The role of ADCYAP1, adenylate cyclase activating polypeptide 1, as a methylation biomarker for the early detection of cervical cancer

SAMIL JUNG[1*], LISHA YI[1*], DONGJUN JEONG[2*], JINSUN KIM[1], SUNGWHAN AN[3], TAE-JEONG OH[3], CHANG-HWAN KIM[2], CHANG-JIN KIM[2], YOUNG YANG[1], KEUN IL KIM[1], JONG-SEOK LIM[1] and MYEONG-SOK LEE[1]

[1]Division of Biological Science and Research Center for Women's Diseases, Sookmyung Women's University, Seoul 140-742; [2]Department of Pathology, College of Medicine, Soonchunhyang University, 366-1, Ssangyong-Dong, Chonan 330-090; [3]Genomictree Inc., Jonmin-Dong 461-8, Daejeon Bioventure Town, Yuseong-gu, Daejeon 305-811, Korea

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Abstract. The *ADCYAP1* gene encodes an adenylate cyclase activating polypeptide 1. ADCYAP1 has been known to be involved in various biological processes. Multiple cytosine guanine dinucleotides (CpG island) are found in the *ADCYAP1* promoter region. Transcriptional silencing by promoter hypermethylation is an important regulatory mechanism in tumorigenesis in many cancers. Therefore, the methylation level of the *ADCYAP1* promoter was investigated in eight cervical cancer cell lines and human tissue samples with a distinctive degree of malignant transformation. While multiple CpG sites in the *ADCYAP1* promoter were highly methylated in CIN III and invasive carcinoma cells as well as seven cervical cancer cell lines, they were rarely methylated in normal cells. Importantly, methylation in the A*DCYAP1* promoter seems to start from CIN I, relatively early stage of multistep carcinogenesis. This fact suggest that *ADCYAP1* can be used as an effective and sensitive methylation biomarker for the early diagnosis of cervical cancer. Moreover, our data imply that the level of the *ADCYAP1* promoter hypermethylation is correlated with cervical cancer development. We also show that *ADCYAP1* gene expression was reactivated by the treatment of a DNA methyltransferase inhibitor of 5'-aza-2'deoxycytidine and/or a histone deacetylase inhibitor of trichostain A in cervical cancer cells suggesting that hypermethylation in the *ADCYAP1* promoter is responsible for the transcriptional silencing of the *ADCYAP1* gene in cervical cancer cells.

Correspondence to: Dr Myeong-Sok Lee, Division of Biological Science and Research Center for Women's Diseases, Sookmyung Women's University, Seoul 140-742, Korea
E-mail: mslee@sookmyung.ac.kr

*Contributed equally

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Introduction

Cervical cancer is an malignant tumor of the cervix uteri or cervical area. In spite of various advanced treatments, cervical cancer still remains a fatal disease worldwide for women (1). When infected by human papillomavirus (HPV), the cervical epithelial cells develop from premalignant cervical lesions to malignant invasive cancer via multistep processes (2-5). The multistep carcinogenesis can be classified into five groups: normal, cervical intraepithelial neoplasia I (CIN I, mild dysplasia), cervical intraepithelial neoplasia II (CIN II, moderate dysplasia), cervical intraepithelial neoplasia III (CIN III, severe dysplasia), and invasive cervical carcinoma (6,7). Fortunately, most cases of CIN do not develop to cervical cancer and only a small percentage of cases becomes cervical cancer if they are not well treated (7). Moreover, this process is a relatively slow event with a long interval between infection and cancer. Therefore, the development of a diagnostic system for early detection has been considered as one of the most powerful tools in the diagnostic area. Several such diagnostic technologies (e.g., Pap smear screening or HPV testing) have been developed. However, their efficiency has been questioned for many reasons including sampling, interpretation errors, cost, and low specificity and sensitivity.

Currently, many researchers are interested in developing diagnostic tools for the early detection of various cancers using epigenetic alterations in the oncogenes and tumor suppressor genes. Representative epigenetic alteration is the hypermethylation at CpG islands of a genomic DNA (8). DNA methylation has been considered to be very useful tool for the detection of various cancers (9). Hypermethylation in the promoter region of potential tumor suppressor genes often leads to a repressed gene expression and therefore the loss of normal function. This epigenetic silencing is now recognized as a frequent event in the

Table I. Human cervix tissue samples used in MSP and BSP analyses.

Number[a]	Diagnosis samples[b] (Age)				
	Normal[c]	CIN I	CIN II	CIN III	Carcinoma
1	08-3488-1d (48)	07-692 (44)	07-4215 (34)	07-852 (51)	7227 (75)
2	08-7568-1b (49)	07-949 (32)	07-4406 (27)	07-1631 (48)	12593 (42)
3	08-3782-1b (40)	07-1573 (40)	07-4556 (36)	07-1858 (56)	10931 (75)
4	08-7275-1a (43)	07-1888 (46)	07-4751 (37)	07-1854 (41)	6956 (59)
5	08-3665-1b (68)	07-1899 (45)	07-4926 (38)	07-2346 (43)	10919 (82)
6	08-5386-1a (44)	07-1857 (42)	07-5660 (39)	07-2914 (53)	8026 (71)
7	08-3513-1a (42)	07-1855 (43)	07-5881 (41)	07-8302 (25)	5739 (38)
8	08-3513-1b (NA)[d]	07-2687 (22)	07-5908 (41)	07-9619 (38)	6851 (56)
9	08-3488-1b (NA)	07-2888 (25)	07-5929 (22)	07-10051 (72)	4321 (81)
10	08-5889-1a (45)	07-3349 (23)	07-6000 (27)	07-10432 (65)	5822 (46)
11		07-3596 (50)	07-6473 (48)	09-153 (41)	09-240 (65)
12		07-3651 (23)	07-6561 (32)	09-640 (35)	09-576 (65)
13		07-5594 (44)	07-6858 (32)	09-796 (57)	09-1183 (46)
14		07-6334 (36)	07-6859 (32)	09-875 (40)	09-1388 (41)
15		07-6474 (32)	07-7288 (36)	09-1877 (48)	09-1645 (48)
16		07-6439 (36)	07-7768 (27)	09-2986 (29)	09-2740 (49)
17		07-6644 (44)	07-8794 (47)	09-3072 (27)	09-2943 (68)
18		07-6665 (23)	07-9302 (29)	09-3431 (41)	09-3671 (62)
19		07-6697 (37)	07-9671 (26)	09-3670 (39)	09-3675 (60)
20		07-7713 (49)	07-9932 (32)	09-3613 (39)	09-4161 (40)
21		07-8301 (37)	07-10724 (50)		
22		07-8663 (26)	07-11282 (37)		
23		07-8899 (36)	07-12365 (28)		
24		07-12017 (24)	07-13050 (40)		
25		07-12230 (38)	07-562 (35)		
26		07-12320 (29)	07-708 (31)		
27		07-12412 (41)	07-879 (21)		
28		07-12620 (34)	07-915 (34)		
29		07-12766 (50)	07-934 (41)		
30		07-13172 (42)	07-1076 (25)		

[a]Number of cases examined. [b]Samples were collected from patient in different histological type of cervical cancer (different tumor grade or clinical stage) in cervical carcinogenesis (Materials and methods). [c]Normal tissue samples are from adjacent tumor tissue. [d]Not available.

carcinogenesis of many cancers including cervical cancer (10-16). In other words, methylated cytosines are absent in the promoter of normal cells but are present in that of cancer cells. Accordingly, DNA methylation can be used as a powerful biomarker for the diagnosis of many cancers (17). A number of different approaches to genome-wide identification of cancer-associated hypermethylated genes have been developed (9). Nevertheless, at the present time only a few cervical cancer specific methylation markers have been identified. Our goal is to identify more effective and sensitive methylation biomarkers. For this purpose, we have initially focused on evaluating the value of *ADCYAP1* for a new methylation-based biomarker.

The *ADCYAP1* gene encodes an adenylate cyclase activating polypeptide 1. This gene belongs as a member of the secretin/glucagons/vasoactive intestinal peptide (VIP) family. ADCYAP1 has been known to be involved in various biological processes including cell growth, proliferation, and differentiation (18). One of the main functions of ADCYAP1 is to stimulate adenylate cyclase and subsequently increases the cAMP level in target cells. It also functions as a hypophysiotropic hormone, neurotransmitter, and neuromodulator. In addition, it plays a vital role in paracrine and autocrine regulation of certain types of cells, in the accumulation of estrogen and progesterone, and synthesis of sulfatides, glycolipid, and sphingolipid. Beyond that, many studies in ADCYAP1 have suggested that *ADCYAP1* gene expression links to cancer. For example, the *ADCYAP1* gene is known to be overexpressed in neuroblastoma (19) and breast cancer (20). ADCYAP1 has shown neurotrophic actions in human neuroblastoma SH-SY5Y tumor cells (21) and also showed anti-apoptotic effects in a schwannoma cell line (22). It was

Table II. Oligonucleotide sequences and conditions for PCR analysis.

Primer name[a]	Primer sequence[b]	Conditions[c]	Amplicon size (bp)	Sources or references
pRT-ADCYAP1-F	GATCTTCACGGACAGCTACAG	69˚C, 30 sec, 35	226	(27)
pRT-ADCYAP1-R	GTTTGGATAGAACACACGAGC			
pMSP-UM-ADCYAP1-F	TAGTGTAGGAATTTGAAGAAGTGT	58˚C, 30 sec, 35	140	This study
pMSP-UM-ADCYAP1-R	TACCAAACAAAAAAATCAACAATCA			
pMSP-M-ADCYAP1-F	TTAGCGTAGGAATTTGAAGAAGC	58˚C, 30 sec, 35	137	This study
pMSP-M-ADCYAP1-R	AAACGAAAAAATCAACAATCGAA			
pBSP-ADCYAP-F	cgt*aagctt*TTATAGTAAGTAAGAAGTGGTAGGG[d]	55˚C, 30 sec, 35	332	This study
pBSP-ADCYAP-R	cta*gaattc*TACCTAAAAAACCCCAACTCCTACT			

[a]F, forward primer; R, reverse primer; M, methylation-specific primers; U, unmethylated primers. [b]All sequences shown in the 5'→3' direction. Restriction enzymes are represented in italic and lower case letters. [c]Conditions are shown as the order of annealing temperature, elongation time, and number of cycles.

reported that ADCYAP1 can promote the growth of the BON neuroendocrine tumor cells (23). On the contrary, it has shown that ADCYAP1 suppresses the proliferation of human κ and λ light chain-secreting multiple myeloma-derived cells, suggesting that ADCYAP1 is an antitumor agent that directly suppresses myeloma cell growth and indirectly affects tumor cell growth (24). Taken together, these facts are consistent with a previous report that the ADCYAP1 has both proliferative and anti-proliferative effects on cancer cell growth (25). Therefore, we examined the effect of promoter methylation on the *ADCYAP1* gene expression during the development of cervical cancer.

We introduce *ADCYAP1* as an effective methylation-based molecular biomarker for the early diagnosis of cervical cancer. Moreover, our further studies suggested that the *ADCYAP1* promoter methylation seems to be responsible for the low levels of gene expression in cervical cancer cells.

Materials and methods

Cervical cancer cell lines and human tissue samples. Eight cervical cancer cell lines were used in this study. C33A, CaSki, HeLa, and SiHa cells were purchased from the American Type Culture Collection (ATCC, USA). The other cell lines, SNU-17, -703, -1160, and -1299 were obtained from the Korean Cell Line Bank (KCLB, Korea). Each cell line was grown in one of the following different media: C33A, HeLa, and SiHa cells in DMEM medium (WelGENE Inc., Korea); CaSki, SNU-703, and SNU-1299 cells in RPMI-1640 medium (Gibco-BRL); SNU-17 and SNU-1160 in AR5 medium (KCLB, Korea). All media were supplemented with 10% fetal bovine serum (Gibco-BRL) and 1% Antibiotic-Antimycotic (Gibco-BRL). All of these cells were cultured at 37˚C in a humidified atmosphere composed of 95% air and 5% CO_2. A total of 110 human tissue samples were kindly provided by Dr Chang-Jin Kim at the Soonchunhyang University Hospital (Cheonan, Korea). These tissue samples originated from cervical cancer patients and their information is represented as the histological tumor grade and age in Table I. These tissue samples for CIN diagnosis were prepared by using micro-excision. Patients provided signed informed consent and the procedure of obtaining tissue samples was approved by the Hospital Clinic's Institutional Review Board.

Reverse transcription (RT)-PCR. Total RNA was extracted from 8 cervical cancer cell lines or human tissue samples using the RNeasy Minikit (Qiagen) following the manufacturer's instructions. For reverse transcription, 1 μg RNA of each sample was subjected to cDNA synthesis using oligo-(dT) primer and RevertAid First Strand cDNA Synthesis Kit (Fermentas, Korea) according to the manufacturer's instruction. PCR amplification was performed using 10 ng cDNA, a primer pair of pRT-ADCYAP1-F and pRT-ADCYAP1-R, and AccuPower PCR PreMix (Bioneer, Korea). The nucleotide sequence of primers and the conditions for gene amplification are shown in Table II. Primers were synthesized by Bioneer. The amplification reaction was carried out using a GeneAmp PCR System 9700 from Applied Biosystems. The amplification products were electrophoresed on a 2% agarose gel stained with ethidium bromide and visualized by using the UV-illuminator or LAS-3000 imaging system.

Methylation-specific PCR (MSP) analysis. Genomic DNA was extracted from eight cervical cancer cell lines or human tissue samples using DNeasy Blood & Tissue Kit (Qiagen). Bisulfite treatment was performed using 1 μg of genomic DNA at 55˚C for 16 h as following the instruction of EZ DNA Methylation Kit (Zymo Research, CA, USA). Bisulfite-treated DNA was then followed by PCR amplification using two pairs of primer for unmethylated (pMSP-UM-ADCYAP1-F and -R) and methylated (pMSP-M-ADCYAP1-F and -R) DNAs. The nucleotide sequence of each primer and the amplification conditions are shown in Table II. Primers used in this study were synthesized by Bioneer. For all MSP analysis, the PCR mixtures contained 10X reaction buffer, dNTP mixture (1.0 mM), primers (final concentration 10 pmole each per reaction), 1 unit of HotStart

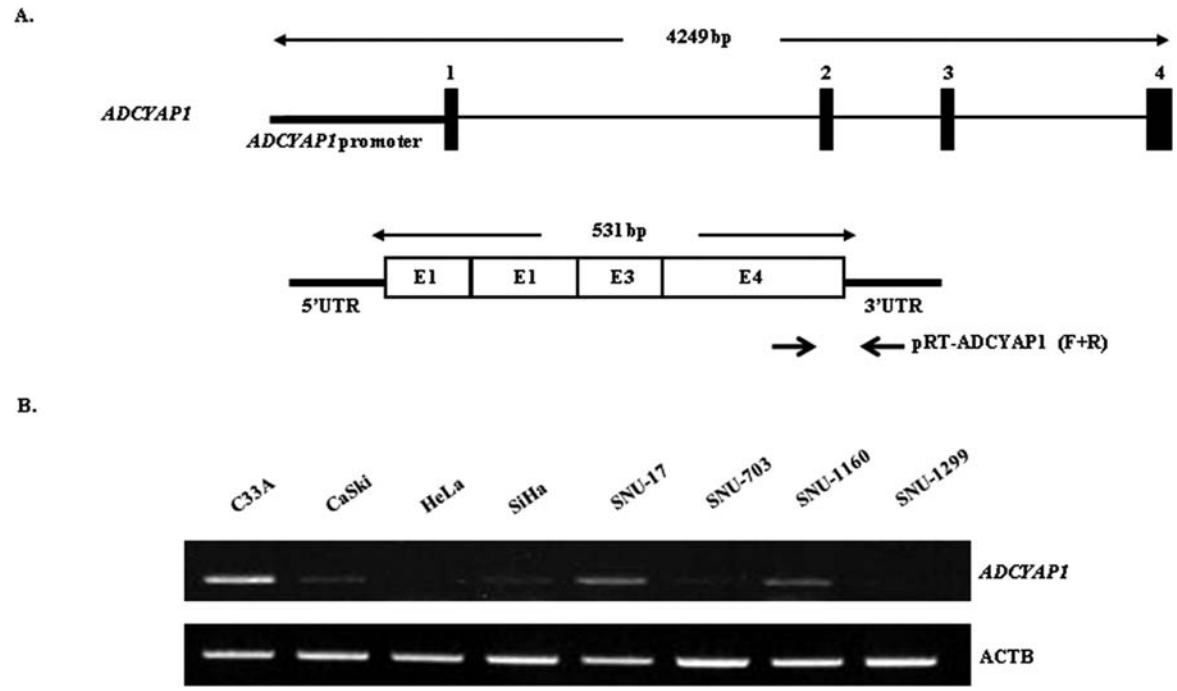

Figure 1. *ADCYAP1* gene expression in eight cervical cancer cell lines. (A) Genomic DNA for the *ADCYAP1* gene is represented as exons (black boxes) and introns (thin lines linking the black boxes). Putative minimal promoter of the *ADCYAP1* gene is indicated by a horizontal thick line. Numbers indicate the position of exons coding the *ADCYAP1* gene. Exons are indicated as boxes and numbers in the full-length mRNA of *ADCYAP1*. Arrows indicate the position of primers used in the RT-PCR (Table II). F and R represent forward and reverse, respectively. (B) The transcriptional level of the *ADCYAP1* gene was measured in eight cervical cancer cell lines. ß-actin served as an internal control for the integrity of the cDNA.

prime Taq (Qiagen), and 0.1 μg of bisulfite-treated DNA. The methylation status was inferred by the presence or absence of bands and its density was represented by the thickness of the bands.

Bisulfite sequencing PCR (BSP) analysis. Genomic DNA (1 μg) extracted from each cell line or human tissue samples was bisulfite-treated by using EZ DNA Methylation Kit (Zymo Research). Bisulfite-treated DNA was subjected to PCR reaction using primers of pBSP-ADCYAP1-F and -R (Table II). Primers were synthesized by Bioneer. The amplified PCR products were cloned into a pBlueScript-SK (+) vector using *Hin*dIII and *Eco*RI restriction enzymes and transformed into DH5α competent cells. Plasmids purified from amphicillin-positive colonies were sequenced using an M13 forward or reverse primer by Solegent (Daejeon, Korea).

Treatment of 5'-aza-2'-deoxycytidine and trichostatin A treatment. Two cervical cancer cell lines, HeLa and SiHa, were treated with a DNA methyltransferase inhibitor of 5'-aza-2'deoxycytidine (DAC, Sigma) and/or a histone deacetylase inhibitor of trichostain A (TSA, Sigma). Briefly, the cells were plated onto 100-mm plates for 24 h before treatment. HeLa cells were treated with 3.0 μM DAC and/or 0.3 μM TSA for 96 or 48 h, respectively. SiHa cells were treated with 10.0 μM DAC and/or 0.5 μM TSA for 72 or 48 h, respectively.

Statistical analysis. Statistical analyses were carried out with the Statistical Package for the Social Sciences (SPSS) software. Associations of the *ADCYAP1* promoter methylation and cervical carcinogenesis were determined using the χ^2 test). Statistical significance was set at $P<0.05$.

Results and Discussion

Levels of ADCYAP1 gene expression in eight cervical cancer cell lines. The genomic structure of the *ADCYAP1* gene is shown in Fig. 1A, which is mapped to chromosome 18p11.32. Nucleotide sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The full-length transcript of the *ADCYAP1* gene is shown below the genomic DNA, in which exons are indicated as boxes and numbers. The *ADCYAP1* transcript basically contains 4 exons, in which 5'UTR and 3'UTR are indicated by horizontal thick lines. RT-PCR was employed to measure the expression level of the *ADCYAP1* gene in eight cervical cancer cell lines (Materials and methods). The positions of pRT-ADCYAP1-F and -R primers are indicated in Fig. 1A. Our result showed that a relatively high level of the *ADCYAP1* gene expression was observed only in the C33A cell line, while low levels of the *ADCYAP1* gene expression were found in most other cell lines (Fig. 1B). Moreover, no transcript was detected in the HeLa and SNU-1299 cell lines (Fig. 1B). Taken together, our result indicates that the *ADCYAP1* gene expression is normally repressed in cervical cancer cell lines.

Methylation status of ADCYAP1 promoter in eight cervical cancer cell lines. Putative CpG islands in the *ADCYAP1* promoter were predicted using the Methprimer program (http://www.urogene.org//methprimer) (26) with the default

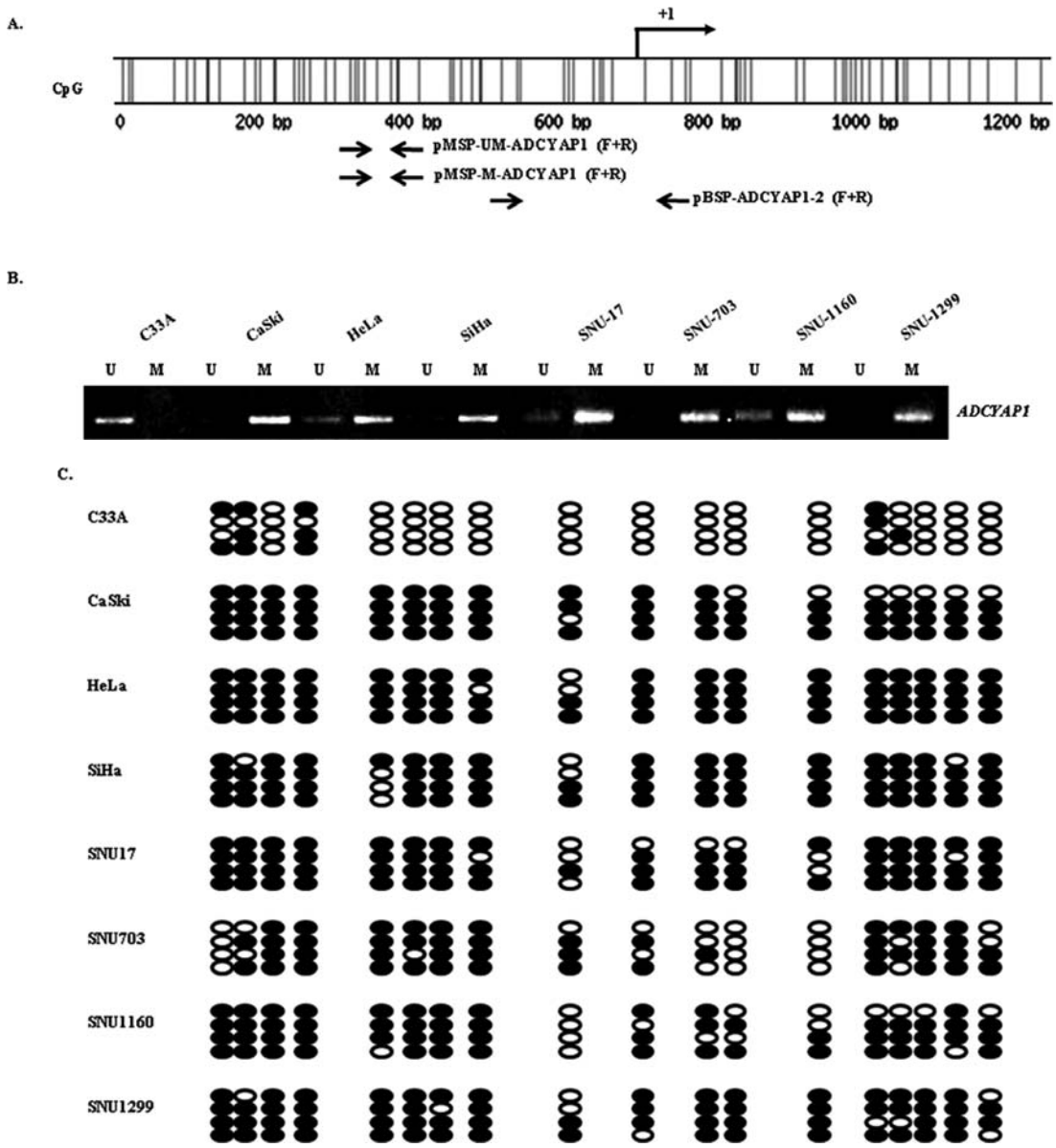

Figure 2. Methylation status of the *ADCYAP1* promoter in eight cervical cancer cell lines. (A) The genomic location and predicted CpG island in the genomic DNA containing the ADCYAP1 gene. CpG dinucleotides in the *ADCYAP1* promoter are shown as vertical lines. Number, +1, indicates the translation start site of the *ADCYAP1* gene. Arrows indicate the position of primers used in the MSP or BSP assays (Table II). The oligonucleotides used in this study are named as indicated. F and R represent forward and reverse, respectively. (B) MSP analysis of the *ADCYAP1* promoter in eight cervical cancer cell lines. Methylation status is presented by the presence or absence of bands. M represents PCR products amplified by oligonucleotide primers specific for methylated DNA whereas U indicates PCR products amplified by primers specific for unmethylated DNA. (C) Bisulfite sequencing of the *ADCYAP1* CpG sites in eight cervical cancer cell lines. Methylated or unmethylated cytosines are represented as closed or open circles, respectively. Their spacing reflects the CpG density of the region. Each row represents an individual cloned allele that was sequenced following sodium bisulfite DNA modification.

setting (%GC >50%, ObsCpG/ExpCpG >0.6) (Fig. 2A). High frequency of CpG dinucleotides is found in the *ADCYAP1* promoter region (Fig. 2A). MSP and BSP assays were employed to investigate the methylation level of the *ADCYAP1* promoter in eight cervical cancer cell lines (Materials and methods). The MSP assay showed that methylation specific bands were detected in all tested cervical cancer cell lines except for C33A, in which only an unmethylation band was observed (Fig. 2B). In the case of HeLa, SNU-17, and SNU-1160 cell lines, both methylated and unmethylated PCR products were amplified from the *ADCYAP1* promoter (Fig. 2B). These results suggest that the *ADCYAP1* promoter is frequently hypermethylated in most cervical cancer cell lines. The accuracy of the MSP method in detecting methylated DNA was verified by using the BSP assay. We found a general concordant result for the methylation status of the *ADCYAP1* promoter in most tested cell lines when compared the MSP and BSP assays. Our data showed that high levels of methylation were exclusively present in most cervical cancer cell lines whereas relatively low levels of methylation were found in the C33A cell line (Fig. 2C). These results suggest that the *ADCYAP1* promoter hypermethylation is a frequent and common event in cervical cancer cell lines. More importantly, our result revealed the inverse relationship between the *ADCYAP1* gene expression and promoter methylation level as shown in Figs. 1B and 2.

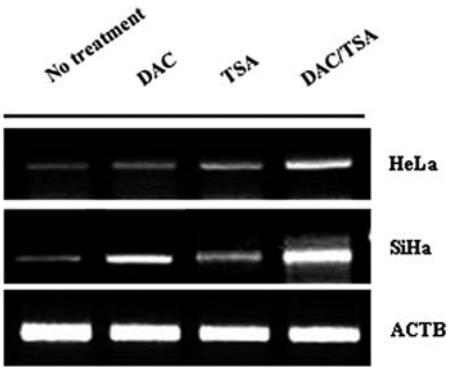

Figure 3. Effect of the demethylating agent 5'-aza-2'-deoxycytidine (DAC) and/or deacetylase inhibitor trichostatin A (TSA) on the *ADCYAP1* gene expression. RT-PCR was carried out using cDNA from each cell line subjected to different concentrations of the drug treatments (Materials and methods).

For example, a high level of the *ADCYAP1* gene expression was detected in the C33A cell line, in which a low level of methylation was observed. Taken together, these results suggested the possible connection between the *ADCYAP1* gene expression and promoter hypermethylation in cervical cancer cells.

Reactivation of the ADCYAP1 gene expression by the treatment of DAC and TSA. Our results implied that the *ADCYAP1* promoter hypermethylation might be responsible for the transcriptional silencing of the *ADCYAP1* gene in cervical cancer cell lines. To test this hypothesis, HeLa and SiHa cell lines were chosen and treated with 5'-aza-2'deoxycytidine (DAC) and/or trichostain A (TSA), in which the total RNA was isolated from each cell line (Materials and

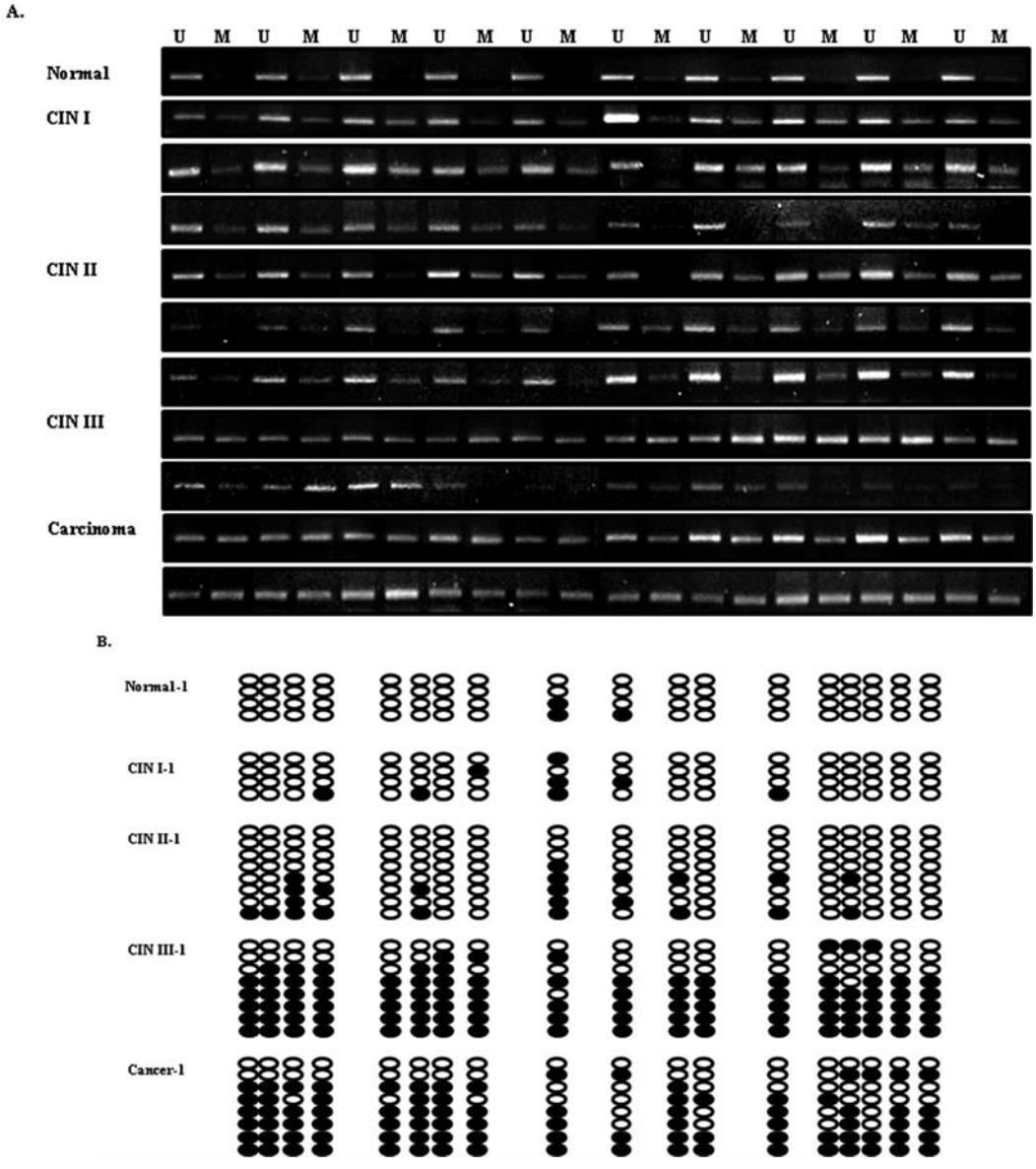

Figure 4. Schematic representation of correlation between *ADCYAP1* hypermethylation and cervical carcinogenesis. (A) MSP analysis of *ADCYAP1* in normal, CIN I, CIN II, CIN III, and invasive cervical carcinoma tissues. The symbols of U or M represent PCR products amplified by oligonucleotide primers specific for unmethylated or methylated DNA, respectively. (B) Bisulfite sequencing of CpG sites in the promoters of *ADCYAP1* in normal, CIN I, CIN II, CIN III, and carcinoma tissue samples. Each row represents an individual cloned allele that was sequenced. Circles represent CpG sites and their spacing reflects the CpG density of the region. Methylated or unmethylated cytosines are represented as closed or open circles, respectively.

methods). The expression level of the *ADCYAP1* gene was measured using RT-PCR. Consistent with our hypothesis, the *ADCYAP1* gene expression was reactivated by the drug treatments in HeLa and SiHa cell lines as shown in Fig. 3. Treatment of either DAC or TSA alone showed no significant effect on *ADCYAP1* expression in HeLa cells. However, treatment with DAC and TSA together greatly increased *ADCYAP1* expression (Fig. 3). In the case of SiHa cells, treatment with DAC alone or with DAC and TSA together resulted in a significanct increase in *ADCYAP1* expression (Fig. 3). These data suggest that hypermethylation in the *ADCYAP1* promoter is associated with transcriptional silencing of *ADCYAP1* gene in HeLa and SiHa cervical cell lines. The *ADCYAP1* gene expression was also found to be reactivated by the DAC and TSA treatments in CaSki and SNU-1299 cervical cancer cell lines (data not shown), confirming the negative effect of methylated or deacethylated DNA on the *ADCYAP1* gene expression. Taken together, we conclude that the *ADCYAP1* promoter hypermethylation is responsible for the low *ADCYAP1* gene expression in cervical cancer cells.

Methylation incidence in ADCYAP1 promoter during multistep processes of cervical carcinogenesis. Discovery of the correlation between *ADCYAP1* methylation and cervical cancer cell lines suggested the possibility of the same situation *in vivo*. Therefore, this investigation was extended to human tissue samples from five different stages of cervical carcinogenesis; normal cervix, CIN I, CIN II, CIN III, and invasive carcinoma. We evaluated the methylation level of the *ADCYAP1* promoter in a total of 110 cervical tissue samples. Briefly, genomic DNA extracted from tissue samples was modified by sodium bisulfite and then subjected to MSP and BSP analysis (Materials and methods). As shown in Fig. 4A, partial or non-methylated *ADCYAP1* promoter was detected in normal tissue samples, whereas a high degree of methylation was observed in CIN III and invasive carcinoma (p=0.00). Methylation density in CIN I and CIN II is clearly higher than those in normal tissues but lower than those in invasive carcinoma samples (p=0.00). This result indicates that methylation in the *ADCYAP1* promoter seems to start from CIN I, relatively early stage of multistep carcinogenesis. BSP assay was performed to confirm the result of MSP (Materials and methods). Four or eight clones from each representative sample were sequenced and evaluated for the frequency of *ADCYAP1* methylation. None of the normal, CIN I, and CIN II cells exhibited hypermethylation. In contrast, CIN III and invasive carcinoma cells showed high levels of methylation in the *ADCYAP1* promoter regions (Fig. 4B). In general, the BSP result was consistent with that of MSP except for the case of CIN I and CIN II, in which relatively high levels of methylation were found in the *ADCYAP1* promoters according to MSP assay. However, our methylation analysis showed a nearly perfect correlation between the *ADCYAP1* hypermethylation and cervical carcinogenesis. The level of *ADCYAP1* hypermethylation is very low at early stages but starts to increase with the tumor development. This fact suggests that the *ADCYAP1* hypermethylation is a tumor-specifc event. It is normally considered that older people have higher levels of methylation compared to younger people, which is consistent with our result. When the *ADCYAP1* methylation status and the age of the patients were compared, a significant association between methylation and age was found, in which older patients showed higher levels of DNA methylation in the *ADCYAP1* promoter (p=0.00). In conclusion, our analysis evaluating methylation in cervical tissues led to the following conclusions. First, *ADCYAP1* methylation is associated with cervical carcinogenesis. Second, this abnormal DNA methylation occurs at the relatively early stages during tumorigenesis of cervical cancer. These results indicate that *ADCYAP1* can be used as an effective bio-marker for early detection of cervical cancer. Third, we showed that hypermethylation of the *ADCYAP1* promoter is responsible for the transcriptional silencing of this gene in cervical cancer cells. Collectively, our study may contribute to the prevention and optimal treatment of cervical cancer by early detection. In addition, studies on the pattern of the methylation event in this gene may lead to a better understanding of its biological function.

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References

1. Pisani P, Parkin DM, Bray F and Ferlay J: Erratum: estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 83: 870-873, 1999.
2. Durst M, Glitz D, Schneider A and Zur HH: Human papillomavirus type 16 (HPV 16) gene expression and DNA replication in cervical neoplasia: analysis by in situ hybridization. Virology 189: 132-140, 1992.
3. Bosch FX, Manos MM, Munoz N, *et al*: Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 87: 796-802, 1995.
4. Zur HH: Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2: 342-350, 2002.
5. Walboomers JM, Jacobs MV, Manos MM, *et al*: Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189: 12-19, 1999.
6. The 1988 Bethesda System for reporting cervical/vaginal cytological diagnoses. National Cancer Institute Workshop. JAMA 262: 931-934, 1989.
7. Agorastos T, Miliaras D, Lambropoulos AF, *et al*: Detection and typing of human papillomavirus DNA in uterine cervices with coexistent grade I and grade III intraepithelial neoplasia: biologic progression or independent lesions. Eur J Obstet Gynecol Reprod Biol 121: 99-103, 2005.
8. Asakawa S, Tsunematsu K, Takayanagi A, *et al*: The genomic structure and promoter region of the human parkin gene. Biochem Biophys Res Commun 286: 863-868, 2001.
9. Laird PW: The power and the promise of DNA methylation markers. Nat Rev Cancer 3: 253-266, 2003.
10. Baylin SB and Ohm JE: Epigenetic gene silencing in cancer, a mechanism for early oncogenic pathway addiction. Nat Rev Cancer 6: 107-116, 2006.
11. Jones PA and Baylin SB: The fundamental role of epigenetic events in cancer. Nat Rev Genet 3: 415-428, 2002.
12. Costello JF, Fruhwald MC, Smiraglia DJ, *et al*: Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 24: 132-138, 2000.
13. Esteller M, Corn PG, Baylin SB and Herman JG: A gene hypermethylation profile of human cancer. Cancer Res 61: 3225-3229, 2001.
14. Issa JP: CpG island methylator phenotype in cancer. Nat Rev Cancer 4: 988-983, 2004.
15. Ushijima T: Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer 5: 223-231, 2005.

16. Egger G, Liang G, Aparicio A and Jones PA: Epigenetics in human disease and prospects for epigenetic therapy. Nature 429: 457-463, 2004.
17. Jones PA and Buckley JD: The role of DNA methylation in cancer. Adv Cancer Res 54: 1-23, 1990.
18. Wolman SR, Heppner GH and Wolman E: New directions in breast cancer research. FASEB J 11: 535-543, 1997.
19. Isobe K, Kaneko M, Kaneko S, *et al*: Expression of mRNAs for PACAP and its receptor in human neuroblastomas and their relationship to catecholamine synthesis. Regul Pept 123: 29-32, 2004.
20. Garcia-Fernandez MO, Bodega G, Ruiz-Villaespesa A, Cortes J, Prieto JC and Carmena MJ: PACAP expression and distribution in human breast cancer and healthy tissue. Cancer Lett 205: 189-195, 2004.
21. Monaghan TK, Pou C, MacKenzie CJ, Plevin R and Lutz EM: Neurotrophic actions of PACAP-38 and LIF on human neuroblastoma SH-SY5Y cells. J Mol Neurosci 36: 45-56, 2008.
22. Castorina A, Tiralongo A, Giunta S, Carnazza ML, Rasi G and D'Agata V: PACAP and VIP prevent apoptosis in schwannoma cells. Brain Res 1241: 29-35, 2008.
23. Germano PM, Lieu SN, Xue J, *et al*: PACAP induces signaling and stimulation of 5-hydroxytryptamine release and growth in neuroendocrine tumor cells. J Mol Neurosci 39: 391-401, 2009.
24. Li M, Cortez S, Nakamachi T, Batuman V and Arimura A: Pituitary adenylate cyclase-activating polypeptide is a potent inhibitor of the growth of light chain-secreting human multiple myeloma cells. Cancer Res 66: 8796-8803, 2006.
25. Hayez N, Harfi I, Lema-Kisoka R, Svoboda M, Corazza F and Sariban E: The neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) modulate several biochemical pathways in human leukemic myeloid cells. J Neuroimmunol 149: 167-181, 2004.
26. Li LC and Dahiya R: MethPrimer: designing primers for methylation PCRs. Bioinformatics 18: 1427-1431, 2002.
27. Garcia-Fernandez MO, Bodega G, Solano RM, *et al*: Expression and distribution of pituitary adenylate cyclase-activating peptide in human prostate and prostate cancer tissues. Regul Pept 110: 9-15, 2002.