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Promoter methylation of PARG1, a novel candidate tumor suppressor gene in mantle cell lymphomas

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Acknowledgments: we thank Britta Hasemeier and Markus Meyer for their expert technical assistance as well as Gillian Teicke for her help in editing the manuscript.

Funding: This study was supported by EU grant QLRT-1999-30687.


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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 ING1, RUNX3 and BNIP3L 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 PARG1 is a strong candidate tumor suppressor gene in MCL.

Key words: mantle cell lymphoma, epigenetic silencing, tumor suppressor gene, PTPL1-associated RhoGAP1 (PARG1).

Haematologica 2007; 92:460-468

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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 8-10% of non-Hodgkin’s lymphomas. 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. The genetic hallmark of MCL is the translocation t(11;14)(q13;q32), which is present in virtually all cases and causes overexpression of cyclin D1. However, there is experimental evidence indicating that additional alterations are required to induce lymphoma genesis. Classical cytogenetic and comparative genomic hybridization (CGH) studies have shown a high number of recurrent chromosomal alterations in t(11;14)-positive MCL. Loss of the chromosomal fragment 11q22-23 harboring the ataxia-telangiectasia mutated (ATM) gene is observed in almost 50% of MCL. 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. 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 and could therefore be used as methylotype models of the primary tumors.

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 with 5-aza-2’-deoxycytidine (5-aza-CdR) to identify epigenetically silenced genes. This inactivating substrate of DNA methyltransferase induces an indirect genomic demethylation and chromatin remodeling and can, therefore, lead to the reactivation of transcriptionally silenced genes. We identified upregulated genes by means of cDNA microarray analysis in a similar manner to that used in recent reports. 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, ING1, PARG1, RUNX3, BNIP3L, PUMA, JUNB, CCNA1, CXCL10, CXCL9 were selected for further investigations in five t(11;14)-positive MCL cell lines. PARG1 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.

**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 Polyvinylidene fluoride membranes (Roth, Karlsruhe, Germany). The membrane was probed with different antibodies and visualized with an enhanced chemilumi-
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 ∆∆-CT method 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.23 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.usc-norris.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: HinfI, MwoI (HpyF10V1) and TaqI (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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cytoband</th>
<th>x-fold upregulated on microarray</th>
<th>x-fold upregulated in real time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>11p22-q23</td>
<td>1.5</td>
<td>1.9</td>
</tr>
<tr>
<td>ING1</td>
<td>13q34</td>
<td>1.5</td>
<td>1.7</td>
</tr>
<tr>
<td>PARG1</td>
<td>1p22.1</td>
<td>1.9</td>
<td>3.3</td>
</tr>
<tr>
<td>RUNX3</td>
<td>1p36</td>
<td>1.9</td>
<td>7.1</td>
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A upregulated genes from frequently lost sites

B upregulated pro-apoptotic BCL2-family members

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cytoband</th>
<th>x-fold upregulated on microarray</th>
<th>x-fold upregulated in real time PCR</th>
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</thead>
<tbody>
<tr>
<td>BNIP3L</td>
<td>8p21</td>
<td>1.8</td>
<td>8.1</td>
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<tr>
<td>PUMA</td>
<td>19q13.3-q13.4</td>
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<td>9.3</td>
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C reported methylation in haematologic malignancies

<table>
<thead>
<tr>
<th>Gene</th>
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<th>x-fold upregulated in real time PCR</th>
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<tr>
<td>JUNB</td>
<td>19p13.2</td>
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<td>4.7</td>
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D highly upregulated genes with discussed cancer connection

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cytoband</th>
<th>x-fold upregulated on microarray</th>
<th>x-fold upregulated in real time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNA1</td>
<td>13q12.3-q13</td>
<td>6.4</td>
<td>71.8</td>
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<tr>
<td>CXCL10</td>
<td>4q21</td>
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<td>25.2</td>
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<tr>
<td>CXCL9</td>
<td>4q21</td>
<td>7.2</td>
<td>57.0</td>
</tr>
</tbody>
</table>

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,3 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 ≥8-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, ING1, PARG1 and RUNX3;2-34 (ii) genes which code for pro-apoptotic members of the Bcl-2 family: BNIP3L and PUMA; (iii) JUNB, for which transcriptional silencing was reported in hematologic malignancies;35 and (iv) the top three upregulated genes: CCNA1, CXCL10 and CXCL9.

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 ΔΔCT method27 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, ING1 and PARG1 was reduced in all studied cell lines (Figure 2A-C). The expression of RUNX3 was clearly reduced in three of five cell lines (Figure 2D). Studying the selected pro-apoptotic genes of the Bcl-2 family, BNIP3L 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...
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, none of these genes was investigated further.

**Methylation analyses**

To investigate the methylation of promoter CpG islands, \(\text{ATM} \), \(\text{RUNX3} \) and \(\text{JUNB} \) were analyzed by MSP and \(\text{ING1} \), \(\text{PARG1} \) and \(\text{BNIP3L} \) by COBRA. Methylated alleles of \(\text{ATM} \) were distinctly amplified in four of five \(t(11;14)\)-positive cell lines, whereas in \(\text{CD19}^+\) PBMC and Hbl-2 only a slight band indicating the methylated state of DNA was observed (Figure 5A). In contrast, \(\text{RUNX3} \) showed constitutive methylation in all investigated cell lines and in \(\text{CD19}^+\) PBMC (Figure 3B). No methylation of the \(\text{JUNB} \) 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 \(\text{ING1} \) showed no evi-
PBMC did not show any methylation, since there were no bisulfite-converted DNA was amplified, was simultaneously digested with the indicated restriction enzyme and is shown as control. (A) ING1, (B) PARG and (C) BNIP3L.

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 (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, we focused on the expression level of the genes located in frequently deleted chromosomal regions in MCL (Table 1A). 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. 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 (11;14)-positive cell lines. Moreover, the extent of gene methylation in MCL cell lines was compared with that of the normal counterpart, CD19B cells from healthy donors. No promoter methylation of JUNB and ING1 was found. Also, no differential methylation was seen for RUNX3 and BNIP3L, which were equally methylated both in control B cells and in MCL cell lines. In contrast, ATM and PARG 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. Patients
with chronic lymphocytic leukemias (CLL) with deletions of 11q, involving the ATM locus, have a significantly poorer prognosis than other CLL patients. 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. 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. However, the ATM gene is a target for epigenetic silencing in advanced breast cancer, head and neck cancer and colorectal cancer. 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.

PARG1 codes for PTPL1-associated RhoGAP1, a GTPase-activating protein (GAP) that exhibits in vitro GAP activity towards the Rho- and Ras-family members RhoA and Rap2, thus promoting a switch from the active to the inactive form and that may, therefore, act as a suppressor of Rho- and Ras-mediated cellular transformation. Recently, Ghobrial and co-workers reported on the overexpression of the guanine nucleotide exchange factor RCC1 (REGulator of chromosome condensation 1) and the RhoA-dependent kinase CRK (citrin Rho-interacting kinase) in MCL, suggesting that
altered by the Rho-Ras-network may be involved in the pathogenesis of MCL. Moreover, PARG1 is located in 1p22.1, one of the most frequently deleted regions in MCL. In this respect, attention should be paid to the data not shown in which a loss of one 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 80% of control levels.

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, VN and DS designed the study, analyzed the data and wrote the paper; TR and RK 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.

**References**


