Alteration of *SMRT* Tumor Suppressor Function in Transformed Non-Hodgkin Lymphomas

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Abstract

Indolent non-Hodgkin lymphomas are characterized by a prolonged phase that is typically followed by a clinical progression associated with an accelerated clinical course and short survival time. Previous studies have not identified a consistent cytogenetic or molecular abnormality associated with transformation. The development of a transformed phenotype, evolving from the original low-grade component, most likely depends on multiple genetic events, including the activation of synergistic dominant oncogenes and a loss of tumor suppressor gene functions. Complex karyotypes and relatively bad chromosome morphology are typical of transformed non-Hodgkin lymphomas, rendering complete cytogenetic analysis difficult. Here, we report the use of transformed non-Hodgkin lymphoma cell lines and primary samples to identify the involvement of the silencing mediator of retinoic acid and thyroid hormone receptor (*SMRT*) gene that maps at chromosome 12q24 in transformed non-Hodgkin lymphomas. We also show that down-regulation of *SMRT* in the immortalized "Weinberg's model" cell lines induces transformation of the cells. Assessment of cDNA array profiles should further help us to design a working model for *SMRT* involvement in non-Hodgkin lymphoma transformation as a novel, nonclassical tumor suppressor. (Cancer Res 2005; 65(11): 4554-61)

Introduction

Non-Hodgkin lymphomas constitute a heterogeneous group of malignant neoplasms that are diverse in their cellular origin, morphology, cytogenetic abnormalities, responses to treatment, and prognoses (1). The Revised European American Lymphoma classification (2) defines different subtypes of lymphoma, offering a list of at least 25 discrete types as well as some provisional ones that seem to correspond to "real" clinical entities (3). Histologic transformation to a higher-grade lymphoma can occur with variable rates (up to 70% in follicular lymphoma) during the course of the disease and carries a poor prognosis (4, 5). Transformed lymphoma characteristically involves the bone marrow, and leukemic manifestations are therefore not uncommon. Both mantle cell and lymphoplasmacytoid lymphoma (6) may present with or develop a transformed leukemic phase, although not as commonly as does follicular lymphoma (7). Many attempts to identify the possible genetic alterations associated with the histologic and clinical transformation of non-Hodgkin lymphomas have been made (5).

A common feature of all transformed lymphomas is the complexity of the karyotypes associated with a relatively poor chromosome morphology that may prevent complete analysis. This makes it especially difficult to identify candidate genes that may be involved in the transformation process. To overcome this difficulty, numerous cell lines have been established and characterized from patients with transformed lymphoma (8–16). High-resolution cytogenetic analysis of some of these cell lines has indicated that chromosome 12q24 is a recurrent breakpoint in transformed non-Hodgkin lymphoma of both B-cell and T-cell lineages (17, 18). The 12q24 region has been documented to be involved in three-way translocations or to appear in sequential follow-up karyotypes of non-Hodgkin lymphoma patients. These observations suggest that these 12q24 abnormalities might be secondary events associated with transformation to aggressive disease.

Recently, this laboratory and others (19, 20) have mapped a gene involved in transcriptional repression to 12q24.3, the silencing mediator of retinoic acid and thyroid hormone receptor (*SMRT*) gene. Transcriptional repression is mediated by a corepressor complex that contains sin3A/B protein and histone deacetylases (21–24). Studies by several groups (25–29) have shown that this complex is recruited to nuclear receptors through *SMRT* and a related corepressor, nuclear receptor corepressor. Chen and Evans (30) in 1995 originally described SMRT as a protein of which association with nuclear receptors is destabilized by ligand. The ability of the "silencing complex" to deacetylate histones results in a condensed chromatin state that is inhibitory to transcription (31, 32).

In this report, we show that *SMRT*, which maps at chromosome 12q24, is recurrently altered by chromosomal rearrangements in transformed non-Hodgkin lymphoma as shown by fluorescence *in situ* hybridization (FISH), and also at the transcript and protein levels. These alterations, observed first in cell lines, have also been detected in patient samples, when obtained pre- and posttransformation. In addition, retroviral transduction of a functionally expressed *SMRT* cDNA caused the death of virtually all non-Hodgkin

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lymphoma cells without affecting endogenous SMRT–producing cells. We also show here that down-regulation of *SMRT* in the immortalized "Weinberg's model" cell lines (33) induces cell transformation. Changes in expression profiles in *SMRT*-deficient non-Hodgkin lymphoma cells on SMRT restoration allowed us to identify several unsuspected new *SMRT* target genes. These data suggest that *SMRT* is a novel, nonclassical tumor suppressor gene and that functional reduction of SMRT is involved in non-Hodgkin lymphoma transformation.

Materials and Methods

Cell lines and patient samples. The cell lines used in this study are listed in Table 1. The lymphoblastoid cell line EBV-LIN was a kind gift from Dr. Thomas Ellis (Blood Center South Eastern Wisconsin, Milwaukee, WI). A small series of transformed lymphoma samples (four follicular lymphomas, three mantle cell lymphomas, and three Burkitt lymphomas) was selected for analysis from the tumor database of the Royal Marsden Hospital, London, United Kingdom. In addition, four matched samples (transformed and untransformed samples from the same patients) were obtained (T.G., University of Arizona, Tucson, AZ/Southwest Oncology Group database). No information was available concerning potential 12q24 abnormalities.

Fluorescence *in situ* hybridization. Probes used in these experiments were described elsewhere (20). FISH experiments were done as previously described (20) with some modifications. Briefly, genomic DNA probes were labeled by nick translation using Spectrum Green-dUTP or Spectrum Red-dUTP (Amersham Biosciences, Piscataway, NJ). Competitive hybridization by preannealing with a 50- to 100-fold excess of Cot-1 DNA to previously denatured DNA was done before overnight hybridization of slides. Probes were visualized after two washing steps at 72°C. Cells and chromosomes were counterstained with 4′,6-diamidino-2-phenylindole and embedded in Vectashield antifade solution (Vector Laboratories, Burlingame, CA). Digital images were captured using a charge-coupled device camera (Sensys) mounted on an Axioplan fluorescence microscope (Zeiss, Thornwood, NY) equipped with selective filters and controlled by a G3 Macintosh computer with Smart-Capture (Digital Scientific, Cambridge, United Kingdom) and MacProbe (PSI/Applied Imaging, League City, TX) software. Two-hundred interphase and 20 metaphase (where applicable) cells were scored per sample.

Western blot analysis. Proteins (50 μg) were resolved on 3% to 7% NuPAGE Tri-Acetate gels (Invitrogen, Carlsbad, CA) and transferred onto polyvinylidene difluoride membrane (Amersham) by using NuPAGE transferring system (Invitrogen). The membranes were probed with anti-*SMRT* (Affinity Bioreagents, Inc, Golden, CO), anti-A20, anti-Id2, anti-A20, anti-BCL2, anti-cFLIP, anti-JUN, and anti-IL-6 (Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibodies. Western blots were developed using BM Chemiluminescence Western Blotting Kit (Roche, Indianapolis, IN) as well as enhanced chemiluminescence reagents (Amersham).

Reverse transcription-PCR. Total RNAs were extracted from cultured cells with RNA isolation kit (Ambion, Austin, TX). The cDNAs were synthesized by using First Strand cDNA Synthesis Kit (MBI Fermentas, Hannover, MD). The gene specific primers were *SMRT*, forward 5′-ACA GTG GCT GAG TGC GTC CTC T-3′ and reverse 5′-ACG TGG AGC TGG ACC GAC ATT C-3′; *JUN*, forward 5′-GCA CAG CCA GAA CAC-3′ and reverse TTC GGC ACT TGG AGG-3′; *A20*, forward 5′-CAA CAC GAG CGA GAG-3′ and reverse 5′-CAC TTC GGC ACA GTC-3′; *Id2*, forward 5′-CCG TGA GGT CCG TTA-3′ and reverse 5′-TGG ACG CCT GGT TCT-3′; *c-Flip*, forward 5′-CGA GCA CCG AGA CTA-3′ and reverse 5′-GAG GCA CTG CAG GTA-3′; *CD44*, forward 5′-CCG CTA TGT CCA GAA-3′ and reverse 5′-CGT GGT GTG GTT GAA-3′; *BCL2*, forward 5′-ACA GCA ACG CAG ATG-3′ and reverse 5′-GTG ACG CAA CGG TTA-3′; *IL-6*, forward 5′-GGG AAC GAA AGA GAA GCT CT-3′ and reverse 5′-ACC AGA AGA AGG AAT GCC CA-3′; and the primer sequences of internal control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were forward 5′-TTA GCA CCC CTG GCC AAG G-3′ and reverse 5′-CTT ACT CCT TGG AGG CCA TG-3′.

Transcriptional activity assay. Cells (10^6) were transfected by electroporation with 2 μg/tube of an renilla luciferase plasmid (Promega, Madison, WI) and the wild-type/mutant CBF1-luciferase-reporter constructs (34). Luciferase expressions were measured after 48 hours by dual-luciferase reporter assay (Promega) according to the instructions of the manufacturer, with chemiluminescence measured, values normalized for both protein content of the samples (Bradford method), and transfection efficiency established by the renilla luciferase intensities measured with the renilla luciferase control plasmid used. Untransfected cell lines were assayed to determine luciferase background.

***SMRT*-retrovirus assay.** A *Bam*HI fragment of the *TRAC2/SMRT* cDNA was subcloned in sense or antisense orientation into a modified NCI (35) retrovirus and pRetro-On (Clontech, Palo Alto, CA) vectors containing a puromycin cassette for eukaryotic selection. The amphotrophic Phoenix producer line was then transfected with the construct and viral supernatant produced. Puromycin was added 2 days after transduction to select for transduced cells. Live cells, purified by Ficoll gradient, were the subject of either cell death experiments (trypan blue and annexin V approaches) after doxycycline induction (pRetro-On) or not (modified NCI), Western blot to assess either expression of the *TRAC2/SMRT* protein or the *SMRT* down-regulation by the antisense constructs, or cDNA array using the lymphochip array (36).

Transformation assays. The human embryonic kidney (HEK) and normal human BJ fibroblasts infected with retroviruses coding for the catalytic subunit of the telomerase holoenzyme (hTERT) or with hTERT and SV40 large T (33) were transfected with the above-described retrovirus vector, alone or expressing antisense *SMRT* in three different experiments. After assessing viability, 1×10^5 cells were seeded in 35-mm^2 plates in 0.7% agar. Colonies present on the plates were scored 2 weeks after soft agar seeding.

Results

The *SMRT* gene is deleted in recurrent 12q24 rearrangements in transformed non-Hodgkin lymphoma. To investigate whether non-Hodgkin lymphoma transformation might be the consequence of a common recurrent genetic event, we did cytogenetic and molecular genetic analysis on a series of high-grade transformed non-Hodgkin lymphoma cell lines randomly selected as examples of transformed non-Hodgkin lymphoma (Table 1). High-resolution cytogenetics on these cell lines revealed chromosome 12q24 to be a recurrent breakpoint in high-grade non-Hodgkin lymphoma of both B-cell and T-cell lineages (17, 18). The observed abnormalities were either complex unbalanced translocations or interstitial deletions (Table 1).

To investigate *SMRT* involvement in the 12q24 rearrangements characterized by high-resolution cytogenetics, we used selected genomic clones (20) in FISH experiments on our panel of transformed non-Hodgkin lymphoma cell lines. All of the lines showed loss of one *SMRT* FISH signal (Fig. 1; Table 1). Additionally, to assess the true involvement of *SMRT* in the genomic rearrangements, we used alternative FISH experiments on our cell lines with a BAC contig around *SMRT*, covering both 5′ and 3′ regions (20). Only the *SMRT*-specific BAC clones show loss of signal; none of the BACs containing the different neighboring genes are affected (data not shown). A small series of bone marrow specimen from patients with transformed non-Hodgkin lymphoma from the tumor database at the Royal Marsden Hospital were studied. No selection was done concerning potential 12q24 abnormalities. All patient samples tested showed loss of one *SMRT* FISH signal in the majority of cells contained in the specimen (Student's t test, $P < 0.0001$).

These data indicate that loss of one *SMRT* allele is a common occurrence *in vivo* and not a cell line artifact. In addition, four paired (untransformed and transformed sample from the same patient) clinical specimens were obtained from the Arizona

Table 1. Partial chromosome 12 karyotype, FISH, and immunohistochemistry results in transformed non-Hodgkin lymphoma cell lines and lymphoma patient specimens

Name	Primary non-Hodgkin lymphoma type	% Cells with loss of 1 *SMRT* FISH signal	Partial karyotype Involving #12	Immunohistochemistry
CTV1	AML	2.4	t(12;16)(q24;q11)	+
HeLa	CC	ND	add(12)(q23)	+
BL58	BL	100	add (12)(q24) = t(12;14;8)	−
PRI	B-NHL	100	add(12)(q24)	−
VAL	B-NHL	100	der(12)t(1;12)(q22;q24)	−
SSK41	BL	100	add(12)(q24)	−
G519	MCL	100	intdel(12)(q24.1;q24.3)	−
DoHH2	FL	100	intdel(12)(q24.1;q24.3)	−
W133	BL	ND	t(8;12;14)	ND
K1106	B-NHL	100	del(12q)	−
LY67	T-NHL	ND	intdel(12)(q24.1;q24.3)	ND
K247	B-NHL	ND	intdel(12q)(24.1;q24.3)	ND
G452	FL	100	intdel(12)(q24.1;q24.3)	−
K417	BL	ND	intdel(12)(q24.1;q24.3)	ND
K384	T-NHL	ND	intdel(12)(q24.1;q24.3)	ND
W135	BL	ND	intdel(12)(q24.1;q24.3)	ND
UCH	MCL	100	intdel(12)(q24.1;q24.3)	−
NITTA	B-NHL	100	intdel(12)(q24.1;q24.3)	−
OZ	FL	100	add(12)(q24)	−
NAMALWA	BL	100	intdel(12)(q24.1;q24.3)	−
OHK	B-NHL	100	intdel(12)(q24.1;q24.3)	−
SU-DHL-1	T-ANA	100	add(12)(q24)	−
SR-786	T-ANA	ND	der(12)t(12;13)(q24.3;q11)	ND
N Control 1	PBL	3.6 *	46, XX	ND
N Control 2	PBL	3.7 *	46, XY	ND
1	BL	83	N12	ND
2	BL	98	N12	ND
3	BL	86	N12	ND
4	FL	97	N12	ND
5	FL	94	N12	ND
6	FL	97	N12	ND
7	FL	88	N12	ND
8	MCL	91	N12	ND
9	MCL	96	N12	ND
10	MCL	94	N12	ND
11	B-NHL unT	2.5	N/A	+
	T	97	N/A	−
12	FL unT	3.6	N/A	+
	T	97.5	N/A	−
13	FL unT	3.5	N/A	+
	T	98.5	N/A	−
14	FL unT	3.2	N/A	+
	T	98	N/A	−

NOTE: The FISH results on cell lines were obtained on metaphase and interphase cells. The FISH results on patient samples 1 to 10 and on the matched (untransformed and transformed components) lymphoma samples (11-14) were obtained on interphase cells only. Samples were hybridized with a *SMRT* genomic probe as well as D13S25 (Vysis, Downers Grove, IL) control probe. Only cells with two FISH signals for the control probe were assessed for the *SMRT* FISH pattern. FISH results represent the percentages of cells with one *SMRT* FISH signal.

Abbreviations: CC, cervix carcinoma; AML, acute myeloblastic leukemia; BL, Burkitt lymphoma; FL, follicular lymphoma; B-NHL, B-cell non-Hodgkin lymphoma; T-NHL, T-cell non-Hodgkin lymphoma; T-ANA, T-cell anaplastic non-Hodgkin lymphoma; MCL, mantle cell lymphoma; PBL, peripheral blood lymphocyte; ND: not done; N12, no chromosome 12 karyotypic abnormalities detected; unT, untransformed; T, transformed, N/A, not applicable; +/−, positive/negative staining with anti-SMRT immunohistochemistry, resp.

*Values for our normal controls represent mean + 3 SD.

Cancer Center/Southwest Oncology Group (T.G., University of Arizona, Tucson, AZ). Immunostaining done with a polyclonal anti-*SMRT* antibody showed the presence of *SMRT* protein in low-grade non-Hodgkin lymphoma samples (Fig. 1*C*; Table 1) whereas the transformed lymphoma samples were composed of larger cells with undetectable *SMRT* protein (Fig. 1*D*; Table 1). We analyzed the same samples by FISH. The nontransformed non-Hodgkin lymphoma samples bore a normal pattern for the

SMRT probe (Fig. 1*C*; Table 1), whereas analysis of the transformed non-Hodgkin lymphoma samples showed a majority of large cells with loss of one *SMRT* FISH signal (Fig. 1*D*; Table 1). These data indicate that there is an *in vivo* correlation between loss of SMRT expression and genomic alteration of the gene, as already shown in our cell lines, and that these changes can be documented in the transformed lymphoma component, but not in the nontransformed lesions.

***SMRT* expression is altered as a consequence of genomic deletion.** Specific primers for the 5′ end of *SMRT* were coamplified with GAPDH-specific primers used as a standard to establish a semiquantitative reverse transcription-PCR (RT-PCR) approach. All cell lines tested showed a reduced amount of *SMRT* transcript compared with the control lines, consistent with the loss of one *SMRT* allele (Fig. 2*A*).

In addition, protein extracts from control cell lines (HeLa, CTV1, and EBV-LIN), as well as representative transformed non-Hodgkin lymphoma lines, were used for Western blot experiments. Whereas the control cell lines showed specific bands of the expected intensity and size, the non-Hodgkin lymphoma lines showed a marked down-regulation of SMRT protein expression (Fig. 2*B*).

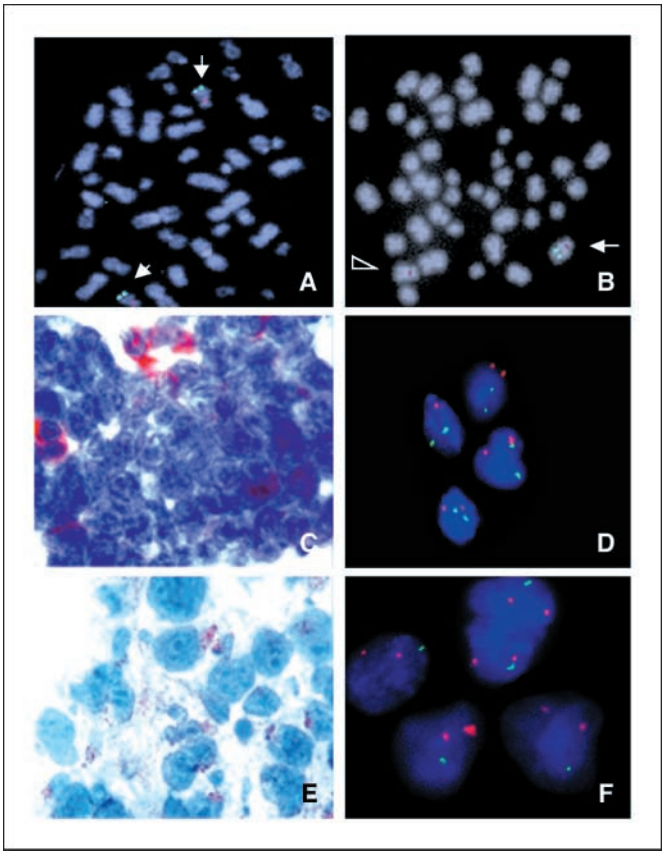

Figure 1. *A,* FISH results with a 3′ *SMRT* phage clone on a normal metaphase cell showing two normal chromosome 12 (*red signals,* centromere 12; *green signals, SMRT* probe); *B,* metaphase cell of the DoHH2 (follicular lymphoma) cell line showing loss of one *SMRT* (*green*) signal (*empty arrow*); *C* and *E,* immunostaining in matched untransformed and transformed non-Hodgkin lymphoma samples (Table 1). The SMRT protein is present in the untransformed non-Hodgkin lymphoma component of patient 13 (*C*), whereas the protein is barely detectable in the transformed component (*E*); *D* and *F,* normal FISH pattern for both the control probe (D13S25, *red*) and *SMRT* genomic probe (*green*) in untransformed cells from patient 13 (*D*), whereas loss of one *SMRT* FISH signal is observed in the transformed sample (*F*).

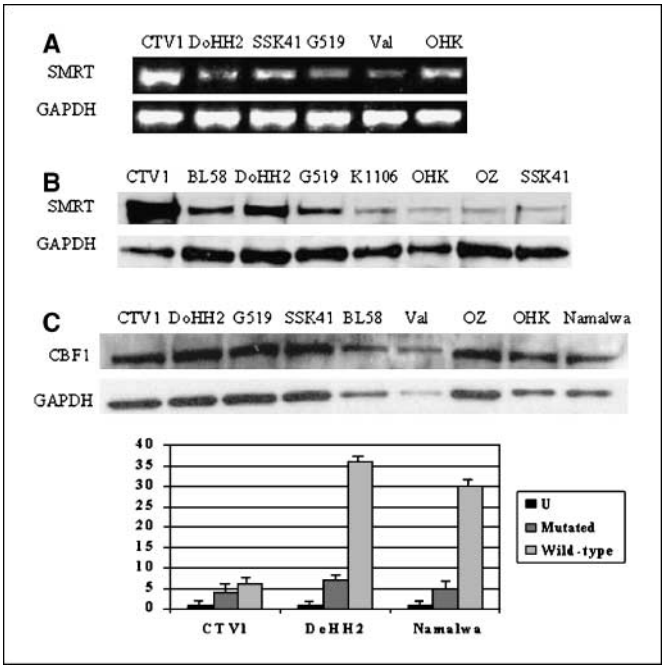

Figure 2. *A,* RT-PCR analysis of representative transformed non-Hodgkin lymphoma cell lines. The upper band represents the *SMRT* specific band, whereas the lower band corresponds to *GAPDH* for loading control and RNA/cDNA integrity. All the transformed cell lines express lower levels of *SMRT* transcript compared with control. *B,* Western blot analysis of nuclear extracts from representative transformed non-Hodgkin lymphoma cell lines using polyclonal anti-*SMRT* and anti-GAPDH antibodies as a loading control. *Top* and *bottom,* SMRT- and GAPDH-specific bands, respectively. *C,* assessment of CBF1 expression levels in our transformed non-Hodgkin lymphoma and control lines showed that CBF1 is uniformly expressed in all lines. Thus, any variation in luciferase activity reflects a variation in CBF1 activation and not an increased level of CBF1 protein expression. *D,* luciferase activity shown with wild-type/mutant luciferase-CBF1-reporter plasmids in representative transformed non-Hodgkin lymphoma cell lines and controls. Luciferase expressions were measured after 48 hours by dual-luciferase reporter assay (Promega), with chemiluminescence measured, values normalized for both protein content of the samples (Bradford method), and transfection efficiency established by the renilla luciferase intensities measured with the renilla luciferase control plasmid used. Untransfected cell lines were assayed to determine luciferase background.

To further confirm our Western blot results, anti-SMRT immunostaining was done on the non-Hodgkin lymphoma cell lines and controls. Only the control lines exhibited positive staining for SMRT protein; none of the transformed non-Hodgkin lymphoma cell lines tested showing detectable SMRT expression (data not shown). As mentioned earlier, patient samples obtained after transformation showed a reduced level of SMRT protein.

Most importantly, we sequenced the remaining *SMRT* allele in two transformed non-Hodgkin lymphoma cell lines (SSK41 and VAL). No sequence alteration, cryptic deletion, or any other genomic event has been detected, suggesting that transformed phenotype correlates with *SMRT* haploinsufficiency (data not shown).

CBF1/RBP-Jκ is a constitutive transcriptional activator as a consequence of *SMRT* haploinsufficiency. *SMRT* is involved in many pathways as a repressor. One of these is represented by CBF1/RBP-Jκ that acts as a transcriptional repressor by recruiting SMRT and the associated silencing complex (36). CBF1-dependent transcriptional activity was measured in representative transformed non-Hodgkin lymphoma, as well as in control cell lines using wild-type and mutant luciferase-CBF1-reporter plasmids (34). The results showed that CBF1 is ubiquitously expressed in all cell lines (Fig. 2*C*) and that elevated luciferase expression in all of the

non-Hodgkin lymphoma cell lines tested (Fig. 2*D*), indicating constitutive activation, is consistent with a reduction of SMRT activity.

***In vitro* down-regulation of *SMRT* induces transformed phenotype to immortalized fibroblasts in the "Weinberg" model.** *SMRT* down-regulation was induced by transfecting normal primary fibroblasts and embryonic kidney cells that have been immortalized by ectopic expression of hTERT and the SV40 large T oncoprotein (33), with a retrovirus construct expressing antisense *SMRT* transcripts (Fig. 3*A*). As shown with ras (33), we showed that *SMRT* down-regulation induces transformation of the immortalized cells with loss of contact inhibition, foci formation, and a 5-fold increase in colony formation (as well as in size of the colonies) in soft-agar assays as compared with the vector alone ($P = 0.0258$; Fig. 3*B*). These results with antisense *SMRT* are comparable with those obtained with the same cells transfected with an activated ras control construct (data not shown), whereas no transformed phenotype was observed when BJ cells immortalized with only hTERT were transduced with *SMRT*-antisense constructs (data not shown).

SMRT function restoration induces apoptosis of *SMRT*-deficient transformed non-Hodgkin lymphoma cell lines. To further test the hypothesis that SMRT may serve a tumor suppressor role, we used a retroviral gene transfer system to transduce expression of the protein in representative non-Hodgkin lymphoma cell lines [SSK41 (Burkitt lymphoma), DoHH2 (follicular lymphoma), G519 (mantle cell lymphoma), and VAL (B-cell non-Hodgkin lymphoma)] that we had shown to present endogenous *SMRT* down-regulation. We first used a modified NCI retroviral vector (35) to express SMRT protein encoded by the *TRAC2* cDNA, which has previously been used in functional studies (38, 39). These experiments were reproduced in two different laboratories (Loyola University Medical Center and National Cancer Institute, Metabolism Branch) with identical results, which showed cells transduced with infectious stocks produced by the empty retrovirus vector to exhibit normal proliferation, whereas all cells transduced with the *SMRT*-containing retroviral stock died within 2 days after puromycin selection (data not shown). The same results were obtained using a Tet-inducible pRetro-on retrovirus vector (Fig. 3*C*), with all cells undergoing apoptosis 2 days after induction of SMRT expression by doxycycline. The same experiments were repeated with an endogenous SMRT–containing cell line (CTV1) and no apoptotic effect was observed with either vector alone or *SMRT*-containing vector (Fig. 3*D*). These experiments clearly showed the survival inhibitory effect of *SMRT* insert in deficient cells, supporting a tumor suppressor capability for the protein.

cDNA array profiles using lymphochip arrays on *SMRT* restoration. To characterize how decreased expression of *SMRT* could play a role in the transformation process, we did cDNA experiments using the lymphochip array (36). Our original intent was to restore partial SMRT function using our *SMRT*-sense (and empty vector as control) retrovirus construct. As mentioned earlier, SMRT restoration induces apoptosis in the transformed non-Hodgkin lymphoma cells. However, we were able to rescue cells for one transduced transformed non-Hodgkin lymphoma cell line (VAL) for which we were able to obtain enough RNA to perform cDNA array experiments. The comparison of expression profiles from VAL cells transduced with either vector alone or *SMRT*-sense construct showed the down-regulation of 82 genes and up-regulation of 49 genes upon on SMRT restoration (genes that were up-regulated and down-regulated in the nontransduced cell line, respectively). Some of the genes with altered expression have been shown to affect cell motility, migration, or adhesion, proliferation, differentiation, and apoptosis or survival (Table 2). To validate and extend our cDNA array results, we assessed transcript and protein expression of six genes down-regulated by SMRT restoration (*CD44*, *ID2*, *A20*, *BCL2*, *cFLIP*, and *AP-1/c-jun*, in addition to *IL-6*) in three transformed non-Hodgkin lymphoma cell lines [VAL (B-cell non-Hodgkin lymphoma), DoHH2 (follicular lymphoma), and G519 (mantle cell lymphoma)] as well as in a lymphoblastoid cell line (EBV-LIN). We chose these genes for their function in apoptosis, motility or adhesion, and proliferation. RT-PCR (Fig. 4*A*) and Western blot (Fig. 4*B*) analysis of the four cell lines confirmed the expression profile observed in the cDNA array in the VAL cell line.

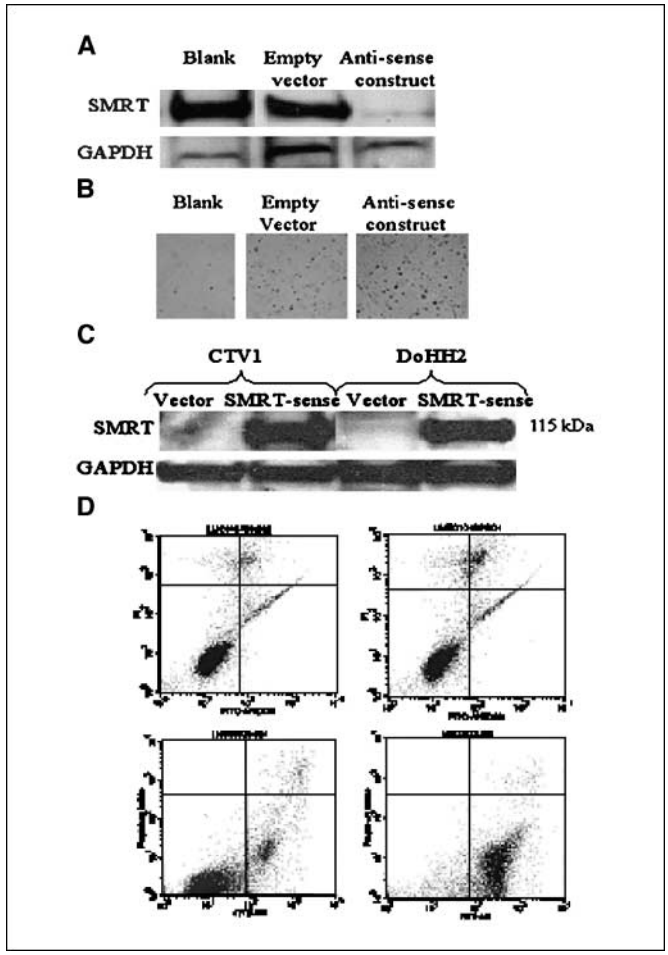

Figure 3. *A,* Western blot analysis of the BJN cells before and after transfection with empty vector or our antisense *SMRT* construct. This shows a marked down-regulation of SMRT expression with our antisense construct, similar to that observed in the transformed non-Hodgkin lymphoma cell lines. *B,* soft agar assay with the BJN cells either without transfection (*left*), empty vector (*middle*), or *SMRT* antisense construct (*right*). Loss of contact inhibition, foci formation, as well as a 5-fold increase in colonies formed are observed with the *SMRT* antisense construct as compared with the assay with vector alone. *C,* Western blot analysis of SMRT-nondeficient (CTV1) and -deficient (DoHH2) cell lines after transduction with vector alone or our *SMRT*-sense construct. As expected, a 115 kDa protein band is detected with the *SMRT*-sense construct. *D,* annexin V and propidium iodide staining/apoptosis analysis of the CTV1 and DoHH2 cell lines on transduction with either vector alone (*left*) or *SMRT*-sense constructs (*right*). The apoptosis profile of CTV1 is not affected by the *SMRT*-sense construct whereas the number of cells undergoing apoptosis increased from 18% (*bottom left, lower right quadrant*) to 91% (*bottom right, lower right quadrant*).

Discussion

No common recurrent genetic abnormalities have been previously shown to clearly be associated with the non-Hodgkin lymphoma transformation. To investigate potential recurrent genomic abnormalities that would correlate with transformation of non-Hodgkin lymphoma to high-grade disease, we originally used high-resolution cytogenetics to unravel any common rearrangements in a large series of transformed non-Hodgkin lymphoma cell lines. We showed that the 12q24.3 region was recurrently involved in deletions and complex rearrangements. Similar breakpoints have been reported by other groups in mediastinal B-cell non-Hodgkin lymphoma (40) and in Richter's syndrome (41), which represents the transformation of chronic lymphocytic leukemia. The nature of these abnormalities suggests that they are secondary "late" events associated with non-Hodgkin lymphoma transformation. This is particularly evident by examination of sequential karyotypes in some patient samples and by identification of three-way translocations in which the initial low-grade translocation is rearranged to a third chromosome (17). The mapping of the *SMRT* gene in this region (19, 20) made this gene a strong candidate gene for its potential involvement and alteration in the observed 12q24 rearrangements. Indeed, as we show in this study, the alteration of the genomic integrity of *SMRT* induces reduction of its function and creates haploinsufficiency. This may drive the transformation process through multiple downstream cascade pathways. Most importantly, correlation between loss of one *SMRT* allele and reduction of SMRT protein and function was documented on (paired) patient samples, thus excluding a potential artifact of cell line/cell culture for the 12q24 rearrangements (Fig. 1).

Table 2. Partial summary of genes of which expression has been altered on SMRT restoration in the *SMRT*-deficient VAL cell line

Genes down-regulated	Genes up-regulated
On SMRT restoration	
Motility/migration/adhesion	Motility/migration/adhesion
TIMP-1	*CD62L*
uPA	*P120*
CX3C chemokine	Apoptosis
CD47	*PARP*
Cathepsin B	Proliferation
CD54	*P57/Kip2*
CD44	*Cyclin B1*
Syndecan 4	*P16-INK4a*
CXCR5	*A-myb*
ELAM-1	Differentiation
Apoptosis/Survival	*INSIG-1*
Galectin-1	Response to oxidative stress
c-jun	*SOD2*
gp34	
Caspase 1	
A20	
cFLIP	
NF-κB2	
BCL2	
Proliferation	
IL7	
FGFR2	
IL15R	
TNFB	
Jun-B	
IRF4/Mum1	
MKP-1	
DP-2	
Id2	
IL8	
Smad7	

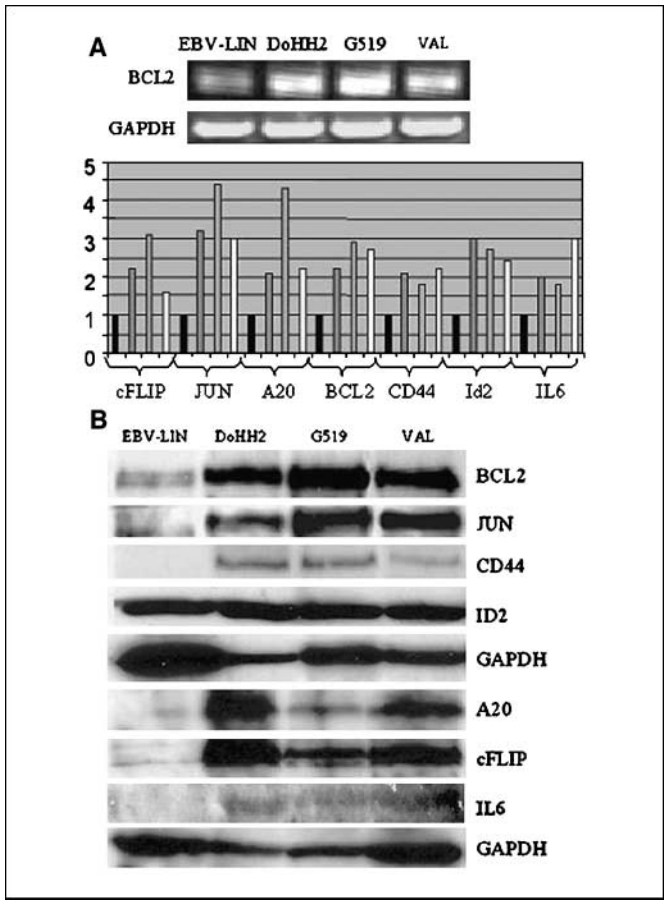

Figure 4. Expression levels for the *BCL2*, *cFLIP*, *JUN*, *A20*, *CD44*, *Id2* and *IL-6* genes in the EBV-LIN lymphoblastoid cell line and three transformed non-Hodgkin lymphoma cell lines [DoHH2 (follicular lymphoma), G519 (mantle cell lymphoma), and VAL (B-cell non-Hodgkin lymphoma)] by RT-PCR (*A*) and Western blot (*B*). The RT-PCR data are displayed as expression normalized with *GAPDH* expression and compared with the normalized expression observed for the EBV-LIN lymphoblastoid cell line.

As earlier mentioned, SMRT is involved in many pathways as a repressor. One of these is represented by CBF1/RBP-Jκκ that acts as a transcriptional repressor by recruiting SMRT and the associated silencing complex. The dissociation of SMRT/silencing complex subsequently converts CBF1 into a transactivator (37). The stimulation of *IL-6* and *NF-κB2* promoter activities can be mediated by CBF1 through the interaction of the protein with a CBF1-responsive element that partially overlaps a binding site (42, 43). Several reports from the literature have shown a strong association between IL-6 overexpression and the malignant phenotype of transformed non-Hodgkin lymphoma, with the IL-6 having been shown to originate from the lymphoma cells (44, 45). Moreover, patient lymphoma samples show a highly significant correlation

between elevated IL-6 expression and elevated mRNA levels of *MMP-9*, *MMP-2*, and *TIMP-1*, suggesting that IL-6 may play an active role in the clinical aggressiveness of human non-Hodgkin lymphoma by stimulating matrix metalloproteinase production (46). Thus, CBF1-mediated induction of IL-6 is one of the several potential mechanisms by which reduction of SMRT expression could promote disease progression. All our cell lines overexpress IL-6 (Fig. 4). Our data indicated that the endogenous CBF1/RBP-Jκ protein present in the transformed non-Hodgkin lymphoma cell lines represents a constitutive activator of transcription, consistent with the reduced amount of SMRT protein available in these cells. In addition, we showed the transformation capabilities of *SMRT* down-regulation using a well-established transformation assay (33). Cells immortalized with only hTERT did not show a transformed phenotype on *SMRT* down-regulation whereas cells bearing both hTERT and large T SV40 presented with a transformed phenotype in soft agar assays (Fig. 3*B*).

The cDNA array results obtained with one of our cell lines correlate well with previously reported results. First of all, some of the genes that are known to be regulated directly or indirectly by SMRT are among the genes altered on SMRT restoration. Indeed, IP10 is regulated by BCL6 (35), BCL6 being a repressor of transcription by recruiting SMRT through its POZ domain (47). Similarly, the *NF-κB2, IL-6*, and *Nur77* genes can be regulated by CBF1/RBP-J (48, 49), which is a repressor of transcription on recruitment of SMRT. Interestingly, we have shown that the CBF1 protein present in transformed non-Hodgkin lymphoma is a constitutive activator of transcription (see above and Fig. 2*D*) and that IL-6 is overexpressed in our cell lines.

To validate our cDNA array results, we chose six genes that were down-regulated on *SMRT* restoration to be assayed in a lymphoblastoid as well as three transformed non-Hodgkin lymphoma cell lines. These genes have different functions, each playing a potential role in cell transformation. Indeed, A20 has been recently shown to confer a broad protection against apoptosis by shutting down cell death pathways initiated by inflammatory and immune offenders (50). Similarly, BCL2 and cFLIP are well-established players of the apoptosis pathway, by forming inactivating heterodimers with Bax/Bak (51) and by blocking caspase-8 activation (52; similar to A20), respectively. The activator protein 1 (AP-1; c-jun and jun-B) and Id2 are involved in cell growth and proliferation. The AP-1 proteins can bind to and induce the expression of their target genes (for example, *IL-8*; see Table 2) whereas Id2 coordinates inhibition of differentiation and stimulation of cell proliferation by inactivating the retinoblastoma tumor suppressor protein, therefore circumventing the block on cell cycle imposed by the retinoblastoma pathway (53, 54). Finally, CD44 has been shown to be involved in the homing of lymphocytes and in their rolling (55) and dissemination of non-Hodgkin lymphoma cells (56). As shown in this study, all tested cell lines showed altered expression for these genes at the cDNA and protein levels (Fig. 4*A* and *B*).

The data presented in this report support the premise that a single genetic abnormality, reduction of SMRT function, may drive the non-Hodgkin lymphoma transformation process through multiple downstream cascade pathways. This implies a nonclassical tumor suppressor role for SMRT through haploinsufficiency. SMRT is known to be involved in several repression pathways (21, 29); therefore, its down-regulation can potentially activate numerous downstream genes that are normally repressed. Our observation of massive apoptosis on reintroduction of a functional SMRT protein into deficient non-Hodgkin lymphoma cell lines can be explained by the fact that SMRT repression activity is at least partially restored, leading to the silencing of genes which expression is necessary for survival in transformed non-Hodgkin lymphoma cells. Interestingly, normal cells do not seem to be affected by the partial SMRT function restoration due to already having a normal endogenous level of SMRT. A similar haploinsufficiency was reported recently in two different studies. The *sno* gene (57), which encodes a component of the histone deacetylase complex (similar to *SMRT*), acts as a tumor suppressor through haploinsufficiency. Indeed, loss of one copy of *sno* increases susceptibility to lymphoma development in mice. B-cells, T-cells, and embryonic fibroblasts had increased proliferative capacity and decreased sensitivity to apoptosis and cell cycle arrest. Similarly, the *Beclin-1* gene (58) is a haploinsufficient tumor suppressor gene. *Beclin-1* +/− mice present a high incidence of tumors due to Beclin-1 insufficiency in its autophagy function.

These observations, taken together with the data presented in this study, strongly support a tumor suppressor role for SMRT and its potential involvement in the transformation process. This is the first report of its alteration and its direct involvement in a specific pathology. Additional expression profile experiments, in different systems, will allow us to analyze more deeply and in greater detail the numerous pathways in which SMRT is involved. These cDNA array data should allow us, in the near future, to draw a mechanistic model for the effects of *SMRT* down-regulation and its implication in the transformation process. This new aspect of the SMRT function will certainly call for closer attention for the development of new potential therapeutic approaches.

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