-312.

37. Li Y, Lyu Z, Zhao L, Cheng H, Zhu D, Gao Y, et al. Prognostic value of MGMT methylation in colorectal cancer: a meta-analysis and literature review. Tumor Biol 2015;36(3):1595-1601.

38. Wang R, Li LW, Wang RL, Fan QX, Zhao PR, Wang LX, et al. [Demethylation of estrogen receptor gene and its re-expression in estrogen receptor-negative breast]. Zhonghua Zhong Liu Za Zhi 2006;28(12):894-897.

39. Wodarz D, Boland CR, Goel A, Komarova NL. Methyltransferase kinetics and CpG-island methylator phenotype status in colorectal cancer cell lines. Biol Direct 2013;8:14.

40. Yoo CB, Jones PA. Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov 2006;5(1):37-50.

41. Reuter S, Gupta SC, Park B, Goel A, Aggarwal BB. Epigenetic changes induced by curcumin and other natural compounds. Genes Nutr 2011;6(2):93-108.

42. Wang LG, Chiao JW. Prostate cancer chemopreventive activity of phenethyl isothiocyanate through epigenetic regulation (review). Int J Oncol 2010;37(3):533-539.

43. Iglesias-Linares A, Yañez-Vico RM, González-Moles MA. Potential role of HDAC inhibitors in cancer therapy: insights into oral squamous cell carcinoma. Oral Oncol 2010;46(5):323-329.

44. Dowden J, Hong W, Parry RV, Pike RA, Ward SG. Toward the development of potent and selective bisubstrate inhibitors of protein arginine methyltrans feres. Bioorg Med Chem Lett 2010;20(7):2103-2105.