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Assessment of Promoter Methylation Identifies *PTCH* as a Putative Tumor-suppressor Gene in Human CLL

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Abstract. *Background: Chronic lymphocytic leukemia (CLL) is characterized by a clonal accumulation of neoplastic lymphocytes, indicating disruption of apoptosis. Patients and Methods: Differential methylation hybridization analysis was performed to identify novel target genes silenced by CpG island methylation in patients with CLL. Results: Patched (PTCH), a tumor-suppressor gene, was found to be frequently methylated in CLL samples compared to samples derived from healthy individuals. De novo methylation of a CpG island region located upstream of PTCH exon 1 was confirmed by pyrosequencing in 17/37 (46%) of peripheral blood mononuclear cells of patients with CLL, but in none isolated from seven healthy individuals. No association was found between PTCH hypermethylation and currently used prognostic CLL factors. Conclusion: Our investigation suggests that epigenetic silencing of PTCH is a mechanism contributing to CLL tumorigenesis.*

Chronic lymphocytic leukemia (CLL) is a frequent malignant disease with a low rate of cure. Major advances have been made in our understanding of the biology and opportunities for treatment of CLL in recent times. Newer treatment regimens have increased the rate of successful remission induction in patients with CLL. Moreover, recent combination of chemoimmunotherapy regimens have produced more frequent complete molecular remissions, and early evidence seems to suggest that this could result in prolonged duration of responses, although this association remains to be clearly demonstrated (1).

Studies of CLL involving epigenetic aberrations have accelerated the search for affected genes, which was initially restricted to commonly deleted chromosomal regions. Many novel genes that are epigenetically silenced in CLL have been identified. Advances in the understanding of post-translational histone modifications and DNA methylation in normal and in CLL cells have proven to be extremely beneficial in finding powerful diagnostic markers, as well as in exploring novel therapies. At present, the field of epigenetics is at an evolving stage, but there is no doubt that further unravelling of its cause and effects in transformed cells will bring a new revolution in cancer therapeutics (2-4). Interestingly, progressive disease in CLL is correlated with the DNA methylation index (5).

In our study, patched (PTCH), the receptor for the hedgehog (Hh) pathway and a familiar tumor-suppressor gene, was selected for further analysis. PTCH is known to be an inhibitor of the Hh pathway and its silencing activates the pathway and promotes growth of various cancer cells including breast cancer, medulloblastomas and basal cell carcinoma.

Materials and Methods

DNA samples. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of 62 unselected patients with CLL at different time points of treatment and buffy coats from seven healthy volunteers by Ficoll density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). Written consent was obtained after Instituion Review Board approval of a protocol to collect blood serially from patients with CLL. Isolated cells were allowed to adhere at a density of 5×10^6 cells/ml for 1 h at 37˚C in RPMI-1640 (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (Invitrogen, Karlsruhe, Germany) in a humidified atmosphere and 5% CO_2. Non-adherent peripheral blood lymphocytes (PBLs) were taken, washed three times in phosphate-

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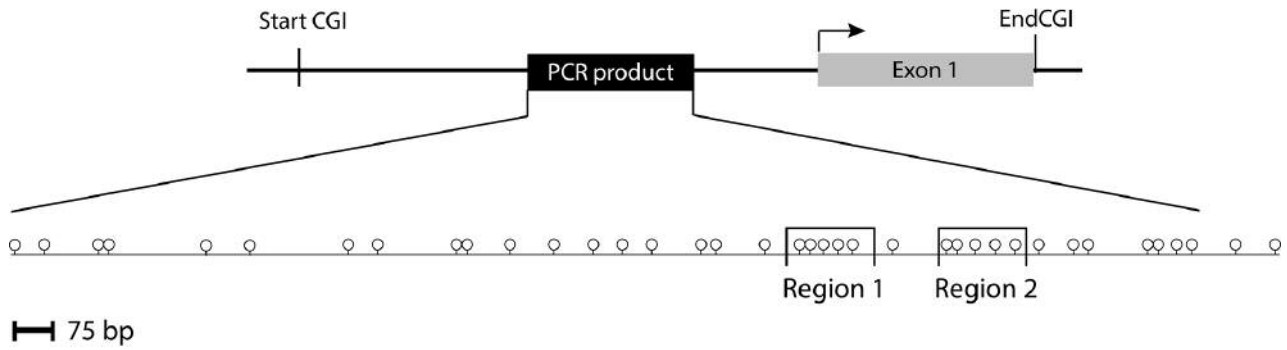

Figure 1. *General map of the CpG island (CGI) spanning the patched (PTCH) promoter region and exon 1b of the PTCH gene. b: Polymerase chain reaction product used for bisulfite sequencing of single clones and bisulfite pyrosequencing. The lollipop diagram shows the CpG positions 1 to 38 at proportional distances, starting on the left side with CpG 1.*

buffered saline/1% bovine serum albumin (PAA). Genomic DNA was extracted from 5×10^6 lymphocytes using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was measured with a photometer (UV mini 1240, Shimadzu, Duisburg, Germany).

Bisulfite modification. Genomic DNA (300-500 ng) from the collected PBLs of the CLL samples and the buffy coats of the healthy volunteers was subjected to bisulfite modification by using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Bisulfite-treated DNA was eluted two times in 20 μl elution buffer.

Bisulfite sequencing of single clones. The primers used for polymerase chain reaction (PCR) amplification of bisulfite-treated DNA were *PTCH*-Bis-fw: 5'-AGGAGTATAAGAAAGTAGAGTT-3' and *PTCH*-Bis-rev: 5'-TTCACTACAAAAAAAACCAA-3'. The amplified region corresponds to GenBank accession number AL161729, nt 51778-52078. PCR was carried out in a 96-well plate and a final volume of 20 μl, containing 10 pmol of each primer, 200 μM of each dNTP, 0.5 U Platinum Taq DNA Polymerase (Invitrogen) in the appropriate buffer containing 2 mM $MgCl_2$ and 1 μl of bisulfite-treated DNA as template. The initial denaturation (95˚C for 10 min) was followed by 40 cycles of 45 s at 94˚C, 45 s at 54˚C, 45 s at 72˚C and a final extension step at 72˚C for 10 min.

PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen) and individual clones were subjected to Sanger sequencing employing the BigDye V.1.1 Cycle Sequencing chemistry (Applied Biosystems, Foster City, CA, USA) and separated on a 3130 Genetic Analyzer (Applied Biosystems). Single clone sequences were analyzed with BiQ Analyzer software (6).

Bisulfite pyrosequencing. The results of the bisulfite sequencing were used to design a pyrosequencing assay allowing quantitative assessment of DNA methylation of the identified region within the CpG island. The primers used for PCR amplification of bisulfite-treated DNA were *PTCH*-Bis-fw and *PTCH*-Py-rev: 5'-Biotin-TTCACTACAAAAAAAACCAA-3'. PCR was carried out in a 96-well plate and a final volume of 50 μl, containing 10 pmol of each primer, 200 μM of each dNTP, 2.5 U Platinum Taq DNA Polymerase (Invitrogen) in the appropriate buffer containing 2 mM $MgCl_2$ and 3 μl of bisulfite-treated DNA as template. The initial denaturation (95˚C, 10min) was followed by 40 cycles of 45 s at 94˚C, 45 s at 54˚C, 45 s at 72˚C and a final extension step at 72˚C for 10 min.

PCR product (40 μl) was then subjected to bisulfite pyrosequencing. The primers used for primer extension reaction were *PTCH*-1: 5'-GGTATTTTYGGATTT-3' and *PTCH*-2: 5'-GTTTTGYGTTTGTTT-3', which belong to the sequence context: 5'-YGYGGYGGYGGYGGT-3' (CpG position 19 to 23) and 5'-TTYGYGGTYGTTYGGGYGA-3' (CpG position 25 to 29), respectively. The pyrosequencing reaction was performed on an automated PSQ 96MA System (Biotage, Uppsala, Sweden) using Pyro Gold Reagents Kit (Biotage). Purification and subsequent processing of the biotinylated single-stranded DNA was performed according to the manufacturer's instructions. Resulting data were analyzed and quantified with PSQ 96MA 2.1 software (Biotage). Each sample was analyzed in duplicate for both regions by individual PCR reactions using the same bisulfite preparation as template.

Determining the DNA methylation status. The DNA methylation status of the individual samples was calculated by MethMarker software (7). The calculation was based on the 10 individual mean values obtained for each CpG position for each sample analyzed by bisulfite pyrosequencing. A refined DNA methylation-based candidate biomarker model was developed according to the recommended guideline of the MethMarker software. Briefly, firstly a biomarker model was calculated for the 10 CpG positions based on DNA methylation profiles of individual clones for six samples classified as unmethylated and for three samples classified as methylated. In a second step, the biomarker model was refined, based on experimental bisulfite pyrosequencing data obtained for five unmethylated samples and for four methylated samples.

Results

By using differential methylation hybridization, we identified a DNA sequence within the 5' region of the *PTCH* gene that indicated significant methylation in patients with CLL.

Using bisulfite sequencing of cloned PCR products, we next determined the methylation frequencies for 38

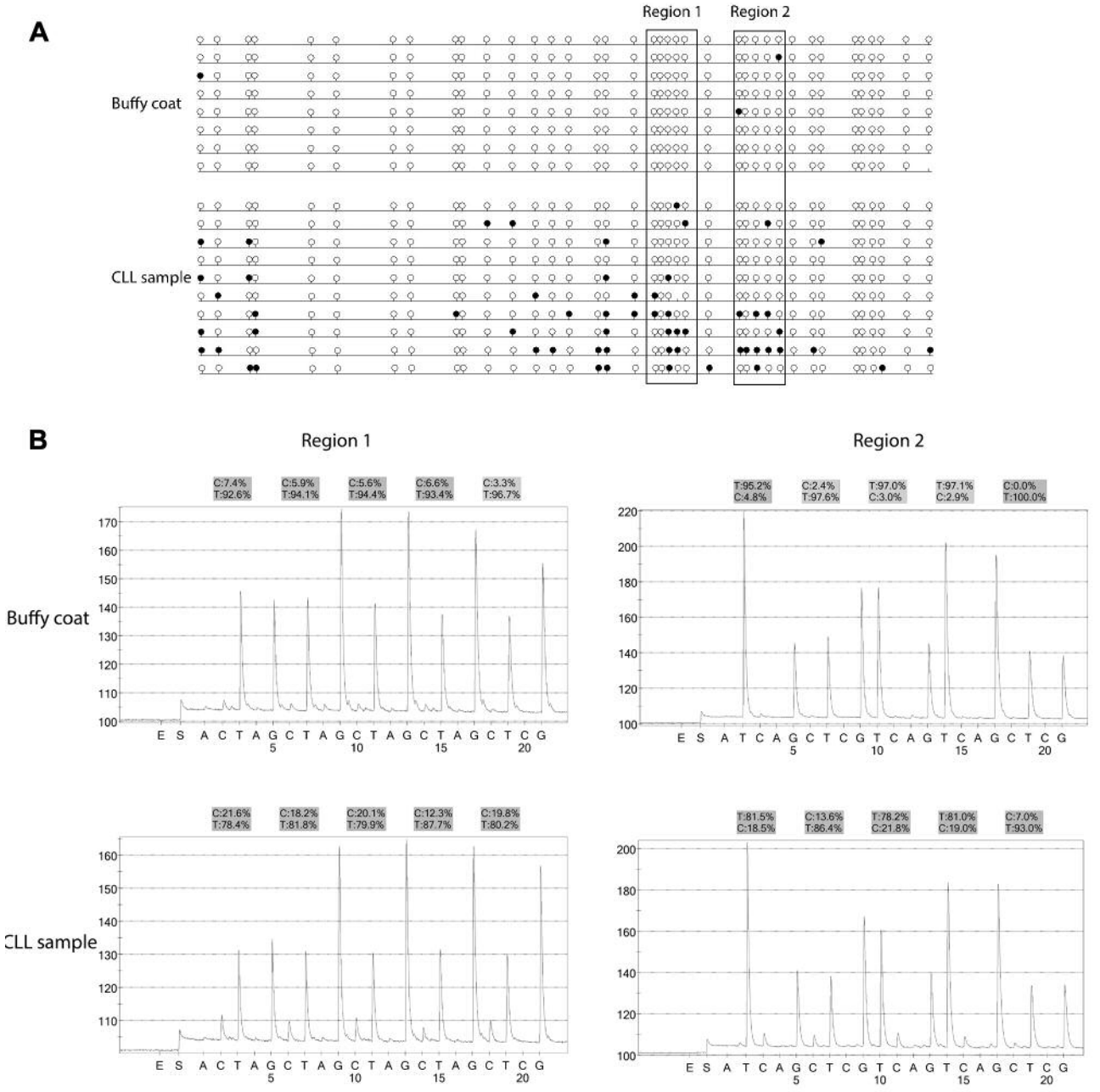

Figure 2. *Typical DNA methylation pattern obtained by bisulfite sequencing of single clones for a control sample (buffy coat) and a chronic lymphocytic leukemia (CLL) sample (A). Filled circles correspond to methylated CpG positions, unfilled circles correspond to unmethylated CpG positions, and the vertical lines without a circle corresponds to CpG positions not determined in the sequence. The diagrams were generated with the BiQ Analyzer software (6). The regions analyzed by bisulfite pyrosequencing are shown below and the pyrograms obtained (B) correspond to the samples and their DNA methylation pattern above.*

individual CpG dinucleotides contained in the interrogated promoter region of *PTCH* in chronic lymphocytic leukemia (CLL) samples (Figure 1). Bisulfite sequencing of single clones was performed on six CLL samples and three buffy coat samples, that served as unmethylated control samples. The DNA extracted from three buffy coats of the control PBMCs showed no methylation in most of the investigated CpG positions and exhibited only trace methylation of a single CpG site within a minor number of alleles. These samples were, therefore, classified as unmethylated. Three

out of six CLL samples showed several methylated CpG sites within most of the sequenced individual clones of the PCR products, indicating hypermethylation of the majority of the tumor cells in PBMCs of these patients and were classified as hypermethylated. The remaining three CLL samples had a DNA methylation pattern similar to the unmethylated buffy coat samples. A typical DNA methylation profile obtained by bisulfite sequencing of single clones for an unmethylated control sample (Buffy coat) and a methylated CLL sample is shown in Figure 2a.

DNA methylation is accumulated in the region of CpG 16 to CpG 29. We therefore, designed two bisulfite pyrosequencing primers which allowed us to investigate CpG 19 to 23 and CpG 25 to 29, respectively (Figure 2b). The quantitative bisulfite pyrosequencing results were in good agreement with the DNA methylation profiles of the samples analyzed by bisulfite sequencing of single clones (Figure 2b).

We interrogated *PTCH* promoter methylation in 37 CLL samples by bisulfite pyrosequencing. Their DNA methylation status was calculated from the DNA methylation values obtained from the appropriate pyrograms as described in the Materials and Methods Section. A total of 17 CLL samples (46%) contained a hypermethylated *PTCH* promoter.

Correlation of PTCH methylation status with clinical parameters. Next, we compared methylated and unmethylated samples. No significant differences were found concerning stage of CLL, mutational status], CD38 (cluster of differentiation 38) or ZAP70 (zeta-chain-associated protein kinase 70) expression or treatment of patients (Table I). A total of 18/37 (49%) cases in the cohort had a low stage (0, 1, 2) and 10/17 (59%) of these cases exhibited methylation of the *PTCH* promoter. A slightly lower proportion of patients classified as having low-stage CLL showed no methylation at the investigated *PTCH* fragment 8/20 (40%). The correlation between the two parameters (methylation of *PTCH* and low-stage grading) was not significant (p=0.45).

Table I. *Patients' characteristics*.

	PTCH, n (%)	
	Methylated (n=17)	Unmethylated (n=20)
Age (range; years)	52-85	29-72
Male	12 (71%)	12 (60%)
Female	5 (29%)	8 (40%)
Low stage (Binet A, Rai stage 0, 1, 2)	10 (59%)	8 (40%)
High stage (Binet B & C, Rai stage 3 or 4)	3 (18%)	3 (15%)
Rai stage unknown	4 (23%)	9 (45%)
Mutational status		
Negative	12 (71%)	15 (75%)
Positive	5 (29%)	5 (25%)
CD38+ (>20%)	3 (18%)	5 (25%)
ZAP70+ (>20%)	5 (29%)	9 (45%)
With treatment	10 (59%)	14 (70%)

PTCH: Patched; CD38; ZAP70; n=number of patients.

Discussion

Epigenetic mechanisms play an important role in the pathogenesis of cancer (8). DNA methylation is mediated by DNA-cytosine methyltransferases and is found in CpG islands at the promoter region of various genes. Interestingly, histone deacetylase inhibitors induce apoptosis of CLL cells (9). In patients with CLL, DNA methylation plays an important role (2). This is of major clinical relevance since DNA methylation can be inhibited by drugs such as 5-aza-2'-deoxycytidine, and histone deacetylation can be inhibited by histone deacetylase inhibitors. Recently, various drugs have been developed for clinical use in patients with cancer.

The Hh pathway plays an important role in the initiation and propagation of neoplastic diseases. In the absence of a ligand, PTCH acts catalytically to inhibit its downstream target, Smoothened [SMO; (10)]. The inhibited SMO interacts with a multiprotein complex that includes a member of the glioblastoma (GLI) family of transcription factors. Activation of the Hh pathway is associated with numerous malignant diseases. Recent data suggest epigenetic silencing of *PTCH* in malignancies, *e.g*. *PTCH* silencing through DNA promoter methylation has been found in acute myeloid leukemia and breast cancer (11, 12). A role for *PTCH* in CLL has not yet been established, however, the Hh signaling pathway has recently been suggested as an interesting therapeutic target for the treatment of patients with CLL (13). Interestingly, the expression of *PTCH* and *GLI1*, two target genes of the Hh signaling pathway, varied significantly among CLL samples and those tumors with high level expression of *PTCH* and *GLI1,* as well as trisomy 12 are characterized by a good response to the treatment with SMO (smoothened) inhibitors (13). Thus, hypermethylation of the *PTCH* promoter observed here for the first time in CLL may hamper the transcriptional activation of *PTCH* and consequently lead to overactivity of the canonical Hh signaling pathway. If a treatment strategy involving modulators of the Hh signaling pathway is developer, the methylation status of *PTCH* will be of significance in evaluating treatment response of patients with CLL.

PTCH, ortholog of a gene first described in Drosophila as a segment polarity gene, is located at 9q22.3. Interestingly, one region on chromosome 9 in the zebrafish genome is of potential interest. Within chromosome 9, five genes and two microRNAs were identified with shared synteny to the minimal-deleted regions in B-CLL (two genes to human chromosome 11, three to human chromosome 13 and two chromosome 13 microRNAs). The critical region on

zebrafish chromosome 9 maps to the minimal-deleted regions for both human chromosomes, suggesting a common ancestry for B-CLL tumor-suppressor genes (14). Interestingly, Richter's syndrome, an aggressive form of CLL, is associated with an increased number of chromosomal losses and deletions in chromosome 9 (15).

Nevoid basal cell carcinoma syndrome, also known as Gorlin syndrome, is an autosomal dominant disorder predisposing to basal cell carcinoma, medulloblastomas and ovarian fibromas (16). In patients with Gorlin syndrome, loss of heterozygosity for *PTCH* was found in several cases and variability in *SMO* in one case (17). *PTCH* appears to function as a gatekeeper gene in the epidermal cell type from which basal cell carcinomas arise (18). Patients with Gorlin syndrome are hypersensitive to radiation therapy.

Recently, an international prognostic index for patients with CLL (CLL-IPI) was generated. The CLL-IPI combines genetic, biochemical, and clinical parameters into a prognostic model, discriminating four prognostic subgroups. The CLL-IPI will allow for more targeted management of patients with CLL in clinical practice and in trials testing novel drugs (19).

In conclusion, we identified frequent DNA methylation of the *PTCH* promoter in samples from patients with CLL, suggesting *PTCH* to be a methylated tumor-suppressor gene with relevance in CLL.

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