Plakoglobin is differentially expressed in alveolar and embryonal rhabdomyosarcoma and is regulated by DNA methylation and histone acetylation

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Plakoglobin (γ-catenin) and β-catenin are pivotal components of cell–cell adherent junctions that link cadherin receptors to the actin cytoskeleton. Whereas β-catenin overexpression induces cell proliferation and tumor formation, plakoglobin induces tumor suppressor activity. We investigated the expression of plakoglobin in alveolar (ARMS) and embryonal (ERMS) rhabdomyosarcoma (RMS) cell lines and tumors, and found that plakoglobin is present both in the cytoplasm and in the nucleus of ERMS cells, whereas it is absent or detectable at extremely low levels in ARMS. As gene silencing can be mediated by methylation and/or deacetylation of promoter regions, we assessed the effects of the DNA demethylating agent 5-Aza-2'-deoxycytidine (5AzadC) and of the histone deacetylase inhibitor Trichostatin A (TSA), and obtained restoration of plakoglobin expression in ARMS cells cultivated in the presence of 5AzadC and TSA. By methylation-specific PCR, ARMS cells were shown to contain methylated CpG dinucleotides in CpG islands located around the transcriptional start site of one or both alleles, whereas ERMS cells did not. Furthermore, we demonstrated that promoter regions (P1–P3) of plakoglobin gene were associated with hypoacetylated H4 histone in ARMS cells RH4, suggesting that aberrant DNA methylation of the 5' CpG island and histone deacetylation play key roles in silencing the plakoglobin gene. These results demonstrate that plakoglobin is differentially expressed in ARMS and ERMS and that its expression depends on the methylation and acetylation status of the gene.

Introduction

Rhabdomyosarcoma (RMS) includes two major histological subtypes: the more frequent embryonal RMS (ERMS), and the more aggressive alveolar RMS (ARMS) (1,2). The latter harbors a reciprocal chromosomal translocation t(2;13) (q35;q14), or, less frequently, the variant translocation t(1;13)(p36;q14) (3,4) that generates a powerful chimeric transcriptional activator PAX3–FKHR or PAX7–FKHR (5) with oncogenic activity (6). The PAX3–FKHR fusion protein causes aberrant expression of a set of PAX3-target genes normally regulated during development by the wild-type protein, including c-Met (7,8) or the HER/erbB family members (EGFR and erbB2) (9), which have been closely linked with tumor aggressiveness and poor prognosis in many cancer types. These receptor tyrosine kinases, which are expressed in RMS cell lines (7–9), are involved in proliferation, migration and metastasis through the phosphorylation of different proteins contained in cellular junctions with consequent tissue destruction and the increase in invasiveness.

β-Catenin is a multifunctional protein that plays a pivotal role both in cell–cell interactions and transcriptional activation in Wnt/Wingless signaling during development (10). The regulation of membrane, cytoplasmic and nuclear pools of β-catenin is crucial for modulating its adhesion and signaling functions (11).

The catenin family member plakoglobin, also known as γ-catenin, is an adhesion molecule with structure, cellular distribution and protein-binding specificity similar to β-catenin. In addition to its involvement in the desmosome structure, plakoglobin is a component of the adherens junctions and binds β-catenin and α-catenin (12).

β-Catenin expression level is high in cancers of various origin (13), mostly as a result of point mutations in β-catenin phosphorylation sites necessary for its ubiquitination or in key components of the degradation machinery (14,15). Unlike β-catenin, plakoglobin mutations are rare. However, the human plakoglobin gene can display the loss of heterozygosity in certain tumors and this has been associated with cancer progression (16). Moreover, plakoglobin overexpression was shown to suppress tumorigenicity in various transformed cell lines (17).

Methylation of cytosine residues at CpG dinucleotides repeats is an important biological phenomenon for gene silencing and regulation of chromatin structure. Cytosine hypermethylation in the promoters of tumor suppressor genes such as RASSF1A and HCJ has been linked to transcriptional repression (18,19). Recent work has demonstrated the importance of post-translational modification of histone proteins as another epigenetic mechanism in the organization of chromosomal domains and gene regulation (20,21). Acetylation of lysine residues on histone H3 and H4 leads to the formation of open chromatin structure (22), whereas deacetylation is associated with repressed chromatin (20,23). There is growing evidence that an interplay exists between cytosine methylation and histone modification. The methyl-CpG binding protein, MeCP2, has been associated with histone deacetylase (HDAC) activity, suggesting that histone modification can be induced by DNA methylation changes (24,25).

In this study, we report that plakoglobin was downregulated in a subset of RMS and hypothesized that this may be a consequence of hypermethylation of CpG islands and
hypocacytation of histones within plakoglobin promoter region. To test these hypotheses, the expression and the methylation/acetylation status of the plakoglobin promoter were analyzed in RMS cell lines and biopsies.

Materials and methods

Cell cultures and tumor samples
The human RMS cell lines RH30 and RD were purchased from ATCC (Manassas, VA); RH4, RH18 and RH28 were a gift of Dr P.J. Houghton (St Jude Children’s Hospital, Memphis, TN); RC2 was provided by Prof. P.L. Lollini (University of Bologna, Bologna, Italy); SMS-CTR, RH36, CCA and the undifferentiated sarcoma cell line A-204 were obtained from Dr M. Tsokos (NCl, Bethesda, MD); the neuroblastoma cell line LAN-5 was a gift of Dr M. Ponzioni (Gaslini Hospital, Genova, Italy). Cells were maintained in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mM/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin under standard tissue-culture conditions. Specimens were obtained from the Italian Association of Pediatric Hematology and Oncology Soft Tissue Sarcoma Bank at the Department of Pediatrics, University of Padova (Padova, Italy).

Reagents and antibodies
Anti-β-catenin and anti-plakoglobin (γ-catenin) monoclonal antibodies were purchased from BD Transduction Laboratories (Lexington, KY), Actinin IgM monoclonal antibody was obtained from Oncogene Research Products (San Diego, CA). Anti-γ-tubulin was purchased from Sigma-Aldrich (St Louis, MO). Anti-HDAC1 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit antibodies were purchased from Amersham Bioscience (Buckinghamshire, UK). Bicinchoninic acid protein assay reagents and western blot chemiluminescence reagents were obtained from Pierce Chemical (Rockford, IL).

Protein extraction and western blot analysis
Cell lines were treated with either Trichostatin A (TSA) (Sigma-Aldrich) 200 ng/ml for 16 h or 5aza-2-deoxycytidine (5AzadC) (Sigma-Aldrich) 2 μM for 72 h. Cells were washed twice in ice-cold 1× PBS and incubated on ice for 20 min with lysis buffer [20 mM Tris (pH 7.5), 140 mM NaCl, 25 mM NaF, 25 mM glycerophosphate, 5 mM sodium orthovanadate, 5 mM EDTA, 5 mM EGTA, 1 mM sodium pyrophosphate, (pp), 1 mM DTT, 1.5 mM MgCl2, 10% glycerol, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 20 μg/ml leupeptin and 20 μg/ml aprotinin] containing 1% Triton X-100. Cell lysates were clarified by centrifugation at 4°C for 14 000 g for 30 min. Protein concentration was determined by the bicinchoninic acid. Aliquots of cell lysates, diluted to a final concentration of 10 μg/ml, were separated on a 10% SDS–PAGE gel and electrotransferred to nitrocellulose membranes. Membranes were exposed to Hyperfilm (Amersham) and signals were quantified by using the NIH image analysis software.

Semi-quantitative RT–PCR analysis
Total RNA was extracted from cultured cells and tumor specimens using RNAzol-Be reagent (Tel-Text, Friendstown, TX) according to the manufacturer’s instructions. From each sample 1 μg of total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random hexamers. The cDNAs were then amplified by PCR using primers specific for plakoglobin (5′-ATGGAGGTGTTAGAAGACCTGTT-3′ and 5′-TGGCAATAGCAGAGGACCAGGACT-3′) and for β2-microglobulin, used as an internal control to standardize the amount of DNA in each sample, (5′-TGGGAGGACCTTTGTCGTTCA-3′ and 5′-TCCATCATTACCAATGCGCATCT-3′). Based on preliminary experiments, we identified the PCR exponential phase and selected 33 cycles for amplification of plakoglobin and 26 cycles for β2-microglobulin. PCR conditions included initial denaturation at 94°C for 2 min followed by 94°C for 45 s, 61°C for 30 s, 72°C for 45 s, for plakoglobin, and by 94°C for 15 s, 60°C for 15 s, 72°C for 15 s, for β2-microglobulin, and a final extension step at 72°C for 5 min, on a GeneAmp PCR 9600 System (ABI Applied Biosystems, Foster City, CA). PCR products were electrophoresed through 2% agarose gels and quantified by densitometric analysis of band intensity using Quantity One Quantitation Software (Bio-Rad, Milan, Italy).

Nuclear and cytoplasmic protein fractions
To obtain purified cytoplasmic and nuclear fractions, cells were processed according to the protocol described in ref. 26. Cells grown on Petri dishes were washed three times in ice-cold 1× PBS, frozen on dry ice and scraped into hypotonic lysis buffer (10 mM HEPES, 10 mM KCl, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT and a cocktail of protease inhibitors) with 2% NP-40. Cell lysis were incubated on ice for 10 min, passed through a 22 gauge needle and then centrifuged for 2 min at 10 000 g. The supernatant was removed as cytoplasmic fraction and the pellets were resuspended in hypertonic buffer (20mM Heps, 0.4M NaCl, 1mM EDTA, 1mM DTT and a cocktail of protease inhibitors) and triturated with a pestle. The nuclei were incubated for 30 min on ice and then microcentrifuged at 4°C for 5 min at 10 000 g. The supernatant containing nuclear extract was collected and aliquoted.

Analysis of plakoglobin gene hypermethylation by MSP and bisulfite DNA sequencing
The plakoglobin gene methylation was studied by methylation-specific PCR (MSP) assay as reported previously (27). Briefly, genomic DNA was isolated using a commercial kit (Qiagen, Hilden, Germany) and bisulfite modification was obtained using CpGenome DNA Modification Kit (Chemicon International, Temecula, CA). The modified DNA was subjected to PCR in separate reactions using the primer pairs specific for the methylated sequence, PG-M (5′-TAATGTTGTCATTGCGGTC-3′ and 5′-AACCG-GAATCGAAATCGAAGCGC-3′), and for the unmethylated sequence, PG-U (5′-TTGGATTGTGTTGTTATTGTTGTT-3′ and 5′-CAACAAATTCAAATCAAACCCCGG-3′) as described previously (28). The PCR mixtures contained 100 μM PCR buffer, 200 μM dNTPs, 2 mM MgCl2, 10 pmol of each primer, 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 200 ng DNA in a final volume of 50 μl. Amplification was carried out after an initial enzyme activation step (5 min at 95°C) for 40 cycles (30 s at 95°C, 45 s annealing, 30 s at 72°C). Annealing temperatures of 62 and 60°C were used for PG-M and PG-U reactions, respectively. Bisulfite-modified lymphocyte DNA from healthy volunteers served as a positive control for the unmethylated allele. This DNA was methylated in vitro with SssI methyltransferase (New England Biolabs, Beverly, MA), subjected to bisulfite modification, and then used as a positive control for amplification of methylated alleles. PCR products were analyzed by gel electrophoresis and ethidium bromide staining on a 3.5% agarose gel. Results were confirmed by repeating the bisulfite reaction and MSP for all samples.

Bisulfite-modified DNA was amplified using Pan-S (5′-GGTTGTTGGTGTAGTTTTT-3′ and Pan-AS (5′-CACTCAATACCTTAACTAAAC-3′) primers. This primer set does not cover any CpG sites within the primer sequences, as reported previously (39). PCR products obtained, was amplified by a second round of nested PCR using primers BS-S (5′-TCCTCAATGTTGTTAGTCACTAC-3′) and BS-A (5′-AAATATAAACCTTAAAC-3′). PCR products were then sequenced using the ABI PRISM 377 DNA sequencer (Applied Biosystems) using BS-S and BS-AS primers.

Chromatin immunoprecipitation (ChIP) analysis
Cells grown in 100 mm dishes were treated with or without TSA (200 ng/ml) for 16 h. Before harvesting, the cells were treated with 1% formaldehyde for 10 min at room temperature to crosslink histones to DNA. Crosslinking was quenched by the addition of glycine at a final concentration of 0.125 M and 5 min incubation at room temperature. Cells were scraped with ice-cold PBS containing 0.5 mM PMSF and centrifuged for 10 min at 720 g at 4°C. ChIP assay was performed using ChIP Assay Kit (Active Motif, CA), according to the manufacturer’s instructions. Briefly, the chromatin solutions were sonicated using a sonicator to reduce the DNA length to 200–1000 bp, pre-cleared with a salmon sperm DNA/protein A agarose slurry, and then immunocaptured using antibody with acetyl-H4 antibody (Upstate, Charlottesville, VA) overnight at 4°C with gentle rotation. Aliquots of each chromatin solution were saved for no-antibody controls. Immune complexes were then collected with a salmon sperm DNA/protein A agarose slurry and washed with the kit wash buffer. DNA was eluted twice with elution buffer containing 1% SDS and 0.05 M NaHCO3. Combined eluates were treated with 5 M NaCl to reverse the DNA/histone crosslinks and RNA was removed by RNase treatment overnight at 65°C. Samples were treated with Proteinase K at 42°C for 2 h to digest the proteins. DNA from each eluate was purified through DNA purification mini-columns and collected into microcentrifuge tubes. Specific promoter sequences from no-antibody control and immunoprecipitated samples were detected by PCR analysis using the following primer sets: 5′-AAATGAGAGGGAGGAGGAGGAGG-3′ and 5′-GTTTGGTGTGTTTACCAATCACTAC-3′ for P1, 5′-AAATTTTCATTACCTTACGAG-3′ and 5′-TTGTTTTAGGACAGTTTACTT-3′ for P2, and 5′-CATCTCTTCCTACGACTCT-3′ and 5′-AAAGCCAGCAACTCAGTAT-A-3′ for P3.
Results

Plakoglobin protein expression, cellular localization and semi-quantitative analysis of mRNA in RMS cell lines

We studied nine RMS cell lines including three ARMS carrying the translocation t(2;13) (RH4, RH28, RH30); one ARMS with translocation t(1;13) (RC2); one ARMS without translocation (RH18); four ERMS (RD, RH36, SMS-CTR, CCA); an undifferentiated sarcoma cell line (A-204) and a neuroblastoma cell line (LAN-5). Western blot analysis of lysates from log-phase growing cells revealed a consistent and homogeneous β-catenin expression in all of the cell lines, whereas plakoglobin, detectable in the positive control neuroblastoma cell line (LAN-5) and in the ERMS cell lines, was absent or barely detectable in ARMS cells (Figure 1A). Unexpectedly, RH18 cells demonstrated a high level of plakoglobin expression similar to ERMS cell lines. The A-204 cells were negative for plakoglobin expression, whereas β-catenin was almost undetectable.

A semi-quantitative RT–PCR assay was established to assess the expression of plakoglobin-specific mRNA in the tumor cell lines described above (Figure 1B). Consistent with protein expression shown in Figure 1A, RT–PCR originated a single band of 700 bp that was expressed at lower levels in ARMS compared with ERMS cell lines (Figure 1C). A similar result was observed in the A-204 undifferentiated sarcoma cell line, whereas the plakoglobin mRNA level in RH18 cells was comparable with ERMS, underlining the peculiarity of this ARMS cell line.

β-Catenin-mediated signaling depends on the balance among its membrane, cytoplasmic and nuclear pools; therefore, we analyzed the subcellular distribution of plakoglobin and β-catenin in ARMS (RH4, RH28, RH30) and ERMS cell lines (RD, RH36, SMS-CTR). As shown in Figure 1D, β-catenin was distributed in comparable amounts between the cytoplasm and the nucleus in all of the RMS cell lines tested (with the exception of RH28 cells, where nuclear β-catenin was significantly lower), whereas in ERMS cells plakoglobin, although detected in both compartments, was mostly localized in the cytoplasmic fraction. As expected, we were unable to detect plakoglobin both in the cytoplasm and in the nucleus of the ARMS cell lines RH4, RH28, RH30 (Figure 1D).

Restoration of plakoglobin expression in ARMS cells treated with the demethylating agent 5AzadC and the HDAC inhibitor TSA

Methylation and acetylation are well-known mechanisms of gene inhibition and activation, respectively (30,31). To assess whether the loss of plakoglobin expression in ARMS was due to methylation, ARMS RH30 cells were treated with different concentrations of the demethylating cytosine analog 5AzadC for 72 h (Figure 2A). Western blot analysis demonstrated partial restoration of plakoglobin expression in 5AzadC-treated RH30 cells in a dose-dependent manner, and this effect appeared specific as the expression of β-catenin remained unaffected under the same experimental conditions (Figure 2A). In addition, we examined plakoglobin expression in RH30 cells cultured in the presence of the HDAC inhibitor TSA. We observed an upregulation of the protein level that reached the highest level after 16 h. Expression levels decreased when cells were treated for longer time intervals (24 h) possibly because of TSA cytotoxicity (Figure 2B, see β-catenin steady-state in the lower panel). To confirm these results, the ARMS cell lines RH4, RH30 and RH18, and ERMS RD cells were treated with 5AzadC and TSA alone or in combination, and plakoglobin gene expression was assessed at both mRNA and protein levels. As shown in Figure 2C, combination treatment with 5AzadC and TSA synergistically augmented mRNA and protein expression in the PAX3–FKHR-positive ARMS cells RH4 and RH30, although the effects were quantitatively different in the two cell lines. In contrast, plakoglobin expression was minimally affected in the ERMS RD and in the PAX3–FKHR-negative ARMS RH18 cells, when the two compounds were used alone or in combination. These results support the hypothesis that transcription of plakoglobin gene in RMS cells is regulated by histone acetylation and CpG methylation of its promoter region.

Analysis of methylation and acetylation status of plakoglobin promoter in RMS cell lines and tumor biopsies

To further investigate plakoglobin expression regulation, genomic DNA of RMS cell lines was extracted, modified by bisulfite treatment and subjected to PCR with primer pairs PG-U and PG-M specific, for unmethylated and methylated promoter-associated CpG islands of plakoglobin gene, respectively. As shown in Figure 3A, RD cells, which endogenously expressed plakoglobin and were 5AzadC unresponsive, contained only unmethylated alleles, whereas plakoglobin-negative and 5AzadC-responsive RH4 and RH30 cells displayed both methylated alleles or coexistence of methylated and unmethylated alleles, respectively. However, the PAX3–FKHR-negative ARMS RH18 cells, which constitutively expressed plakoglobin and were 5AzadC unresponsive, showed a methylated and an unmethylated allele.

To assess the methylation status in more detail, a promoter region of plakoglobin gene that allows the study of 9 CpG islands by direct sequencing was amplified by a two-step PCR. As demonstrated by sequence analysis of the amplicons (Figure 3B), the RH4 cell line which showed two methylated plakoglobin alleles, was heavily methylated at all 9 CpG sites tested. In contrast, RD cells that harbor only unmethylated alleles (Figure 3A), completely lacked methylated CpG sites (Figure 3B). RH30 cells showed the presence of both methylated and unmethylated CpG islands. RH18 possessed all of the CpG islands unmethylated except CpG +54 that was methylated. In addition, the sequence analysis of cloned PCR products that allows to study a higher number of CpG islands, showed that CpG island +33, included in the primer sequence used for MSP assay, was methylated in 1/6 RH18 clones (data not shown).

To investigate histone deacetylation as an epigenetic mechanism contributing to gene silencing, we determined the binding of hyperacetylated histones to plakoglobin promoter in TSA-treated RMS cells by ChIP assay. Inhibition of HDAC by TSA causes local hyperacetylation of histones that can be immunoprecipitated; thus, allowing purification of bound DNA sequences. Acetylated histone H4 was immunoadsorbed with a specific monoclonal antibody from TSA-treated and untreated RMS cells, and co-precipitated DNA fragments were assessed by PCR, using three specific primer pairs spanning the P1 (−945−745), P2 (788−646) and P3 (−34+14)’s flanking promoter regions of the plakoglobin gene (GeneBank accession no. AJ 276892) (32). As shown in Figure 3C, co-precipitation of all three plakoglobin
5’ flanking promoter regions (P1–P3) in RH4 cells, which possess both methylated alleles, was obtained only after TSA treatment. When the analysis was extended to RD cells and to the two cell lines displaying the coexistence of methylated and unmethylated alleles (RH30 and RH18), no significant difference was seen between TSA-treated and untreated cells, suggesting that deacetylation of H4 histone is a particularly relevant phenomenon in RH4.

**Fig. 1.** Differential expression and cellular localization of catenins in RMS cell lines. (A) Nine RMS cell lines, one ARMS with translocation t(1;13) (RC2), three ARMS with t(2;13) (RH4, RH28, RH30), one ARMS without translocation (RH18) and four ERMS (RD, RH36, SMS-CTR, CCA) were studied for plakoglobin and β-catenin expression together with the undifferentiated sarcoma cell line A-204 and the neuroblastoma cell line LAN-5. Cells lysates were analyzed by western blot using anti-β-catenin and anti-plakoglobin (γ-catenin) specific antibodies. (B) Plakoglobin mRNA expression in RMS cell lines was determined by semi-quantitative RT-PCR where β2-microglobulin transcript was co-amplified. Amplification of plakoglobin and β2-microglobulin cDNAs originated single bands of 700 and 220 bp, respectively. In the PCR-negative control (H2O) no RNA was added to the reactions, whereas LAN-5 cells represented the positive control. (C) The signal intensity of plakoglobin mRNA of all tumor cell lines, normalized to β2-microglobulin, was quantified and expressed as arbitrary units as described in Materials and methods. (D) Cytoplasmic (C) and nuclear (N) plakoglobin and β-catenin were studied by differential cell lysis, followed by immunoblotting of protein lysates. Cell fractions of three ARMS (RH4, RH28, RH30) and three ERMS (RD, RH36, SMS-CTR) cell lines were obtained and 30 μg of protein lysates from each fraction were analyzed. To verify the integrity of the fractions, blots were reprobed for actin and HDAC1 (histone deacetylase), as markers of the cytoplasmic and nuclear compartments, respectively.
Fig. 2. 5AzadC and TSA treatments restore plakoglobin expression in ARMS cells. (A) To determine the role of hypermethylation in plakoglobin expression, RH30 cells were treated with increasing concentrations (0–10 μM) of 5AzadC for 72 h. Total protein extracts containing equal amounts of proteins of treated/untreated cells were analyzed by western blot using antibodies specific for plakoglobin and β-catenin. RD cell lysate was included as positive control for plakoglobin expression. (B) Time course analysis of plakoglobin protein expression in RH30 cells treated with (+) or without (−) TSA 200 ng/ml. RD cells were used as positive controls. (C) mRNA and protein expression in RMS cell lines. Bar graphs represent plakoglobin expression after treatment with 5AzadC 2 μM for 72 h and with TSA 200 ng/ml for 16 h, alone or in combination, reported as optical density units (mRNA) or as folds of plakoglobin expression levels relative to untreated cells (protein).
Fig. 3. Methylation and acetylation status of plakoglobin gene in RMS cell lines. (A) Methylation of plakoglobin gene was characterized in plakoglobin-positive and -negative RMS cell lines using MSP. Genomic DNA of four cell lines (RH4, RH18, RH30, RD) was extracted, modified by bisulfite treatment and subjected to PCR with primer pairs PG-U (UM) and PG-M (M) specific for unmethylated and methylated plakoglobin alleles, respectively. PCR products were analyzed by 3.5% agarose gel electrophoresis. Band size of plakoglobin allele(s) amplicons is indicated. Normal lymphocyte DNA and normal lymphocyte DNA treated with Sss I methyl transferase were used as unmethylated (ctr UM) and methylated gene promoter (ctr M) controls, respectively. (B) Bisulfite mapping of CpG islands in the plakoglobin promoter and gene was carried out on PCR products containing nine CpG sites. All cytosines in the nine CpG islands analyzed are numbered on top of each panel, starting from the transcription start site based on the sequence and GeneBank data (accession no. AF233882). All of the CpG islands in RH4 cells were methylated (the presence of an unmodified ‘C’). In RD cells all CpG islands were non-methylated (‘C’ were converted into ‘T’). RH30 cells showed all CpG islands partially methylated (‘C’ covered with ‘T’). In RH18 all of the CpG islands were unmethylated with the exception of CpG island +54 that was methylated. (C) Acetylation status of histone H4 in plakoglobin promoter in RMS cell lines treated with or without TSA 200 ng/ml for 16 h. Results of a representative ChIP assay carried out at least in triplicate with the antiacetyl-H4 antibody followed by PCR with the indicated primer sets are shown. ProG represents no-antibody control. The amount of DNA used was monitored by a PCR that amplified DNA prepared from the input solution. Ladder 50 (M) was used as DNA size marker. The presence of PCR products demonstrates the association of acetylated H4 histone with a specific promoter region.
Finally, we studied 11 RMS tumor biopsies: 4 PAX3–FKHR-positive and 2 PAX3–FKHR-negative ARMS and 5 ERMS (Figure 4A). Results in Figure 4A and B confirmed that β-catenin was expressed at high levels in all specimens, whereas plakoglobin, detected in all of the ERMS biopsies, was barely detectable in the ARMS biopsies. We investigated the methylation status of the plakoglobin gene promoter in 7 of the 11 biopsies by MSP analysis. The hemi-methylated pattern, was predominant irrespective of the histological subtype (Figure 4C) with the exception of sample no. 6 (ARMS) that showed a higher signal intensity for the methylated allele and sample no. 8 (ERMS) where the unmethylated allele was more evident.

Discussion

Plakoglobin overexpression is known to suppress cell proliferation and tumorigenicity, consistent with its ability to act as a tumor suppressor (17,33). Low or absent plakoglobin expression in cancer implies changes in intercellular junctions, and
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In accordance with this scenario, we observed restoration of plakoglobin expression in PAX3–FKHR-expressing RH30 cells following treatment with either the demethylating agent 5AzadC or the deacetylase inhibitor TSA (Figure 2C). Owing to the fact that TSA treatment increased plakoglobin expression, although ChIP assay did not show any difference between TSA-treated and untreated cells, we cannot exclude that other histones (i.e., H3) or a partial acetylation of histone H4 may be involved.

Interestingly, the two single agents 5AzadC and TSA had a less pronounced effect on plakoglobin protein expression in RH4 cells that possess both hypermethylated alleles, but were highly effective when used in combination, suggesting a marked increase of the acetylation/methylation ratio achieved synergistically by the two agents in a mechanism involving both histone methylation/acetylation status and methylated plakoglobin promoter regions, as previously reported (44). In contrast, treatment with 5AzadC and/or TSA had only a limited effect on RMS cells that constitutively expressed plakoglobin, such as RD and RH18 cells, independent of the histological subtype.

From further studies based on MSP assay and sequencing, lack of unmethylated allele as in RH4 cells would suggest that gene inactivation might be the consequence of the methylation of both alleles, possibly associated with a deacetylation of histone H4 that may increase plakoglobin gene silencing. However, we cannot exclude that the presence of one methylated allele, and loss of the other may also occur.

The PAX3–FKHR positive RH30 cell line showed a loss of plakoglobin expression despite the presence of methylated and unmethylated alleles. DNA sequencing of a promoter region that enabled us to study 9 CpG islands confirmed these findings. In this case, we may hypothesize that inactivation of the unmethylated allele might be achieved through different mechanisms such as nonsense or frame-shift mutations, or deletion. In addition we cannot rule out uniparental isodisomy. This is also suggested by the observation that the PAX3–FKHR negative RH18 cell line harboring both unmethylated and methylated alleles does express plakoglobin, although the methylation status of plakoglobin gene promoter region analyzed by sequencing differs from RH30 cells.

The RD cell line did not show either methylation or acetylation of the plakoglobin promoter in agreement with the expression profile.

Finally, in the tumor samples we found both unmethylated and methylated alleles as in some RMS cell lines (44 C and 3A). Interestingly, case no. 6 (ARMS) and case 8 (ERMS) showed a higher intensity of the methylated and unmethylated alleles, respectively, in agreement with the plakoglobin protein expression. In other tumors though, plakoglobin expression was very low despite the presence of both alleles similar to the case of RH30 cell line. In this regard, we need to consider that mechanisms other than selected promoter methylation may influence plakoglobin expression in vivo. In addition, the heterogeneity of tumor biopsies, due to the presence of some normal cells intermixed with tumor cells, may also influence the results of the MSP assay making it more difficult to fully appreciate the methylation status in vivo.

Given the relationships and interactions between catenins and cadherins, their role in proliferation and transformation, and the recent findings suggesting that impairment...
of cadherin-mediated adhesion might contribute to the rapid growth and metastatic properties of RMS (42), our results highlight a further differential feature that may contribute to the more aggressive behavior of ARMS compared with ERMS. The availability of drugs that can modify the methylation and acetylation status of the cell should encourage their preclinical evaluation as novel therapeutic agents in RMS.

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