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Epigenetic regulation of microRNA expression in renal cell carcinoma

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ABSTRACT

The underlying mechanisms of microRNA deregulation in cancer cells include epigenetic modifications, which play a crucial role in carcinogenesis. We demonstrate that numerous microRNAs are induced in renal cell carcinoma cell lines after treatment with inhibitors of the DNA-methyltransferase (5-aza-2′-deoxycytidine) and the histone-deacetylase (suberoylanilide hydroxamic acid). We provide evidence that enrichment of H3 and H3K18 acetylation at the miR-9 promoter is causative for re-expression, while DNA hypermethylation remains unchanged. Our experiments show that the treatment with the epigenetic drugs causes re-expression of silenced microRNAs with putative tumor suppressive function in ccRCC cell lines.

1. Introduction

MicroRNAs are non-coding, small single-stranded RNA-species. They modulate fundamental cell processes (e.g. cell growth, proliferation and apoptosis) post-transcriptionally [1]. Thereby, microRNAs regulate approximately 30% of human genes [2]. Dysregulation of microRNAs contributes to carcinogenesis. Alterations of specific microRNA expression profiles have been observed in many tumors, enabling to distinguish between healthy and malignant tissue as well as between different tumor entities [3]. Up- and down-regulated microRNAs are likely to function as oncogenes and tumor suppressors, respectively [3,4].

Little is known about the mechanisms causing dysregulation of microRNA expression in RCC, but both, genetic and epigenetic alterations are possible causes; epigenetic mechanisms include DNA methylation and histone modifications [5]. Aberrant DNA methylation usually occurs in CpG rich regions at the 5′-cytosine and is associated with gene silencing [6]. Histone lysine acetylation at the N-terminal leads to gene activation whereas histone lysine methylation causes transcriptional activation or repression depending on the position of the methylated lysine rest [7]. For a number of tumor suppressor genes silencing by aberrant promoter hypermethylation and histone modifications is already depicted as a common way of epigenetic regulation in carcinogenesis [8,9]. Taking advantage of the fact that epigenetic alterations are reversible, numerous studies evaluated epigenetic therapeutic agents such as DNA-methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors and demonstrated re-expression of epigenetically silenced tumor suppressor genes [8,10]. Thus, the approach of targeting microRNA expression might be promising for future therapies. However, the influence of such reagents has not been investigated in the context of microRNA re-expression in RCC.

2. Materials and methods

2.1. Cell culture

We studied five ccRCC cells lines with different von Hippel–Lindau (VHL) gene characteristics: Caki-1 and ACHN are wild-type VHL, Caki-2 and A-498 have a mutated VHL and 769-P has a methylated VHL gene. The cell lines (Caki-1, Caki-2, A-498) were obtained from the DSMZ (Braunschweig, Germany), and ACHN and 769-P were from Cell Lines Service (Eppelheim, Germany). All cell lines were maintained at 37 °C, 5% CO_2 in RPMI 1640 culture medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM Glutamine and 0.4% Penicillin/Streptomycin (PAA, Pasching, Austria).

5-Aza-2′dC, a DNMT-inhibitor, was dissolved in DMSO and was stored as a 50 mM stock solution at −20 °C. SAHA, a HDAC inhibitor, was dissolved and kept under the same conditions as 5-Aza-2′dC; both reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA). For treatment 4×10^6 cells were seeded in 175 cm^2 flasks and were allowed to adhere overnight. Stock solutions and DMSO as the control, were then dissolved in medium in order to obtain the following concentrations: DMSO 1:10,000, 5 μM of 5-Aza-2′dC, 5 μM of SAHA (Caki-1, Caki-2), 2.5 μM SAHA (769-P,

Abbreviations: ccRCC, clear cell renal cell carcinoma; DNMT, DNA methyltransferase; HDAC, histone deacetylase; miR, microRNA; 5-Aza-2′dC, 5-aza-2′-deoxycytidine; SAHA, suberoylanilide hydroxamic acid; RIN, RNA integrity numbers; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TBST, TBS-Tween; ChIP, chromatin immunoprecipitation.

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ACHN), 7.5 μM SAHA (A-498) and a combination of 5 μM 5-Aza-2′dC and 2.5 μM, 5 μM or 7.5 μM SAHA depending on the cell line as indicated above. Since 5-Aza-2′dC is not very stable in aqueous solution, 5 μM 5-Aza-2′dC was added every 24 h for three times, whereas cells treated with SAHA were treated once for 72 h.

2.2. Cell proliferation assay

All RCC cell lines were seeded at a concentration of 1×10^4 cells per well into a 96-well plate. Treatment was started after 24 h allowing the cells to adhere. Cells were treated for 72 h in six replicates as mentioned above; the concentrations of 5-Aza-2′dC and SAHA were tested in preliminary experiments (data not shown). 5-Aza-2′dC was added every 24 h. The EZ4U assay (Biomedica, Vienna, Austria) was then used to assess cell viability according to the manufacturer's protocol using the 340 ATTC SLT photometer (Crailsheim, Germany).

2.3. RNA purification

Total RNA was extracted from cell pellets using the mirVana PARIS Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany) at 260 nM. For quality control, the ratio 260/280 and 260/230 was determined. RNA integrity numbers (RIN) were measured on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to verify a high-quality RNA.

2.4. Western blot

The protein lysates obtained during RNA isolation with the mirVana PARIS Kit were used for protein extraction. The lysate was sonified (5 min, high level, 30 s on–off time interval) with the Bioruptor Sonicator (Diagenode, Liège, Belgium). Protein concentrations were measured via Bradford Assay Kit (Pierce, Rockford, IL, USA). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 5 μg protein with a 16% polyacrylamid gel (100 V, 100 min). The gel was blotted onto 0.2 μm nitrocellulose membranes (80 V, 90 min). The membrane was blocked in 0.025% TBS-Tween 20 (TBST) with 5% milk and then incubated with 1 μg/ml monoclonal primary antibody (both: Abcam, Cambridge, UK) against acetylated histone H3 and H4 and alpha-tubulin as loading control at 4 °C overnight. After washing, membranes were incubated with the secondary antibody conjugated with horseraddish-peroxidase (Bio-Rad Laboratories, Munich, Germany). The ECL detection system (Thermo Fisher Scientific, Rockford, Il, USA) was used for visualization.

2.5. DNA Dot blot

DNA was extracted from cell pellets using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany); DNA concentration and purity was measured with a NanoDrop spectrophotometer. Each DNA spot on the 0.45 μm nitrocellulose membrane consisted of 150 ng. After UV-crosslink for 4 min and blocking in 0.025% TBST plus 5% BSA, the membrane was incubated with the primary antibody 5-methylcytosine (Abcam) in TBST + 1% BSA at 4 °C overnight. After washing the membranes were incubated with the secondary antibody conjugated with horseraddish-peroxidase (Bio-Rad Laboratories). Visualization was performed with the ECL detection system.

2.6. TaqMan low density arrays

TaqMan Low Density Array experiments were essentially performed as described earlier (see MIQE-compliant description in Wulfken et al. [11]). In brief, total RNA (1 μg) of Caki-1 and Caki-2 cells of the DMSO control, 5-Aza-2′dC-, SAHA- and 5-Aza-2′dC + SAHA-treated cells was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT Primers. Preamplification was interposed prior to the real-time PCR to obtain an adequate amount of target microRNA (TaqMan PreAmp Mastermix). TaqMan Low Density Array (TaqMan Array human MicroRNA A + B cards set v3.0) were used to profile the expression of 754 microRNAs on an ABIPrism7900HT (Applied Biosystems). All experiments were performed according the manufacturer's recommendations (all reagents: Applied Biosystems, Foster City, CA, USA). We used miR-422a, miR-589_2, miR-103a and miR-28 as reference genes due to their stability on the array cards. Furthermore, the latter two were reported as suitable reference genes for microRNA analysis in RCC [12]. Quantification was performed using the delta-delta Cq formula with the DataAssist v2.0 software.

2.7. Quantitative real-time PCR

We used the Qiagen miScript System (Qiagen, Hilden, Germany) for validation experiments; see Supplementary Methods S1 for the detailed protocol. Briefly, RT-PCR (miScript Reverse Transcription Kit) and qPCR (miScript SYBR Green PCR Kit) was performed according the manufacturer's protocol. qPCR was carried out on an ABIPrism7900HT and dissociation-curve analysis was performed to confirm the specificity of the PCR products. We determined the expression level of miR-9–1, miR-95, miR-184, miR-642, miR-142, miR-211, miR-34b using the delta-delta Cq formula; miR-28, miR-103a, miR-422a and miR-589_2 were used as reference genes.

2.8. Methylation-specific qPCR

DNA (1 μg) was bisulphite treated with the EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA). Methylation levels of miR-9–1 (primer sequences: methylated: forward 5′-TTT-TAT-TTT-CGT-TGA-CGG-GC-3′, reverse 5′-CCC-GCC-TCC-TAA-CTA-CTA-TCG-3′; unmethylated: forward 5′-TTT-TTT-TAT-TTT-TGT-TGA-TGG-GT-3′; reverse 5′-CCC-ACC-TCC-TAA-CTA-CTA-TCA-CC-3′; published by Lujambio et al. [13]) were determined by quantitative methylation-specific PCR on an ABIPrims7900HT; an ACTB primer set without CpGs served as reference gene (forward 5′-TGG-TGA-TGG-AGG-AGG-TTT-AGT-AAG-T-3′; reverse 5′-AAC-CAA-TAA-AAC-CTA-CTC-CTC-CCT-TAA-3′; see Ellinger et al. [14]). In brief, 1 μl of the bisulphite-converted DNA was amplified 1× Power SYBR Green MasterMix (Invitrogen), 10 pmol forward/reverse primer in a total volume of 10 μl. Specificity of the PCR products was confirmed with dissociation curve analysis. Relative miR-9–1 methylation levels were calculated using the delta-delta Cq method; normalization was performed against ACTB. 20 μl of the PCR products were loaded on a 2% agarose gel (100 V, 90 min),

2.9. Chromatin immunoprecipitaion

Chromatin immunoprecipitation (ChIP) was carried out as described previously [8]. In brief, 4×10^6 cells were cross-linked in 1% formaldehyde at 20 °C for 3 min, stopped by addition of 0.125 M glycine. After washing the cells twice with cold PBS, the cells were resolved with 200 μl lysis buffer (0.1% SDS, 50 nM Tris–HCl, 10 mM EDTA; pH 8.1) and incubated on ice for 10 min. Chromatin was sheared by sonication using a Bioruptor (Diagenode, Liège, Belgium; setting: 30 s on/off for 10 min), followed by centrifugation (10 min, 4 °C, 13 000 rpm) and collection of the supernatants. Successful sonication (DNA fragmentation to approximately 100 bp) was controlled by agarose gel electro-