Promoter methylation of *PARG*1, a novel candidate tumor suppressor gene in mantle cell lymphomas

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ABSTRACT

Background and Objectives

Mantle cell lymphoma (MCL), a mature B-cell neoplasm, is genetically characterized by the translocation t(11;14)(q13;q32). However, secondary alterations are required for malignant transformation. The identification of inactivated tumor suppressor genes contributing to the development of MCL may lead to further elucidation of the biology of this disease and help to identify novel targets for therapy.

Design and Methods

Whole genome microarray-based gene expression profiling on treated versus untreated MCL cell lines was used to identify genes induced by 5-aza-2'-deoxycytidine. The degree of promoter methylation and transcriptional silencing of selected genes was then proven in MCL cell lines and primary cases by methylation-specific polymerase chain reaction (PCR) techniques, real-time PCR and gene expression profiling.

Results

After 5-aza-2'-deoxycytidine treatment, we identified more than 1000 upregulated genes, 16 of which were upregulated ≥3-fold. Most of them were not known to be silenced by methylation in MCL. A low expression of *ING*1, *RUNX*3 and *BNIP*3*L* was observed in three of the five the MCL cell lines. In addition, the expression of *PARG1*, which is located in the frequently deleted region 1p22.1, was substantially reduced and displayed at least partial promoter methylation in all investigated MCL cell lines as well as in 31 primary MCL cases.

Interpretation and Conclusions

In summary, we identified interesting novel candidate genes that probably contribute to the progression of MCL and suggest that *PARG*1 is a strong candidate tumor suppressor gene in MCL.

Key words: mantle cell lymphoma, epigenetic silencing, tumor suppressor gene, *PTPL*1-associated RhoGAP1 (*PARG*1).

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In the current World Health Organization classification of tumors, mantle cell lymphoma (MCL) is defined as a distinct subtype of the mature B-cell neoplasms and comprises approximately 3-10% of non-Hodgkin's lymphomas.[1] Clinically, MCL is characterized by an aggressive disease course and a poor prognosis with a median survival of 3 years and only 10-15% long-term survivors.[2] The genetic hallmark of MCL is the translocation t(11;14)(q13;q32), which is present in virtually all cases[3,4] and causes overexpression of cyclin D1.[5,6] However, there is experimental evidence indicating that additional alterations are required to induce lymphoma genesis.[7] Classical cytogenetic and comparative genomic hybridization (CGH) studies have shown a high number of recurrent chromosomal alterations in t(11;14)-positive MCL.[8-10] Loss of the chromosomal fragment 11q22-23 harboring the ataxia-telangiectasia mutated (*ATM*) gene is observed in almost 50% of MCL.[11,12] However, in many other regions of genomic loss, the putative tumor suppressor genes have not been identified.

Today, it is widely accepted that, in addition to inactivation of a tumor suppressor gene by deletion and mutation, DNA hypermethylation of promoter CpG islands represents a third mode to satisfy Knudson's hypothesis.[13] Although tumor cell lines may display a greater extent of CpG island hypermethylation than the primary tumors, they still carry the characteristic aberrant methylation modifications[14] and could therefore be used as methylotype models of the primary tumors.[15]

To search for novel candidate genes probably involved in the progression of t(11;14)-positive MCL, we treated the human MCL cell line Granta-519[16,17] with 5-aza-2'-deoxycytidine (5-aza-CdR) to identify epigenetically silenced genes. This inactivating substrate of DNA methyltransferase[18] induces an indirect genomic demethylation[19] and chromatin remodeling[20] and can, therefore, lead to the reactivation of transcriptionally silenced genes.[21] We identified upregulated genes by means of cDNA microarray analysis in a similar manner to that used in recent reports.[22-26] The selection of potential candidate genes from the list of reexpressed genes was based on their location in frequently deleted regions, and their relevance in regulation of proliferation, apoptosis or DNA repair. *ATM*, *ING*1, *PARG*1, *RUNX*3, *BNIP*3L, *PUMA*, *JUNB*, *CCNA*1, *CXCL*10, *CXCL9* were selected for further investigations in five t(11;14)-positive MCL cell lines. *PARG*1 was identified as a strong candidate tumor suppressor gene on 1p22.1 and was subsequently investigated in primary MCL samples.

Design and Methods

Cell culture and 5-aza-CdR treatment

The Granta-519 and Jvm-2 cell lines were received from H.G. Drexler (DSMZ, Braunschweig, Germany), NCEB-1 and JeKo-1 were obtained from M. Daibata (Kochi Medical School, Kochi, Japan) and HBL-2 was obtained from M. Abe (Fukushima Medical College, Fukushima, Japan). All cell lines were cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and additional 4 mM N-acetyl-L-alanyl-L-glutamine (all from Biochrom, Berlin, Germany). For treatments, Granta-519 cells were incubated with 0.01, 0.1 and 0.5 μM 5-aza-CdR (Fluka, Seelze, Germany) dissolved in 0.1% DMSO. A solvent control was simultaneously incubated with 0.1% DMSO. All treatment experiments were performed using extensively characterized Granta-519 cells in the exponentially growing phase.[17]

Patients' samples

In total, 31 MCL samples were analyzed. In most cases the origin of the analyzed tissue was lymph node, except for one spleen (sample 6), three bone marrow (sample 4) and one blood sample. In all patients, the diagnosis of MCL was confirmed by histology and immunophenotype. Except for one blastoid MCL all cases were classical MCL. Informed consent was provided according to the Declaration of Helsinki.

DNA extraction

DNA from bone marrow, blood and cell cultures was extracted using the DNA Isolation Kit for Mammalian Blood (Roche, Mannheim, Germany). When extracting DNA from cell lines the step of red cell lysis was omitted. DNA from MCL frozen sections was extracted according to standard procedures with proteinase K digestion and sodium chloride precipitation. The DNA was further purified using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Isolation of $CD19^+$ peripheral blood mononuclear cells (PBMC)

PBMC from healthy donors (n=5) were separated by density gradient centrifugation using Leucosep tubes (Greiner bio-one, Solingen-Wald, Germany) and Ficoll paque PLUS solution (Amersham Biosciences, Freiburg, Germany). To isolate $CD19^+$ PBMC, magnetic activated cell sorting (MACS) CD19 microbeads and MACS separation columns (both from Miltenyi Biotec, Bergisch-Gladbach, Germany) were used to perform immunomagnetic bead selection according to the manufacturer's protocol.

Western blotting

Western blotting was performed using standard procedures. Briefly, total protein extracts from harvested cells (40 μg) were separated by 15% sodium dodecylsulfate polyacrylamide gel electrophoresis and blotted on Roti-polyvinylidene fluoride membranes (Roth, Karlsruhe, Germany). The membrane was probed with different antibodies and visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Freiburg, Germany) in an LAS-1000 luminescence image analyser (Fuji Photo Film Co., Tokyo, Japan). To test the uniform loading of all lanes, membranes were probed with a goat polyclonal antibody against actin. A goat polyclonal antibody for actin (C-11, sc-1615, Santa Cruz Biotechnology, Heidelberg, Germany), and a mouse monoclonal antibody against 14-3-3σ (clone 1433S01, Labvision-Neomarkers, Microm, Walldorf, Germany) were used as primary antibodies. A horseradish peroxidase-conjugated goat-anti-mouse (sc-2005, Santa Cruz Biotechnology) or donkey-anti-goat (sc-2020, Santa Cruz Biotechnology) antibody (both Santa Cruz Biotechnology) was used as a secondary antibody.

cDNA microarray analysis

For cDNA microarray experiments Granta-519 cells were treated with 0.1 μM 5-aza-CdR for 4 days in three parallel experiments as described above. Total RNA was isolated using Trizol Reagent (Invitrogen, Karlsruhe, Germany) followed by RNeasy Mini Kit (Qiagen, Hilden, Germany) and on-column DNase digestion with RNase-free DNase I (Qiagen). One microgram of total RNA was linearly amplified using a MessageAmp aRNA kit (Ambion, Huntingdon, Cambridgeshire, UK). Quantification and validation for integrity was performed on an Agilent 2100 Bioanalyzer using an RNA 6000 NanoLabChip kit according to the manufacturer's protocol (Agilent Technologies, Böblingen, Germany). Three micrograms each of amplified RNA from the 5-aza-CdR treated cells (sample) and the solvent control (reference) were labeled with Cy5-dUTP and Cy3-dUTP (Amersham Biosciences), respectively. In each of the three parallel experiments, fluorescence-labeled RNA probes of the sample and the corresponding reference were comparatively hybridized on a spotted cDNA microarray (Stanford Functional Genomics Facility, Stanford, CA, USA) according to the posted protocol (*http://brownlab.Stanford.edu/protocols.html*). The fluorescence intensities of Cy5 and Cy3 were measured on a GenePix 4000 scanner (Axon Instruments, Foster City, CA, USA) and analyzed using GenePix Pro 4.1 software (Axon Instruments). Areas of the microarray or spots exhibiting obvious damages due to technical failures were excluded from subsequent analysis. Single spots were only considered as well-measured and included in the further investigation when the mean fluorescent intensity of Cy5 and Cy3 was ≥3-fold more than the local background and the regression correlation was ≥0.8. Genes were classified as upregulated when the corresponding spot met these quality criteria and a log (base 2) ratio of Cy5 relative to Cy3 revealed a value of ≥0.3 in at least two of the three parallel experiments.

Quantitative real-time PCR for relative quantification

In a total volume of 120 μL, 1 μg total RNA was reverse-transcribed by M-MuLV reverse transcriptase using random hexamer primers (RevertAid First Strand cDNA synthesis kit; Fermentas, St-Leon-Rot, Germany). Two microliters of each cDNA sample were amplified on an iCycler iQ real-time detection system (Bio-rad Laboratories, München, Germany) using a QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). Melting curve analyses were performed to verify the amplification specificity. Relative quantification of gene expression was performed according to the ΔΔ-CT method[27] using iCycler iQ real-time detection system software version 3.1 (Bio-rad Laboratories). SDHA was used as an internal control gene. Detailed PCR conditions and primer sequences are listed as supplemental data.

Bisulfite modification of genomic DNA

One microgram of DNA was denatured by NaOH and modified by sodium bisulfite using the CpGenome DNA modification kit (Intergen Company, Purchase, NY, USA). The modified DNA was purified, treated with NaOH to desulfonate, washed with ethanol and finally resuspended in 30 μL TE buffer.

Methylation-specific polymerase chain reaction (MSP)

The MSP technique used in the present study was originally described by Herman *et al.*[28] Two microliters of the modified DNA samples were amplified. Detailed PCR conditions and primer sequences are listed as supplemental data. MSP products were analyzed on 3% agarose gels or on an Agilent 2100 Bioanalyzer using a DNA 1000 LabChip kit (Agilent Technologies) according to the manufacturer's protocol.

Combined bisulfite restriction analysis (COBRA)

In order to carry out COBRA, a technique originally described by Xiong and Laird,[29] CpG islands were defined using the CpG island searcher (http://www.uscnorris.com/cpgislands2/cpg.aspx) according to the revised criteria of Takai and Jones.[30] For bisulfite-PCR, 2 μL of the modified DNA samples were amplified followed by restriction digestion using the following restriction enzymes: *Hinf*1, *Mwo*1 (HpyF10V1) and *Taq*1 (all purchased from Fermentas GmbH, St.Leon-Rot, Germany). Restriction maps were designed using Discovery Studio Gene version 1.5 (Accelrys Inc., Cambridge, UK). Detailed PCR conditions, primer sequences and the position of investigated CpG islands are listed as supplemental data.

Bisulfite sequencing

The *PARG1* PCR product of 295 bp was generated as described for COBRA. Fresh PCR products were cloned into plasmids using the TOPO TA cloning Kit (Invitrogen, Karlsruhe, Germany). Individual clones were sequenced using the CEQ™ DTCS-Quick start Kit (Beckman Coulter GmbH, Krefeld, Germany) with uni-

Table 1. List of investigated genes. Upregulated genes were selected for further investigations according to their putative function in tumorigenesis. The displayed x-fold upregulation represents the mean value of triplicate determinations.

Gene	*Cytoband*	*x-fold upregulated on microarray*	*x-fold upregulated in real time PCR*
A *upregulated genes from frequently lost sites*			
ATM	11q22-q23	1.5	1.9
*ING*1	13q34	1.5	1.7
*PARG*1	1p22.1	1.9	3.3
*RUNX*3	1p36	1.9	7.1
B *upregulated pro-apoptotic BCL2-family members*			
*BNIP*3L	8p21	1.8	8.1
PUMA	19q13.3-q13.4	2.1	9.3
C *reported methylation in haematologic malignancies*			
JUNB	19p13.2	1.7	4.7
D *highly upregulated genes with discussed cancer connection*			
*CCNA*1	13q12.3-q13	6.4	71.8
*CXCL*10	4q21	8.9	25.2
*CXCL*9	4q21	7.2	57.0

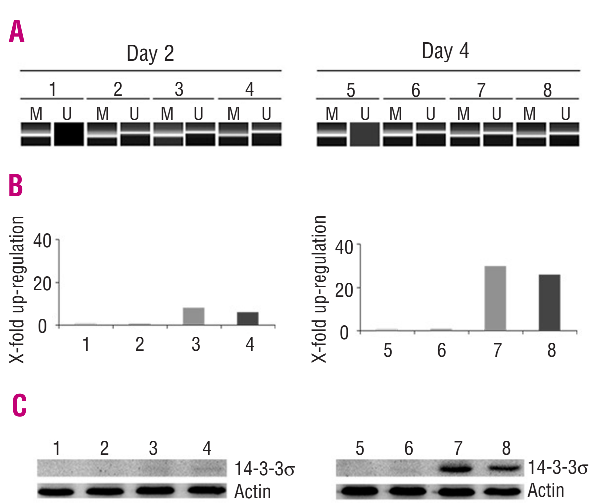

Figure 1. **Reactivation of 14-3-3σ in Granta-519. Cells treated with different doses of 5-aza-CdR for 2 and 4 days. Data are representative of one of two independent experiments. Solvent control (lanes 1 and 5), 0.01 μM (lanes 2 and 6), 0.1 μM (lanes 3 and 7), 0.5 μM (lanes 4 and 8) 5-aza-CdR. (A) Methylation-specific PCR (Agilent analysis. M: methylation-specific primers, U: unmethylation-specific primers). (B) Quantification of 14-3-3σ expression in 5-aza-CdR treated cells relative to the cells treated with solvent control using real-time PCR and the ΔΔ-CT method. (C) Western blotting.**

versal T3 and T7 primers after amplification of the insert with M13 forward and reverse primers. Sequence reactions were analyzed using the CEQ 8000 Sequencing System (Beckman Coulter GmbH, Krefeld, Germany).

Results

5-aza-CdR treatment of Granta-519 cells

To establish a demethylation protocol utilizing non-cytotoxic doses of 5-aza-CdR on Granta-519 cells, the cells were treated with 0.01, 0.1 or 0.5μM 5-aza-CdR for up to 6 days. In line with the results of an earlier study on the anti-tumor effect of 5-aza-CdR,[31] time- and dose-dependent inhibition of cell proliferation was seen with a concomitant increase of G1-phase cells and a decrease of S-phase cells. Possible induction of apoptosis or cytotoxicity could be excluded at all dose levels examined, as ensured by negative propidium iodide/annexin V double staining and identification of normal levels of marker genes p53, p21, Bcl-2, Bax and caspase-9 upon Western blotting (*data not shown*). Re-expression of the 14-3-3σ gene, which is constitutively methylated in the Granta-519 cell line (*data not shown*), served as a functional control for successful demethylation (Figure 1).

cDNA microarray analysis

The microarray analysis displayed more than 1000 upregulated genes, 16 of which were upregulated ≥3-fold. For further studies, upregulated genes that met the quality criteria were selected according to the following criteria (Table 1): (i) genes are located in chromosomal regions that are frequently deleted in MCL: *ATM*, *ING*1, *PARG*1 and *RUNX*3;[32-34] (ii) genes which code for pro-apoptotic members of the Bcl-2 family: *BNIP*3L and *PUMA*; (iii) *JUNB*, for which transcriptional silencing was reported in hematologic malignancies;[35] and (iv) the top three upregulated genes: *CCNA*1, *CXCL*10 and *CXCL*9.

Quantitative real-time PCR analysis in untreated t(11;14)-positive cell lines

To determine whether identified candidate genes are down-regulated by transcriptional silencing, their expression was evaluated by quantitative real-time PCR analysis in untreated t(11;14)-positive cell lines Granta-519, Hbl-2, Jvm-2, JeKo-1 and NCEB-1. Using the ΔΔ-CT method[27] and succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*) as an internal control, relative quantification was performed. The expression level in CD19+ PBMC obtained from five healthy donors was set as 1 (Figure 2). The expression of *ATM, ING*1 and *PARG*1 was reduced in all studied cell lines (Figure 2A-C). The expression of *RUNX*3 was clearly reduced in three of five cell lines (Figure 2D). Studying the selected pro-apoptotic genes of the Bcl-2 family, *BNIP*3L was downregulated in all t(11;14)-positive cell lines except in JeKo-1 (Figure 2E). A distinct reduction of *PUMA* expression was seen in Hbl-2, JeKo-1 and NCEB-1 (Figure 2F). The expression of *JUNB* was only reduced in HBL-2 and JeKo-1 (*data not shown*). Interestingly, none of the most highly upregulated genes (Table 1D) was

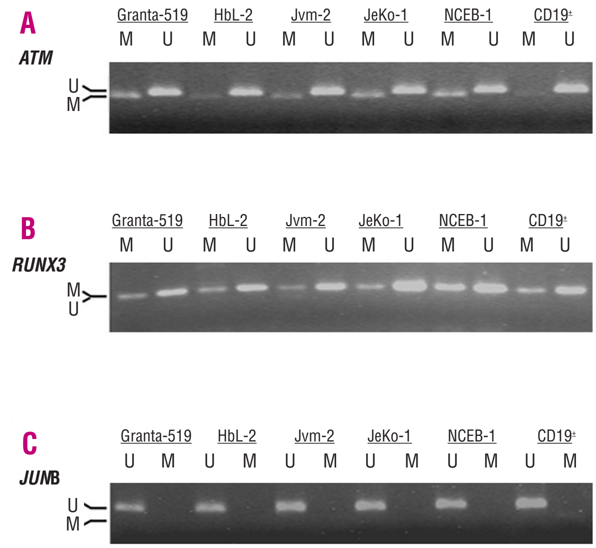

Figure 3. MSP in t(11;14)-positive MCL cell lines and CD19+ PBMC. To confirm epigenetic changes in the t(11;14)-positive MCL cell lines in comparison to CD19+ PBMC from healthy donors, MSP was performed (M: methylation-specific primers, U: unmethylation-specific primers) (A), *ATM*. (B) *RUNX*3. (C) *JUN*B.

downregulated in untreated MCL cell lines. Since this seems to be attributable to demethylation-independent influences of 5-aza-CdR, secondary changes and/or the reactivation of endogenous retroviruses or retroviral elements,[22-24,26,36,37] none of these genes was investigated further.

Methylation analyses

To investigate the methylation of promoter CpG islands, *ATM*, *RUNX*3 and *JUNB* were analyzed by MSP and *ING*1, *PARG*1 and *BNIP*3L by COBRA. Methylated alleles of *ATM* were distinctly amplified in four of five t(11;14)-positive cell lines, whereas in CD19+ PBMC and Hbl-2 only a slight band indicating the methylated state of DNA was observed (Figure 3A). In contrast, *RUNX*3 showed constitutive methylation in all investigated cell lines and in CD19+ PBMC (Figure 3B). No methylation of the *JUN*B promoter site was found (Figure 3C).

Performing COBRA, restriction digestion demonstrates the methylation of the investigated CpG site. Thus, partial and full digestion indicate incomplete and complete methylation, respectively, and no digestion corresponds to an unmethylated state. Analyses of *ING*1 showed no evi-

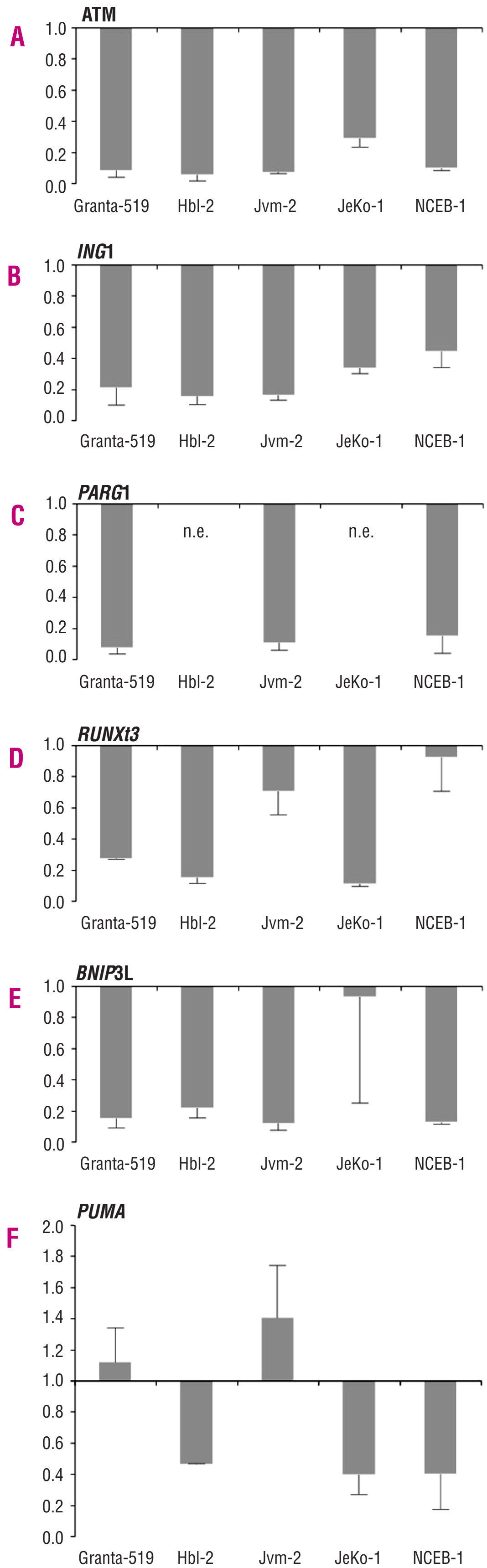

Figure 2 (left). Relative quantification of gene expression in untreated t(11;14)-positive cell line. The expression of selected genes in t(11;14)-positive cell lines was quantified relative to the expression in CD19+ PBMC of healthy donors using real time PCR and the ΔΔ-CT method. The expression level in the CD19+ PBMC of healthy donors was set as 1. The displayed columns represent the mean value and standard deviation of triplicate determinations. A, *ATM*. B, *ING*1. C, *PARG*1. D, *RUNX*3. E, *BNIP*3L and F, *PUMA*. (n.e. – no expression was detected in the real-time PCR).

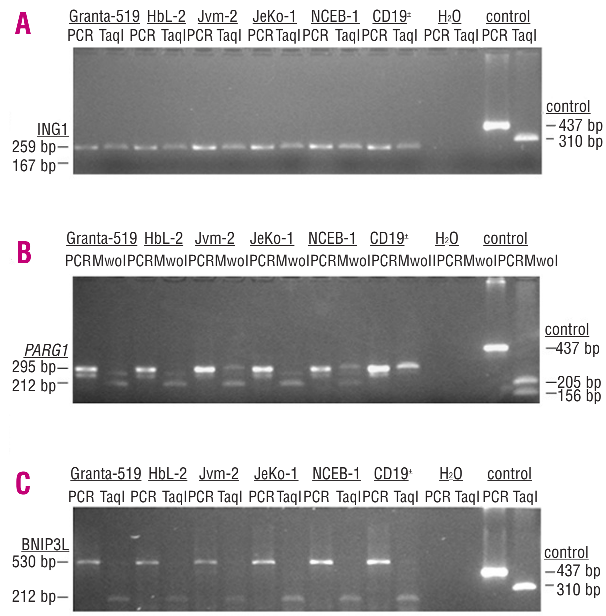

Figure 4. **COBRA in t(11;14)-positive MCL cell lines and $CD19^+$ PBMC. In consideration of the known demethylation-independent effects of 5-aza-CdR, selected genes were investigated by COBRA, to test for epigenetic changes in the t(11;14)-positive cell lines in comparison to $CD19^+$ PBMC from healthy donors. To control the restriction enzyme digestion, the PCR-product of a PCR, in which non-bisulfite-converted DNA was amplified, was simultaneously digested with the indicated restriction enzyme and is shown as *control*. (A) *ING*1. (B) *PARG*1 and (C) *BNIP*3L.**

dence of methylation (Figure 4A). COBRA of *PARG*1 in $CD19^+$ PBMC did not show any methylation, since there was no digestion of the PCR product (295 bp) leading to the expected fragment of 212 bp by *Mwo*1 in the case of methylation. In contrast, full methylation was found in Granta-519, Hbl-2 and JeKo-1 cells. Partial methylation was demonstrated in Jvm-2 and NCEB-1 (Figure 4B). Finally, investigations of *BNIP*3L displayed a complete digestion of the PCR product (530bp) by *Taq1* that leads to a fragment of 212 bp in all studied cell lines as well as in the investigated $CD19^+$ PBMC (Figure 4C).

Methylation in primary MCL samples

To investigate further whether *PARG*1 is a candidate tumor suppressor gene, inactivated in MCL by methylation, 31 primary MCL samples were analyzed by COBRA and bisulfite sequencing. The PCR products (295 bp) obtained from bisulfite converted DNA of all samples were at least partially digested by *Hinf*1 that leads to the expected fragments of 181bp and 114bp (Figure 5). Finally, promoter methylation of *PARG*1 in primary tumor samples was confirmed by bisulfite sequencing (Figure 5). In conclusion, clear differences between the control $CD19^+$ PBMC and the t(11;14)-positive MCL cell lines as well as the primary tumor cells regarding promoter methylation were demonstrated for *PARG*1, which is located in the frequently deleted chromosomal region 1p22.1.

PARG1 expression in primary tumor cells

Finally, we sought to determine whether aberrant methylation of the *PARG*1 promoter leads to reduced expression of the *PARG*1 gene in primary tumor cells. Expression data for *PARG*1 were obtained from a gene expression profiling analysis of primary MCL cases that was performed on the GeneCHIP Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, USA) (*Schraders, M. et al., submitted*). Since the tumor load was more than 90%, microdissection was not necessary. Comparing *PARG*1 expression in control cells, using cells from lymph nodes with follicular hyperplasia, with the expression in MCL cells, a significant down-regulation could be demonstrated in the tumor cells (Figure 5D).

Discussion

To screen for epigenetic aberrations in MCL, we combined a cDNA microarray approach with non-cytotoxic, demethylating 5-aza-CdR treatment of Granta-519, the most frequently used MCL cell line. From the upregulated genes with putative function in MCL tumorigenesis, we selected ten candidate genes for further investigations in five untreated t(11;14)-positive cell lines, to validate whether these genes are indeed transcriptionally downregulated and methylated in MCL.

Because epigenetic changes can co-operate with genetic aberrations, leading to the inactivation of tumor suppressor genes,[13] we focused on the expression level of the genes located in frequently deleted chromosomal regions in MCL (Table 1A).[32-34] Real time PCR confirmed the findings of the microarray analyses for all ten genes. However, the observed upregulation may occur not only through demethylation of otherwise methylated promoter regions but also due to other transcriptional or translational regulation or activation of retroviral elements.[22-24,26,36,37] Therefore, it is essential to determine the extent of methylation of the promoter regions of these genes directly. For this purpose, bisulfite DNA modification was applied. This treatment leads to a conversion of unmethylated but not methylated cytosines to uracils. Subsequently, MSP or COBRA was carried out for interesting genes in the five untreated t(11;14)-positive cell lines. Moreover, the extent of gene methylation in MCL cell lines was compared with that of the normal counterpart, $CD19^+$ B cells from healthy donors. No promoter methylation of *JUNB* and *ING*1 was found. Also, no differential methylation was seen for *RUNX*3 and *BNIP3L*, which were equally methylated both in control B cells and in MCL cell lines. In contrast, *ATM* and *PARG*1 were at least partially methylated in the MCL cell lines, but only slightly or completely unmethylated in the control B cells, respectively.

ATM, which plays a key role in DNA repair and G2/S checkpoint control, is frequently inactivated by deletions and point mutations in MCL.[11,12,32,33,38-41] Patients

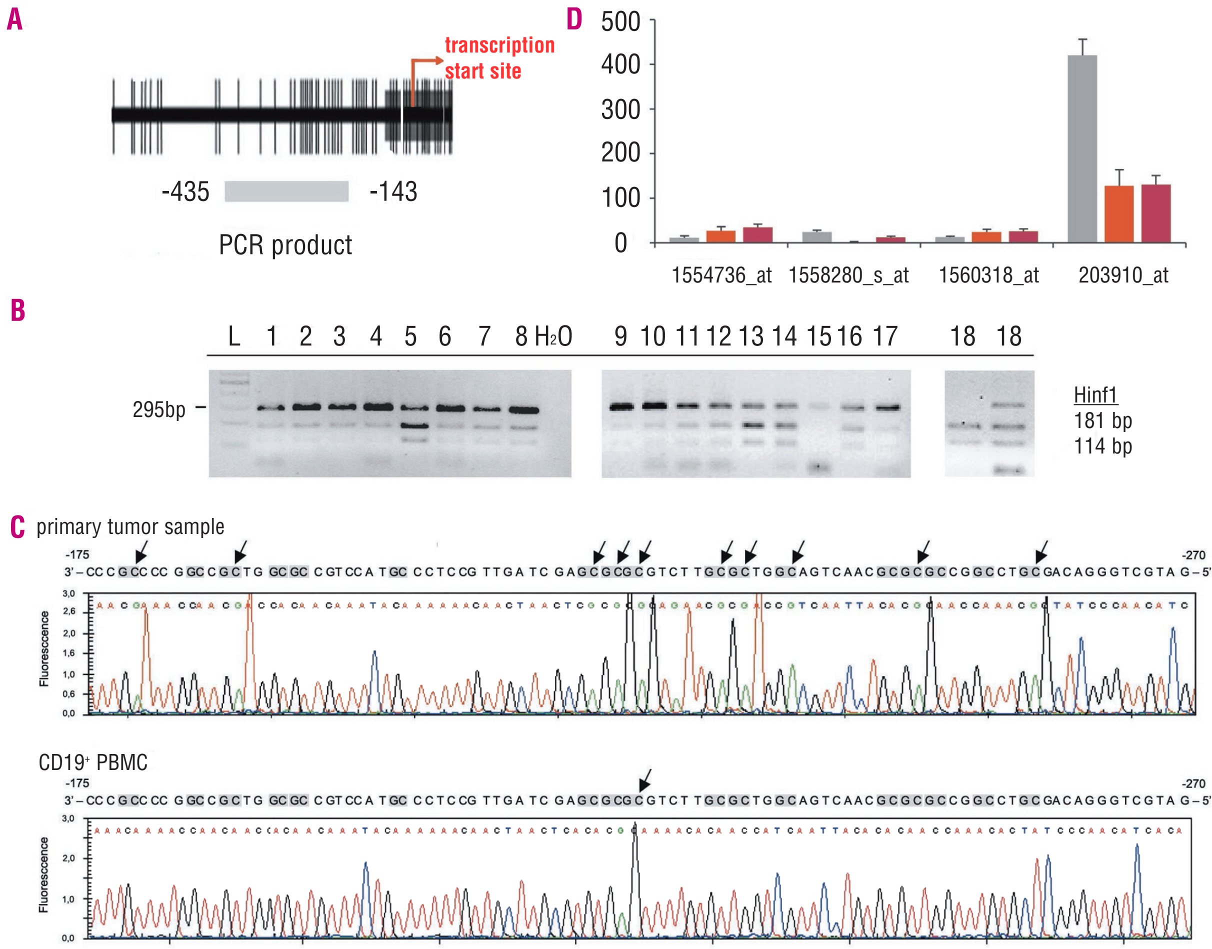

Figure 5. COBRA and bisulfite sequencing of PARG1 in primary MCL samples. (A) A section (-692 to +90) of a CpG island in the 5' region of *PARG1* (-692 to +777) with the location of the COBRA PCR product (-435 to - 140) near the transcription start site (TS) is shown. Vertical lines indicate individual CpG sites. The dark gray box represents exon 1. (B) COBRA on 19 primary MCL samples. Restriction digestion of the 295 bp PCR fragment with *Hinf*1 leads to products of 181 bp and 114 bp indicating at least partial methylation in all cases. (C) Bisulfite sequencing results of the converted antisense strand of a selected tumor sample showing strong methylation (No. 5) and CD19+ PBMC. The original sequence (-175 to -270), with CpG islands indicated by gray boxes, is shown above the electropherograms. Methylated CpG sites are indicated by arrows. (D) *PARG1* gene expression (pool of six control lymph nodes with follicular hyperplasia– gray, MCL without 1p loss (mean values of five cases) – light red, MCL with 1p loss (mean values of six cases) – dark red): GeneCHIP Human Genome U133 Plus 2.0 Array contains four probes for *PARG1* (Locus Link 9411). Three probes were not informative because there was no expression at all in either MCL cases with or without 1p loss or in controls (1554736_at, 1558280_s_at, 1560318_at). Probe 203910_at showed a much lower expression in primary MCL than in controls.

with chronic lymphocytic leukemias (CLL) with deletions of 11q, involving the *ATM* locus, have a significantly poorer prognosis than other CLL patients.[42] So far, there is only one study about epigenetic mechanisms leading to the inactivation of *ATM* in MCL showing the absence of *ATM* hypermethylation in eight primary cases as well as in Granta-519 cells.[43] The discrepancy between these and our results may be explained by the fact that although the identical promoter region was analyzed (position -4578 to -4799 on the genomic level based on the *ATM* transcription start site), different primers, leading to a larger PCR fragment, were used. Since methylation of a tumor suppressor gene does not mean that each CpG site is methylated and that methylation is 100% at each site, primer design has a big impact on the results.[44] However, the *ATM* gene is a target for epigenetic silencing in advanced breast cancer,[45] head and neck cancer[46] and colorectal cancer.[47] Future studies will have to clarify whether promoter methylation, which was shown in four of the five cell lines for the first time, induces relevant inactivation of *ATM* in patients with MCL and CLL and whether it is associated with the clinical outcome.

*PARG*1 codes for *PTPL*1-associated RhoGAP1, a GTPase-activating protein (GAP) that exhibits *in vitro* GAP activity towards the Rho- and Ras-family members Rho[48] and Rap2,[49] thus promoting a switch from the active to the inactive form[50] and that may, therefore, act as a suppressor of Rho- and Ras-mediated cellular transformation.[50] Recently, Ghobrial and co-workers reported on the overexpression of the guanine nucleotide exchange factor *RCC*1 (regulator of chromosome condensation 1) and the RhoA-dependent kinase *CRIK* (citron Rho-interacting kinase) in MCL, suggesting that alterations of the Rho-Ras-network may be involved in the pathogenesis of MCL.[51] Moreover, *PARG1* is located in 1p22.1, one of the most frequently deleted regions in MCL.[32] In this respect, attention should be paid to the observation that array-CGH revealed a monoallelic deletion in Granta-519 and JeKo-1.[34] In addition to this allelic loss, our studies demonstrated a downregulation and full methylation of *PARG1* in these t(11;14)-positive MCL cell lines. The other MCL cell lines also showed downregulation due to full or partial methylation. Finally, methylation of *PARG1* was demonstrated in 31 primary MCL samples. Two of the cases were also investigated by array-CGH[32] in which a loss of one *PARG1* allele was shown. Furthermore, for two additional cases a deletion of 1p11-p31 and 1p13-p33 was detected by routine karyotyping (*data not shown*). A drastic reduction of gene expression should be the consequence of the observed epigenetic silencing. Unfortunately, due to shortage of material, RT-PCR could not be performed on the same cases. However, array-based genome-wide gene expression profiling was done on an independent set of MCL cases, demonstrating a significant down-regulation of *PARG1*. *PARG1* is down-regulated to approximately 30% of control level.

In conclusion, *PARG1*, coding for a RhoGTPase activation protein, was found to be methylated in all studied MCL cell lines as well as in all studied primary MCL samples. Furthermore, a strong down-regulation of *PARG1* was observed in all MCL cases analyzed. Thus, *PARG1* may be a crucial candidate tumor suppressor gene in the frequently deleted region 1p22.1, satisfying Knudson's hypothesis by monoallelic deletion and transcriptional silencing of the other allele. Further investigations are required to define the proportions and functional consequences of genetic and epigenetic mechanisms that may lead to a biallelic knockdown of *PARG1* in MCL.

Authors' Contributions

TR, Nvn and DS designed the study, analyzed the data and wrote the paper; TR and KK performed all experiments; ME, UL, MT, BS, CR and BS gave critical comments and assistance with technical performance; EC-B, PG, MS and HvK were involved in patient acquisition and delivery of clinical data; MS, PG and HvK contributed Affymetrix expression data.

Conflict of Interest

The authors reported no potential conflicts of interest.

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