

# 5-Aza-2'-deoxycytidine stress response and apoptosis in prostate cancer

Aditi Patra · Moonmoon Deb · Rajvir Dahiya · Samir Kumar Patra

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**Abstract** While studying on epigenetic regulatory mechanisms (DNA methylation at C-5 of –CpG– cytosine and demethylation of methylated DNA) of certain genes (FAS, CLU, E-cadh, CD44, and Cav-1) associated with prostate cancer development and its better management, we noticed that the used in vivo dose of 5-aza-2'-deoxycytidine (5.0 to 10.0 nM, sufficient to inhibit DNA methyltransferase activity in vitro) helped in the transcription of various genes with known (steroid receptors, AR and ER; ER variants, CD44, CDH1, BRCA1, TGF $\beta$ R1, MMP3, MMP9, and UPA) and unknown (DAZ and Y-chromosome specific) proteins and the respective cells remained healthy in culture. At a moderate dose (20 to 200 nM) of the inhibitor, cells remain growth arrested. Upon subsequent challenge with increased dose (0.5 to 5.0  $\mu$ M) of the inhibitor, we observed that the cellular morphology was changing and led to death of the cells with progress of time. Analyses of DNA and anti-, pro-, and apoptotic factors of the affected cells revealed that the

molecular events that went on are characteristics of programmed cell death (apoptosis).

**Keywords** Epigenome · CpG hypermethylation · DNMT · 5-Aza-2'-deoxycytidine · Apoptosis · Prostate cancer

## Abbreviations

|         |  |
|---------|--|
| DNMT(s) | DNA methyltransferase(s)                 |
| HDAC(s) | Histone deacetylase(s)                   |
| MBD(s)  | Methyl–CpG–DNA binding domain protein(s) |
| CaP     | Cancer of prostate                       |
| AzadC   | 5-Aza-2'-deoxycytidine                   |

## Introduction

Overexpression of DNA (cytosine-5-carbon) methyltransferases and hypermethylation of CpG islands at the regulatory regions of certain genes (for example, ER, CDH1, CD44, Casp3, GSTP1, MDR1, FAS, RASSF1A, P15INK4B, and P16INK4A) and global genome-wide hypomethylation are well known to be associated with prostate and other multiple cancer development and metastasis (Graff et al. 1995; Ferguson et al. 1997; Li et al. 2000; Szyf et al. 2000; Li et al. 2001; Nojima et al. 2001; Patra et al. 2001; Dasari et al. 2002; Jones and Baylin 2002; Patra et al. 2002; Sasaki et al. 2002; Chen et al. 2003; Jaenisch and Bird 2003; Patra et al. 2003; Patra 2008a; Patra et al. 2008a; Patra and Szyf 2008; Patra and Bettuzzi 2009). The CpG sites in these gene promoter regions are rarely methylated in normal cells except in, e.g., inactivated X-chromosome and imprinted genes. Some non-regulatory site CpG island methylation has no direct effect on gene

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A. Patra  
Department of Animal Science, Bidhan Chandra Krishi  
Viswaidyalaya,  
Mohanpur Campus and Kalyani,  
West Bengal, India

M. Deb · S. K. Patra (✉)  
Epigenetics and Cancer Research Laboratory,  
Department of Life Science, National Institute of Technology,  
Rourkela, Orissa, India  
e-mail: skpatra\_99@yahoo.com

S. K. Patra  
e-mail: samirp@nitrrkl.ac.in

R. Dahiya  
Department of Urology, University of California at San Francisco  
and VA Medical Center,  
San Francisco, CA, USA

activity (Patra 2008a; Patra et al. 2008a; Patra and Szyf 2008). It is now revealed that abnormal methylation of CpG islands is not restricted to cancer cells but can also occur during aging and during early stages of tumor development (Patra et al. 2008a). While the co-existence of genome-wide hypomethylation and site-selected hypermethylation are well documented by chemical analyses of bases from total genomic DNA and gene-specific DNA segment, the specific mechanisms at the level of enzymes, co-substrates, and repressor proteins are subject to immense interest of current investigations (Ferguson et al. 1997; Bhattacharya et al. 1999; Cervoni et al. 1999; Ramchandani et al. 1999; Fuks et al. 2000; Ng et al. 2000; Peterson and Logie 2000; Rhee et al. 2000; Szyf et al. 2000; Misteli 2001; Ohki et al. 2001; Patra et al. 2001; Cervoni et al. 2002; Di Croce et al. 2002; Jones and Baylin 2002; Patra et al. 2002, 2003; Pogribny and James 2002; Jaenisch and Bird 2003; Patra 2008a; Patra et al. 2008a; Patra and Szyf 2008; Patra and Bettuzzi 2009).

5-Aza-2'-deoxycytidine (AzadC), when incorporated into the genomic DNA in sites to be occupied by cytosine during replication, is best known as an inhibitor of DNA methyltransferases (DNMTs—DNMT1, DNMT3A, and DNMT3B) through covalent adduct formation with the enzyme (Patra and Bettuzzi 2009). Since the methylation target for the mammalian maintenance methyltransferase, DNMT1, is CpG in hemi-methylated sites, significant inhibition of DNA synthesis, even due to complete lack of repair of DNMT1–AzadC adducts, does not occur for at least two cell cycles. In contrast, DNMT1 becomes bound to DNA and inactivated as soon as AzadC is incorporated into CpG sites opposite to methylated CpG sites on the template strand, i.e., within a few hours of initiating treatment with AzadC. This in turn leads to rapid passive loss of methylation. There is plenty of evidence that treatment of cancers with AzadC leads to reactivation of function in tumor cell lines in which one copy of a gene for a tumor suppressor, a cell cycle regulator, or a DNA repair enzyme is muted and the complementary is normal (wild type) but inactivated by methylation (Graff et al. 1995; Li et al. 2000; Wijermans et al. 2000; Li et al. 2001; Nojima et al. 2001; Christman 2002; Patra et al. 2002; Chen et al. 2003; Claus et al. 2005; Yoo and Jones 2006). This suggests that loss of methylation induced by AzadC treatment can lead to reactivation of the same gene whose inactivation was programmed/selected during development of a specific tumor and has stimulated interest in revisiting the use of AzadC and analogs in therapeutic intervention of cancer (Wijermans et al. 2000; Christman 2002; DeSimone 2004; Claus et al. 2005; Lavelle et al. 2006; Yoo and Jones 2006; Lavelle et al. 2008; Patra and Bettuzzi 2009). But along with expression of desired curative genes (for example, ER, AR, and CDH1), some unwanted genes that

code proteins enhancing metastasis (lipid raft associated genes, UPA and MMPs) could be expressed even at a low non-toxic dose of the drugs, thus creating complications in the use of this chemotherapeutic agent (Sato et al. 2003; DeSimone 2004; Lavelle et al. 2006; Hellebrekers et al. 2007; Patra and Bettuzzi 2009). Since the 1970s, there have been many studies on the toxicity of AzadC. The early studies were mostly devoted to understanding various toxic effects on the basis of physiology and genetics (Christman 2002). The present study was designed to investigate the systematic molecular and epigenetic effects of 5-azadC on the DNMT activity and expression, and on cell growth. The data presented in this work provide strong evidence that the cells died by epigenetic stress-induced apoptosis when challenged with higher dose (~5.0  $\mu$ M, which is far above the DNMT inhibitory concentration) of the drug 5-azadC. The results show that Bcl2 and DNMT1 genes are not repressed; rather, they overexpressed dose dependently of 5-azadC when supplemented with the cell culture media. DNMT1 enzyme activity was reduced, perhaps due to adduct formation with DNA containing 5-azadC replacing cytosine. Gradual increase of 5-azadC in cell culture medium caused dose-dependent overexpression of apoptosis genes like Bak, Bax, and caspase-3, including many other genes in TSUPr1 and DuPro cell lines (Table 3).

## Experimental procedures

### Cell culture and drug(s) treatment

Prostatic cancer cell lines of human origin PC3, TSUPr1, and DuPro were purchased from American Type Culture Collection (Manassas, VA, USA). Cell lines were cultured and maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamate, and 0.1  $\mu$ M of penicillin and streptomycin. Media and supplements were obtained from the University of California at San Francisco Cell Culture Facility. For drug treatment, exponentially growing cells were seeded at a density of  $\sim 10^6$  cells/75 cm<sup>2</sup> flask. After 6 h, the cells were treated at different concentrations (nanomolar to micromolar) of 5-azadC (Sigma Chemical Co., St. Louis, MO, USA) and marked as zero “0” time. Trichostatin A was dissolved in PBS and diluted with the required volume of the medium, and cells were also treated at different concentrations (4, 10, and 50 nM) of MG-132 (Calbiochem, San Diego, CA, USA) inhibitor of proteasome, dissolved in DMSO and diluted with the required volume of medium for 8–12 h prior to harvest for further investigations. All cells were harvested after 96 h unless otherwise mentioned.

## Assays of enzymes

The DNMT enzymatic activity was measured following a previous protocol as standardized in our laboratory (Patra et al. 2001, 2002, 2003). Poly•dI–dC duplex or poly•dG–dC duplex was used as substrate and *S*-adenosylmethionine (tritiated methyl) as methyl group donor. All assays were performed in duplicates in three sets of independent experiments. Background levels were determined in assays in which the template DNA was omitted. Statistical analyses were performed using Student's *t* test.

Caspase-3-like activity was measured according to the kit supplied by Calbiochem. Caspase-3 assay kit employs the colorimetric substrate DEVD-pNA that upon cleavage exhibits increased absorption at 405 nm. A caspase-3 inhibitor (Ac-DEVD-CHO) is also used as a prototypic control inhibitor. The DEVD amino acid sequence is derived from the caspase-3 cleavage site in PARP. All the kit components were thawed and immersed in an ice bath until use. Briefly, caspase-3 inhibitor I was warmed to room temperature. In a separate microcentrifuge tube, it was diluted in assay buffer (1:200). Caspase-3 (15  $\mu$ l, approximately 30 U) diluted in assay buffer (1:50) was used as control. Caspase-3 was not used in assay buffer to blanks. Caspase-3 inhibitor (20  $\mu$ l) was added in triplicate tubes. Reaction was started by adding 200  $\mu$ M (final concentration) caspase-3 substrate I and pre-equilibrated to assay temperature at 25°C and measured in a colorimeter/microplate reader.

Histone deacetylase (HDAC) enzymatic activity was measured using fluorescent amide, *N*-(4-methyl-7-coumarinyl)-*N*- $\alpha$ -(*tert*-butyloxy-carbonyl)-*N*- $\Omega$ -acetyllysineamide (MAL) (Calbiochem) as a potential substrate following the published protocol of ours and other laboratories (Patra et al. 2001; Cervoni et al. 2002) as well as using a radioactive kit (Upstate Biotechnology). The background fluorescence was kept significantly low using filters, and nanomolar (nM) concentration of MAL was used to avoid interference (inner-filter effects). In the presence of trichostatin A (Calbiochem), a potent inhibitor of HDAC (Taunton et al. 1996; Hoffmann et al. 1999), no activity was observed (see also Patra et al. 2001, 2003). Statistical analyses were performed using Student's *t* test.

## Nucleic acid extraction and reverse transcription–PCR

Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Cytosolic and nuclear RNA were prepared using TRI reagent after control lysing of cells and separation by centrifugations of those contents. RNA (1–2  $\mu$ g) was reverse transcribed using random hexamer primers and Superscript II, reverse transcriptase (Life Technologies Inc., Gaithersburg, MD, USA) in a 25- $\mu$ l reaction volume. cDNA was amplified by PCR using primers specific for the genes Bcl2, Bak, Bax, Bcl-X<sub>L</sub>, and caspase-3.  $\beta$ -Actin, glyceraldehyde-3-phosphatedehydrogenase (GAPDH), and histone 4 (H4) genes were also amplified as internal controls to ensure high quality. Primer sequences specific for  $\beta$ -actin gene (TCTACAATGAGCTGCGTGTG, sense; ATTCCTTCTGCATCCTGTC, antisense), GAPDH gene (GAAGGTGAAGGTCGGAGTC, sense; GAAGA TGGTGATGGGATTTTC, antisense), and H4 gene (CAA CATTTCAGGGGCATCACCAA, sense; CCCGAATCA CATTCTCCAAGAA, antisense) were used and resulted in 682-, 226-, and 131-bp products, respectively. The PCR sample mixtures, in a 10- $\mu$ l volume, contained 1 $\times$  PCR buffer (Sigma), 0.2 mM of each dNTP (Sigma), 4 ng of TaqStart antibody (Clonetech, Palo Alto, CA, USA), 0.2  $\mu$ M primers, and 0.5  $\mu$ l RedTaq DNA polymerase (Sigma). PCR reactions were performed in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA) at 94°C for 1 min, 26 cycles at 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s, followed by an extension step at 72°C for 5 min. The PCR products were electrophoresed through a 1.2% agarose gel containing ethidium bromide and were visualized by UV detection.

## Immunocytochemistry

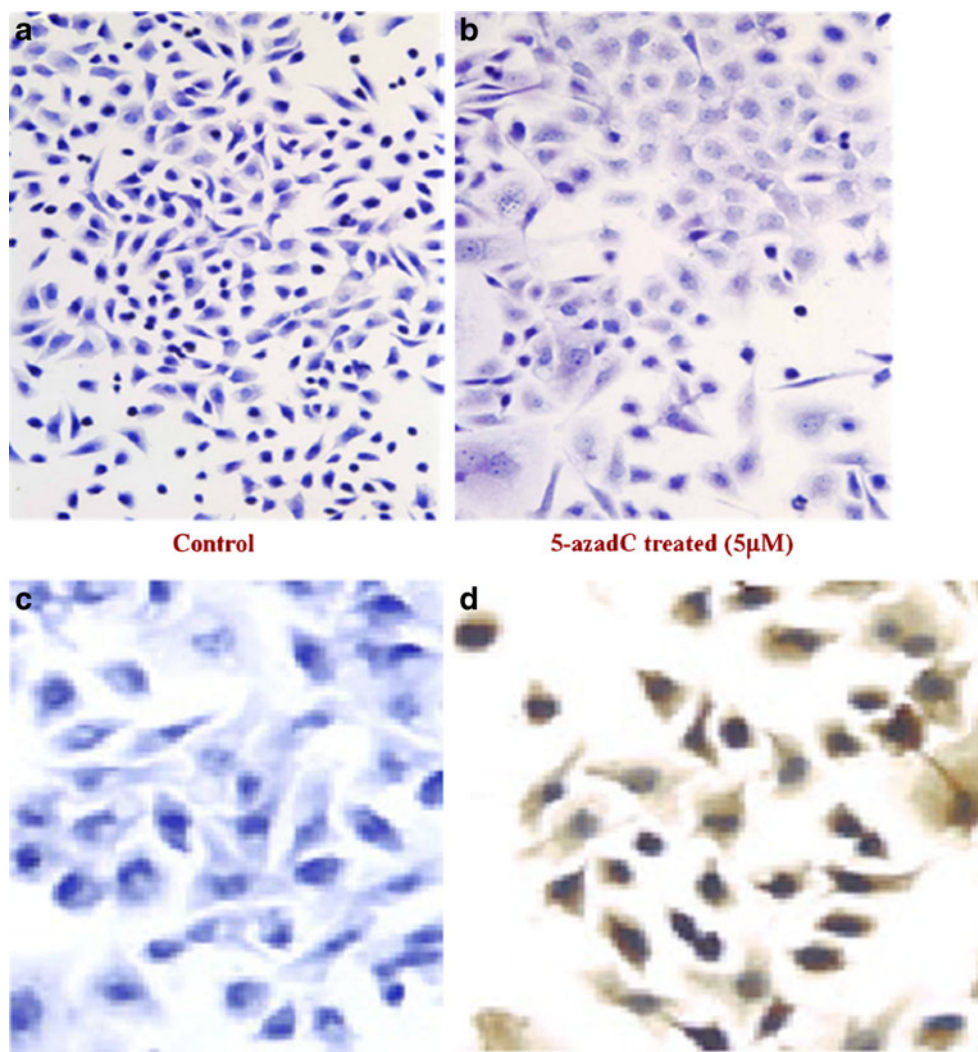
Prostatic cancer cell lines were grown in chamber slides and stained as our standardized protocol (Patra et al. 2001, 2002, 2003). In brief, sub-confluent cells were fixed by 10% formalin and were permeabilized by 0.1% triton X-100 in PBS. The endogenous peroxidase activity was blocked by incubation in 5% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min. The cells were pre-blocked using Ultra V block (Lab

**Table 1** Comparison of % activity (average of three sets of independent experiments) of the enzymes without and with increasing concentration of drug

| Enzymes   | Control | 5-AzadC (nM) |       |      |       |                |                 |                 |                 |
|-----------|---------|--------------|-------|------|-------|----------------|-----------------|-----------------|-----------------|
|           |         | 10           | 20    | 35   | 50    | 75             | 100             | 250             | 500             |
| DNMT      | 100     | 71.23        | 50.36 | 5.14 | 2.32  | –              | –               | –               | –               |
| HDAC      | 100     | 90.62        | 81.07 | nd   | 73.34 | 61.58          | nd              | nd              | nd              |
| Caspase-3 | –       | –            | –     | –    | –     | 2.3 $\pm$ 1.73 | 5.67 $\pm$ 3.46 | 27.5 $\pm$ 8.40 | 41.49 $\pm$ 7.0 |

nd not detected

**Fig. 1** Hematoxylin and eosin (H&E) staining of TSUPr1 cells showing apoptotic damage to the drug treated cells (**a** without 5-azadC; **b** with 5.0  $\mu$ M 5-azadC) and immunocytochemistry of TSUPr1 cells directed against caspase-3 antibodies. Sub-confluent cells were fixed by 10% formalin and were permeabilized by 0.1% triton X-100 in PBS. The endogenous peroxidase activity was blocked by incubation in 5%  $H_2O_2$  in methanol for 20 min. The cells were pre-blocked using Ultra V block (Lab Vision) for 10 min and incubated overnight at 4°C with goat polyclonal antibody against caspase-3. No expression of caspase-3, control (**c**); expression of caspase-3 in 0.5  $\mu$ M 5-azadC-treated cells (**d**)



Vision Corporation, Fremont, CA, USA) for 10 min and incubated for overnight at 4°C with goat polyclonal antibody against caspase-3 (Santa Cruz Biotech, CA, USA) New Zealand white rabbits polyclonal antibody against DNMT1 peptide (I0015801K; Research Genetics, Inc., Huntsville, AL, USA; or met-cat 2) and goat polyclonal antibody against Bcl2, Bak, Bax, BclXs, and MBD1 (Santa Cruz Biotech). The antibody for HDAC1 (1:100), DNMT1 (1:1,000), and others was diluted according to the manufacturer's instructions (and several titration) with primary antibody dilution buffer (Bio-medica, Foster City, CA, USA). The cells were washed in PBS then incubated with anti-goat biotinylated secondary antibody (Santa Cruz Biotech) for 30 min followed by another wash and incubation with HRP-conjugated streptavidin. Finally, reactions were visualized by incubation with DAB (substrate and chromogen) and counterstaining with Harris hematoxylin. For negative control, cells were incubated overnight with dilution buffer (no primary antibody).

#### TUNEL assays

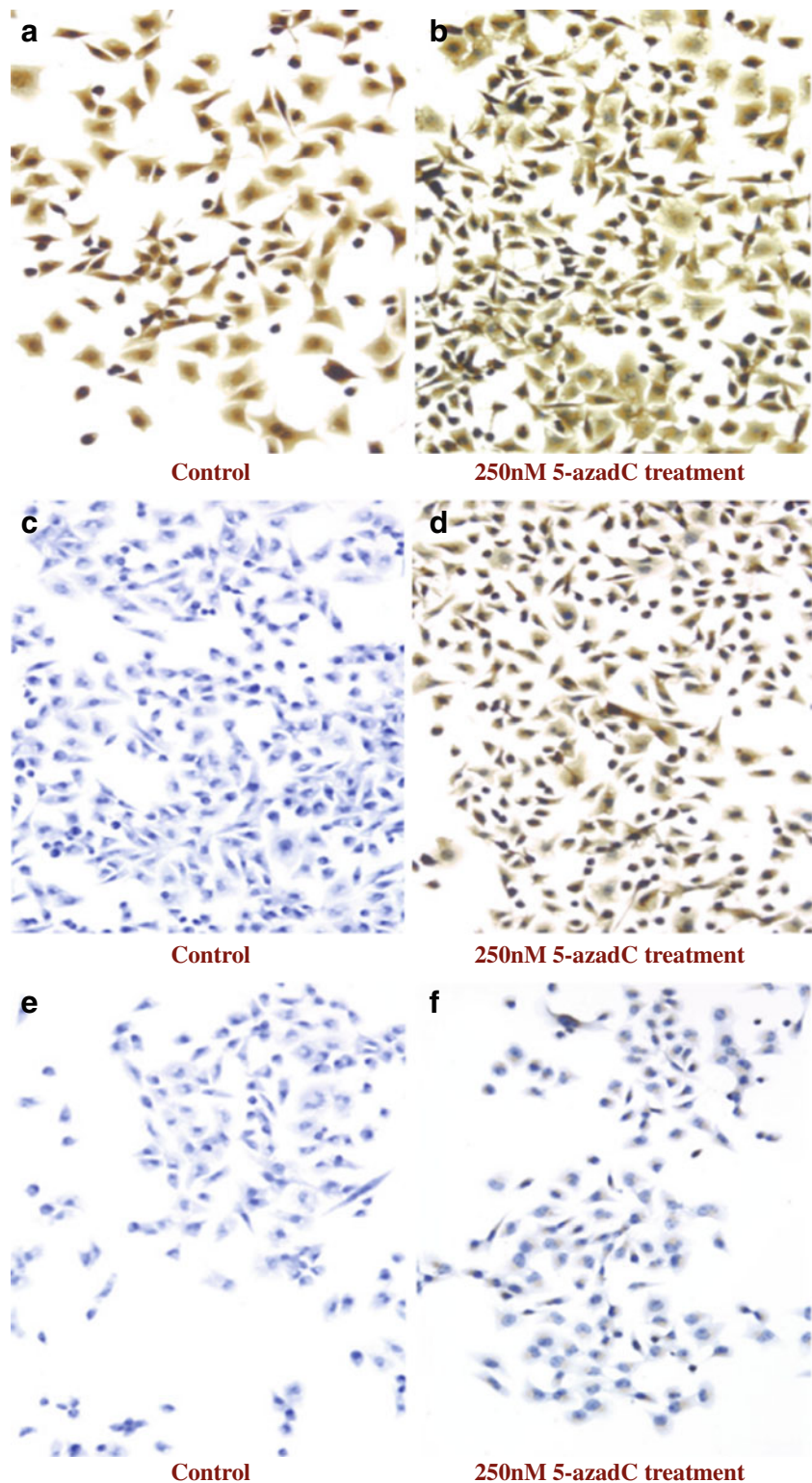
Cells were permeabilized with 0.1% Triton for 2 min, fixed for 10 min in 4% paraformaldehyde in PBS, and stored in 70% ethanol before assay. Slides were equilibrated in terminal transferase (Roche) and incubated for 60 min with TdT (200 U/ml) and biotin-16-dUTP (10  $\mu$ M; Roche). Following two washes with 4 $\times$  SSC, TUNEL-positive cells were detected with avidin–Texas Red (1:100; Jackson).

#### Results

**In vitro DNMTase, HDACs, and caspase-3 activity: inhibition of DNMT activity is not sufficient for induction of caspase-3 activity**

The measurement of DNMTase enzyme activity, using poly (dI–dC) and poly (dG–dC) duplex as substrates, in cancer cell lines (PC3, TSUPr1, and DUPro) and tissues (post-

**Fig. 2** Immunocytochemistry. Sub-confluent cells were fixed by 10% formalin and were permeabilized by 0.1% triton X-100 in PBS. The endogenous peroxidase activity was blocked by incubation in 5% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min. The cells were pre-blocked using Ultra V block (Lab Vision) for 10 min and incubated for overnight at 4°C with respective antibodies. The cells were washed in PBS then incubated with anti-goat biotinylated secondary antibody (Santa Cruz Biotech) for 30 min followed by another wash and incubation with HRP-conjugated streptavidin. Finally, reactions were visualized by incubation with DAB (substrate and chromogen) and counterstaining with Harris hematoxylin. For negative control, cells were incubated overnight with dilution buffer (no primary antibody). Representative examples of expression of Bcl<sub>2</sub> in untreated TSUPr1 (a). In 250 nM 5-azadC-treated TSUPr1 cells, the amount of expression of Bcl<sub>2</sub> was very high (b). Caspase-3: there was no expression in control/untreated TSUPr1 (c). The amount of expression in 5-azadC 250 nM 5-azadC-treated TSUPr1 cells was very high (d). Bax: there was no expression in control/untreated TSUPr1 (e), reasonable in 5-azadC 250 nM 5-azadC-treated TSUPr1 cells (f)



operated tissues obtained from UCSF and VA Medical Center, San Francisco) showed the increased activity in cancer, and it is clear that in prostate cancer expression of DNMT is very high as compared to BPH-1 cell line and BPH tissues. Enzyme activity of HDAC in prostate cancer

is also higher than normal or BPH (Patra et al. 2001, 2003). There was no expression of caspase-3 activity in prostate cancer cell lines. Treatment of cells with 10, 20, 35, 50, 75, 100, 250, and 500 nM 5-azadC respectively supplemented in culture medium abolished the in vitro DNMT activity

**Table 2** Immunocytochemistry results

| Proteins           | Cells                       |        |       |                                 |        |       |
|--------------------|-----------------------------|--------|-------|---------------------------------|--------|-------|
|                    | Control (no drug treatment) |        |       | 5-Aza-2'-deoxycytidine (250 nM) |        |       |
|                    | PC-3                        | TSUPr1 | DuPro | PC3                             | TSUPr1 | DuPro |
| Caspase-3          | –                           | –      | –     | na                              | +++    | +++   |
| Bcl2               | nd                          | ++     | ++    | na                              | +++    | +++   |
| Bak                | nd                          | –      | –     | na                              | ++     | ++    |
| Bax                | nd                          | –      | –     | na                              | +      | +     |
| Bcl-X <sub>S</sub> | nd                          | –      | –     | na                              | ++     | ++    |
| DNMT1              | ++                          | ++     | ++    | na                              | +++    | +++   |
| HDAC1              | ++                          | ++     | ++    | na                              | +      | +     |

+++ High, ++ medium, and + low expression, – no expression, *nd* not detected, *na* not applicable; PC3 cells died at very low dose (35 nM)

around 75 nM and significantly decreased HDAC activity in BPH-1, DuPro, and other cancer cell lines. Although in the TSUPr1 cells the DNMT enzyme activity was blocked at 50/75 nM of 5-azadC, the significant caspase-3 activity was traced around 250 nM (see Table 1 for details).

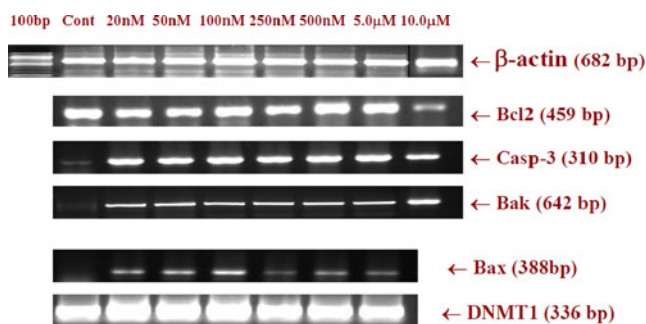
Effect of drugs (5-azadC, DEVD-CHO, and MG-132) on cell growth

The hypomethylating agent 5-azadC is known to block DNMTs activity by forming covalent adducts with the daughter strand in replicating cells/organisms (Patra and Bettuzzi 2009). We found that in culture medium supplemented with 5-azadC at concentrations of 10, 20, 35, 50, 75, and 100 nM, the cells grow well. A gradual decrease in growth rate (i.e., increase in cell doubling time) was observed in all cases beyond 75 nM. All prostate cancer cell lines showed a significantly similar pattern of decrease in enzyme activity and growth rate, except PC3 cell line. The cancer cell line PC3 was found to be highly prone to death at low, 35-nM concentrations of 5-azadC compared to other cancer cell lines (for example, TSUPr1 and DuPro) that needed the drug concentration at 350 to 500 nM range (about 100 times higher) for growth arrest and very high concentrations (1.0–5.0 μM) of drug to undergo death. We

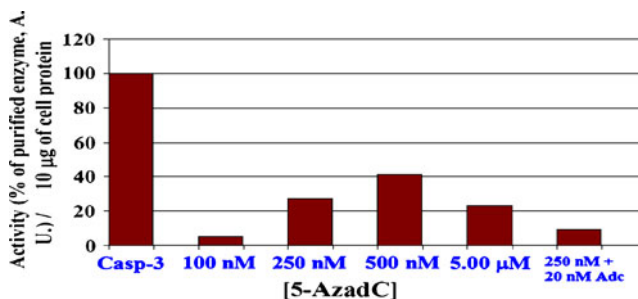
found that the use of 50-nM concentration of 5-azadC in the culture medium of growing cells (thus incorporated into the genomic DNA) completely inactivated the enzyme DNMT activity to methylate either of the substrates poly(dI–dC) and poly(dG–dC) in vitro, but the cells grew well. At higher dose around 250 nM, cells were growth arrested and changes in morphology were observed. The morphology of the cells (Fig. 1a, b and inset) around 250 nM to 5 μM indicates that this change is due to apoptosis induced by the drug. At lower dose (below 50 nM) of MG-132 (proteasome inhibitor) and DEVD-CHO (caspase-3 inhibitor), no effective cell growth retardation or arrest and no change in morphology have been observed.

Inactivation of DNMT1 causes DNA hypomethylation and activation of caspase-3, Bak, Bax, and Bcl-X<sub>L</sub> and over-expression of Bcl<sub>2</sub> and DNMT1

The expression of Bak, Bax, Bcl-X<sub>L</sub>, caspase-3, DNMT1, and HDAC1 as visualized by immunocytochemistry method is represented in Fig. 2a–f and summarized in Table 2. It is clear that the expression of pro-apoptotic factors and caspase-3 was triggered by higher doses of the 5-azadC and



**Fig. 3** RT–PCR: expression of mRNA of Bak, Bax, Bcl2, and caspase-3 at various concentration of 5-azadC. β-Actin gene was used as positive control for this experiment (see “Experimental procedures” for further details)



**Fig. 4** Caspase-3 activity of purified enzyme, and 100, 250, 500 nM, and 5.0 μM 5-azadC-treated TSUPr1 cells lysates, and of 250 nM 5-azadC-treated TSUPr1 cell lysates in presence of caspase-3 inhibitor (Ac-DEVD-CHO). Among different TSUPr1 cell lysates, activity is maximum at 500-nM concentration of 5-azadC. A control (without drug treatment) TSUPr1 cell lysate had no caspase-3 activity and not shown in the figure

**Table 3** Re-expressed genes in prostate cancer cells by application of 5-azadC in culture

| Low-dose (<10 nM) genes          | High-dose (>20 to 500 nM) genes |
|----------------------------------|---------------------------------|
| Steroid receptors (SR—AR and ER) | Bak                             |
| ER variants                      | Bax                             |
| CD44                             | Bcl-X <sub>s</sub>              |
| CDH1                             | Caspase-3                       |
| BRCA1                            | MDR1                            |
| TGFβR1                           |                                 |
| UPA                              |                                 |
| DAZ                              |                                 |
| Y-chromosome specific            |                                 |

the anti-apoptotic factor Bcl<sub>2</sub>, and DNMT1 expressions were increased significantly. This is understandable and can be explained in the way that, as DNA methylation is a post-replicative process and DNMT1 is trapped by DNA at N-5 of 5-azadC, cells produce more and more DNMT1. On the other hand, Bcl<sub>2</sub> overexpression can be explained in the way that anti-apoptotic factor is fighting to keep the cell to strive under the threat imposed by higher concentration of the drug. mRNA expression of DNMT1 and Bcl<sub>2</sub> was not decreased due to 5-azadC treatment up to 500 nM. We also tested the HDAC activity of the 50-nM-drug-treated cell lines and found a 15–20% decrease in cancer cell lines. The decrease in HDAC1 mRNA expression in cancer cell lines by 50 nM 5-azadC was confirmed by RT–PCR analyses. The 5-azadC dose-dependent expression of Bak, Bax, Bcl-X<sub>L</sub>, and caspase-3 mRNA in prostate cancer cell line TSUPr1 is represented in Fig. 3. DuPro cell line also showed similar pattern. The proteasome inhibitor MG-132 at considerably higher concentration (>250 nM) induced cell death by blocking the ubiquitin–proteasome pathway and thus by the accumulation of unwanted protein components with the cytosol which eventually disturbs intracellular trafficking. We found that at reasonably lower concentration up to 50 nM of MG-132, the cell growth was not arrested.

#### Caspase-3 activity

Caspase-3 was induced in PC3, TSUPr1, and DuPro cell lines using 250 nM 5-azadC in culture medium. For drug treatment, exponentially growing cells were seeded at a density of ~10<sup>6</sup> cells/75 cm<sup>2</sup> flask. After 6 h, the cells were treated at different concentrations (nanomolar to micromolar) of 5-azadC and cells were collected for biochemical investigation when they were 90% confluent. Induction of caspase-3 gene and expression of the protein are visualized by RT–PCR and immunocytochemistry, respectively. However,

it did not confirm that caspase-3 was functionally active. To confirm that caspase-3 was active, we measured the enzyme activity of the cellular extracts of cultured TSUPr1 cells at different concentrations of 5-azadC (Fig. 4 and Table 1). DEVD-pNA was used as the substrate and purified caspase-3 (Calbiochem) used as reference/positive control. It is apparent that even complete inhibition of DNMTase activity using AzadC in between 50 and 75 nM in culture (as reflected by in vitro measurement of DNMTase activity) does not result in caspase-3 activity (Table 1). Higher dose (>75 nM) of the drug is necessary for significant expression and activity of caspase-3, and we observed that at 250 nM concentration of 5-azadC the caspase-3 activity is optimum for characterizing apoptotic factors and reaches a maximum around 500 nM of 5-azadC. Use of DEVD-CHO as inhibitor of caspase-3, simultaneously with 5-azadC, prevented the cell death and in vitro caspase activity partially. Hence, our result supports that death was due to caspase-3 activation. TUNEL assay also confirmed that the cells died due to apoptosis (figure not shown).

#### Discussion

Overexpression of active DNA methyltransferase and deregulation of cell proliferation in conjunction with the suppression of apoptosis constitutes a minimal common platform for the initiation and progression of most neoplastic lesions. Cells of the normal adult prostate or those constituting primary prostate cancer are dependent on androgen/estrogen for survival and proliferation. Upon withdrawal of androgen, the rate of apoptosis overtakes the rate of cell proliferation, thereby causing involution of the normal prostate and regression of the tumor. On the other hand, estrogen has a palliative effect on prostate cancer. The essential role for aberrant DNA methylation, especially repression of androgen, estrogen, and progesterone, and many Y-chromosome specific genes associated with CpG-island promoter hypermethylation in the development of prostate cancer, and E-cadherin and CD44 inactivation associated with metastasis, led us to address the requirement for demethylation/hypomethylation induced re-expression and reversal of prostate cancer (Li et al. 2000, 2001; Wijermans et al. 2000; Nojima et al. 2001; Patra et al. 2001, 2002, 2003, 2008a; Dasari et al. 2002; Sasaki et al. 2002; Sato et al. 2003; DeSimone 2004; Lavelle et al. 2006; Yoo and Jones 2006; Hellebrekers et al. 2007; Lavelle et al. 2008; Table 3). In view of this, we had revisited the effects of 5-azadC as potential therapeutic agent for the treatment of prostate cancer. Our results clearly indicate that the enhanced expression of DNA methyltransferase and hyperactivity of the enzyme is associated with the disease prostate cancer (Patra et al.

2001, 2002, 2003). 5-Aza-dC cannot prevent expression of DNMT (Patra and Bettuzzi 2009) or anti-apoptotic factor Bcl<sub>2</sub> but helps in the expression of various steroid receptors like AR, ER, PR and E-cadherin, CD44 tumor suppressor, and cell cycle regulatory and various Y-chromosome-specific genes with unknown protein function (Table 3). The re-expression can lead to remission of the cancer. A higher dose of the drug can induce the expression of apoptotic factors and hence can be used for retardation of tumor growth. Apoptosis is characterized by stereotypic morphological changes like chromatin condensation, cytoplasmic shrinkage, lipid like phosphatidylserine (PS) exposure on cell membrane, membrane rupture and segregation of lipid raft-associated cell surface marker proteins, zeiosis, and formation of apoptotic bodies (Ferguson et al. 1997; Gurumurthy et al. 2001; Jackson-Grusby et al. 2001; Patra and Patra 2003). The biochemical and molecular pathways involved in apoptosis can be approximately grouped as “private” pathways and “common” pathways. The “private” pathways induced by diverse insults converge on a “common” pathway comprising of a set of molecular components collectively known as caspases that activate a cascade of proteolytic events compelling to DNA fragmentation. Among the various insults by chemical agents and UV radiation, induced damage or FasL–FADD induced activation of pro-caspase-8 or p53→Bax→cytochrome *c* induced activation of Apaf-1/caspase 9 ultimately leads to activation of caspase 3 and leads to death (apoptosis) (Gurumurthy et al. 2001; Patra 2008b). We have demonstrated that treatment of prostate cancer cell lines with 5-azadC overexpressed Bcl2 and DNMT1 in prostate cancer cell lines TSUPr1, DuPro, and PC3 dose dependently. The Bcl-2 gene has been implicated in a number of cancers, including melanoma, breast, prostate, and lung carcinomas, as well as schizophrenia and autoimmunity. It is also thought to be involved in resistance to conventional cancer treatment. This supports a role for Bcl2 in decreased apoptosis in the pathogenesis of cancer, including prostate cancer. DNMT1 overexpression in prostate and other cancers have been implicated to repress various cell cycle regulatory genes by promoter methylation and inactivation of genes. However, these two proteins could not prevent apoptosis of the respective cells (TSUPr1, DuPro, and PC3) at higher concentrations of the drug. The morphology of the 5-azadC-treated TSUPr1 and other cells is characteristic for apoptotic cells, a biochemical process initiated by expression of Bak and Bax and finally completely destructed by activated caspase-3 activity. 5-Aza-2'-deoxycytidine, an analog of cytosine base (Wijermans et al. 2000; Christman 2002; Sato et al. 2003; DeSimone 2004; Claus et al. 2005; Lavelle et al. 2006; Yoo and Jones 2006; Hellebrekers et al. 2007; Lavelle et al. 2008; Patra and Bettuzzi 2009), has the potential to kill tumor cells. The genotoxic stress imposed to

the surrounding normal cells and epigenetic reprogramming due to inclusion of 5-azadC in the genome of the cells has bedeviled and questioned its therapeutic use (Ferguson et al. 1997; Ng et al. 2000; Kress et al. 2001; Li et al. 2004; Pakneshan et al. 2004; Jones and Baylin 2007; Patra and Bettuzzi 2007; Zhu and Yao 2007; Patra et al. 2010). Potent inhibitors of DNA methylation with less side effects and toxicity originating from bioflavonoids, including tea catechins and other death-inducing membrane raft orchestrating drugs, are emerging (Fang et al. 2003; Miyaji et al. 2005; Mollinedo and Gajate 2006; Patra and Bettuzzi 2007; Patra 2008b; Patra et al. 2008b; Patra and Szyf 2008).

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## References

- Bhattacharya SK, Ramchandani S, Cervoni N, Szyf M (1999) A mammalian protein with specific demethylase activity for mCpG DNA. *Nature* 397:579–583
- Cervoni N, Bhattacharya S, Szyf M (1999) DNA demethylase is a processive enzyme. *J Biol Chem* 274:8363–8366
- Cervoni N, Detich SS-B, Chakravarti D, Szyf M (2002) The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. *J Biol Chem* 277:25026–25031
- Chen H, Toyooka S, Gazdar AF, Hsieh JT (2003) Epigenetic regulation of a novel tumor suppressor gene (hDAB2IP) in prostate cancer cell lines. *J Biol Chem* 278(5):3121–3130
- Christman JK (2002) 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 21:5483–5495
- Claus R, Almstedt M, Lubbert M (2005) Epigenetic treatment of hematopoietic malignancies: in vivo targets of demethylating agents. *Semin Oncol* 32:511–520
- Dasari VK, Deng D, Perinchery G, Yeh CC, Dahiya R (2002) DNA methylation regulates the expression of Y chromosome specific genes in prostate cancer. *J Urol* 167(1):335–338
- DeSimone J (2004) The evolving role of DNA demethylating agents in the treatment of sickle cell disease and thalassemia. *Semin Hematol* 41:1–3
- Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M, Faretta M, Fuks F, Lo Coco F, Kouzarides T, Nervi C, Minucci S, Pelicci PG (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* 295:1079–1082
- Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, Welsh W, Yang CS (2003) Tea polyphenol Epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res* 63:7563–7570



- Ferguson AT, Vertino PM, Spitzner JR, Baylin SB, Muller MT, Davidson NE (1997) Role of estrogen receptor gene demethylation and DNA methyltransferase–DNA adduct formation in 5-aza-2'-deoxycytidine induced cytotoxicity in human breast cancer cells. *J Biol Chem* 272:32260–32266
- Fuks F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T (2000) DNA methyltransferase DNMT1 associates with histone deacetylase activity. *Nat Genet* 24:88–91
- Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, Pitha PM, Davidson NE, Baylin SB (1995) E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* 55:5195–5199
- Gurumurthy S, Basudevan KM, Rangnekar VM (2001) Regulation of apoptosis in prostate cancer. *Cancer Metastasis Rev* 20:225–243
- Hellebrekers DM, Melotte V, Viré E, Langenkamp E, Molema G, Fuks F, Herman JG, Van Criekinge W, Griffioen AW, van Engeland M (2007) Identification of epigenetically silenced genes in tumor endothelial cells. *Cancer Res* 67:4138–4148
- Hoffmann K, Brosch G, Loidl P, Jung M (1999) A non-isotopic assay for histone deacetylase activity. *Nucleic Acids Res* 27:2057–2058
- Jackson-Grusby L, Beard C, Possemato R, Tudor M, Fambrough D, Csankovszki G, Dausman J, Lee P, Wilson C, Lander E, Jaenisch R (2001) Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. *Nat Genet* 27:31–39
- Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 33(Suppl):245–254
- Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 3(6):415–428
- Jones PA, Baylin SB (2007) The epigenomics of cancer. *Cell* 128:683–692
- Kress C, Thomassin H, Grange T (2001) Local DNA demethylation in vertebrates: how could it be performed and targeted? *FEBS Lett* 494:135–140
- Lavelle D, Vaitkus K, Hankewych M, Singh M, DeSimone J (2006) Effect of 5-aza-2'-deoxycytidine (Dacogen) on covalent histone modifications of chromatin associated with the epsilon-gamma- and beta-globin promoters in *Papio anubis*. *Exp Hematol* 34:339–347
- Lavelle D, Sauntharajah Y, Desimone J (2008) DNA methylation and mechanism of action of 5-azacytidine. *Blood* 111:2485
- Li LC, Chui R, Nakajima K, Oh BR, Au HC, Dahiya R (2000) Frequent methylation of estrogen receptor in prostate cancer: correlation with tumor progression. *Cancer Res* 60(3):702–706
- Li LC, Zhao H, Nakajima K, Oh BR, Filho LA, Carroll P, Dahiya R (2001) Methylation of the E-cadherin gene promoter correlates with progression of prostate cancer. *J Urol* 166(2):705–709
- Li LC, Okino ST, Dahiya R (2004) DNA methylation in prostate cancer (review). *Biochim Biophys Acta* 1704:87–102
- Misteli T (2001) Protein dynamics: implications for nuclear architecture and gene expression. *Science* 291:843–847
- Miyaji M, Jin ZX, Yamaoka S et al (2005) Role of membrane sphingomyelin and ceramide in platform formation for Fas-mediated apoptosis. *J Exp Med* 202:249–259
- Mollinedo F, Gajate C (2006) Fas/CD95 death receptor and lipid rafts: new targets for apoptosis-directed cancer therapy. *Drug Resist Updat* 9:51–73
- Ng H-H, Jeppesen P, Bird A (2000) Active repression of methylated genes by the chromosomal protein MBD1. *Mol Cell Biol* 20:1394–1406
- Nojima D, Li LC, Dharia A, Perinchery G, Ribeiro-Filho L, Yen TS, Dahiya R (2001) CpG hypermethylation of the promoter region inactivates the estrogen receptor-beta gene in patients with prostate carcinoma. *Cancer* 92(8):2076–2083
- Ohki I, Shimotake N, Fujita N, Jee J-G, Ikegami T, Nakao M, Shirakawa M (2001) Solution structure of the methyl-CpG binding domain of human MBD1 in complex with methylated DNA. *Cell* 105:487–497
- Pakneshan P, Szyf M, Farias-Eisner R, Rabbani SA (2004) Reversal of the hypomethylation status of urokinase (uPA) promoter blocks breast cancer growth and metastasis. *J Biol Chem* 279:31735–31744
- Patra SK (2008a) Ras regulation of DNA methylation and cancer. *Exp Cell Res* 314:1193–1201
- Patra SK (2008b) Dissecting lipid raft facilitated cell signaling pathways in cancer. *Biochim Biophys Acta* 1785:182–206
- Patra SK, Bettuzzi S (2007) Epigenetic DNA methylation regulation of genes coding for lipid raft-associated components: a role for raft proteins in cell transformation and cancer progression (review). *Oncol Rep* 17:1279–1290
- Patra SK, Bettuzzi S (2009) Epigenetic DNA-(cytosine-5-carbon) modifications: 5-aza-2'-deoxycytidine and DNA-demethylation. *Biochem (Mosc)* 74(6):613–619
- Patra SK, Patra A (2003) Lipid rafts in cancer metastasis—a working hypothesis. *Proc Am Assoc Cancer Res* 44:64–65 (1st edition, March 2003)
- Patra SK, Szyf M (2008) DNA methylation mediated nucleosome dynamics and oncogenic Ras signaling: insights from FAS, FASL and RASSF1A. *FEBS J* 275:5217–5235
- Patra SK, Patra A, Dahiya R (2001) Histone deacetylase and DNA methyltransferase in human prostate cancer. *Biochem Biophys Res Commun* 287:705–713
- Patra SK, Patra A, Zhao H, Dahiya R (2002) DNA methyltransferase and demethylase in human prostate cancer. *Mol Carcinog* 33:163–167
- Patra SK, Patra A, Zhao H, Carroll P, Dahiya R (2003) Methyl-CpG-DNA binding proteins in human prostate cancer: expression of CXXC sequence containing MBD1 and repression of MBD2 and MeCP2. *Biochem Biophys Res Commun* 302:759–766
- Patra SK, Patra A, Rizzi F, Ghosh TC, Bettuzzi S (2008a) Demethylation of (cytosine-5-C-methyl) DNA and regulation of transcription in the epigenetic pathways of cancer development. *Cancer Metast Rev* 27:315–334
- Patra SK, Rizzi F, Silva A, Rugina DO, Bettuzzi S (2008b) Molecular targets of (–)-epigallocatechin-3-gallate (EGCG): specificity and interaction with membrane lipid rafts. *J Physiol Pharmacol* 59 (Suppl 9):217–235
- Patra SK, Deb M, Patra A (2010) Molecular marks for epigenetic identification of developmental and cancer stem cells. *Clin Epigenet*. doi:10.1007/s13148-010-0016-0
- Peterson CL, Logie C (2000) Recruitment of chromatin remodeling machines. *J Cell Biochem* 78:179–185
- Pogribny IP, James SJ (2002) De novo methylation of the p16INK4A gene in early preneoplastic liver and tumors induced by folate/methyl deficiency in rats. *Cancer Lett* 187:69–75
- Ramchandani S, Bhattacharya SK, Cervoni N, Szyf M (1999) DNA methylation is a reversible biological signal. *Proc Natl Acad Sci USA* 96:6107–6112
- Rhee I, Jair K-W, Yen R-WC, Lengauer C, Herman JG, Kinzler KW, Vogelstein B, Baylin SB, Schuebel KE (2000) CpG methylation is maintained in human cancer cells lacking DNMT1. *Nature* 404:1003–1007
- Sasaki M, Tanaka Y, Perinchery G, Dharia A, Kotcherguina I, Fujimoto S, Dahiya R (2002) Methylation and inactivation of estrogen, progesterone, and androgen receptors in prostate cancer. *J Natl Cancer Inst* 94(5):384–390
- Sato N, Maehara N, Su GH, Goggins M (2003) Effects of 5-aza-2'-deoxycytidine on matrix metalloproteinase expression and pancreatic cancer cell invasiveness. *J Natl Cancer Inst* 95:327–330

- Szyf M, Knox DJ, Milutinovic S, Slack AD, Araujo FD (2000) How does DNA methyltransferase cause oncogenic transformation? *Ann NY Acad Sci* 910:156–174
- Taunton J, Hassing CA, Schreiber SL (1996) A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science* 272:408–411
- Wijermans P, Lübbert M, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A (2000) 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* 18:956–965
- Yoo CB, Jones PA (2006) Epigenetic therapy of cancer: past, present and future. *Nat Rev Drug Discov* 5:37–50
- Zhu J, Yao X (2007) Use of DNA methylation for cancer detection and molecular classification. *J Biochem Mol Biol* 40:135–141