Epigenetic Inactivation of the Groucho Homologue Gene TLE1 in Hematologic Malignancies

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Abstract

An undifferentiated status and the epigenetic inactivation of tumor-suppressor genes are hallmarks of transformed cells. Promoter CpG island hypermethylation of differentiating genes, however, has rarely been reported. The Groucho homologue Transducin-like Enhancer of Split 1 (TLE1) is a multitasked transcriptional corepressor that acts through the acute myelogenous leukemia 1, Wnt, and Notch signaling pathways. We have found that TLE1 undergoes promoter CpG island hypermethylation–associated inactivation in hematologic malignancies, such as diffuse large B-cell lymphoma and AML. We also observed a mutual exclusivity of the epigenetic alteration of TLE1 and the cytogenetic alteration of AML1. TLE1 reintroduction in hypermethylated leukemia/lymphoma cells causes growth inhibition in colony assays and nude mice, whereas TLE1-short hairpin RNA depletion in unmethylated cells enhances tumor growth. We also show that these effects are mediated by TLE1 transcriptional repressor activity on its target genes, such as Cyclin D1, Colony-Stimulating Factor 1 receptor, and Hairy/Enhancer of Split 1. These data suggest that TLE1 epigenetic inactivation contributes to the development of hematologic malignancies by disrupting critical differentiation and growth-suppressing pathways.

Introduction

Genetic lesions impairing cell differentiation in human cancer are exemplified by the chromosomal translocations involving the transcription factor AML1 in acute myelocytic human leukemia (AML; refs. 1, 2) and NOTCH1 in T-cell acute lymphoblastic leukemia/lymphoma (3). Transcriptional silencing of tumor-suppressor genes associated with promoter CpG island hypermethylation has emerged as another hallmark of human tumors (4–6). To date, however, similar hypermethylation events in the AML1 or NOTCH1 pathways that could compromise their differentiating capacities have not been identified in cancer cells. To address this issue, we have turned our attention to a Groucho homologue, the Transducin-like Enhancer of Split 1 (TLE1).

The Groucho/TLE/Grg family of corepressors operates in many signaling pathways (7, 8). The human homologue of Groucho, TLE1 (9), has critical transcription factor partners such as TCF/LEF-1 in the case of the Wnt signaling pathway (10), Hairy/Enhancer of Split 1 (HES1) in the case of Notch (11), and the AML/CBFα runt domain transcription factor family in hematopoiesis (12, 13). TLE1 expression has been associated with immature cells that are progressing toward a terminally differentiated state, suggesting a role during differentiation (14). At the end of hematopoiesis, when committed progenitors begin to acquire the characteristic differentiation status of mature hematopoietic cells, target genes from the AML1, Wnt, and Notch signaling pathways (15, 16) need to be repressed by TLE1 (12, 13). Hence, we examined whether epigenetic silencing of TLE1 was involved in the development of human malignancies.

Materials and Methods

Human cancer cell lines and primary tumor samples. The 49 human cancer cell lines examined in this study were obtained from the American Type Culture Collection. The cell lines represented 14 types of malignancy (leukemia, lymphoma, breast, cervix, colon, condrosarcoma, liver, lung, muscle, neuroblastoma, osteosarcoma, prostate, skin, and testis). Cell lines were maintained in appropriate medium and treated with 1 μmol/L 5-aza-2'-deoxycytidine (Sigma) for 3 d to achieve demethylation. One-hundred-six primary hematologic malignancies were obtained from the Spanish National Cancer Research Centre Tumour Bank, the University of Navarra, and the University Hospital Schleswig-Holstein Campus Kiel. The study was approved by the corresponding institutional review boards.

DNA methylation analysis of the TLE1 gene. We established TLE1 CpG island methylation status by PCR analysis of bisulfite-modified genomic DNA. First, methylation status was analyzed by bisulfite genomic sequencing of both strands of the CpG island. The primers used were 5'-GAG AAT TTG GTA GTG GGT TGG-3' (sense) and 5'-CAA TCC TAA ACA AAG TCC A-TG-3' (antisense). The second analysis used methylation-specific PCR with primers specific to either the methylated or modified unmethylated DNA. Primer sequences for the methylated reaction were 5'-GAG TTT CCG AGT ATT CGG TC-3' (sense) and 5'-GAA CTT TCC CGG AAA CGA C-TG-3' (antisense), and for the unmethylated reaction, primer sequences were 5'-TGG TAT TTT GTT ATG ATT TTG TT-3' (sense) and 5'-TCA AAT TCC CCA AAG AAC-3' (antisense). Bisulfite genomic sequencing and methylation-specific PCR oligos were designed to be able to discriminate between TLE1 and its pseudogene (LOC 389863) located on
the X chromosome. To achieve this aim, the MSP oligos contain several polymorphisms of TLE1 and its pseudogene, and bisulfite sequencing oligos contain a region of 88 bp located 456 bp upstream of the transcriptional start site of the pseudogene that it is absent from the TLE1 promoter.

These bisulfite sequencing oligos generate 2 PCR products of 587 and 676 bp that correspond to TLE1 and its pseudogene, respectively (Supplementary Table S1). All the bisulfite sequencing analyses presented were developed using the 587 bp PCR that corresponds to TLE1.

TLE1 RNA and protein analysis by conventional reverse transcription-PCR and immunofluorescence. RNA was isolated by using TRIZOL (Life Technologies). Two micrograms of RNA were reverse-transcribed using SuperScript II reverse transcriptase (Life Technologies/Bethesda Research.

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**Figure 1.** Analysis of TLE1 CpG island promoter methylation status and expression. **A,** schematic depiction of the TLE1 CpG island around the transcription start site ([long black arrow]. Short vertical lines, CpG dinucleotides. Location of bisulfite genomic sequencing PCR primers ([black] and methylation-specific PCR primers are indicated (white). Results are shown of bisulfite genomic sequencing of 12 individual clones in normal lymphocytes (NL1 and NL2) and the cell lines Molt-4, KG1A, Hut-78, and Raji. Presence of a methylated (black square) or unmethylated cytosine (white square) is indicated. An illustrative fragment of the sequencing electropherogram is shown for NL1 and KG1A. A representative methylation-specific PCR for the TLE1 gene is also shown. The presence of a PCR band under lanes M or U indicates methylated or unmethylated genes, respectively. In vitro methylated DNA (IVD) is used as a positive control for methylated DNA. **B,** RT-PCR analysis of TLE1 expression (top). Treatment with the demethylating agent (ADC + lanes) reactivates TLE1 gene expression. Bottom, immunofluorescence analysis of TLE1 expression. The methylated cell line Hut-78 does not stain for the TLE1 protein, unlike the unmethylated Molt-4 cell. Treatment with the ADC restores protein expression. The histone deacetylase inhibitor trichostatin A reinforces the ADC reactivation effect. **C,** methylation-specific PCR for the TLE1 gene in human primary malignancies. The presence of a PCR band under lanes M or U indicates methylated or unmethylated genes, respectively. In vitro methylated DNA is used as a positive control for methylated DNA.
Figure 2. Tumor suppressor–like properties of TLE1. A, effect of TLE1 transfection on the in vitro growth of KG1A, Hut-78, and Raji cells. Left, RT-PCRs of TLE1 in untransfected and transfected cells. Middle, decrease in the number of cells after TLE1 transfection; right, recovery of TLE1 expression by immunofluorescence analysis. The plots show the monitoring over time of the number of cells after transfecting the cell lines KG1A, Hut-78, and Raji with TLE1. B, colony formation assay (left). Two (R1 and R2) independent experiments were carried out. Example of the colony focus assay after a 2-wk selection with G418. Bottom, detailed images from the top panels. C, effect of TLE1 transfection on the growth of KG1A and Raj cells in nude mice. Tumor weight was monitored over time. Shown are female athymic nude mice 45 d after injection of 10^7 KG1A or Raji cells. Note the large tumor on the left flank, corresponding to empty vector cells, and the absence of visible tumor on the opposite flank, corresponding to TLE1-transfected cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Laboratories) and amplified using specific primers for TLE1 (forward, 5'-CCC ATA TCC TGC TCC TTT TG-3; reverse, 5'-GGT TGA GGG TGT TGA TCT GG-3). PCR was performed for 25 cycles (94°C for 30 s, 57°C for 30 s, and 72°C for 30 s). Immunofluorescence experiments were developed using the TLE1 antibody (Abcam).

**Results and Discussion**

TLE1 has a typical CpG island around its transcription start site (Fig. 1A). We first determined the TLE1 CpG island methylation status of a panel of 49 human cancer cell lines from 14 tumor types by bisulfite genomic sequencing of multiple clones and methylation-specific PCR (Supplementary Fig. S1; Fig. 1A). Strikingly, TLE1 CpG island hypermethylation was only found in hematologic malignancies (Supplementary Fig. S1; Fig. 1A). It was present in AML (43%, 3 of 7), non–Hodgkin’s lymphoma (100%, 5 of 5), and chronic myeloid leukemia (100%, 1 of 1) cell lines (Supplementary Fig. S1; Fig. 1A). However, it was unmethylated in acute lymphocytic leukemias (ALL; n = 7) and in all solid tumor cell lines (Supplementary Fig. S1). The hypermethylation event was cancer specific because all normal peripheral leukocytes analyzed (n = 12) were completely unmethylated at the TLE1 CpG island (Supplementary Fig. S1; Fig. 1A). Most importantly, lymphoma and leukemia cell lines with TLE1 CpG island hypermethylation, such as Hut-78 and KG1A, did not express TLE1 transcript and protein (Fig. 1B). However, TLE1 unmethylated leukemia cell lines, such as Molt-4, strongly expressed TLE1 transcript and protein (Fig. 1B). We established a further link between TLE1 CpG island hypermethylation and its gene silencing by the treatment of the methylated cell lines with a DNA demethylating agent. The treatment of the TLE1-hypermethylated Hut-78 and KG1A lymphoma and leukemia lines with the demethylating drug 5-aza-2'-deoxycytidine restored the expression of the TLE1 RNA transcript and protein (Fig. 1B).

The observed epigenetic disruption of TLE1 in undifferentiated leukemia and lymphoma cells was not just an *in vitro* cell culture phenomenon. We extended our TLE1 CpG island methylation analysis to 106 patients with hematologic malignancies, including 47 diffuse large B-cell lymphomas (B-DLCL), AML, and 34 ALL. We
observed that TLE1 CpG island hypermethylation was a common event in B-DLCLs (36%, 17 of 47) and AMLs (24%, 6 of 25), whereas it was much less prevalent in ALLs (2.9%, 1 of 34; Fig. 1C). This is a similar scenario than the one encountered in the cancer cell lines described above.

It is interesting to remember that TLE1 binds to the AML/CBFα runt domain transcription factor family (12, 13), and that the imbalance of this signaling pathway by the presence of the AML1-ETO translocation occurs in a subset of AMLs (1, 2). Importantly, the AML1 binding site for TLE1 is located at its carboxilic end, which is lost when AML1 is translocated with ETO (12, 13).

Thus, if it is widely accepted that the probability of simultaneous hits in the same molecular pathway in a given tumor is very low, simultaneous molecular lesions in TLE1 and AML1 in the same leukemia sample should be a rare event. To test whether this inverse association exists, we have analyzed the TLE1 CpG island promoter methylation status in 24 primary AMLs carrying the AML1-ETO translocation. We have observed an unmethylated TLE1 CpG island in all AMLs positive for AML1-ETO (0%, 0 of 24) compared with the 24% (6 of 25) frequency of TLE1 hypermethylation in the primary AMLs without translocation described above (P = 0.02, Fisher’s exact test). Thus, the mutual exclusivity of
the epigenetic alteration of TLE1 and the cytogenetic alteration of AML1 suggests that they play a critical and cooperative role in human leukemogenesis.

From a functional standpoint, we next wanted to determine whether epigenetic inactivation blocked growth suppression and differentiation in those hematologic malignancies with TLE1-methylation–associated silencing. We adopted a double approach in addressing this question. First, we transfected TLE1 in hematologic malignancies with TLE1 hypermethylation, such as the cell lines KG1A, Hut-78, and Raji. Upon restoration of TLE1 expression, as showed by reverse transcription PCR (RT-PCR) and immunofluorescence analyses (Fig. 2A), all three leukemia/lymphoma cell lines experienced reduced cell growth (Fig. 2A). We further showed the growth inhibitory features of TLE1 reintroduction in colony focus assays and nude mouse models. In the colony formation assay, we observed that TLE1 re-expression revealed tumor-suppressor activity, whereby there was a marked 78% lower colony formation density with respect to the empty vector (Fig. 2B). We next tested the ability of TLE1–transfected KG1A and Raji leukemia cells to form tumors in nude mice compared with empty vector–transfected cells (Fig. 2C). Cells transfected with the empty vector formed tumors rapidly, but cells infected with the TLE1 expression vector had much lower tumorigenicity (Fig. 2C). At the time of sacrifice, tumors were 6 to 10 times larger in mice with the empty vector than in the TLE1-transfected xenografts (Fig. 2C). Finally, we also knocked down TLE1 expression by RNA interference in an expressing leukemia cell line unmethylated at the TLE1 CpG island, Molt-4. We observed that significantly reduced TLE1 expression (Fig. 3A) was associated with increased cell growth (Fig. 3B). Further evidence of the tumor-suppressor function of TLE1 is provided by its location in a commonly deleted segment in del(9q) AML (17).

Finally, we wanted to characterize the putative mechanisms mediating the described tumor-suppressor activity of TLE1 in hematologic malignancies. TLE1 is a multitasked transcriptional co-repressor that acts through the AML1, Wnt, and Notch signaling pathways (12, 13). The TLE1-related silencing effects on gene transcription might be associated with its interaction with chromatin proteins involved in transcriptional repression, such as Histone Deacetylase 1 (HDAC1; refs. 18, 19), Sirtuin 1 (20), and Polycym members (21). Thus, we examined whether the expression of characteristic target genes of each pathway was altered by the reintroduction of TLE1 in leukemia/lymphoma cell lines (KG1A, Hut-78, and Raji) with TLE1 CpG island hypermethylation. AML1 expression itself was used as a negative control because it is known not to be regulated by TLE1 (12, 13). Upon efficient re-expression of TLE1 in transfected cells (Fig. 4A), we observed down-regulation of the AML1 pathway growth-promoting factors Colon-Stimulating Factor 1 receptor (CSF1R; refs. 22, 23) and B-Cell Lymphoma 2 (BCL2; ref. 24), the oncogene myc (C-MYC; ref. 25), and Cyclin D1 (CDY1; ref. 26) from the Wnt pathway; and the HES1 (27) from the Notch signaling pathway (Fig. 4A). A further link between TLE1 re-expression and repression of the described target genes was established by the development of ChIP experiments for those particular promoters. TLE1-transfected cells exhibited occupancy by the TLE1 protein in the 5′-regulatory regions of the CYD1, CSFIR, and HES1 genes, recruitment of the transcriptional repressor HDAC1, and an associated reduction of a histone modification marker associated with transcriptional activation, histone H3 acetylation (Fig. 4B). These findings are evidence of the proposed role of TLE1 as a transcriptional repressor that targets HDAC1 to promoters and mediates gene repression through histone H3 deacetylation (18, 19).

Overall, our results indicate that TLE1 promoter CpG island methylation-mediated silencing occurs in leukemia and lymphoma cells, where it might prevent the development of growth inhibitory and differentiating programs mediated by three critical pathways, AML1, Wnt, and Notch.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

18. Palaparti A, Baratz A, Stifani S. The Groucho/


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