PROGRESS IN BIOLOGICAL SCIENCES

Study of promoter CpG island hypermethylation of cyclin-dependent kinase inhibitor gene *p21*$^{waf1/cip1}$ on some breast carcinoma cell lines

Mohsen Alipour[1], Seyed Jalal Zargar[1, *], Shahrokh Safarian[1], Shamileh Fouladdel [2], Ebrahim Azizi [2, 3], Naser Jafargholizadeh [1]

[1]Department of Cell & Molecular Biology, School of Biology, College of Science, University of Tehran, Tehran, Iran.
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
[3]Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran.

Received: November 5, 2012; Accepted: January 30, 2013

Abstract

The *p21* belongs to the CIP/KIP family of CDK inhibitors involved in cell cycle arrest at specific stages of the cell cycle progression. DNA methylation is the best studied epigenetic mark that have been evidently associated to chromatin condensation, and repression of gene transcription. The CpG island hypermethylation in promoter region of certain genes occurs in cancer cells and affects tumorigenesis. The aim of the current study was to assess DNA methylation pattern of *p21* gene promoter region in the MCF7, T47D, MDA-MB-231 and MDA-MB-468 human breast carcinoma cell lines. The methylation status of cancer associated gene, *p21*$^{waf1/cip1}$ was analyzed at CpG sites in the promoter region using a sensitive methylation-specific PCR (MSP) technique. The total genomic DNA from each cell line was isolated and subjected to the sodium bisulfite treatment to differentiate between methylated and unmethylated CpG islands. Then MSP was performed using designed primers for methylated (M-MSP) and unmethylated (U-MSP) forms of CpG islands in the promoter region of *p21* gene. The results of the MSP indicated that promoter of *p21* gene was consistently unmethylated in tested human breast cancer cell lines. Therefore, methylation inactivation of the *p21*$^{waf1/cip1}$ does not commonly happen in all cancer cell lines.

Keywords: *p21*$^{waf1/cip1}$, Tumor suppressor gene, DNA methylation, MSP, Breast cancer

Introduction

Breast cancer (BC) is the most common malignancy in females worldwide. BC is a heterogeneous disease that results from the presence of multiple genetic and epigenetic alterations involved in different cellular pathways (1). To develop novel strategies for cancer therapy, it is required to identify molecular mechanisms involved in pathways that are differentially regulated in normal and cancer cells. DNA methylation is one of the most common epigenetic events that occurs almost exclusively at cytosine residues within the symmetric dinucleotide CpGs, and has crucial roles in the regulation of gene activity (2). Aberrant methylation of normally unmethylated CpG islands in the promoter regions of tumor suppressor genes is a major event in the origin of various human malignancies including breast cancer (3). It serves as an alternative mechanism (in addition to mutations or deletions) for gene transcription silencing, via changes in chromatin conformation and can provide selective advantages to cancer cells (4). In recent years, the number of tumor suppressor genes that are inactivated by CpG-island methylation rather than classic genetic mutation inactivation events has increased.

Cellular proliferation is mediated by the control of cell cycle progression (i.e. the progression through

* Corresponding authorzargar@ut.ac.ir; zargar@khayam.ut.ac.ir
Tel.: +98-21- 61113646

G1, S, G2 and M phases), which involves the periodic activation and inactivation of cyclin-dependent kinase complexes (CDKs). CDKs are activated through association with positive regulators (cyclins) and inactivated by CDK inhibitors (5). *p21* [*CDKN1A* (cyclin-dependent kinase inhibitor 1A)/cip1/waf1] is a potent tumor suppressor gene that is located on chromosome 6q21, and is comprised of four exons. The *p21*waf1/cip1 belongs to the cip and kip family of CDK inhibitors that mediates a broad spectrum of biological activities primarily by binding to and inhibiting CDKs activity, leading to cell cycle arrest at multiple phases of cell cycle progression, both in unstressed cells and after genotoxic stresses. Though best known for its cell cycle-inhibitory functions, *p21* also protects cells from apoptosis, which might account for its inconsistent oncogenic activities (6). In addition, *p21*waf1/cip1 by binding to PCNA , proliferating cell nuclear antigen , and several other factors involved in process of DNA synthesis such as DNA methyl-transferase (DNMT) interferes with DNA polymerase δ activity, and as a result it directly inhibits DNA synthesis. It is also involved in DNA repair processes (7).

Transcription of the *p21*waf1/cip1 gene can be induced by the wild-type p53, following oncogenic or genotoxic stress (8). The *p21* knock-out mice developed a variety of tumors, including sarcomas and lymphomas (9). It may also regulate the transcription of the genes involved in growth arrest, senescence, aging, or apoptosis after DNA damage (10). Many studies have shown that *p21*waf1/cip1 is often epigenetically silenced in a variety of human neoplastic cell lines (11, 12). For instance, histone deacetylation could be a good candidate to the gene inactivation, as has been shown in many solid tumors (13), because using histone deacetylase (HDAC) inhibitors leads to activation of *p21* transcription (14). Additionally, a recent study has shown that although this gene is hypermethylated in some human lung cancer cell lines, its hypermethylation is rare in tumors in general and the gene is unmethylated in most types of human cancers (15). On the other hand, an inverse correlation of *p21*waf1/cip1 expression and DNMT levels was found between ER-positive, T47D and MCF-7, and ER-negative, MBD-MA-231 and MDA-MB-468, breast cancer cell lines as previously reported (16). In the present study, we aimed to test the relationship of CpG island hypermethylation and changes in expression of *p21*waf1/cip1 gene in ER-positive, T47D and MCF-7, and ER-negative, MBD-MA-231 and MDA-MB-468, breast carcinoma cell lines by methylation specific polymerase chain reaction (MSP). *p21*waf1/cip1 expression level in these cells have previously been established. Here, we assess methylation levels in the promoter region of the gene.

Materials and methods

cell lines and cell culture

Human breast carcinoma cell lines T47D, MCF7, MDA-MB-231 and MDA-MB-468 were obtained from the National Cell Bank (Pasteur Institute of Tehran, Iran) and routinely grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and in a 37°C, humidified incubator under 5% CO_2.

Genomic DNA isolation and sodium bisulfite modification

The total genomic DNA from each cell line was isolated using Qiagen Genomic DNA Isolation Kit (Qiagen Inc. FlexiGene DNA kit, Germany) and was used immediately or stored at -20°C. The sodium bisulfite treatment was performed using the Imprint DNA Modification Kit (Sigma-Aldrich Inc., UK) according to the manufacturer's protocol. Bisulfite treatment was used to differentiate between methylated and unmethylated DNA sequences and it was followed by MSP as described by Herman et al. (17). In addition, total genomic DNA from human peripheral white blood cells (WBCs) was isolated and treated *in vitro* with excess bacterial enzymes *Sss*I methyltransferase (New England Biolabs, UK), to generate completely methylated DNA at all CpGs to be used as positive control for methylated alleles of each gene. The untreated DNA from human WBCs was also modified with sodium bisulfite to be used as an unmethylated control.

Methylation specific PCR assay

The MSP for the analysis of *p21*$^{waf1/cip1}$ gene promoter methylation was carried out as described in detail previously (18). Briefly, DNA methylation patterns in the CpG island , -227 to +1294 relative to transcription start site, existing in promoter region of the *p21* gene were determined by chemical modification of unmethylated but not the methylated cytosines to uracil and subsequent PCR using primers specific for either methylated or unmethylated DNA. MSP was performed in a thermal cycler (Verriti, ABI, USA) with the following cycling conditions: The amplifications consisted of a denaturation step at 95°C for 5 min followed by 40 amplification cycles (95°C for 30 s, annealing temperature for 45 s, and 72°C for 30 s) and a final incubation at 72°C for 5 min. The PCR mixture contained 100-200 ng of bisulfite-treated DNA, 1 × Sinagen PCR Buffer, 1.5 mM MgCl, 0.2 mM of each dNTP, 10 pmol of each primer set, and one unit of Taq (Cinnagen Inc., Tehran, Iran) in a final volume of 25 μl. Amplified products were electrophoresed on 2.5% agarose gels and visualized by ethidium bromide staining. All MSP assays were repeated at least twice. The primer sequences for MSP of *p21* gene are listed in Table 1. Methylated CpG-specific primers amplify 171 bp fragment spanning nucleotides -139 to +32, with respect to transcription initiation site. Unmethylated CpG-specific primers amplify 161 bp fragment spanning nucleotides -125 to +36, with respect to transcription initiation site (Table 1).

Table 1. Primer sequences for *p21* promoter region.

Primer	Sequence (5′→3′)*
Forward	TACGCGAGGTTTCGGGATC $^{\alpha}$
Reverse	CCCTAATAT ACAACCGCCCCG $^{\alpha}$
Forward	GGATTGGTTGGTTTGTTGGAATTT $^{\beta}$
Reverse	ACAACCCTAATATACAACCACCCCA $^{\beta}$

*Gen Bank accession no: NC_000006.11; α: methylated CpG-specific primers; β: unmethylated CpG-specific primers.

Results

Genomic DNA sequence from *p21* gene was downloaded from public databases available and subjected to CpG island discovery software. A typical CpG island in promoter of *p21*$^{waf1/cip1}$ gene showing ≥60% CpG content and an observed vs. expected CpG frequency of ≥0.65 was detected using the CpGPlot/CpGreport website (http://www.ebi.ac.uk/emboss/cpgplot/) with a high probability of being subjected to epigenetic control. This CpG island largely spans the core promoter, exon1 and 5′end of intron 1. The CpG islands in promoter regions of genes represent a major target for DNA hypermethylation. A DNA region with an observed/expected CpG ratio of equal or more than 0.65 and a GC content of equal or more than 60% is considered a CpG island (19). Our results demonstrated that, the *CDKN1A* proximal promoter, -214 to +20 relative to transcription start site, partially overlaps with the CpG island (Fig. 1).

Next, we investigated whether the observed down-regulation of *p21*$^{waf1/cip1}$ expression in the four breast carcinoma cell lines T47D, MCF7, MDA-MB-231 and MDA-MB-468 may possibly be due to an increased methylation of the *p21*$^{waf1/cip1}$ promoter region, considering the CpG island which exists in that region. Thus, MSP method was used to determine the methylation status of *p21*$^{waf1/cip1}$ promoter in the four breast carcinoma cell lines. In this assay, bisulfite modification results in a C to U conversion when the C is unmethylated in the context of a CpG dinucleotide. However, when the cytosine is methylated, bisulfite modification leaves the methylated cytosine unchanged. Therefore, after bisulfite modifications, methyl-ation specific and unmethylation-specific primers were used in MSP. Primer sets were designed to specifically amplify either methylated (M) or unmethylated (U) bisulfite-modified sequences in the *p21* promoter-associated CpG islands and promoter methylation status was detected by PCR. Amplification with either the methylated or unmethylated set of primers results in either the presence or absence of a PCR product depending on the methylation status of the CpG dinucleotides interrogated by that primer pair. As shown in Figure 2, MSP revealed the investigated CpG dinucleotides to be unmethylated in all breast carcinoma cell lines. This result is illustrated by no methylated *p21*$^{waf1/cip1}$ band of MSP products in all breast carcinoma cell lines (Fig. 2).

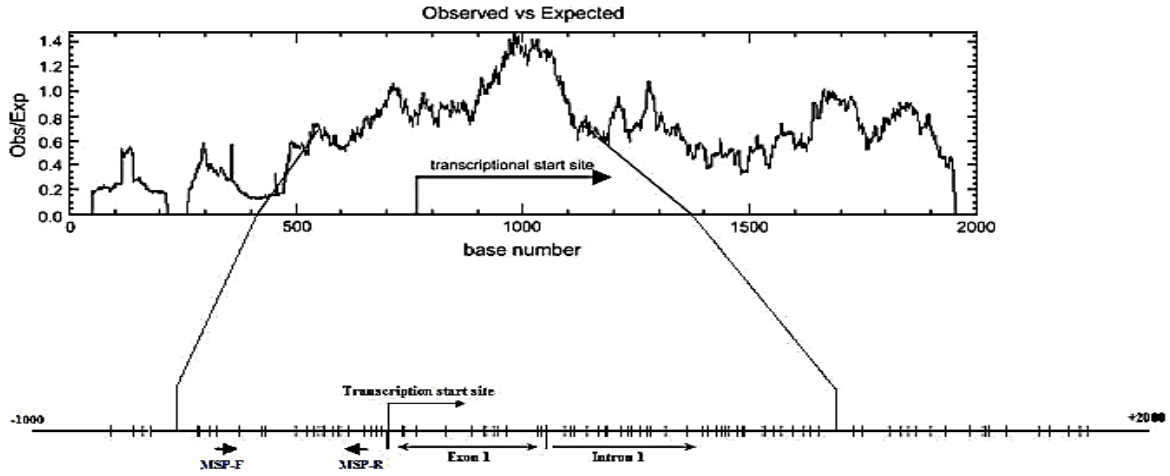

Figure 1. Map of the CpG island consisting promoter, exon 1 and 5′ end of intron 1 regions of *p21*waf1/cip1 gene. MSP-F and MSP-R primers anneal near transcription start site. Each vertical mark indicates a CpG pair. The transcription start site is indicated by the bent arrow.

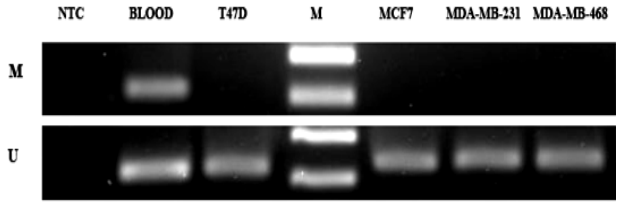

Figure 2. Gel electrophoresis analysis of methylation specific PCR amplifications showing methylation status of *p21*$^{waf1/cip1}$ gene promoter in various breast cancer cell lines (T47D, MCF7, MDA-MB-231 and MDA-MB-468). In panel M, DNA was amplified using primers specific for methylated status. In panel U, DNA was amplified with primers specific for unmethylated status.

Discussion

The cell cycle is notably conserved in different living organisms and is controlled by Cyclin-dependent kinases (CDKs). CDK function can be inhibited by binding of CDK inhibitory proteins, referred to as CKIs (20). The CDKs are negatively regulated by the activity of CDKIs through direct interactions. CDKIs are divided into two major families: the INK4 (inhibitor of CDK4) family consisting of p16^{INK4a}, p15^{ink4b}, p18 ^{ink4c} and p19 ^{ink4d} and the cip/kip, kinase inhibitor protein, family including p21$^{cip1/waf1}$, p27 ^{Kip1} and p57 ^{kip2} (21). The *p21* is a tumor suppressor gene with multiple functions that is encoded by CDKN1A gene, and promotes mammalian cell cycle arrest in response to various stimuli.

There are reports of both genetic and epigenetic modulations of tumor suppressor genes (TSGs) during tumor formation, and hypermethylation of CpG islands is accepted as a frequent mechanism for loss of TSG activity in human cancers (22). Thus, information about methylation patterns throughout the genome can help to recognize key TSGs which are inactivated in human tumorigenesis. Several reports have been published about abnormal hypermethylation of CpG islands in the promoter region of TSG genes involved in DNA repair, cell cycle control, cell-cell adhesion and apoptosis in breast cancer, which is associated with the transcriptional silencing of these genes (23).

A large amount of our understanding about the role of *p21* in cancer is from knockout mouse studies integrated with biochemical and functional analysis of cells in culture (6). In contrast to other CDKI genes, approximately no point mutations and deletions in coding region of *p21*$^{waf1/cip1}$ have been reported in tumor cells so far (24). Extensive screening of hundreds of various tumors has revealed, this phenomenon occurs very rarely in human cancers. However, a role for deletions and loss of expression of *p21*$^{waf1/cip1}$ in aggressive lymphomas has been proposed (25), but others have been unsuccessful to identify mutations of this gene in a large series of human cancers (26).

Although, the actual role of the p21$^{CIP1/WAF1}$ protein in the development and progression of breast cancer is not completely known, but it is possible

that the transcriptional silencing of CDKN1A by MYC plays an important role in the development of ER-positive breast tumors in which estrogen-dependent overexpression of myc and the following downregulation of *p21* promotes cell proliferation (27). *p21* expression was reported to be relatively low in different tumors. Therefore, it has been postulated that the gene is repressed via epigenetic alterations (28). However, investigation of many of solid tumors for *p21*$^{waf1/cip1}$ promoter methylation have shown variable, and in some cases, controversial results (13, 29, 30).

It had been previously demonstrated that most ER negative breast cancer cell lines (e.g. MDA-MB-231, MDA-MB-468) expressed little or no *p21*$^{waf1/cip1}$ gene, while expression levels were quite high in most ER-positive cell lines (e.g. T47D, MCF7) and these cell lines show differential expression of p21$^{WAF1/CIP1}$ proteins (15). The cell cycle inhibitor p21 can disrupt the connection between DNMT and PCNA, possibly disturbing the activity of these two important proteins and interfering with the DNA replication process (6). Moreover, it was found that DNMT protein level was inversely associated with the level of p21$^{WAF1/CIP1}$ in breast cancer cells. These observations, and the lack of p21$^{WAF1/CIP1}$ protein expression in selected breast carcinoma cell lines, led us to evaluate whether the lack of *p21*$^{waf1/cip1}$ expression could be the result of methylation of its promoter region. Therefore, we examined the hypermethylation status of *p21*$^{waf1/cip1}$ gene in four breast carcinoma-derived cell lines T47D, MCF7, MDA-MB-231 and MDA-MB-468. As shown in Fig. 2, MSP showed unmethylation and there was no level of *p21*$^{waf1/cip1}$ promoter methylation in any of the selected breast carcinoma cell lines. Thus aberrant CpG island hypermethylation of promoter regions of *p21*$^{waf1/cip1}$ gene sequence is not the most common mechanism for down-regulation or silencing of the gene in selected breast cancer cell lines. Though, our study still does not rule out the likelihood of epigenetic suppression of this gene, through methylation-independent mechanisms such as chromatin structure modifications, since several histone deacetylase inhibitors such as trichostalin A can also activate *p21* expression. In summary, in this study, we analyzed the methylation status of the control region of the *p21*$^{waf1/cip1}$ gene in the four breast carcinoma-derived cell lines T47D, MCF7, MDA-MB-231 and MDA-MB-468 with previously reported differential expression levels of p21$^{WAF1/CIP1}$ proteins, under normal culture conditions. This study demonstrated that the *p21*$^{waf1/cip1}$ CpG island remained completely unmethylated in all of these breast carcinoma cell lines. There was no correlation between down-regulation of *p21*$^{waf1/cip1}$ expression and CpG islands hypermethylation.

Acknowledgments

Authors would like to thank the Office of Vice-Chancellor for Research of University of Tehran and Tehran University of Medical Sciences for joint financial support of this research project.

References

1. Simpson,P. and Reis-Filho,J. (2005) Molecular evolution of breast cancer. *J. Pathol.*, **205**, 248-254.
2. Esteller,M. (2008) Epigenetics in cancer. *N. Engl. J. Med.*, **358**, 1148-1159.
3. Esteller,M. (2007) Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum. Mol. Genet.*, **16**, 50-59.
4. Virmani,A., Rathi,A. and Heda,S. (2003) Aberrant methylation of the cyclin D2 promoter in primary small cell, nonsmall cell lung and breast cancers. *Int. J. Cancer.*, **107**, 341-345.
5. Besson,A., Dowdy,S. and Roberts,J. (2008) CDK inhibitors: cell cycle regulators and beyond. *Dev. Cell.*, **14**, 159-169.
6. Abbas,T. and Dutta,A. (2009) p21 in cancer: intricate networks and multiple activities. *Nat. Rev. Cancer.*, **9**, 400-409.
7. Mortusewicz,O. (2005) Recruitment of DNA methyltransferase I to DNA repair sites. *P. NATL. ACAD. SCI. USA.*, **102**, 8905-9.
8. El-Deiry,W., Tokino,T. and Velculescu,V. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell.*, **75**, 817-25.
9. Chim,C., Wong,A. and Kwong,Y. (2005) Epigenetic inactivation of the CIP/KIP cell-cycle control pathway in acute leukemias. *AM. J. Hematol.*, **80**, 282-287.
10. Coqueret,O. (2003) New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends. Cell. Biol.*, **13**, 45-70.
11. Shiohara,M. and El-Deiry,W. (1994) Absence of WAF1 mutations in a variety of human malignancies. *Blood.*, **84**, 3781-4.
12. Malkowicz,S. and Tomaszewski,J. (1996) Novel

p21WAF1/CIP1 mutations in superficial and invasive transitional cell carcinomas. *Oncogene.*, **13**, 1831-7.

13. Roman-Gomez,J., Castillejo,J., Jimenez,A., Gonzalez,M., Moreno,F., Rodriguez,M.C., Barrios,M., Maldonado,J. and Torres,A. (2002) CpG island hypermethylation is associated with transcriptional silencing of the p21CIP1/WAF1/SDI1 gene and confers poor prognosis in acute lymphoblastic leukemia. *Blood.*, **99**, 2291-96.

14. Ocker,M. and Schneider-Stock,R. (2007) Histone deacetylase inhibitors: signaling towards P21waf1/cip1. *INT. J. Biochem. CELL B.*, **39**, 1367-74.

15. Majid,S., Kikuno,N., Nelles,J. and Noonan,E. (2008) induces the p21WAF1/CIP1 and p16INK4a tumor suppressor genes in prostate cancer cells by epigenetic mechanisms involving active chromatin modification. *Cancer. Res.*, **68**, 2736-42.

16. Nass,S., Ferguson,A. and El-Ashry,D. (1999) Expression of DNA methyl-transferase (DMT) and the cell cycle in human breast cancer cells. *Oncogene.*, **18**, 7453-61.

17. Herman,J. and Graff,J. (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *P. NATL. ACAD. SCI. USA.*, **93**, 9821-6.

18. Brakensiek,K., Langer,F., Kreipe,H. and Lehmann,U. (2005) Absence of p21 CIP1, p27 KIP1 and p57 KIP2 methylation in MDS and AML. *Leukemia. Res.*, **29**, 1357-60.

19. Takai,D. and Jones,P. (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *P. NATL. ACAD. SCI. USA.*, **99**, 3740-5.

20. Clercq,A. and Inze,D. (2006) Cyclin-dependent kinase inhibitors in yeast, animals, and plants: a functional comparison. *CRIT. REV. Biochem. Mol.*, **41**, 293-313.

21. Dai,Y. and Grant,S. (2003) Cyclin dependent kinase inhibitors. *Curr. Opin. Pharmacol.*, **3**, 362-70.

22. Kim,M., Lebron,C. and Nagpal,J. (2008) Methylation of the DFNA5 increases risk of lymph node metastasis in human breast cancer. *Biochem. Bioph. Res. Co.*, **370**, 38-43.

23. Szyf,M., Pakneshan,P. and Rabbani,S. (2004) DNA methylation and breast cancer. *Biochem. Pharmacol.*, **68**, 1187-97.

24. Allan,L., Duhig,T., Read,M. and Fried,M. (2000) The p21 Promoter Is Methylated in Rat-1 Cells: Stable Restoration of p53-Dependent p21waf1/cip1 Expression after Transfection of a Genomic Clone Containing the p21waf1/cip1 Gene. *Mol. Cell. Biol.*, **20**, 1291-8.

25. Pinyol,M., Hernandez,L., Cazorla,M., Balbin,M., Jares,P., Fernandez,P., Montserrat,E., Cardesa,A., Lopez-Otin,C. and Campo,E. (1997) Deletions and loss of expression of p16INK4a and p21Waf1INK4a/ARF ALTERATIONS IN PCNSL genes are associated with aggressive variants of mantle cell lymphomas. *Blood*, **89**, 272-80.

26. Nakamura,M., Sakaki,T. and Hashimoto,H. (2001) Frequent alterations of the p14ARF and p16INK4a genes in primary central nervous system lymphomas. *Cancer. Res.*, **61**, 6335-9.

27. Mukherjee,S. and Conrad,S. (2005) c-Myc suppresses p21WAF1/CIP1 expression during estrogen signaling and antiestrogen resistance in human breast cancer cells. *J. Biol. Chem.*, **280**, 17617-25.

28. Scott,S., Kimura,T. and Dong,W. (2004) Methylation status of cyclin-dependent kinase inhibitor genes within the transforming growth factor beta pathway in human T-cell lymphoblastic lymphoma/leukemia. *Leukemia. Res.*, **1293-1301**, 28.

29. Zheng,Q., Ma,L. and Zhu,W. (2006) p21Waf1/Cip1 plays a critical role in modulating senescence through changes of DNA methylation. *J. Cell. Biochem.*, **98**, 1230-8.

30. Ying,J., Srivastava,G. and Gao,Z. (2004) Promoter hypermethylation of the cyclin-dependent kinase inhibitor (CDKI) gene p21WAF1/CIP1/SDI1 is rare in various lymphomas and carcinomas. *Blood*, **103**, 743-6.