Wnt/β-catenin pathway activation and silencing of the APC gene in HPV-positive human cervical cancer-derived cells

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Abstract. Although persistent infections with high-risk human papilloma virus (HPV) constitute the most significant cofactor for the development of cervical cancer, they are insufficient on their own. Mutations or epigenetic inactivation of the tumor suppressor adenomatous polyposis coli (APC), the two acting as prominent oncogenic mechanisms in a number of types of cancer, are frequently associated with aberrant activation of the Wnt/β-catenin pathway. According to these observations, it was hypothesized that APC alteration may lead to β-catenin deregulation and the abnormal expression of direct targets of the Wnt pathway in HPV-infected cervical cancer cells. The present study confirmed that the stabilization of β-catenin correlates with enhanced transcriptional activity of the β-catenin/T-cell factor complex in cervical cancer cell lines. Sequence analysis of the 'hot-spot' in the mutation cluster region did not exhibit genetic alterations that may be associated with APC gene inactivation. In addition, it was identified that there was a good correlation with the hypermethylation status of the APC promoter 1A and the abnormal accumulation of endogenous β-catenin in cell lines and biopsies infected with HPV16, although not HPV18. Removal of the epigenetic markers led to an increase in APC levels and a reduction of β-catenin expression in two transcriptional targets of the Wnt pathway: Matrix metalloproteinase-7 and vascular endothelial growth factor. The present study suggested that the increase in Wnt activity in certain cervical cancer-derived cells may be associated with an alteration in the methylation status of the APC gene promoter 1A.

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Introduction

The Wnt/β-catenin pathway serves critical functions in the sequential development of the neoplastic process from initiation, proliferation and transformation in a broad range of types of cancer. Aberrant activation of the Wnt pathway may lead to β-catenin stabilization and accumulation in the cytoplasm, allowing its translocation into the nucleus to act as a transcriptional co-activator of members of the T-cell factor (TCF)/lymphoid enhancing factor (LEF) family of transcription factors. It is known that the β-catenin/TCF-LEF complex induces the transcription of genes involved in carcinogenesis including c-myc, matrix metalloproteinase-7 (MMP-7) and the vascular endothelial growth factor (VEGF) (1). The tumor suppressor adenomatous polyposis coli (APC), an essential part of a multi-protein complex together with protein phosphatase 2A (PP2A), axin, glycogen synthase kinase 3β (GSK-3β), and casein kinase I (CK1), serves an important function in turning off the β-catenin signaling pathway. When this complex is active, GSK-3β and CK1 phosphorylate the amino terminal serine and threonine residues of β-catenin, an initial step which targets it for degradation by the proteasomal machinery (2).

Cervical carcinoma, one of the most common malignancies in women worldwide (3), is associated with persistent infection with oncogenic or high-risk human papillomavirus (HR-HPV). Although HR-HPV infection is required, it is insufficient on its own to cause cervical cancer (4). Several lines of evidence have indicated a potential association between activation of the Wnt signaling pathway and HPV-mediated cervical cancer. For instance, Uren *et al* (5) and Bulut *et al* (6) generated two models that mimicked the effects of HPV infection on the human cervical epithelium for cancer progression: i) HPV-immortalized human keratinocytes, either expressing mutated S37A β-catenin or overexpressing Wnt1; and ii) double-transgenic mice overexpressing β-catenin and HPV16-E7 oncogenes. In addition, β-catenin has been observed to be localized and expressed in the cytoplasm and nucleus in human cervical carcinoma samples (7,8) and cell lines (9), indicating the activation of the Wnt pathway. The results of these previous studies suggested that activation of

the canonical Wnt pathway may represent one of the key events required for the malignant transformation of HPV-infected epithelial cells.

Previous studies have demonstrated that β-catenin deregulation is frequently associated with alterations in the APC gene, and ~80 to 90% of all APC gene mutations are confined to codons 1,286 to 1,513, a region known as the mutation cluster region (MCR), these mutations typically lead to a truncated protein lacking the β-catenin binding and regulatory sites, as is frequently observed in colon cancer (10,11). In addition, transcriptional silencing via promoter hypermethylation may impair APC function, a phenomenon of epigenetic inactivation observed in tumors in the absence of APC gene mutations. For example, in gastric cancer, a decrease in or loss of APC expression has been identified to be associated with the APC gene promoter A1 hypermethylation (12,13).

According to these observations, it has been suggested that the stabilization and activation of β-catenin in cervical cancer-derived cells are a consequence of alterations in APC gene expression. To test this hypothesis, the present study first sought to determine the transcriptional activity of β-catenin/TCF among a group of cervical cancer cell lines. Subsequently, MCR-region mutational and methylation status analyses of the APC gene and its promoter 1A, respectively, were performed and the impact of APC gene mutations and/or its epigenetic silencing on two known Wnt target genes in cervical carcinoma cell lines was analyzed.

Materials and methods

Cell cultures and clinical samples. Human cervical cancer-derived cell lines CaSki, SiHa and HeLa, in addition to C33A and the colon cancer cell line SW480 were obtained from the American Type Culture Collection (Manassas, VA, USA). The gastric cancer cell line KATOIII was provided by Dr Angel Zarain-Herzberg (Facultad de Medicina-UNAM, CDMX, México). Human foreskin fibroblasts (HFF) were provided by Dr. María Luisa Villarreal Ortega (Centro de Investigaciones en Biotecnología-UAEM, Cuernavaca, Morelos, México). HFF and CaSki cells were grown in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); SiHa, HeLa, C33A, SW480 and KATOIII cells were grown in Dulbecco's modified Eagle's medium/F-12 (Invitrogen; Thermo Fisher Scientific, Inc.), and all the cultured cells were supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 2 mM L-glutamine (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. Normal tissue samples and tumors from the uterine cervix were obtained from archives of the Department of Gynecology at the Hospital General Manuel Gea González and the Department of Pathology at the Instituto Nacional de Cancerología-SSA (CDMX, México). This set of samples has been characterized previously for clinical pathological parameters, type of HPV and β-catenin protein status (7). All the samples were frozen in liquid nitrogen immediately following resection and stored at -70˚C until processing.

Reporter assay. The TOPflash/FOPflash TCF reporter system (Upstate Biotechnology, Inc., Lake Placid, NY, USA) was used to directly measure the levels of Wnt signaling in human cervical cancer cell lines. A total of 4,000 cells were cultured in 96-well plate and transfected, with either TOPflash or FOPflash (100 ng) and the internal control plasmid pRL-Renilla (5 ng; Promega Corporation, Madison, WI, USA) expressing *Renilla* luciferase, using Lipofectamine reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The TOPflash and FOPflash reporters contain two sets of three copies of wild-type (TOP) or mutant (FOP) β-catenin/TCF binding sites, respectively, in addition to the thymidine kinase minimal promoter upstream of the firefly luciferase open reading frame. Luciferase activity was measured with Dual-Glo™ Luciferase Assay System (Promega Corporation) 48 h following transfection, according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity. All results are expressed as TOPflash/FOPflash correlation mean ± standard deviation for independent triplicate cultures.

Mutational analysis of APC. Genomic DNA was isolated from cells using the standard Proteinase K treatment followed by phenol/chloroform extraction (14). Briefly, the cells were incubated in the lysis buffer (Tris 10 mM, EDTA 20 mM, SDS 0.5% and Proteinase K 100 μM/ml) for 20 min at 45˚C. The lysate was extracted by phenol/chloroform (1:1, v/v), DNA was precipitated with isopropanol (1:1, v/v), then washed with 75% ethanol and the pellet DNA was dissolved in water. The mutation cluster region of the APC gene was amplified as three overlapping fragments (codons 1032-1703) in a nested polymerase chain reaction (PCR) strategy. In addition to the previously described primers (15), new primers were used to adapt the size of different amplicons (Table I). PCR was performed in a 50-μl volume containing 100 ng genomic DNA and 0.25 μM each primer using Taq DNA Polymerase-recombinant (Fermentas; Thermo Fisher Scientific, Inc.). The PCR reactions were run at 95˚C for 5 min followed by 35 cycles at 95˚C for 40 sec, followed by 54˚C, 63˚C and 60˚C for 60 sec as the annealing step of the three overlapping fragments, respectively, and finally at 72˚C for 60 sec. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen GmbH, Hilden, Germany). Mutational analysis by direct sequencing was performed using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). All sequencing analyses were performed at least twice on two independent PCR products.

Methylation analysis. DNA methylation patterns in the CpG islands of the APC gene promoter A1 were determined by methylation-specific PCR (MSP), according to protocols described previously (16). Genomic DNA (1 μg) was treated with sodium bisulfite using the EZ DNA methylation kit (Zymo Research Corp., Irvine, CA, USA). For the MSP analysis of APC gene promoter 1A, the primers for the unmethylated reaction amplify a 108-bp PCR product, and the primers for the methylated reaction amplify a 98-bp product; all primer sequences used are listed in Table I. PCR amplification was carried out at 95˚C for 5 min followed by 35 cycles at 95˚C for 60 sec, 60˚C for 40 sec and 72˚C for 60 sec. PCR was run in a 50-μl volume containing 100 ng genomic DNA, 0.25 μM each primer using Taq DNA Polymerase-recombinant (Fermentas; Thermo Fisher Scientific, Inc.). Each experiment was repeated twice.

Table I. Primer sequences used in the present study.

Gene and method	Sense 5'-3'	Antisense 5'-3'	Product length (bp)
Mutational analysis			
Fragment A	CCCCTCGAGTCAGATGAGCAGTTG	CCGGATCCCTGCTTCCTGTGTCG	796
Fragment B	CCCCTCGAGCAGCTCCATCCAAG	CCGGATCCCCATCTGGAGTAC	809
Fragment C	CCCCTCGAGCCAGATAGCCCTGG	CCGGATCCCCTCCTTGAGCCTC	839
MSP			
APCprom1AUNMet	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	108
APCprom1AMet	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGAA	98
RT-PCR			
MMP7	TGTTAAACTCCCGCGTCATA	GCGTTCATCCTCATCGAAGT	379
VEGF	GAGGGCAGAATCATCACGAA	AACGCTCCAGGACTTATACC	395
β-globin	CAACTTCATCCACGTTCACC	GAAGAGCCAAGGACAGGTAC	260
APC-exon 15	CCCCTCGAGTCAGATGAGCAGTTG	CCGGATCCCTGCTTCCTGTGTCG	796

bp, base pair; RT-PCR, reverse transcription-polymerase chain reaction; MMP-7, matrix metalloproteinase-7; VEGF, vascular endothelial growth factor; APC, adenomatous polyposis coli.

Treatment with 5-aza-2'-deoxycytidine (Aza) and trichostatin-A (TSA). Cells were cultured at 60% confluence and incubated for 48 h in a medium containing 3 μM DNA methyltransferase (DNMT) inhibitor Aza (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The medium was refreshed every 24 h. The cells were treated with the histone deacetylase (HDAC) inhibitor TSA (Sigma-Aldrich; Merck KGaA) at 0.5 and 3 μM of decitabine for further 24 h to complete 72 h of treatment. The medium was removed and the cells were processed to perform assays by reverse transcription (RT)-PCR and western blot analysis.

RNA isolation and RT-PCR. Total RNA was extracted from cells by using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. For RT-PCR, total RNA (5 μg) was used for cDNA synthesis by reverse transcription using a RevertAid H. Minus First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, in a total volume of 20 μl. The PCR reactions of APC, VEGF, MMP7 and β-globin genes were performed using the specific primers presented in Table I, and standard PCR conditions were used: 95˚C for 5 min, followed by 35 cycles at 95˚C for 60 sec, annealing at 60˚C, 62˚C, 58˚C and 55˚C for each gene, respectively, for 60 sec, and extension at 72˚C for 60 sec. The PCR products were visualized by electrophoresis in 2% agarose gels.

Western blot analysis. Cells were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS), containing protease inhibitors cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentration was determined by the bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.). Total proteins (25 μg) was separated by a 7% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The antibodies and dilutions used included anti-β-catenin (cat. no. C19220; 1:2,000; BD Biosciences, San Jose, CA, USA) and anti-β-actin (cat. no. 81178, 1:100; Santa Cruz Biotechnology Inc., Dallas, TX, USA), both incubated for 5 h at room temperature and following extensive washing the membranes were incubated with anti-mouse immunoglobulin G-horseradish peroxidase-conjugated antibody (cat. no. Nr7074, 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature and revealed with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Inc., Waltham, MA, USA). Membranes were probed for β-actin to normalize for loading.

Statistical analysis. Data were analysed using the GraphPad Prism 5.0 statistical program (GraphPad Software, Inc., La Jolla, CA, USA) and statistical differences were evaluated using one-way analysis of variance followed by Tukey's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

β-catenin expression and transcriptional activity of the β-catenin/TCF complex in cervical cancer cell lines. β-catenin levels in different cell lines were analyzed using western blotting and there was abundant β-catenin in CaSki cells, low levels in HeLa cells, intermediate levels in SiHa and none detected in C33A cancer-derived cells (Fig. 1A). The transcriptional activity of the β-catenin/TCF complex in these four cell lines (CaSki, SiHa, HeLa and C33A) was subsequently analyzed using a luciferase reporter assay with TCF reporter constructs (TOPflash and FOPflash). The results demonstrated that the activity of the TCF reporter TOPflash was increased compared with the FOPflash reporter in all cell lines except C33A cells. The transcriptional activity of the β-catenin/TCF complex was higher in CaSki cells, with higher β-catenin levels, compared with the transcriptional activity observed in SiHa and Hela cells (Fig. 1B). The CaSki cells, infected with HPV16, thus exhibited the highest levels

of β-catenin expression and transcriptional activity. These results demonstrated that the activation of the Wnt/β-catenin signaling pathway responded in a concentration-dependent manner to the levels of β-catenin in these cell lines.

APC gene mutations in cervical cancer cell lines. To investigate the existence of sequence variation in APC, one of the most important members of the β-catenin degradation complex, the MCR region, was evaluated via PCR and direct sequencing. As a positive control, DNA extracted from the colon cancer cell line SW480 was used, homozygous for a mutation at codon 1338 that generates a truncated APC protein (17), leading to abnormal accumulation/delocalization of β-catenin. The sequence analysis of the MCR did not reveal any mutations that were associated with the activation of APC in the cervical cancer cell lines CaSki, SiHa, HeLa and C33A (Fig. 2). None of the known 'hot-spots' in codons 1061, 1338 and 1450 were mutated, the most common sites of mutation in colorectal cancers (18). The mutation at codon 1338 (CAG/TAG) in SW480 cell line was detected (Fig. 2), confirming the single base specificity of the sequence analysis detection level. These results suggested that APC mutations are not responsible for the β-catenin accumulation observed in the cervical cancer cell lines analyzed.

Methylation of the APC gene promoter 1A in cervical cancer cell lines and biopsies. It has been demonstrated that APC downregulation may be associated with epigenetic rather than genetic alterations in other tumor cell types (19,20). To investigate that possibility, the methylation status of the APC gene promoter 1A in cervical cancer cell lines was analyzed by MSP. The gastric cancer cell line KATOIII, which exhibits bi-allelic methylation (12) and HFF, without methylation, were used as positive and negative controls, respectively. The APC gene promoter 1A did not exhibit DNA methylation at CpG dinucleotides in the C33A, HeLa and SiHa cell lines. However, CaSki cells demonstrated heterozygous signals, indicating methylated and unmethylated alleles of the APC gene promoter 1A. (Fig. 3A).

To further investigate a possible association between the methylated status of the APC promoter, the levels of expression of β-catenin or its aberrant localization and HPV infection, the same MSP analysis was applied to a small group of biopsies from tumors of the uterine cervix. Different samples, with variations in the delocalization/accumulation of β-catenin and the types of HPV (HPV-16 or -18), were analyzed (7). Notably, the MSP results demonstrated that methylated alleles (homozygous) or unmethylated/methylated alleles (heterozygous) of the APC gene promoter 1A were only amplified in cervical tumor samples that exhibited abnormal subcellular localization of β-catenin and were infected by the HPV16 type (Fig. 3B). These observations indicated a clear association between the methylation status of the APC gene promoter 1A and the abnormal localization of β-catenin observed in cervical carcinoma specimens infected by HR-HPV16.

APC expression and Aza/TSA treatment. To determine how the methylation patterns of APC gene promoter 1A identified in certain cervical cancer cell lines may affect APC transcript levels, their abundance was examined by quantifying exon

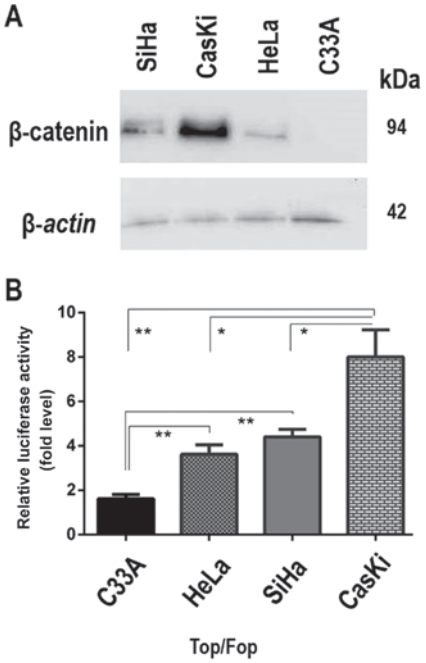

Figure 1. β-catenin expression and transcriptional activity in cervical cancer cell lines. (A) β-catenin protein was determinate by western blot analysis. An anti-β-actin antibody was used as the control for total protein loading. (B) Cell lines were transfected with TOPFLASH or FOPFLASH luciferase reporter constructs. Luciferase activity was determined in triplicate and the results are expressed as TOP/FOP correlation mean ± standard deviation for independent triplicate cultures. Significant induction of β-catenin activity ($^{*}P<0.05$; $^{**}P<0.001$) was calculated using an one-way analysis of variance and Tukey's multiple comparisons test. Top, TOPFLASH; Fop, FOPFLASH.

15 of APC by RT-PCR analysis. Abundant APC transcript expression in C33A, SiHa and Hela cell lines and HFF was identified where the APC promoter 1A was not methylated, whereas the KATOIII cell line, which demonstrated bi-allelic methylation, exhibited undetectable levels of APC mRNA. As expected, a marked reduction of APC transcripts in CaSki cells, which presented a heterozygous methylation pattern, was identified (Fig. 4A). These observations indicated that methylation of the APC gene promoter 1A led to the repression of APC expression. To further confirm this role of the methylation of APC gene promoter 1A in the transcriptional repression of the APC gene, KATOIII cells carrying fully methylated APC alleles and all cervical cancer cell lines were treated with the DNA methyltransferase inhibitor, Aza, together with TSA. Following treatment, the KATOIII cells demonstrated high levels of APC transcripts, a result associated with the demethylation of APC gene promoter 1A and the chromatin remodeling by histone acetylation (13). Of note, CaSki cells also demonstrated a marked increase in APC transcript expression following treatment (Fig. 4B), whereas the HFF, C33A, SiHa and HeLa cells did not demonstrate changes in APC expression levels. Taken together, these results highlighted that the levels of APC gene expression were negatively regulated by the methylation status of the promoter 1A in CaSki and KATOIII cells.

β-catenin expression and Wnt signaling pathway activity. It was observed that low levels of APC were linked to the methylation status of its promoter 1A. Since APC is a direct

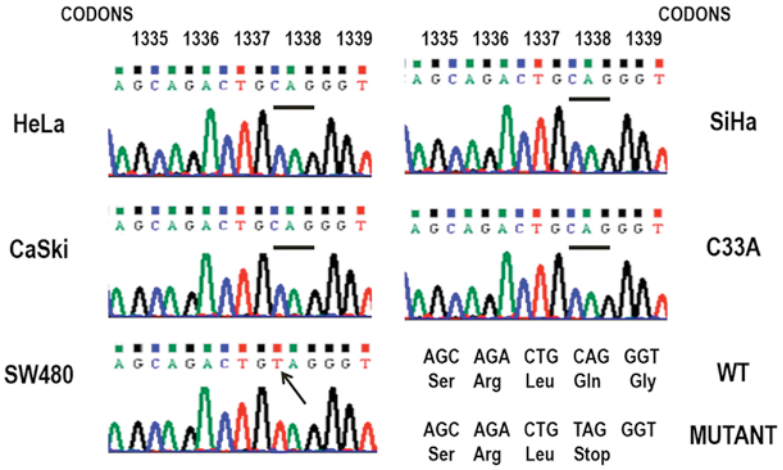

Figure 2. Sequence analysis of the APC gene. The APC mutation cluster regions from HeLa, CasKi, SiHa, C33A and SW480 cells were amplified by polymerase chain reaction and sequenced. The histogram from the codons 1335 to 1339 are shown as representative results of the sequence analysis with a mutation at codon 1338 (CAG/TAG) in the control SW480 cells. APC, adenomatous polyposis coli.

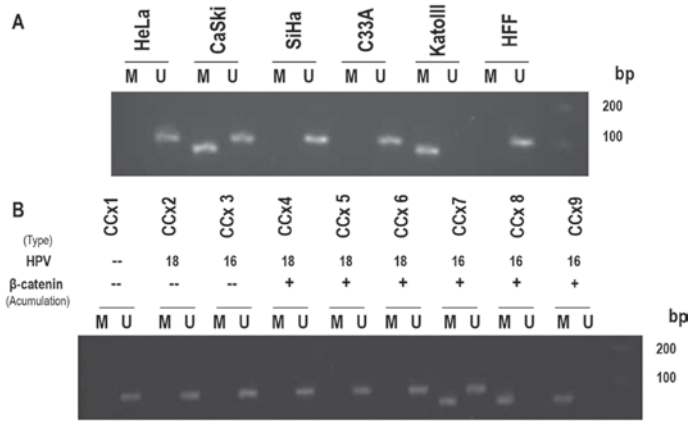

Figure 3. Hypermethylation analysis of APC gene promoter 1A. Methylation-specific PCR analysis were performed to determine APC promoter 1A methylation patterns in (A) cervical cancer cell lines and (B) biopsies. The DNA was treated with sodium bisulphite and PCR-amplified with primer pairs specific for unmethylated (108 bp) and methylated (98 bp) APC gene promoter 1A alleles. The PCR products were separated on an agarose gel. The HPV status of specimens is depicted as the specific HPV type present (16/18) or negative (-). The status of β-catenin is depicted as altered (+) or normal (-). KatoIII cells were included as positive control. APC, adenomatous polyposis coli; PCR, polymerase chain reaction; bp, base pair; HPV, human papilloma virus; U, unmethylated; M, methylated.

regulator of β-catenin, it was hypothesized that the reactivation of APC gene expression may lead to a reduction in β-catenin accumulation and a decrease in Wnt signaling pathway activity. To test this hypothesis, the expression of endogenous β-catenin and the expression of two β-catenin/TCF complex target genes in CaSki cells were evaluated prior to and following treatment with Aza and TSA. Prior to treatment, CaSki cells demonstrated abundant β-catenin protein levels, while following treatment; the β-catenin levels were significantly reduced. Consistently, alterations in β-catenin protein expression levels were observed in KATOIII cells, prior to and following treatment with Aza and TSA. In HFF, the negative control, the β-catenin protein levels did not alter following treatment (Fig. 5A). To further confirm the link between the presence of high β-catenin levels and high β-catenin/TCF complex activity, RT-PCR analysis was used to evaluate the expression of VEGF and MMP-7, two well-known target genes of the Wnt canonical pathway. The VEGF and MMP-7 mRNA levels were notably reduced following treatment in CaSki and KATOIII cells, while no alteration was observed in HFF (Fig. 5B). Taken together, these results confirmed that the methylation of the APC gene promoter 1A in CaSki cells had a direct effect on the expression of β-catenin and on the activity of the β-catenin/TCF complex, leading to increased levels of at least two Wnt target genes: VEGF and MMP-7. This suggested that this epigenetic modification of the APC gene promoter 1A may directly contribute to the expression of Wnt signaling pathway target genes involved in tumor growth and invasion.

Discussion

It has been widely accepted that abnormal activation of the Wnt signaling pathway leads to the stabilization and accumulation of β-catenin in the cytoplasm, then to its translocation to the nucleus, where it promotes the transcription of multiple genes involved in tumor growth and invasion (2,21,22). In certain types of cancer, including colorectal and hepatocellular

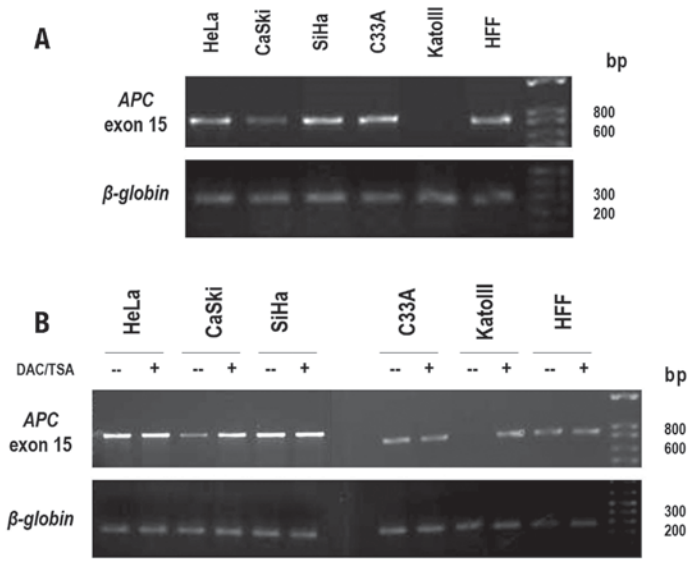

Figure 4. Analysis of APC gene expression in cervical cancer cell lines. (A) The expression of the APC gene in cancer cell lines was analyzed by RT-PCR. (B) The effect of the demethylating agents DAC (3 μM) and TSA (0.5 μM) on APC gene expression was measured by RT-PCR in cancer cell lines. Expression of β-globin mRNA was assessed in all samples as the control. The PCR products were separated on an agarose gel. APC, adenomatous polyposis coli; RT-PCR, reverse transcription-polymerase chain reaction; DAC, decitabine; TSA, trichostatin-A; bp, base pair.

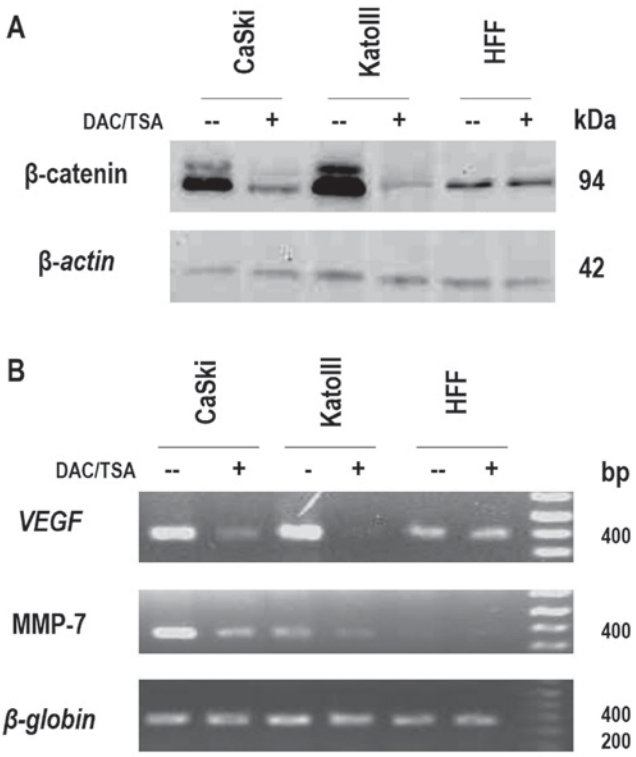

Figure 5. β-catenin repression and Wnt pathway activity. (A) β-catenin protein levels were determined by western blot analysis in CaSki, KatoIII and HFF cells prior to and following treatment with DAC (3 μM) and TSA (0.5 Mm). An anti β-actin antibody was used as the control for total protein loading. (B) VEGF and MMP-7 mRNA levels were analyzed via RT-PCR in CaSki, KatoIII and HFF cells prior to and following treatment with DAC (3 μM) and TSA (0.5 μM). The expression of β-globin mRNA was assessed in all samples as the control. The PCR products were separated on an agarose gel. HFF, human foreskin fibroblasts; DAC, decitabine; TSA, trichostatin-A; VEGF, vascular endothelial growth factor; MMP-7, matrix metalloproteinase-7; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair.

carcinomas, deregulation of this pathway occurs almost invariably through mutation of the two key regulators: APC and β-catenin. These mutations lead to the reduced regulatory activity of APC and enhanced protein stability of β-catenin, respectively (23,24). No mutations in CTNNB1 (β-catenin gene) have yet been detected in cervical neoplasia or cancer-derived cell lines (8,9). Therefore, the present study aimed to identify mutations in the APC gene. However, no significant mutations were observed at the 'hot-spot' codons (1061, 1338 and 1450) within the MCR, the most common site

of alterations that usually lead to a truncated protein lacking β-catenin binding and regulation sites (25).

A number of studies have suggested aberrant methylation in promoter regions as an epigenetic mechanism leading to the deregulation of tumor suppressor genes. Esteller *et al* (16) first demonstrated the mono- or bi-allelic methylation of APC gene promoter 1A in tumors of the gastrointestinal tract, including colon, gastric, pancreatic, esophageal and hepatic carcinomas. Subsequent studies have detected a similar status of promoter 1A methylation in several cancers, although in gastric cancer it has been identified with particularly high frequencies, in >50% of the cases (12,13,26). Fu *et al* (27) suggested that the hypermethylation of APC gene promoter 1A led to moderate activation of Wnt signaling pathway in colorectal cancer, instead of the usual mutations involving the APC and β-catenin genes. In apparent contradiction with these results, studies in cervical cancer samples have reported a great diversity in the APC gene promoter 1A methylation status (28,29), although none evaluated the association between APC promoter methylation, APC gene expression and the aberrant activation of the Wnt signaling pathway. To reconcile these data, the methylation patterns of the APC gene promoter 1A were evaluated in cell lines and biopsies from cervical cancer, in order to determine the frequency of this epigenetic regulation. These samples exhibited different levels of accumulation/delocalization of β-catenin and were either infected by HR-HPV type 16 or 18. The results demonstrated that the methylation frequency ranged from 1/4 (25%) to 3/9 (~33%) in cell lines and biopsies, respectively. These results are in conflict with a previous report, which demonstrated a 90% frequency of APC promoter 1A methylation in biopsies from cervical cancer (30). However, the results of the present study are comparable to other studies, which have proposed that the methylation of the APC promoter 1A, analyzed by MSP or by quantitative MSP, is not as common in cervical cancer cells, ranging between 12 and 35% (31-33). Notably, the results of the present study indicated a strong correlation between the methylation status of the APC gene promoter 1A and the delocalization/accumulation of β-catenin observed in biopsies that were infected by HPV16. Furthermore, alleles of APC promoter 1A which were completely unmethylated (homozygous) were observed in C33A (HPV negative), and HeLa cells (HPV18). The mechanism through which HPV16 promotes Wnt signaling requires further investigation. However, knocking down HPV16 E6/E7 reduced the Wnt signal in CaSki (HPV16) cells, while overexpressing HPV16 E6 and/or E7 increased the Wnt signal in C33A cells (34).

To confirm the role of the methylation of promoter 1A in the transcriptional regulation of the APC gene, APC gene expression was examined over the same panel of cervical cancer cell lines. The results demonstrated no significant difference in APC transcript levels in C33A, SiHa and Hela cell lines when compared with HFF, where the APC gene promoter 1A was not methylated. The absence of APC mRNA in the KATOIII cell line, which presented bi-allelic hypermethylation, was confirmed (12), and it was identified that CaSki cells, which demonstrated a heterogeneous methylation pattern, exhibited a marked reduction of APC mRNA levels. The RT-PCR and MSP analyses thus demonstrated a correlation between APC expression levels and the APC gene promoter 1A methylation status in the cell lines. Additionally, since some studies have demonstrated that the lack of expression of tumor suppressor genes may be reversed by treatment with epigenetic silencing inhibitors (inhibitor of DNMT and HDAC) in cancer cells (35,36), this strategy was employed to evaluate the recovery of APC gene expression. The results demonstrated a significant increase in APC mRNA levels following treatment with Aza and TSA in CaSki cells, whereas no significant alteration was observed in C33A, SiHa and HeLa cells. Similar results were observed in melanoma and gastric cancer cell lines carrying a mono- or bi-allelic methylation of APC gene promoter 1A, in which the low or absent APC expression levels were modified following Aza and TSA treatment, leading to its active transcription and recovery of APC expression (13,37). These results indicated that the presence of promoter methylation suppressed APC gene transcription and contributed to inactivating the APC tumor suppressor function in CaSki cells.

Conversely, previous studies with human cervical carcinoma samples observed an abnormal accumulation/delocalization of β-catenin, which constitutes a hallmark of the activated Wnt pathway (7,8). The present study confirmed through western blot analyses and luciferase reporter assays that the abnormal expression of β-catenin is associated, in a concentration-dependent manner, with the transcriptional activity of the β-catenin/TCF complex in CaSki, SiHa and HeLa cervical cancer cells infected with HR-HPV16 or 18. In CaSki cells (HPV16), with the greatest β-catenin expression, the strongest transcriptional activity was observed, whereas neither expression of β-catenin, nor β-catenin/TCF transcriptional activity was observed in non-HPV infected C33A cervical cancer cells (38,39). To determine the role of the APC promoter methylation in the regulation of Wnt/β-catenin signaling, the expression of endogenous β-catenin and the expression of β-catenin/TCF complex target genes were evaluated following treatment with Aza and TSA. In CaSki cells, a reduction of β-catenin levels was observed, in addition to a decrease in the transcripts of two β-catenin/TCF target genes, VEGF and MMP-7. These results suggested that stimulating the expression of the APC gene via treatment with epigenetic silencing inhibitors reduced the abnormal activation of the Wnt pathway in CaSki cells. This conclusion is further supported by a study from Svedlund *et al* (40), which demonstrated that the treatment of a primary PC cell culture with the DNA hypomethylating agent Aza induced APC expression and reduced β-catenin levels. Taken together, these data suggested that the re-expression of the APC gene in CaSki cells appears to be sufficient to assemble a functional complex of Axin/PP2A/GSK-3β/CK1/APC, capable of phosphorylating the β-catenin protein, eventually leading to its degradation and switching off the Wnt/β-catenin signaling pathway (41).

In conclusion, the present study proposed that methylation-dependent silencing of the APC gene promoter 1A is a mechanism that contributes to the activation of Wnt signaling pathway in cervical cancer cells infected by high risk HPV16. The reduction of APC levels induced by hypermethylation of the APC gene promoter 1A, rather than direct mutations in the APC or β-catenin genes, leads to accumulation of β-catenin, which in turn increases the Wnt/β-catenin transcriptional activity. This mechanism may lead to an increase

in the transcription of genes involved in cancer development. Therefore, the development of an epigenetic therapy to reactivate the expression of *APC* could be advantageous for the treatment of cervical cancer cells-infected with HPV16. However, further studies need to be performed to confirm the above results.

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