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Effects of Promoter Methylation on the Expression Levels of Plakoglobin Gene in Both the ARO Thyroid Cancer Cell Line and Cancer Tissues

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Plakoglobin (PKG) is a protein linking cadherin adhesion receptors to the actin cytoskeleton and its overexpression has been known to suppress cell proliferation and tumorigenesis in thyroid cancer. We investigated the effect of 5-aza-2'-deoxycytidine (5-Aza-CdR), a DNA methyltransferase inhibitor, on the methylation status of the promoter and the expression of the plakoglobin gene in a thyroid carcinoma cell line (ARO) and papillary thyroid carceinoma. In cultures of ARO cell line incubated without 5-Aza-2'-deoxycytidine (5-Aza-CdR), five of the fifteen CpG sites in the promoter spanning -225 and -54 were methylated at 4.2 - 12.5%. When the cells were treated with 5-Aza-CdR, all the methylated CpG sites were induced to be demethylated except one. In addition, a new methylation at one CpG site, CpG4, was identified at level of 12.0%. The expression level of PKG decreased approximately 10-fold in the 5-Aza-CdR treated cells compared to untreated cells. Different pattern of promoter methylation and expression of PKG was also observed in the tissue samples. CpG10 and CpG12 sites were methylated at 9.0-27.0% in normal tissues. However, in cancer tissues, CpG5 and CpG10 sites were methylated at 10.0-22.0%. Three of ten normal thyroid tissue samples and one of thirteen papillary carcinoma tumor samples showed increased PKG mRNA expression level. PKG protein expression analyzed by the immunohistochemical staining showed higher expression in the tumor compared with normal.

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I. INTRODUCTION

Thyroid tumors of follicular cell origin comprise follicular neoplasms (benign follicular adenoma and follicular carcinoma, FTC) and papillary thyroid carcinoma (PTC). PTC is the most prevalent endocrine malignancy in humans (Melillo *et al*, 2005) and many thyroid cancer cell lines have been developed (Roswall *et al*, 2006). A few genetic abnormalities, at the somatic level, are associated with PTC: chromosomal alterations that affect the RET or TRKA tyrosine kinase receptors and oncogenic activation of the RAS or BRAF genes (Melillo *et al*, 2005). The ARO cell line is one that originated from an anaplastic thyroid cancer and is characterized by having a number of genetic abnormalities including mutations of p53 (Fagin *et al*, 1993), p15 (Elisei *et al*, 1998), protein kinase Cε (Knauf *et al*, 1999), and APC (Zeki *et al*, 1994).

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The regulation of thyroid cell proliferation in human is dependent on a number of growth factors such as TSH, EGF, IGF-1, insulin, FGF, and TGF. Cell adhesion receptors and their associated proteins such as β-catenin and plakoglobin (ɣ-catenin) also play critical roles in the progression of thyroid cancer (Rocha *et al*, 2001; Smyth *et al*, 2003). Additional thyroid tumor markers have become available by transcriptosome analysis of thyroid cancer (Reis *et al*, 2005).

For many genes that might be involved in the oncogenesis of the thyroid cancer, promoter methylation has been reported to be related to activation or inactivation of the genes. In cases of TSHR, MT1G, CRABP1, and NIS, hypermethylation of the promoter accompanied the underexpression of the genes (Roth *et al*, 2006; Venkataraman *et al*, 1999; Xing *et al*, 2003). Meanwhile in the case of RASSF1A and Mapsin, hypermethylation induced an overexpression of the genes (Kim *et al*, 2005; Nakamura *et al*, 2005; Ogasawara *et al*, 2004).

Plakoglobin is a structural and functional homologue of β-catenin linking cadherin adhesion receptors at cell to cell adhesion sites to the actin cytoskeleton (Hakimelahi *et al*, 2000). Plakoglobin is associated with transcription factors of the LEF/TCF family to regulate the expression of target genes that are involved in cell death and cell proliferation (Ben-Ze'ev and Geiger, 1998). Although elevated β-catenin expression has been implicated in hyperproliferation and tumor formation (Korinek *et al*, 1997; Morin *et al*, 1997), overexpression of plakoglobin was shown to suppress cell proliferation and cell tumorigenesis in experimental animals (Simcha *et al*, 1996). Consistent with the ability of plakoglobin to act as a tumor suppressor are the findings that reduced plakoglobin expression was observed in tumor tissues (Buchner *et al*, 1998; Nakanishi *et al*, 1997). Even though the plakoglobin gene is a major regulator of the thyroid cell, no study on the methylation status of the promoter of plakoglobin gene have been reported in thyroid tumors. To date, methylation status has been described only in the regions around and downstream of the transcription start site in rhabdomyosarcoma (Gastaldi *et al*, 2006).

The purpose of present study was to evaluate a possible relationship between methylation status of the plakoglobin promoter and expression of the gene in the ARO thyroid cancer cell line and normal and tumor tissue samples of the thyroid. We also compared the expression level of PKG mRNA to the expression of PKG protein by RT-PCR and immunohistochemistry in the normal and papillary carcinoma of thyroid tissues.

II. MATERIALS AND METHODS

1. Cell culture, treatment with 5-Aza-CdR, and tissue samples

A human thyroid cancer cell line, ARO, was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultivated in RPMI medium supplemented with 0.1 mM non-essential amino acids, 2 mM L-glutamine, 10% fetal bovine serum, and 50 U/mL penicillin-streptomycin. Cell cultures were maintained in a humidified 5% CO_2 environment.

For the treatment of ARO cells with the methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-AzaCdR; Sigma, St. Louis, MO), 3×10^5 cells were plated onto 90-mm^2 dishes 18-24 hours prior to adding 5-AzaCdR at a final concentration of 5 μM. More 5-Aza-CdR was freshly added onto cells 24 and 36 hours after the first adding and cells were further cultured for 12 hours before harvest.

Thyroid tissues were obtained from surgically resected primary thyroid tumors of patients diagnosed at Yonsei University Severance Hospital between January 2004 and December 2005. All tumor samples were examined histologically for the presence of tumor cells. Normal thyroid tissues were obtained from histologically normal tissues next to the tumor cells. Sixty fresh tissue samples was collected, and frozen in liquid nitrogen at the time of thyroidectomy under the diagnosis of papillary thyroid

carcinoma, and stored at -75℃.

2. Isolation of nucleic acids

Chromosomal DNA was extracted from the ARO cells cultured in 90-mm^2 and thyroid tissues after lysis in 80 μL of buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% Tween 20, 200 μg/mL of proteinase K) at 55℃ for 72 hours and finally suspended in 50 μL of distilled water.

The total RNA was prepared using Trizol according to the supplier's protocol (GibcoBRL, Carlsbad, CA) (Kang *et al*, 2004). The RNA from cells cultured in a 90-mm^2 dish and a thyroid tissue slice was finally suspended with 20 μL of RNase-free water.

3. Bisulfite genomic sequencing

Bisulfite genomic DNA sequencing was carried out as previously described (Kang *et al*, 2001) with a minor modification. Briefly, chromosomal DNA extracted from the tissues was digested with EcoRI and HindIII, and then subjected to bisulfite treatment. The bisulfite-treated DNA was subjected to two rounds of PCR to amplify a 217 bp DNA fragment of the promoter region. The primer sequences are 5'-AGGATGAGATTATAAGTTAGAG-3' and 5'-AACTCTAACCAAAACAACTC-3' for the primary PCR, and 5'-AGGTTAGAGTTTATTTTTAGAG-3' and 5'-AAAAAACCTAACAACCAAACCA-3' for the nested PCR. All the PCR conditions were 94℃ for 2 min, 35 cycles of 94℃ for 30 sec, 55°C for 1 min, and 72℃ for 30 sec, with a final extension at 72℃ for 5 min. The resulting products were purified using a PCR purification kit (Bioneer, Daejon, Korea), subcloned into the pGEM-T vector (Promega). The DNA sequences were confirmed by analyzing each clone in both directions. The promoter regions of PKG that we selected to investigate included 15 CpG sites (-225 through -54 of GenBank accession no. AF233882, taking the major transcriptional site to be +1 when numbering, Fig. 1).

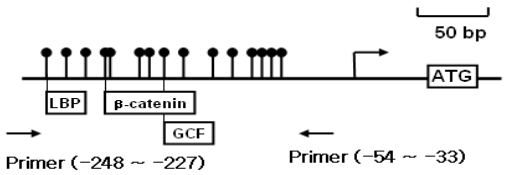

Fig 1. Schematic diagram for the CpG sites in the promoter region of the plakoglobin gene. ATG signal, transcription start site (arrow), and factor-binding sites were indicated. A primer set was used to amplify a 217 bp fragment and the positions of primers are indicated. •, CpG sites amplified by PCR.

4. Real-time and end-point RT-PCR

Reverse transcription was carried out with 10 μg of total RNA using a reverse transcription kit (Promega). Real-time PCR reactions were carried out using 100 ng of the template (cDNA), 300 nM of each forward and reverse primer, a dye-labeled TaqMan probe, and 1× PCR Master Mix (Applied Biosystems, CA; USA) in a volume of 25 μL. Each PCR plate contained triplicates of the test cDNA template and samples, which were used to construct a standard curve. Samples were amplified for 40 cycles in an ABI Prism 7300 Sequence Detection System (Applied Biosystems) with an initial melt at 95℃ for 10 min, followed by 40 cycles, each carried out at 95℃ for 15 sec then 60℃ for 1 min. The partial cycle giving a statistically significant increase in the PKG product was determined and normalized to G3PDH. The primer sequences used for PKG were as follows: forward primer, 5'-GGCATCCCTGCTCTGGTC-3'; reverse primer, 5'-GCGTGGTGATGGCATAGAACA-3'; and probe, 5'-CATGCTCAGCTCCCC-3'. G3PDH was amplified using a probe and primers synthesized by Applied Biosystems.

End-point PCR reactions were carried out using primers 5'-AGGCGGCCATGATTGTGAAC-3' and 5'-TGCGGA-CCAGAGCAGGGATGT-3' to amplify 219 bp fragment of the PKG cDNA. G3PDH primers were used to normalize PKG expression; their sequences are 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCA-CCCTGTTGCTGTA-3', and they amplified a 500 bp cDNA fragment. A total of 25 cycles of the PCR

reaction were performed, each cycle being carried out at 94℃ for 30 sec, 55℃ for 1 min, and then 72℃ for 30 sec.

5. Immunohistochemical staining

Immunohistochemical staining was performed on paraffin embedded tissues and the primary antibody used for this study was monoclonal anti-plakoglobin (Clone, 4F11; Zymed, South San Francisco, CA, USA). Heat-induced epitope retrieval using microwave oven was done for Plakoglobin. Antigen was localized using an EnVision™ Detection System (DAKO, Carpinteria, CA, USA). Paraffin blocks were sectioned into 3㎛ with microtome, and section ribbon was attached to coating slides, which were treated with 3-aminopropyltriethoxy silane (Sigma Chemical, Behring, Germany). After deparaffinization and hydration process, endogenous peroxidase activity was blocked by incubation for 10 minutes in 3% H_2O_2. The slides were put into 10 mM citrate buffer (pH 6.0) that was preheated in microwave for 10 minutes and then boiled for 15 minutes. After cooling at room temperature for 30 minutes, the slides were put into PBS (pH 7.5) for 10 minutes, followed by incubation with anti-plakoglobin (dilution, 1:100) antibody in refrigerator for 18 hours. After being washed slides with PBS (pH 7.5), the slides were reacted with the EnVision™ Detection System for 30 minutes. Counter staining of the slides was performed with Mayer's hematoxylin for 5 minutes. The cells of which cytoplasmic membrane was stained in brown color were evaluated to be positive cell of PKG.

6. Statistical analysis

Differences in the percentages of PKG between PTC and normal thyroid tissues were evaluated using the student's t-test. The significance of differences in the Plakoglobin was tested using student's t-test. Values were indicated in percentage with means ± standard deviation. The program used for analysis was SAS (Statistical Analysis System) v 9.1 (SAS Institute Inc. Cary, NC). P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Methylation status of the plakoglobin promoter in the ARO cells

To examine the methylation status of the plakoglobin promoter in the thyroid cancer cell line, ARO, the promoter region containing a CpG island was amplified by PCR after treatment of the DNA with bisulfite. There appeared 15 CpG sites at the proximal promoter region at -225 to -54 upstream of the transcription starting site (Fig. 1). A 217 bp DNA fragment covering all 15 CpG sites was amplified by nested PCR and subcloned into the pGEM-T vector. In total, 30 clones were sequence analyzed for both directions to determine methylation status of the CpG sites. The result indicated that all the cytosine residues outside of the CpG sites were changed to thymine residues implying that bisulfite induced a proper demethylation status. For the CpG sites, five sites showed methylation levels of 4.2-12.5% (Fig. 2). The other 10 sites showed no methylation. An example of methylated

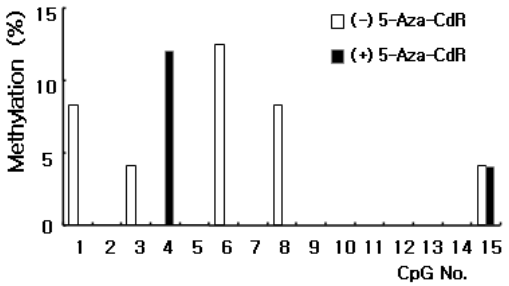

Fig 2. Methylation profiles for the CpG islands in the PKG promoter before and after treatment with 5-Aza-CdR of the ARO cells. The methylation status of the 15 CpG sites in the PKG promoter were examined before (white bar) and after (black bar) treatment with 5-Aza-CdR. CpG no.1 is the most distal CpG from the transcription initiation site. The bisulfitetreated region was PCR amplified, subcloned into the plasmid vector, and sequence analyzed. The graphs are results from 30 plasmid clones per sample.

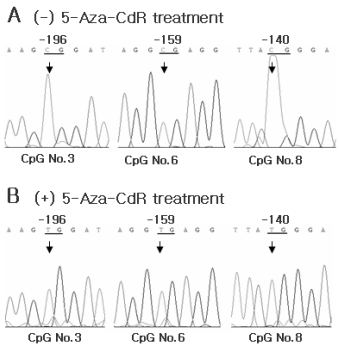

Fig 3. Examples of a bisulfite sequencing chromatogram. DNA was modified by bisulfite and PCR amplified. PCR products were then subcloned into the pGEM-T vector and sequenced on an ABI automated sequencer. The number attributed to CpG sites is the sequence number from the transcription start site. CpG sites are underlined, and the cytosine (C) or thymine (T) peaks are indicated by arrows. A and B are representations for results of no treatment and treatment of 5-Aza-CdR, respectively.

and unmethylated cytosine is presented in Fig. 3.

2. Methylation status of the plakoglobin promoter after treatment with 5-Aza-CdR in the ARO cells

5-Aza-CdR was added to the culture media to inactivate the DNA methyltransferase activity in the ARO cells, and the methylation of the CpG sites was examined for 30 clones. The five CpG sites that had shown methylation before 5-Aza-CdR treatment were completely demethylated except for one (CpG15) that is most proximal to the transcription initiation site. Interestingly, the cytosine residue of the CpG4 site that had no methylation before treatment with 5-Aza-CdR appeared newly methylated at a level of 12.0%. The other 13 CpG sites remained totally demethylated or unmethylated status (Fig. 2).

3. Expression level of plakoglobin gene after demethylation of the promoter in the ARO cells

To examine the relationship between promoter methylation and expression level of the PKG gene, PKG expression was monitored by real-time RT-PCR. As shown in Fig. 4, The expression level of PKG gene of ARO cells that were not treated with 5-Aza-CdR showed approximately 10 times higher than those treated with 5-Aza-CdR. Considering that cells treated with 5-Aza-CdR had lower methylation as well as a different methylation pattern at the promoter level, it may be considered that altered methylation pattern of the promoter provoked the lowered expression of the PKG gene.

4. Methylation status of promoter and expression level of the PKG gene in normal and tumor samples of the thyroid

To examine whether there also exists a different methylation profile in the PKG promoter between thyroid cancer and normal tissues, the methylation status of the 15 CpG sites were deciphered in 34 cancer and normal tissue sets. As indicated in Fig. 5, CpG sites identified that the cancer tissue showed different CpG profile from the normal tissue. In the normal tissues, CpG10 and CpG12 sites were methylated at 9.0-27.0%. In the cancer tissues, CpG5 and CpG10 were methylated at 10.0-22.0%. The methylation level in tissues (9.0-27.0%) is a little higher than that of the ARO cells (4.2-12.5%). The methylated

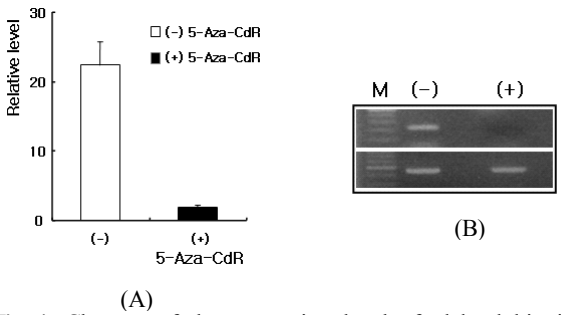

Fig 4. Changes of the expression level of plakoglobin in the ARO cell by treatment with 5-Aza-CdR. Total cellular RNA (5 μg) from the ARO cell cultured in the absence (-) and presence (+) of 5-Aza-CdR was reverse transcribed. A. The resulting cDNA was amplified by real-time PCR using the PKG-specific TaqMan probe. The expression levels are the averages of three independent reactions with standard error bars after normalization with G3PDH. B. End-point PCR was performed using a primer set spanning exon 3 of PKG to amplify a 219 bp DNA fragment. The figure at the bottom is the result for G3PDH as a control. M is a molecular weight marker.

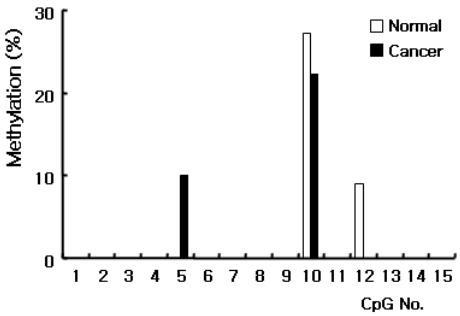

Fig 5. Methylation profiles for the CpG islands in the PKG promoter of the normal and cancer thyroid tissues. The methylation status of the 15 CpG sites in the PKG promoter were examined in the normal (white bar) and cancer thyroid tissues (black bar). CpG no.1 is the most distal CpG from the transcription initiation site. The bisulfite-treated region was PCR amplified, subcloned into the plasmid vector, and sequence analyzed. The graphs are results from eight plasmid clones per sample.

CpG sites are also different in the ARO cells and thyroid tissues (Fig. 2, 5).

The expression level of PKG was monitored by real time PCR analysis of total RNAs from 13 cancer and 10 normal thyroid tissues. One cancer and three normal samples showed detectable expression levels (Fig. 6). Comparing the different methylation profiles between the normal and cancer tissues, it can be considered that the altered methylation profile induced change of expression level.

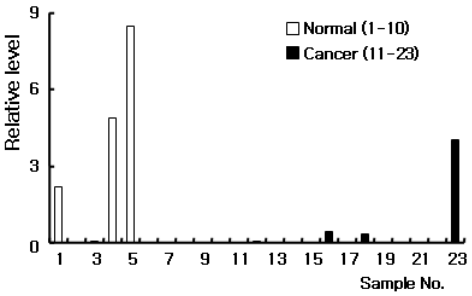

Fig 6. Changes of the expression levels of plakoglobin in the normal and cancer thyroid tissues. Total cellular RNA (5 μg) from the tissue was reverse transcribed, and the resulting cDNA was amplified by real-time PCR using the PKGspecific TaqMan probe. The expression levels are presented after normalization with GAPDH.

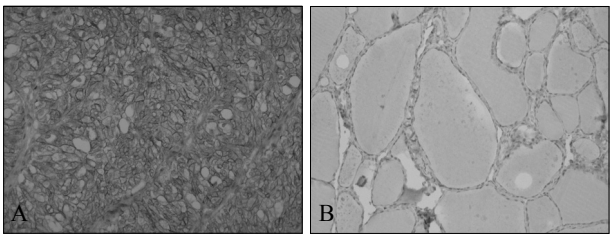

Fig 7. Immunohistochemical stain using a monoclonal antibody for plakoglobin antigen in the thyroid tissues. Immunoreactivity was considered as positive when brown staining of the cytoplasm and membrane, or both was observed. A. Most of cell membranes were strongly stained with brown color in the PTC (×400). B. The plakoglobin was localized to the small follicles in the normal thyroid tissue (×200).

5. Immunohistochemical analysis of the PKG protein expression in normal and papillary cancer of the thyroid tissues

PKG protein expression was compared in the normal and papillary carcinoma tissue sections. The PKG protein positive cells demonstrated brown color reaction in the cytoplasm and membrane, or both (Fig. 7A). In normal tissues, positive reactions were usually confined to follicular epithelial cells lining small follicles (Fig. 7B). In the PTCs, about 49.53±14.56 % of the cells showed positive staining for PKG protein, while only 1.64±0.67 % in the normal tissues. There was no statistically significant difference in the PKG protein expression rate between PTCs and normal positive cells ($p>0.05$)

IV. DISCUSSION

Plakoglobin is known to be down-regulated in many cancers, including the thyroid cancer (Cerrato *et al*, 1998). Unlike the ß-catenin, plakoglobin mutations are rare. However, the human plakoglobin gene can display loss of heterozygosity as in breast and ovarian tumors and this has been associated with cancer progression (Aberle *et al*, 1995). Epigenetic alteration of the plakoglobin promoter can be another crucial mechanism regulating expression of the gene. The CpG methylation status of cancer-related

genes has been reported in a few tumor cell lines (Potter *et al*, 2001). In the cases of renal cancer (Breault *et al*, 2005) and rhabdomyosarcoma (Gastaldi *et al*, 2006) expression of PKG is regulated by promoter CpG methylation, however, the examined CpG sites were very restricted to regions downstream of the transcription start site. Expression of plakoglobin is regulated by many transcriptional factors acting on the cis-elements in the promoter (Potter *et al*, 2001; Samady *et al*, 2006) where CpG sites are abundant and comprise the CpG island. In this study, we have examined the methylation status of the 15 CpG sites in the PKG promoter region spanning -225 to -54 for the ARO cells and thyroid tissues. Although change of methylation status is related with the variation of expression level in both samples of the ARO cells and thyroid tissues, their methylation profiles are not the same (Fig. 2, 5). This fact indicates that the ARO cells do not represent the characteristics of the thyroid cancer.

Of the four CpG sites (CpG1, 3, 6, 8) that showed methylation in the ARO cells cultured without 5-Aza-CdR, CpG1 and 8 located in or near the transcriptional factor binding sites, LBP (Sato *et al*, 2005) and GCF (Takimoto *et al*, 1999), respectively. The CpG4 site, the only CpG site showing methylation after treatment of 5-Aza-CdR, is the β-catenin binding site (Schwartz *et al*, 2003). Considering that plakoglobin and β-catenin are structural and functional homologues linking the cadherin adhesion receptors at cell to cell adhesion sites to the actin cytoskeleton (Hakimelahi *et al*, 2000), it is interesting that regulation of plakoglobin expression can be modulated through the methylation at the CpG site that is located close to the β-catenin-binding DNA element. Taken together, these results imply that the methylation profile in this region also participates to modulate the expression level of plakoglobin.

When 5-Aza-CdR was added to the culture media of ARO cells, complete demethylation of the methylated CpG sites was induced, except CpG15. In addition, a new methylation at one CpG site emerged from the 5-Aza-CdR treatment. This result indicates that 5-Aza-CdR is not sufficient to inhibit activity of the DNA methyltransferase and other factors, including an inhibitor of histone deacetylase are needed. Thus, the modified methylation pattern after 5-Aza-CdR treatment is thought to come from the partial inhibition of methyltransferase and this in turn might induce a local change of chromosome structure at the modified promoter region. A further assay using histone deacetylase inhibitors such as Trichostatin A in combination with 5-Aza-CdR would be useful to identify the factors that induced modified CpG methylation.

In a few tumor cells, a DNA demethylation reagent was shown to restore plakoglobin expression (Gastaldi *et al*, 2006). In our study, however, 5-Aza-CdR induced a further down-regulation of plakoglobin. We speculate that this result may come from the newly arisen CpG4 site where β-catenin may potentially bind. These alternative methylation patterns, as demonstrated for ARO cancer cell line and thyroid tissues, can provide useful information regarding the methylation profile of the plakoglobin promoter and its expression level in thyroid cancer. By immunohistochemical analysis, about 49.53±14.56% of the tumor cells showed positive staining for PKG protein in the PTCs, while only 1.64±0.67% of the thyroid follicular cells in normal tissue express the protein ($p>0.05$).

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