

CpG Island Methylation and Expression of the Secreted Frizzled-Related Protein Gene Family in Chronic Lymphocytic Leukemia

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Abstract

B-cell chronic lymphocytic leukemia (CLL) is characterized by a clonal accumulation of mature neoplastic B cells indicating disruption of apoptosis. Restriction Landmark Genome Scanning was done to identify novel target genes silenced by CpG island methylation in CLL. Secreted frizzled-related protein 4 (SFRP4), a negative regulator of the Wnt signaling pathway, was found to be frequently methylated in CLL samples. Wnt signaling has been shown to control normal apoptotic behavior and is required for normal B-cell development whereas aberrant activation of this pathway has been observed in CLL. We show aberrant DNA methylation and silencing of SFRP4, as well as of additional SFRP family members, in primary CLL samples. Induction of their expression in a dose-dependent manner following treatment with a demethylating agent, 5-aza-2'-deoxycytidine, was shown. Of the five SFRP family members studied in detail, SFRP1 was hypermethylated and down-regulated in all CLL patient samples studied, suggesting that this epigenetic event is a critical step during leukemogenesis. Our results suggest that silencing of SFRPs by CpG island methylation is one possible mechanism contributing to aberrant activation of Wnt signaling pathway in CLL. (Cancer Res 2006; 66(2): 653-8)

Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common adult leukemias in the Western world and represents a clonal accumulation of mature neoplastic B cells characterized by low proliferation rates and disruption of apoptotic pathways. Wnt pathway has been shown to regulate early B-cell development (1) and aberrant activation has been described for CLL, suggesting a potential role of Wnt signaling in CLL (2, 3). Wnt signaling is activated on binding of the Wnt ligand to the frizzled (Fz) membrane receptor (4). A family of five secreted frizzled-related glycoproteins (SFRP1-5) were identified as modulators of Wnt signaling (5). The SFRP family members contain an NH₂-terminal domain homologous to the cysteine-rich domain (CRD) of Fz receptor but lack a transmembrane region or cytoplasmic domain required for transduction of signals into the cells (6, 7). Thus, SFRPs

compete with the Fz receptors for Wnt binding through the CRD domain or by binding directly to Fz, resulting in the formation of inactive complexes with the receptor (8, 9). Using a genome-wide methylation scanning method called Restriction Landmark Genome Scanning (RLGS), our lab reported that aberrant CpG island methylation is prevalent in CLL and identified *SFRP4*, a member of SFRP family, as a methylation target in CLL (10). Epigenetic modifications, such as DNA methylation, have emerged as additional and equally important mechanisms besides genetic alterations to silence genes. In this study, we show extensive methylation of SFRP gene family in CLL. In addition, significant methylation and silencing of *SFRP1* in all the CLL samples studied suggest its potential as a major player in leukemogenesis.

Methods

Patient selection and sample collection. Blood was obtained from patients with B-cell CLL through the CLL Research Consortium (CRC) Tissue Bank. The six paired diagnostic/relapse CLL blood samples were obtained from patients enrolled on a phase II study of fludarabine and rituximab (11) in previously untreated CLLs that were obtained through Cancer and Leukemia Group B leukemia tissue bank protocol 9665. Leukocyte reduction filters were obtained from the Red Cross and B cells were isolated according to Weitkamp and Crowe (12). Culture conditions and treatment of the CLL cell line Wac3CD5 have previously been described (10).

RLGS and RLGS profile analysis. DNA isolation, RLGS, and RLGS gel analysis were done as previously described (10). A detailed description can also be found in the Supplementary data.

Southern hybridization. Southern blots were done as described by Rush et al. (10). Probes were obtained by PCR amplification. The *SFRP4* forward primer was 5'-GGAAGTGGGAGCAGAGATGG-3' and the reverse primer was 5'-TAGGAGCTAGCGGAGAGCAC-3'.

Bisulfite genomic sequencing and combined bisulfite restriction analysis. One microgram of genomic DNA from CLL samples, mice samples, and normal controls was treated with sodium bisulfite according to published protocols with modifications (13). Primer sequences and reaction condition are listed in Supplementary Table S1.

Statistical analysis. The methylation levels in CLL patients and normal B cells were compared by nonparametric Mann-Whitney test.

Reverse transcription-PCR. RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) and further purified by Rneasy Mini Kit (Qiagen, Valencia, CA). Superscript First-Strand Synthesis kit (Invitrogen) was used for reverse transcription. Triplicates of semiquantitative SYBR Green PCR were done with IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in a Bio-Rad iCycler. Primer sequences and reaction condition are available in the Supplementary data. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for RNA integrity. The expression data were analyzed by comparative Ct method. Ct represents the cycle number at which the fluorescent signal first exceeds the threshold. The Δ Ct value was obtained by subtracting the Ct value of *GAPDH* from the Ct value of the target gene. The expression of *SFRP1*, *SFRP2*, and *SFRP4* in each sample is shown relative to the average expression of the three normal CD19+ B cells.

Note: C. Plass is a Leukemia and Lymphoma Society Scholar and J.C. Byrd is a Leukemia and Lymphoma Society Clinical Scholar.

Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Results

RLGS of CLL patients identifies CpG island methylation of *SFRP4*. In a previous study, RLGS analysis on 10 CLL patient samples established the prevalence of aberrant CpG island methylation in CLL WaC3CD5 (10). Of 194 novel sequences aberrantly methylated in CLL, RLGS fragment A2D39 was found methylated in 2 of 10 CLL patients and the CLL cell line WaC3CD5 (Fig. 1A). The sequence of this clone overlapped with the 5' CpG island of *secreted frizzled-related protein 4* (*SFRP4*), a secreted protein and a putative inhibitor of the Wnt signaling pathway (Fig. 1B). Examination of the second set of RLGS profiles, comprising six pairs of diagnostic and relapsed immunoglobulin V_H (IgV_H) unmutated CLL samples, showed decreased intensity of

RLGS fragment A2D39 (*SFRP4*) in two pairs (patients 12 and 15) as compared with normal CD19+ B cells, indicating DNA methylation of the *SFRP4* CpG island in CLL (Fig. 1A). A differential increase in the fragment intensity within one pair (patient 16) was also observed, suggesting progressive increase in aberrant DNA methylation from a partial methylation state at diagnosis to complete methylation in relapse samples (Fig. 1A).

Confirmation of DNA methylation of *SFRP4*. To confirm DNA methylation at the restriction landmark site, *AscI*, Southern blotting was done with DNAs from normal peripheral blood, and CD19+ selected normal B cells, 10 CLL samples, and 6 paired CLL samples were digested with *EcoRV* alone or in combination with *AscI*. Figure 1B shows the structure of *SFRP4* and the location of the probe used

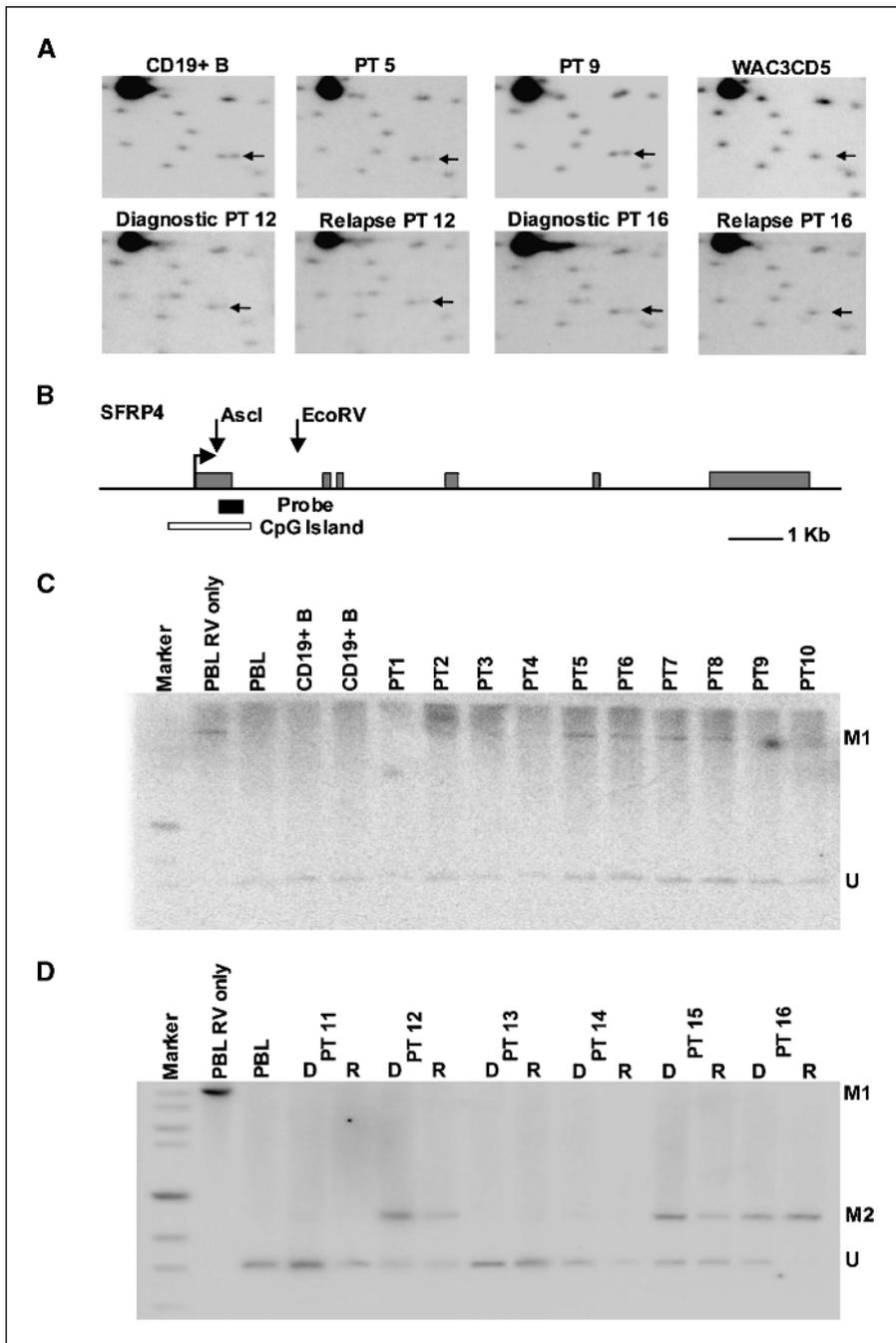
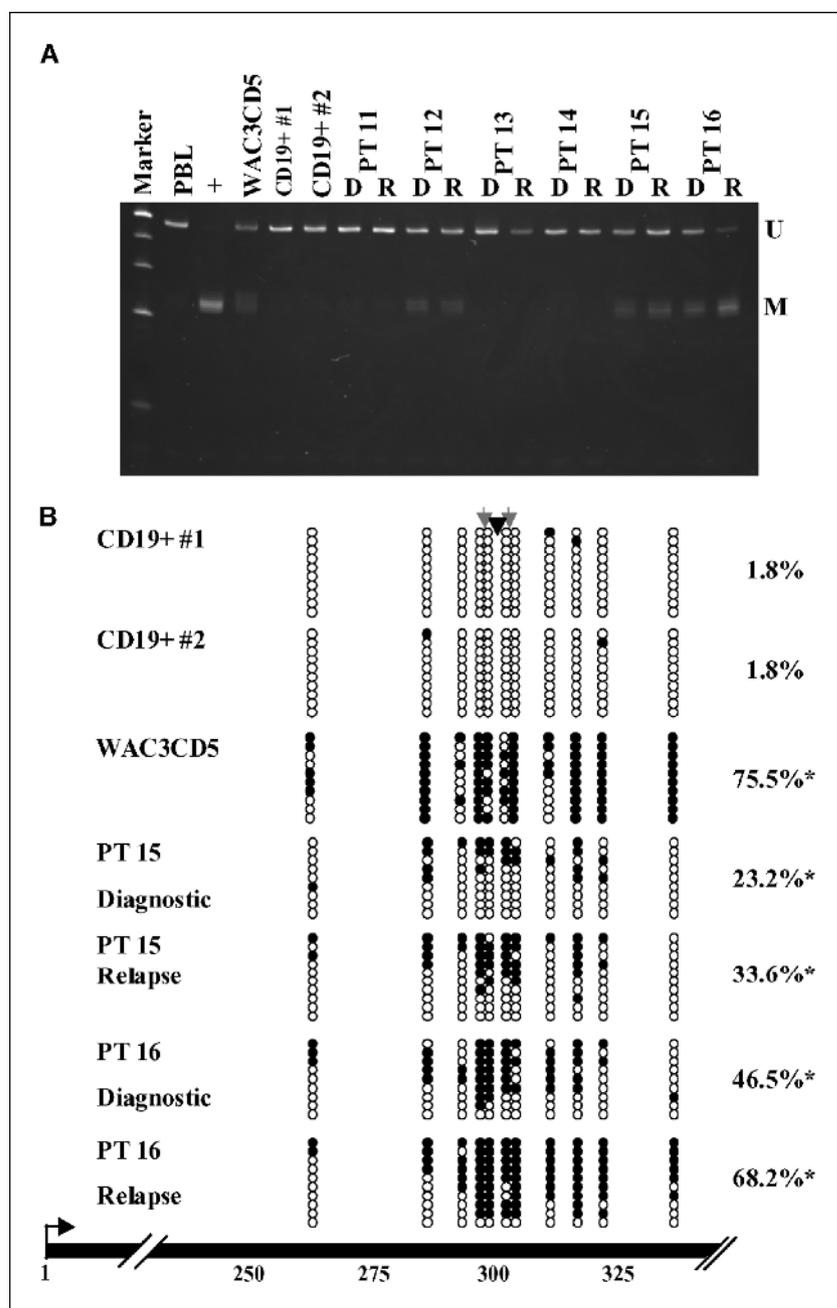


Figure 1. RLGS analysis. A, sections of *AscI-EcoRV-HinfI* RLGS profiles are shown from normal CD19+ B cells, CLL cells of patient (PT) 5, patient 9, CLL cell line WaC3CD5, and two paired CLL patient samples, patients 15 and 16, taken at diagnosis of the disease and following relapse. Arrows, fragment A2D39. B, *SFRP4* gene structure and CpG island (shaded box). Location of the *AscI-EcoRV* fragment detected by RLGS and the probe used for Southern blotting is indicated. C, Southern blot of 10 CLL patients (patients 1-10), CD19+ B cells, and peripheral blood lymphocytes (PBL). Samples were digested with *AscI* and *EcoRV* except for the first lane in which PBL digested with *EcoRV* only was loaded. The 10.8-kb band (M1) represents the undigested *EcoRV* fragment. The 1.5-kb fragment (U) shows the size of the fragment produced when the unmethylated DNA is digested by *AscI*. D, Southern blot of *SFRP4* in six CLL patients (patients 11-16) from which both diagnostic and relapse CLL cells were collected. Samples were digested with *AscI* and *EcoRV* except for the first lane in which PBL digested with *EcoRV* only was loaded. The 10.8-kb (M1) and 2.5-kb (M2) bands represent the undigested *EcoRV* fragment. The 1.5-kb fragment (U) shows the size of the fragment produced when the unmethylated DNA is digested by *AscI*.

Figure 2. COBRA and bisulfite sequencing of *SFRP4* in paired CLL patient samples. *A*, COBRA analysis of DNA from PBL, *Sss*I-treated PBL (+), WAc3CD5, CD19+ B cells, and six CLL patients collected both diagnostic (*D*) and after relapse (*R*) of the disease. Undigested and digested fragments correspond to unmethylated (*U*) and methylated (*M*) DNA, respectively. *B*, bisulfite sequencing of *SFRP4* from two CD19+ B cells and two paired CLL patients (patients 15 and 16). Each row of circles represents the sequence of an individual clone. *Open and filled circles*, unmethylated and methylated CpG site, respectively. *Arrows*, *Bst*UI restriction sites used in the COBRA assay. *Arrowhead*, *Asc*I site. Location of the CpG sites relative to the transcription start sites is shown below. Average percentage of DNA methylation is shown on the right hand side. *, $P < 0.05$, statistically significant differences in DNA methylation relative to CD19+ samples.



for the Southern blot analysis. DNA from normal peripheral blood cells and two CD19+ B cells showed only a 1.5-kb fragment, indicating that the *Asc*I site was not methylated (Fig. 1C). The presence of both the 10.8- and 1.5-kb fragments in four CLL patients (patients 5-8) indicated partial methylation of *SFRP4* (Fig. 1C). Figure 1D shows the Southern blot with six pairs of diagnosis and relapse CLL samples. DNA from patients 12 and 15 showed partial methylation of *SFRP4* in diagnostic and relapse samples as indicated by the presence of both 2.5- and 1.5-kb fragments (Fig. 1C). The appearance of a 2.5-kb band instead of the expected 10.8-kb band is most likely due to a polymorphic *Eco*RV sequence. The Southern blot is consistent with the RLGS results wherein both patients 12 and 15 showed decreased intensity of A2D39. Partial methylation of diagnostic DNA from patient 16 and complete methylation of relapse DNA are consistent with complete loss of

A2D39 in RLGS. Southern blotting confirmed that the *Asc*I site within *SFRP4* was methylated in 7 of 16 (43.75%) CLL samples.

Combined bisulfite restriction analysis and bisulfite genomic sequencing of *SFRP4*. To further characterize aberrant CpG island methylation of *SFRP4* in the paired CLL samples, we used combined bisulfite restriction analysis (COBRA) and bisulfite genomic sequencing on DNA from normal blood, B cells, CLL cell line WAc3CD5, and paired diagnostic and relapsed samples from CLL patients. Initially, we evaluated the region from +242 to +357 containing the *Asc*I site. Normal blood lymphocytes and CD19+ selected B cells had no or very low levels of DNA methylation whereas WAc3CD5 and three of six CLL patient samples (patients 12, 15, and 16) showed different degrees of DNA methylation in both the diagnostic and relapse DNA. Moreover, the relapse DNA of patient 16 showed a much higher degree of DNA methylation as

compared with its paired diagnostic DNA sample. The COBRA result was consistent with RLGs and Southern blotting data and indicated that the 5' region of *SFRP4* was extensively methylated in three CLL patients (Fig. 2A). Subsequent bisulfite sequencing on two different sources of CD19+ B cells showed virtually no methylation at any of the CpG sites (Fig. 2B). In the two paired samples, patients 15 and 16, both diagnostic and relapse showed significant methylation compared with CD19+ B cells ($P < 0.05$). Thus, bisulfite sequencing results further validated *SFRP4* CpG island methylation

Methylation and expression of *SFRP1*, *SFRP2*, *SFRP3*, *SFRP4*, and *SFRP5* in CLL cell line WaC3CD5. To investigate the importance of all SFRP family members in B cells, *SFRP1*, *SFRP2*, *SFRP3*, *SFRP4*, and *SFRP5*, we evaluated DNA methylation and expression in CD19+ B cells and in CLL cell line WaC3CD5. Bisulfite sequencing on WaC3CD5 showed that the CpG dinucleotides in the 5' region of *SFRP1*, *SFRP2*, and *SFRP4* are densely methylated whereas *SFRP5* showed a much lower degree of aberrant DNA methylation (Fig. 3A). *SFRP3* does not possess a CpG island and was not included in the bisulfite sequencing study. To determine whether expression of the SFRP family members in WaC3CD5 is regulated by DNA methylation, we examined the expression in untreated and 5-aza-2'-deoxycytidine-treated WaC3CD5. 5-aza-2'-deoxycytidine has been shown previously to deplete the cellular content of noncovalently bound DNMT1 in a dose-dependent manner (10). Semiquantitative reverse transcription-PCR (RT-PCR) showed that expression of *SFRP1*, *SFRP2*, and *SFRP4* was induced even at low dosages (0.05, 0.1, and 0.5 $\mu\text{mol/L}$) of 5-aza-2'-deoxycytidine treatment with induction of expression levels up to 20- to 25-fold (Fig. 3B). Both *SFRP3* and *SFRP5* expression increased up to 2.6- and 1.8-fold, respectively (Fig. 3B). These data suggest that DNA methylation is involved, either directly or indirectly, in gene regulation for *SFRP1*, *SFRP2*, and *SFRP4*. However, DNA methylation does not play a significant role in the regulation of *SFRP3* and *SFRP5* expression in CLL.

DNA methylation and expression of *SFRP1*, *SFRP2*, and *SFRP4* in CLL patient samples. We next investigated the methylation frequency of *SFRP1*, *SFRP2*, and *SFRP4* by COBRA

and bisulfite sequencing in 20 CLL samples which were divided in two subgroups: 10 each of IgV_H unmutated and mutated samples. Frequencies of methylation as evaluated by COBRA were 100% (*SFRP1*), 55% (*SFRP2*), 30% (*SFRP4*), and 15% (*SFRP5*). Semiquantitative RT-PCR was done to measure the expression levels on the same set of CLL samples used to study DNA methylation. All 20 samples showed aberrant promoter methylation of *SFRP1* whereas no methylation was observed in CD19+ selected B cells from healthy individuals. Bisulfite sequencing of two CD19+ selected B cells from healthy donors and three CLL patients (CRC7, CRC10, and CRC50) further confirmed that *SFRP1* CpG island was methylated in CLL samples (Fig. 4A). The methylation levels in patient samples were statistically different from those in CD19+ B cells ($P < 0.05$). *SFRP1* was silenced in 17 of 20 samples and was almost undetectable in the remaining three CLL samples compared with the expression from three individual sources of CD19+ selected normal B cells (Fig. 4B).

In case of *SFRP2*, four samples (CRC1, CRC5, CRC7, and CRC55) with normal *SFRP2* expression showed no methylation by COBRA, and bisulfite sequencing confirmed the absence of DNA methylation in CRC7 (Fig. 4C). Sixteen of 20 (80%) CLL samples showed down-regulation or silencing compared with normal CD19-selected B cells (Fig. 4D). Five CLL samples (CRC10, CRC14, CRC33, CRC23, and CRC53) had no or very low amounts of *SFRP2* mRNA whereas results from our COBRA assay suggested that the *SFRP2* CpG islands in these samples were either unmethylated or methylated at very low degree. Bisulfite sequencing of CRC10 revealed that there was significant methylation of the *SFRP2* promoter ($P < 0.05$); however, this methylation was not within the sequences recognized by COBRA. Eleven CLL patient DNA samples, including CRC50, that showed methylation of the *SFRP2* CpG island by COBRA assay had no or very low level of *SFRP2* expression. Bisulfite sequencing also confirmed that CRC50, which had no detectable *SFRP2* expression, was hypermethylated ($P < 0.05$) as shown by the COBRA assay.

To study CpG island methylation in *SFRP4*, COBRA assay was designed that covered the region between +42 and +261

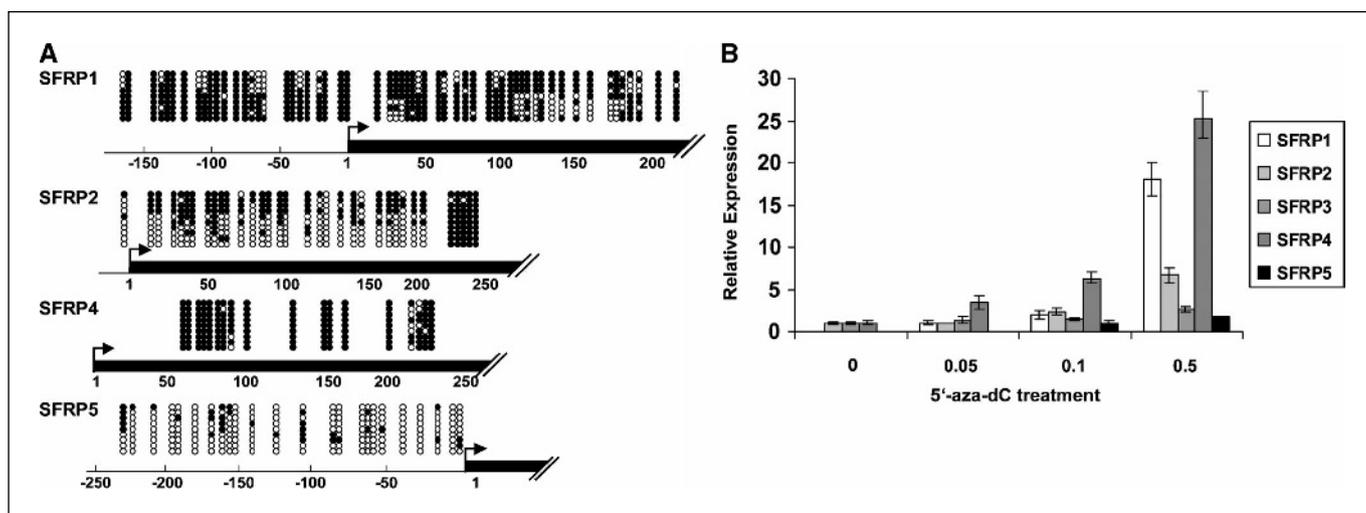


Figure 3. Analysis of methylation and expression of the SFRP gene family in CLL cell line WaC3CD5. A, bisulfite sequencing of four SFRP gene family members, *SFRP1*, *SFRP2*, *SFRP4*, and *SFRP5*, from WaC3CD5. Each row of circles represents the sequence of an individual clone. Open and filled circles, unmethylated and methylated CpG sites, respectively. The location of CpG sites relative to the transcription start sites is shown below. Solid bars, first exons. B, expression levels of *SFRP1*, *SFRP2*, *SFRP3*, *SFRP4*, and *SFRP5* quantified using semiquantitative RT-PCR in untreated WaC3CD5 cells and following treatment with the demethylation agent 5-aza-2'-deoxycytidine at various concentrations (0.05, 0.1, and 0.5 $\mu\text{mol/L}$). GAPDH expression was used as an internal control. The relative expression levels were determined by normalizing the Ct values against the average Ct values of untreated (*SFRP2* and *SFRP4*) WaC3CD5 cells or first detectable expression (*SFRP1* at 0.05 $\mu\text{mol/L}$, *SFRP5* at 0.1 $\mu\text{mol/L}$) for the specific genes.

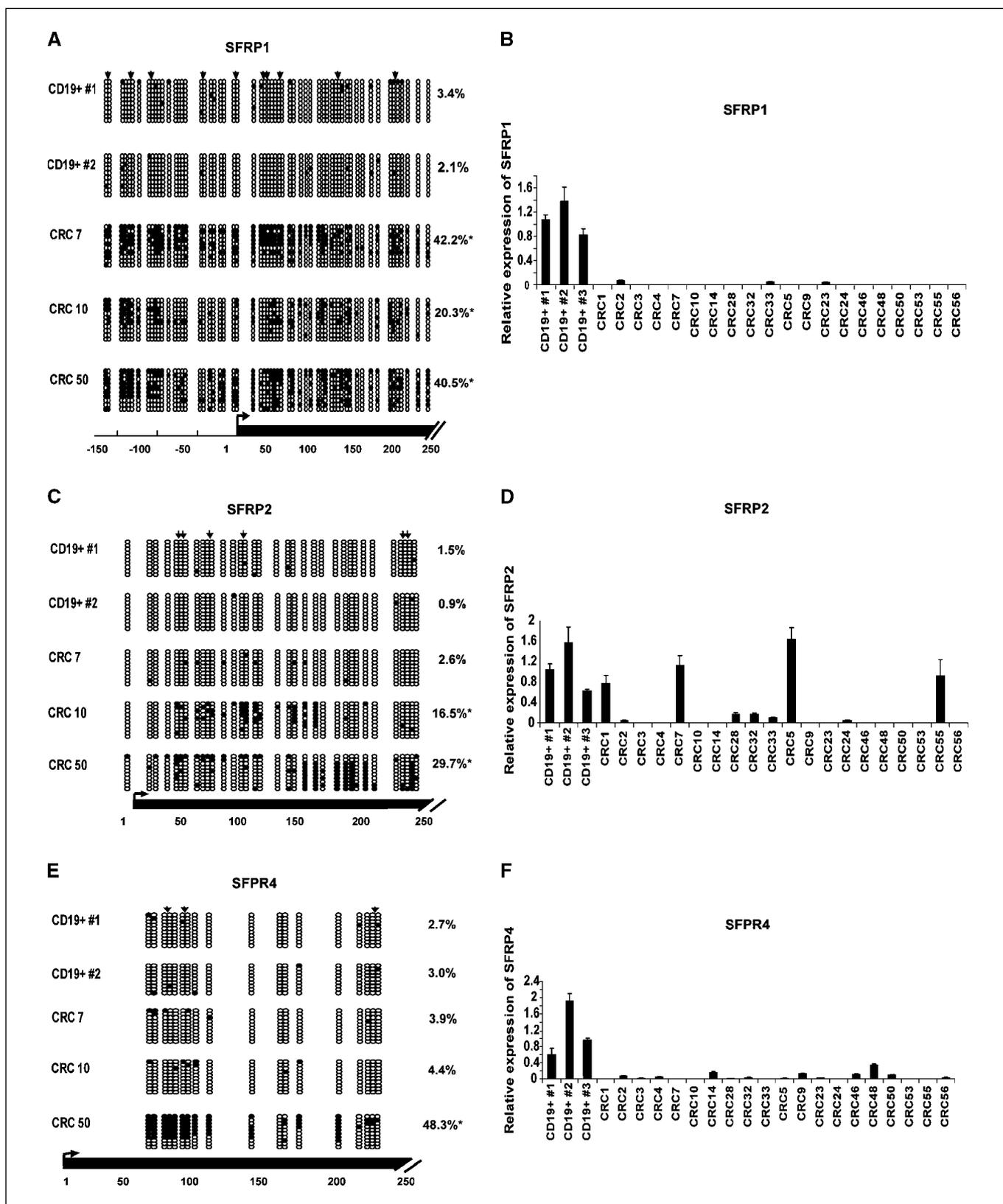


Figure 4. Methylation and expression analysis of *SFRP1*, *SFRP2*, and *SFRP4* in CLL patients. **A**, **C**, and **E**, bisulfite sequencing of *SFRP1*, *SFRP2*, and *SFRP4* on two normal CD19+ B cells and three selected CLL patients (CRC7, CRC10, and CRC50). Each row of circles represents the sequence of an individual clone. *Open and filled circles*, unmethylated and methylated CpG sites, respectively. The location of CpG sites relative to the transcription start sites is shown below. *Solid bars*, first exons. *Arrows*, *Bst*UI restriction sites used in the COBRA assay. Average percentage of DNA methylation is shown on the right hand side. *, $P < 0.05$, statistically significant differences in DNA methylation relative to CD19+ samples. **B**, **D**, and **F**, relative expression of *SFRP1*, *SFRP2*, and *SFRP4* in CD19+ B cells and CLL patients quantified by semiquantitative RT-PCR. GAPDH expression was used as the internal control.

comprising 18 CpG sites. The COBRA assay identified methylation of *SFRP4* in 6 of 20 (30%) patient samples. Bisulfite sequencing on three patients confirmed the COBRA results that CRC7 and CRC10, similar to two CD19+ B cells, showed virtually no methylation whereas CRC50 showed significant methylation ($P < 0.05$) at the 5' region of *SFRP4* (Fig. 4E). However, semiquantitative RT-PCR showed that expression of *SFRP4* was down-regulated in all 20 CLL patients when compared with the expression level of normal CD19+ B cells (Fig. 4F). For example, there was no detectable expression of *SFRP4* in CRC7 and CRC10 whereas the 5' region of *SFRP4* was unmethylated. We further examined the upstream (−120 to +132) and downstream (+242 to +375) regions of *SFRP4* 5' (data not shown) in patient samples and no prevalent methylation of CpG sites was found. Thus, 14 of 20 CLL patients showed no correlation between CpG island methylation and *SFRP4* expression. This indicates that CpG island methylation does not participate in the down-regulation of *SFRP4* expression in CLL cells. DNA methylation and expression data for the patient samples are summarized in Supplementary Table S2.

Discussion

We describe for the first time the aberrant DNA methylation and expression of five members of the SFRP gene family, *SFRP1*, *SFRP2*, *SFRP3*, *SFRP4*, and *SFRP5*, in CLL. This work was initiated, using a genome-wide scan (RLGS) in 10 diagnosis and 6 pairs of diagnosis and relapse CLL samples, to identify aberrantly methylated genes, and *SFRP4* emerged as one of the loci exhibiting altered DNA methylation. Of five SFRP family members, four have dense CpG islands covering the promoter and the first exon; hence, we extensively studied CpG island methylation and expression of four family members (i.e., *SFRP1*, *SFRP2*, *SFRP4*, and *SFRP5*) in CLL. *SFRP3* is the only member without a 5' CpG island. Interestingly, we found marked differences in the levels of aberrant DNA methylation between these genes, 100% (*SFRP1*), 55% (*SFRP2*), 30% (*SFRP4*), and 15% (*SFRP5*), suggesting that epigenetic silencing of these SFRPs and especially *SFRP1* could be important in the onset of CLL. Our data also show that additional SFRP family members like *SFRP2* and *SFRP4* are also frequently silenced in CLL although CpG island methylation does not seem to be the main cause of this

silencing. Although this study did not examine alternative mechanisms of silencing of *SFRP2* and *SFRP4*, it is possible that these are targeted by genetic alterations as it has been described for *SFRP1* in colon cancers (14).

The potential significance of SFRP silencing in CLL relates to its involvement in the Wnt signaling pathway. Recent studies show that Wnt signaling pathway plays a role in hematopoietic development (1, 15). Aberrant regulation of Wnt signal pathway has been reported in several hematoneoplasia, including acute lymphoblastic leukemia and acute myeloid leukemia (16, 17). The function of the SFRP family members has not been studied in lymphoid cells; however, CpG island methylation, together with down-regulation of *SFRP1*, *SFRP2*, *SFRP4*, and *SFRP5*, has been reported in several cancers, such as colorectal cancer, ovarian cancer, and mesothelioma (18–20).

According to the mutation status of IgV_H, CLL can be divided into two subtypes: unmutated IgV_H, which is a predictor for poor prognosis, and mutated IgV_H, which is associated with a better outcome. This suggests that SFRP expression is not differentially regulated between the two CLL subtypes. The function of SFRPs as antagonist of the Wnt pathway provides a potential mechanism to suppress the abnormal activation of this pathway, which, in turn, results in the restoration of the apoptotic machinery. Because epigenetic-based therapy with demethylation agents is currently under development, understanding the role of SFRPs and its effects in influencing the activity of the Wnt pathway in CLL is critical and could lead to candidates for epigenetically targeted therapy.

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