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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.

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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-

phoresis. Antibodies against H3, H3Ac, H3K18Ac or IgG negative control were pre-incubated with Magnetic Dynabead Protein A (Invitrogen). The dynabeads facilitated washing and elution of the immunoprecipitates by magnetic separation. The sonicated samples were incubated with a protein inhibitor (Complete25X, Hoffmann LaRocheAG, Basel, Switzerland), salmon sperm DNA (Stratagene, La Jolla, CA, USA) and the dynabead-antibody complex overnight for immunoprecipitation. Afterwards, the antibody-bead-complex was washed (10 nM TrisHCl, 1 mM EDTA, 0.01% Tween20; pH 8.0) and eluted (elution buffer: 20 nM Tris–HCl, 5 nM EDTA, 50 nM NaCl, 1% SDS; pH 7.5). DNA was heated overnight (65 °C) for reverse-crosslinking. Finally, the DNA was purified by ethanol-ammoniumacetate immunoprecipitation. Reagents not otherwise specified were purchased from Merck (Darmstadt, Germany). The amount of a specific histone modification at the miR-9–1 promoter was determined using real-time PCR on an ABI PRISM 7900HT using 1xSYBR GreenER qPCR Supermix (Invitrogen) and 10 pmol forward/reverse primer in 10 µl reactions. GAPDH served as a positive control and IGX1A as a negative control. Primer sequences were: miR-9–1: forward 5′-TGG-CTC-CAA-GAA-TTT-TGA-CA-3′; reverse 5′-TGC-TGT-GAT-GCT-TCT-GGC-TA-3′; IGX1A: forward 5′-CCT-CCC-TCT-CAC-AGT-TGG-TC-3′; reverse 5′-CCC-AA T-GGT-AAA-ACC-CAC-AC-3′; GAPDH: forward 5′-CGG-GAT-TGT-CTG-CCC-TAA-T-3′, reverse 5′-GAG-GTT-TCT-GCA-CGG-AAG-G-3′. Enrichment of H3, H3Ac, H3K18Ac or IgG at the miR-9–1 promoter was quantified relative to the input DNA using the delta-delta Cq formula.

3. Results

3.1. Cell proliferation

5-Aza-2′dC and SAHA reduced cell proliferation in a dose-dependent manner (data not shown). Based on these findings we chose a concentration of 5 µM of 5-Aza-2′dC (all cell lines), 2.5 µM (769-P, ACHN), 5 µM (Caki-1, Caki-2), 7.5 µM (A-498) of SAHA and the respective concentrations for the combination of 5-Aza-2′dC and SAHA for further experiments. 5-Aza-2′dC induced a rather modest decrease of cell viability (∼10%) compared to SAHA treatment, which reduced cell viability by about 50% in all cell lines tested. The combination of 5-Aza-2′dC and SAHA worked additive leaving ∼40% viable cells; see Fig. 1. Values represent the mean of six parallel samples.

3.2. Global DNA methylation and histone acetylation levels

Demethylating effects in 769-P cells were stronger than in the other cell lines. 5-Aza-2′dC decreased the levels of global DNA methylation in Caki-1, Caki-2, 769-P, A-498 and ACHN cells. The combination of 5-Aza-2′dC and SAHA lowered DNA methylation in Caki-1, 769-P, A-498 and ACHN. SAHA had even stronger demethylating effects than 5-Aza-2′dC in Caki-1 and Caki-2 cells. The global histone H3 and H4 acetylation increased upon treatment with SAHA: SAHA and 5-Aza-2′dC + SAHA post-treatment levels were markedly increased in Caki-1, 769-P and A-498. However, in Caki-2 and ACHN the combined treatment had a mild effect on H4 acetylation levels. 5-Aza-2′dC did not induce histone acetylation. Representative blots of histone H4 acetylation, see Fig. 2.

3.3. Identification of epigenetically regulated microRNAs in Caki-1 and Caki-2

In Caki-1 cells, 283 out of 754 analyzed microRNAs were expressed. Among these microRNAs, 21 were upregulated and 20 were down-regulated (defined as 5-fold expression change) upon treatment with 5-Aza-2′dC, SAHA or 5-Aza-2′dC + SAHA compared to the control. Most microRNAs (e.g. miR-891a, miR-9#, miR-184, miR-642, miR-542–5p, miR-95 and miR-139–3p) were up-regulated by SAHA and 5-Aza-2′dC + SAHA, therefore SAHA appears to have a dominant role in reactivation. See Fig. 3A.

We have similar findings for microRNA up-regulation in Caki-2 (Fig. 3B): 293 microRNAs were detectable, out of which 18 were up-regulated and 15 down-regulated after treatment. Concerning decreased microRNA levels, SAHA had the strongest impact down-regulating 13 out of 20 microRNAs in Caki-1 and 9 out of 15 microRNAs in Caki-2 (Supplementary Table S2). The detailed expression level of each microRNA is shown in Supplementary Table S3.

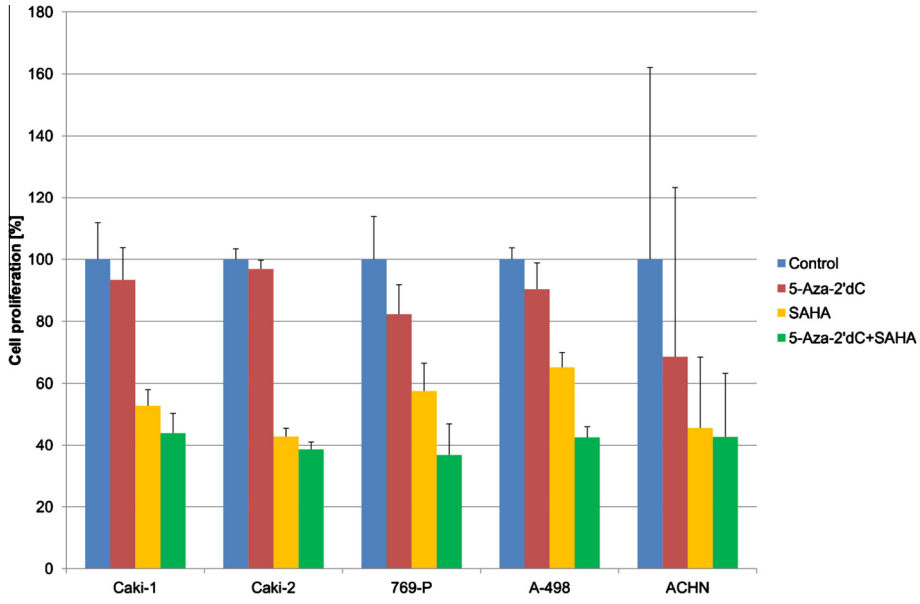

Fig. 1. Cell viability tested via cell proliferation assay EZ4U. Decrease in cell proliferation after 5 µM 5-Aza-2′dC by about 10%, 2.5 µM, 5 µM or 7.5 µM SAHA (769-P, A-498/Caki-1, Caki-2/ACHN) by about 50% and the combination of 5 µM 5-Aza-2′dC and 2.5 µM, 5 µM or 7.5 µM SAHA (769-P, A-498/Caki-1, Caki-2/ACHN) by about 60% in all RCC cell lines.

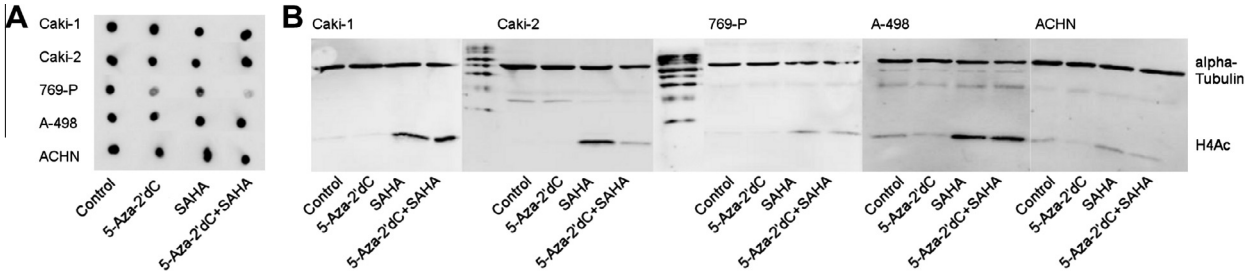

Fig. 2. Effect of 5-Aza-2′dC and/or SAHA on global DNA methylation and histone H4 acetylation levels. A. DNA Dot blot results using a 5-Methylcytosine antibody. The global methylation levels decreased by 5-Aza-2′dC, SAHA and the combination. B. Western blots using an antibody against acetylated histone H4. The H4 acetylation levels increased by SAHA and 5-Aza-2′dC + SAHA.

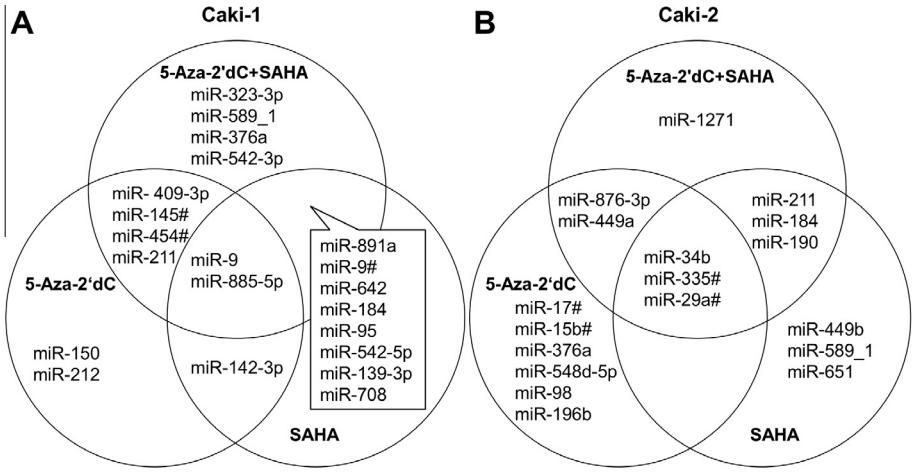

Fig. 3. Venn diagram of TaqMan LDA re-expressed microRNAs upon treatment in Caki-1 (A) and Caki-2 (B). microRNAs specified according to the treatment causing up-regulation. The combination was the most effective treatment.

3.4. Validation of the low density array experiments

Conventional qPCR was performed to validate seven candidate microRNAs showing elevated expression levels. For example, an increase of miR-9–1 was also observed by qPCR detecting for 5-Aza-2′dC (7-fold), SAHA (15-fold) and their combination (25-fold) in Caki-1 cells. Similar results were also seen for miR-184, miR-642, miR-95, miR-142, miR-211 and miR-34b in the validation experiments (see Supplementary Table S4).

In order to further consolidate our findings, we examined the ccRCC cell lines 769-P, A-498 and ACHN. The combination of 5-Aza-2′dC and SAHA was the most effective treatment re-expressing every of the seven candidate microRNAs in at least one cell line, and miR-95 and miR-184 in all five cell lines. Interestingly, SAHA was more effective for re-expression than 5-Aza-2′dC, and the combination of both agents was most successful in inducing re-expression of silenced microRNAs; see Table 1.

3.5. DNA hypermethylation and histone acetylation at the miR-9–1 promoter region

In silico analysis of the DNA sequence of miR-9–1 demonstrated the presence of a CpG island in the promoter region of the microRNA. Hence, miR-9–1 is a candidate for gene silencing by DNA CpG island hypermethylation. We observed a methylated and an unmethylated allele in the miR-9–1 promoter region of 769-P cells. Treatment with 5-Aza-2′dC, SAHA and 5-Aza-2′dC + SAHA enhanced the level of the unmethylated allels in 769-P (see Fig. 4A). However, we did not detect a relevant change in promoter DNA methylation levels in Caki-1, although 5-Aza-2′dC and 5-Aza-2′dC + SAHA allowed miR-9–1 up-regulation (data not shown). We observed a considerable enrichment of H3 acetylation and H3K18 acetylation at the miR-9–1 promoter in 769-P cells after treatment with 5-Aza-2′dC + SAHA (Fig. 4B).

Table 1
Changes of microRNA expression after treatment with 5-Aza-2′dC and/or SAHA in RCC cell lines.[a]

	miR-9			miR-95			miR-184			miR-211			miR-642			miR-34b			miR-142		
	A	S	A+S	A	S	A+S	A	S	A+S	A	S	A+S	A	S	A+S	A	S	A+S	A	S	A+S
Caki-1	↑	↑	↑	-	↑	↑	-	-	↑	-	-	-	↑	↑	↑	-	-	-	↑	-	-
Caki-2	-	-	-	-	↑	↑	↑	↑	↑	-	↑	↑	-	-	-	-	↑	↑	-	↑	↑
769-P	↑	-	↑	-	↑	↑	↑	↑	↑	-	↑	↑	↑	↑	↑	-	-	-	-	-	-
A-498	-	-	-	-	↑	↑	-	↑	↑	-	-	-	-	-	-	-	-	-	-	-	-
ACHN	-	-	-	-	↑	↑	-	-	↑	-	↑	↑	-	↑	↑	-	↑	↑	-	-	-

[a] ↑: Indicates elevated expression levels upon treatment, -: indicates no re-expression; A = 5-Aza-2′dC, S = SAHA, C = Combination of 5-Aza-2′dC + SAHA.

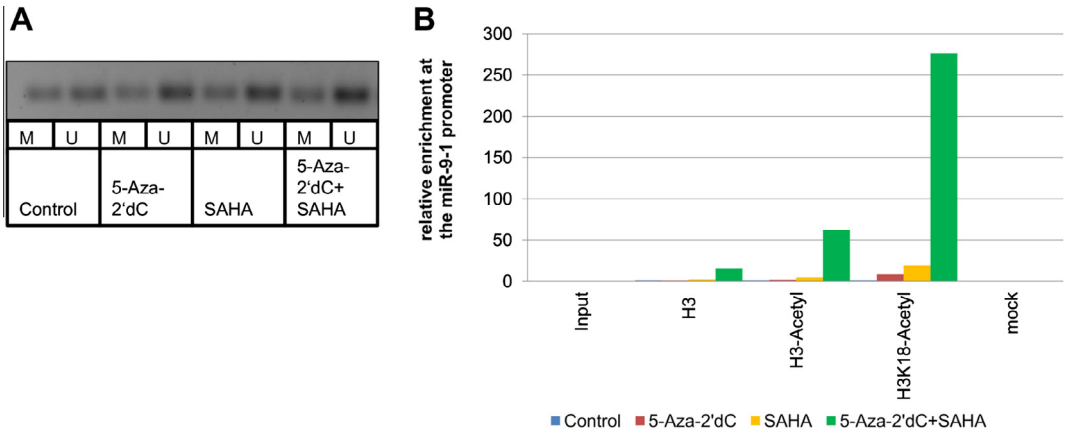

Fig. 4. Analysis of DNA methylation and histone acetylation at the miR-9–1 promoter. A. miR-9–1 promoter DNA methylation level in 769-P cells. M indicating methylated sequences, U indicating unmethylated sequences. A methylated and an unmethylated allel can be detected. However, promoter methylation did not change after treatment. B. Chromatin Immunoprecipitation: Input, H3 levels, acetylated H3 levels, acetylated H3K18 levels and the mock control at the miR-9–1 promoter in 769-P cells. Levels of acetylated H3 and H3K18 markedly increased after treatment with the combination.

4. Discussion

Epigenetic alterations have a strong impact on carcinogenesis by down-regulating tumor suppressor genes [15]. Recent studies found that epigenetic alterations also affect microRNA dysregulation in cancer [16,17]. A better understanding of the mechanisms causing microRNA dysregulation would contribute to a profound comprehension of the pathogenesis of cancer.

Drugs such as inhibitors of DNMT (5-Aza-2′dC) and HDAC (SAHA) have been shown to be able to reverse epigenetic alterations [15]. 5-Aza-2′dC has already been approved for myelodysplastic syndrome and SAHA for cutaneous T-cell lymphoma by the U.S. Food and Drug Administration in 2006. We observed antiproliferative effects of both drugs in our ccRCC cell lines; similar findings were made in pancreatic cancer stem cells [18] and ovarian cancer cells [19] in a dose-dependent manner. We and others found 5-Aza-2′dC to decrease cell proliferation by about 10% [20,21] and SAHA by almost 50% [22] in RCC cell lines.

Both reagents have already been applied to restore known tumor suppressor genes (e.g. SFRP2) in RCC [9]. Recently, the interest in targeting microRNAs therapeutically started to grow. Saito et al. [23] were the first to demonstrate microRNA reactivation upon treatment with 5-Aza-2′dC and 4-phenylbutyric acid, another HDAC inhibitor. 5-Aza-2′dC treatment lead to re-expression of miR-9–1 in breast cancer cell lines [24] and miR-124a in colon cancer cell lines [25]. To our knowledge, this is the first study investigating microRNA-restoration by 5-Aza-2′dC and/or SAHA in the context of ccRCC. Out of 754 microRNAs which were screened using the TaqMan Low Density Arrays, 21 microRNAs were up-regulated (at least 5-fold) by the treatment with 5-Aza-2′dC and/or SAHA compared to untreated Caki-1 cells and 18 microRNAs compared to untreated Caki-2 cells.

Besides microRNA up-regulation, we also detected down-regulated microRNAs after treatment, which might be due to indirect regulatory mechanisms. As one possible mechanism we can consider that restored microRNAs or genes might have initiated down-regulation of these microRNAs. Interestingly, miR-17, miR-18, miR-19 and miR-92, which form a microRNA-cluster and have been described as oncogenes in literature [26], were silenced by our treatment. Thus, we could presume that the up-regulated microRNAs might fulfil tumor suppressive functions by targeting oncogene microRNAs. However, we did not trace down-regulated microRNAs further.

We were able to validate the array experiments using conventional qPCR as exemplarily shown for miR-9–1, miR-184, miR-95, miR-642, miR-211, miR-34b and miR-142. Furthermore, we investigated additional ccRCC cell lines (769-P, A-498, ACHN) and showed that the treatment with epigenetic drugs has not only cell line specific effects but can be generalized to all RCC cells irrespective of the VHL gene status. Thus, the treatment does not seem to be impaired by VHL, although some microRNAs are regulated by VHL [27].

Out of the 21 microRNAs up-regulated in Caki-1, the promoter regions of miR-9–1, miR-184, miR-95, miR-642 and miR-142 are located within or near a CpG island. In addition, miR-9–1, miR-184 and miR-642 could be restored by 5-Aza-2′dC in at least two cell lines (Caki-1, Caki-2 or 769-P) suggesting that these microRNAs are potentially down-regulated by DNA-hypermethylation. Surprisingly, the promoter CpG island of miR-9–1 was not demethylated after treatment in Caki-1, although miR-9–1 was up-regulated by 5-Aza-2′dC (>20-fold), SAHA (>30-fold) and 5-Aza-2′dC + SAHA (>50-fold) in Caki-1. Only in 769-P we detected increased levels of the unmethylated allel after treatment. The promoter of miR-9–1 was methylated in RCC tumor tissue and associated with RCC progression [28]. miR-9–1 has already been identified as a tumor suppressor microRNA, which is methylated in breast [24] and gastric cancer [29], and Lehmann et al. also reported miR-9–1 demethylation by 5-Aza-2′dC. Earlier studies suggest that 5′-Aza-2′dC as a solitary treatment is not adequate to enhance microRNA expression levels and that adding a HDAC inhibitor to 5′-Aza-2′dC re-expression was more successful [23].

Histone modifications also play an important role in epigenetics; global H3K18 acetylation levels have been implicated in RCC progression [30]. We detected enhanced acetylated H3 and H3K18 levels at the miR-9–1 promoter region after treatment with Aza-2′dC and SAHA. Recently, Saito et al. [31] also detected increased levels of acetylated H3 at the miR-9–1 gene upon 5′-Aza-2′dC and 4-phenylbutyric acid treatment. Thus, miR-9–1 seems to be regulated by histone acetylation and SAHA contributes to gene re-expression by deacetylation. Interestingly, SAHA also had an effect on global DNA-hypermethylation. Indeed, silencing by DNA-methylation and histone-modifications is molecularly linked [32] making it reasonable to target epigenetic gene silencing with a combination of a DNMT and HDAC inhibitor. Our experiments underline this suggestion as the combination was most successful reactivating microRNAs in all cell lines and also had the best impact on histone acetylation.

In summary, the combination of 5-Aza-2′dC and SAHA emerged as an effective treatment for reactivation of microRNAs with putative tumor suppressor features, in particular miR-9–1.

4.1. Limitations

We would like to mention some limitations: The TaqMan Low Density Array gave only single replicates. However, we were able to validate the results by conventional qPCR and could broaden our results for more ccRCC cell lines in triplicate.

In order to rule cytotoxic effects out we used DMSO as a control, as it is already cytotoxic. In Caki-2 cells decreased global DNA methylation can hardly be detected, which might be a cause of remaining cytotoxic effects. However, 5-Aza-2′dC was able to reactivate microRNAs in Caki-2. Thus, there seem to be more complex mechanisms involved in microRNA regulation.

Demethylation seems to play a minor role at the miR-9 promoter, as it could hardly change DNA methylation levels. Only in 769-P, the demethylated allel could be elevated after treatment. Also, the global DNA methylation level only slightly decreased upon treatment. However, strong effects could be seen in 769-P, which is in accordance with the elevated demethylated allel upon 5-Aza-2′dC in 769-P. The ChIP results indicate a trend only, since the enrichment of H3Ac and H3K18Ac by Aza + SAHA is not replicated. Further studies are necessary in order to explore the effect of histone acetylation on microRNA restoration more detailed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.061.

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