Down-regulation of specific miRNAs enhances the expression of the gene Smoothed and contributes to T-cell lymphoblastic lymphoma development

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Inappropriate activation of the GLI/hedgehog (GLI/Hh) signalling occurs in several human cancers, including haematological neoplasms. However, little is known about its relevance in precursor T-cell lymphoblastic lymphomas (T-LBL) development. Moreover, the mechanisms whereby GLI/Hh signalling is activated in haematological malignancies are not fully clear. Here, we show that the gene Smoothed (SMO), the only non-redundant gene of this pathway, is up-regulated in mouse and human T-LBL. Interestingly, down-regulation of micro-RNAs mmu-miR-30a and mmu-miR-141 as well as hsa-miR-193b clearly contributes to enhance the expression of this gene in mouse and human lymphomas and, subsequently, to activate the GLI/Hh signalling. Activation of the GLI/Hh signalling in T-LBL promotes cell survival and proliferation, since inhibition of the pathway using short-hairpin-RNA-mediated SMO knockdown, or cyclopamine as a specific antagonist, significantly reduces these cellular processes. These findings suggest that sustained SMO up-regulation may contribute to T-LBL development and advocate the use of specific SMO inhibitors or microRNAs-based therapies as an attractive possibility to treat an important subset of T-LBL.

Introduction

T-cell lymphoblastic neoplasms are aggressive diseases that mainly develop in children and adolescents (1). Molecular pathogenesis of these neoplasias, in particular T-cell lymphoblastic lymphomas (T-LBL), involves genetic and epigenetic alterations in multiple coding and non-coding genes affecting most of the known signalling pathways, but there was no evidence involving the GLI/hedgehog (GLI/Hh) pathway in the development of T-LBL (1–7). This signalling pathway is implicated in vertebrate developmental patterning and cell-fate induction (8). GLI/Hh signalling is required as well for primitive haematopoiesis (9); it regulates intrathymic T-cell development and is one of the survival signals provided by follicular dendritic cells to prevent apoptosis in germinall centre B cells (10). However, conflicting data have been reported regarding the role of this pathway in adult haematopoiesis (11). Particularly relevant from the standpoint of our work is that (i) stromally produced Hh ligands-proteins have a key role in thymocyte development (12), (ii) in the thymus, the GLI/Hh pathway controls thymic cellularity as well as the development of CD4+CD8+ thymocytes (13), (iii) one ligand of the pathway, Sonic hedgehog, promotes cell cycle progression in activated peripheral CD4+ T lymphocytes (14), (iv) Sonic hedgehog signalling modulates cytokine production of human peripheral CD4+ T cells (14) and (v) Hh signals regulate cell proliferation and differentiation during development, assuming homeostatic roles in adults to maintain stem cells (15).

Inappropriate activation of the GLI/Hh signalling pathway occurs in several human cancers, including haematological neoplasms. Three models of mechanisms whereby GLI/Hh signalling can be aberrantly activated in cancer have been described [reviewed in ref. (11)]. First, a hyperactivation of the pathway is induced by mutation of members of the pathway (PTCH1, SUFU and SMO). Second, autocrine and/or paracrine stimulation of cancer cells is caused by excessive and/or inappropriate expression of Hh ligands. A third model is defined by the activation of GLI/Hh signalling in the stroma induced by secretion of Hh ligands by the tumour cells. In response to tumour-secreted Hh ligands, stromal cells secrete growth factors contributing to proliferation and/or survival of the tumour cells.

Despite the abundant data on the inappropriate activation of the GLI/Hh signalling in cancer, the contribution of this pathway in haematological malignancies has not been thoroughly examined. Emerging data indicate that the GLI/Hh signalling pathway is active in some haematopoietic malignancies and that this activation contributes to the biology of these neoplasms. Abnormal GLI/Hh signalling pathway activation has been described in chronic lymphoctic leukaemia/small lymphocytic lymphoma, plasma cell myeloma, mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), ALK-positive anaplastic large cell lymphoma, chronic myelogenous leukaemia and acute leukaeemias (16–25). In these neoplasms, Hh signalling promotes proliferation and survival, contributes to the maintenance of cancer stem cells and enhances tolerance or resistance to chemotherapeutic agents. In particular, evidence regarding lymphoblastic lymphomas or leukaemias show that inhibition of GLI/Hh signalling affects highly clonogenic B-cell acute lymphoblastic leukaemia cells primarily by limiting their self-renewal potential. As well, data suggest that GLI/Hh signalling promotes the growth of T-cell acute lymphoblastic leukaemia (20,21). Regarding the mechanisms underlying the activation of the pathway in haematological malignancies, they are not fully clear at the moment. Notably, no activating or inhibiting mutations in GLI/Hh signalling members have been found to date in these malignancies. Gains at GLI1 locus and/or extra copies of chromosome 12 have been reported in B-cell lymphomas including splenic and nodal marginal zone lymphoma, DLBCL and MCL (16,22,23,25). Extra copies of GLI1, DHH or SMO genes have been found in DLBCL cell lines and in patient samples (11), which may contribute to GLI/Hh activation but, according to the authors, does not fully explain activation of the pathway. In cerebellar granule cell precursors and in medulloblastoma, it has been shown that the microRNA-17/92 cluster synergizes with GLI/Hh signalling (26). However, it is not known to date whether specific miRNAs are involved in the activation of GLI/Hh signalling in lymphomas and leukaemias.

Recently, we have reported on gene expression profiles in murine γ-radiation-induced T-LBLs using cDNA microarrays and miRNA arrays. Interestingly, we detected a significant increase in the level of expression of the gene Smoothed (Smo), the only non-redundant gene of GLI/Hh signalling pathway, and discovered a panel of 41 miRNAs consistently down-regulated in these tumours. We showed

Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; GLI/Hh, GLI/hedgehog; mRNA, messenger RNA; miRNA, microRNA; qRT–PCR, quantitative reverse transcription–polymerase chain reaction; shRNA, short hairpin interfering RNA; T-LBL, T-cell lymphoblastic lymphomas; UTR, untranslated region.
that the up-regulation of critical oncopgenes, as Abl1 and c-Myc, is controlled by down-regulation of specific miRNAs (5,7). Thus, the aim of this work was (i) to validate the up-regulation of Smo in mouse and human T-LBLs, (ii) to investigate if the up-regulation of this gene could be attributed to the down-regulation of specific miRNAs, (iii) to explore whether the sustained up-regulation of this gene may contribute to the aberrant activation of the GLI/Hh pathway and (iv) to prove whether this activation may contribute to the biology of T-LBLs development.

Material and methods

Mouse colony and induction of tumours

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animal experiments were carried out according to the European Commission Guidelines (Directive 86/609/CEE) on the use of laboratory animals. T-cell lymphoma was induced as described previously (27) following the protocol approved by the Ethic Committee of Autonomous University of Madrid (CEI 22-478). All analyzed tumours were double positive (CD4+CD8+) blast cells expressing nuclear terminal deoxynucleotidyl transferase.

Human T-LBL samples

Four human T-LBLs were obtained from the Spanish Tumour Bank Network of the Spanish National Cancer Research Centre (CNIO, Madrid, Spain). These samples derive from mass lesions in mediastinum or lymph nodes and are composed of small- to medium-sized blast cells expressing nuclear terminal deoxyribonucleotidyl transferase. Institutional review board approval was obtained for these studies (reference RNDT 10/073), and all participants provided a written informed consent in accordance with the Declaration of Helsinki.

Cell lines

JURKAT and NIH-3T3 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Human thymocytes immortalized with Saimiri Herpes virus were a gift of Dr Regueiro [Universidad Complutense de Madrid, UCM, Spain (28)]. Cell lines were cultured using standard procedures as suggested by the suppliers.

Isolation and purification of DNA and RNA

DNA from mouse and human samples was extracted using standard phenol–chloroform methods. Total RNA was isolated from cells and tissues using Tripure Isolation Reagent (Roche Applied Science, Westerlo, Belgium) or 10 µM dimethyl sulfoxide was used as baseline controls.

RNA interference against SMO

A pool of short hairpin interfering RNA (shRNA) lentiviral particles containing three target-specific constructs was used to knock-down the expression of SMO in JURKAT cells (Santa Cruz Biotechnology). Transduction with lentiviral particles was performed according to the supplier and selection of SMO deficient cells was done using 4 µg/ml puromycin (Santa Cruz Biotechnology).

Viability assay

Treated and non-treated JURKAT cells were analyzed daily 15 min after incubation with 7-aminoactinomycin D (BD Pharmaniing, Franklin Lakes, NJ). The percentage of dead cells was analyzed by flow cytometry in a FACSCanto™ cytometer with Cellquest software (Becton Dickinson, San Jose, CA).

Proliferation assay

Treated and non-treated JURKAT cells were incubated with a carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Carlsbad, CA) dilution, and the proliferation assay was performed according to standard procedure. Proliferation was measured by loss of CFSE dye by flow cytometry in a FACSCanto™ cytometer with Cellquest software (Becton Dickinson, San Jose, CA).

miRNA expression analysis

Total RNA from tumours was isolated using Trizol Reagent (Invitrogen) or Plus™ Reagent (Invitrogen). Luciferase assays were performed 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase was normalized to Renilla luciferase activity.

Blocking SMO activity

The blockade of SMO activity was carried out in JURKAT cells by treatment with 10 µM KAAD-cyclopamine for 5 days. In addition, 10 µM tomatidine (Sigma) or 10 µM dimethyl sulfoxide was used as baseline controls.

Western blot

Thirty micrograms of protein lysates were separated on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The following antibodies were used: polyclonal Smoothened Drosophila Homolog (MBL International Corporation, Woburn, MA), monoclonal anti-c-tubulin DM1A (Sigma) clone mAb-1D4 (Enzo Life Sciences, Farmingdale, NY), polyclonal anti-rabbit secondary antibody (Cell Signaling Technology, Carlsbad, CA) and sheep anti-mouse secondary antibody (GE Health Care, Piscataway, NJ).

Real-time quantitative reverse transcription–polymerase chain reactions

Gene expression levels were quantified by quantitative reverse transcription–polymerase chain reaction (qRT–PCR) using the LightCycler system (Roche Applied Science). Primers are included in Supplementary Table 1, available at Carcinogenesis Online.

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miRNA expression analysis

Total RNA from tumours was isolated using Trizol Reagent (Invitrogen). Quantifications of miRNA expression were performed using the miRCURY LNA Universal RT microRNA PCR system (EXIQON, Woburn, MA) and LightCycler system (Roche Applied Science). Specific forward and reverse primers were designed by EXIQON. The reference genes used were mmu-miR-451 (mouse) and U6 small RNA (human).

Statistical analysis

Statistical significances were determined using a one-way analysis of variance with a Tukey post-test when multiple comparisons were performed or a Student’s t-test in the case of two-group comparisons. The Pearson correlation coefficient was calculated to assess the linear relationship between normalized expression values of Smoothened and normalized expression values of each miRNA. All statistical tests were performed using the Statistical Package for the Social Sciences software (version 15.0; SPSS, IBM Corporation, Somers, NY).
Results

Smoothened is up-regulated in T-LBL

To identify specific alterations involving the GLI/Hh pathway, we first interrogated mRNA expression profiles of murine T-LBLs induced by γ-irradiation (7) (microarray data deposited in GEO DataSets, GSE10891 record). Interestingly, among the members of the GLI/Hh pathway, only Smo was significantly up-regulated in these lymphomas. This result was validated in eight mouse primary samples of T-LBL (Table I). As well, we analysed four human primary samples of T-LBL with optimum quality status, where SMO was up-regulated. Their SMO expression levels, together with those of the T-cell lymphoblastic leukaemia/lymphoma-derived JURKAT cell line, are shown in Table II. Strikingly, the levels of SMO expression in human lymphomas are markedly higher than the ones observed in mouse lymphomas.

DNA sequencing neither revealed any change in the coding sequence of Smo in the mouse lymphoma samples nor in the coding sequence of SMO in the JURKAT T-cell line (data not shown). Since GLI expression is the ultimate output of the GLI/Hh signalling, we studied the expression levels of GLI1 in the same tumour samples. Six out of the eight mouse tumour samples exhibited a significant up-regulation of Glil (Supplementary Table 2, available at Carcinogenesis Online). All of the human samples analysed showed a significant up-regulation of GLI1 (Supplementary Table 3, available at Carcinogenesis Online).

Up-regulation of Smoothened in T-LBLs may be controlled by specific miRNAs

Comparative genomic hybridization array using the same T-cell lymphoma samples as for the present work had not shown specific chromosomal amplifications involving the Smo locus (7). Therefore, we decided to investigate whether the up-regulation of Smo may be controlled, at least in part, by specific miRNAs. Firstly, we interrogated a miRNA array we had performed previously in these samples (5,7) (GEO DataSets, GSE10891 record: γ-irradiation-induced T-cell lymphomas, miRNA study). Interestingly, a repertoire of 41 miRNAs was found to be significantly down-regulated, 7 of them (mmu-miR-30a, mmu-miR-141, mmu-miR-345-5p, mmu-miR-324, mmu-miR-125b, mmu-miR-326, and mmu-miR-200a) being putative controllers of murine Smo expression according to bioinformatics predictions [TARGETSCANS and PICTAR databases (29)]. However, only two out of these miRNAs, mmu-miR-30a and mmu-miR-141, were confirmed to be significantly down-regulated in all the mouse tumour samples used in this study and to show an adequate correlation with the expression levels of Smo (Table I). Correlation factor analysis indicates that, in six out of eight mouse lymphomas, the up-regulation of Smo tends to be related to a down-regulation of any of the two miRNAs selected (r = −0.50 for mmu-miR-30a; r = −0.55 for mmu-miR-141). However, since negative correlation coefficients are higher than −1.0, other factors, probably additional down-regulated miRNAs not included in our miRNA array, may also be involved.

To check whether these two miRNAs target Smo by interaction with its 3′-UTR, as predicted from the alignment, we transfected NIH-3T3 cells with a luciferase reporter vector carrying the mouse Smo 3′-UTR (pG-Smo 3′-UTR). As expected, co-transfection of these cells with any of the two miRNAs resulted in significant reductions of luciferase activity, unlike what resulted from the co-transfection with miR-203, irrelevant for Smo expression (Figure 1A). Then, we analysed the expression levels of Smo in cells transfected with vectors carrying mmu-miR-30a (miR-Vec-30a), miR-141 (miR-Vec-141) or miR-203 (miR-Vec-203) (Figure 1B). As indicated in Figure 1B and C, a significant reduction in the expression levels of Smo was observed both through qRT–PCR (Figure 1B) and western blot (Figure 1C), thus validating that Smo is actually a target for these miRNAs. To verify that Smoothened regulation by these miRNAs has an effect in the GLI/Hh signalling pathway, we studied the expression levels of mouse
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Gli1 after exogenous expression of mmu-miR-30a and mmu-miR-141 (Figure 1D). Our results indicate a significant down-regulation of Gli1.

In human samples, the scenario appears to be more complex, as the SMO 3′-UTR is different from that of the mouse gene and subsequently, it may be recognized by other miRNAs. Nonetheless, 2 of the 41 miRNAs down-regulated in the mouse miRNA arrays, hsa-miR-193b and hsa-let-7e, emerged as putative controllers of human SMO expression (TARGETSCANs and PICTAR databases (29)). It, therefore, led us to check the expression levels of these miRNAs by qRT–PCR in the aforementioned human samples. Interestingly, miR-193b was significantly down-regulated in three out of four human samples, similarly as in the JURKAT cell line (Table II). The lack of negative correlation observed in the primary sample #11010521 indicated that other miRNAs could be involved in controlling the up-regulation of SMO in this sample. Unfortunately, hsa-let-7e showed inconsistent results and was not considered for further study (data not shown).

To verify whether SMO may be a target for hsa-miR-193b, we transfected NIH-3T3 cells with a construct carrying the human SMO 3′-UTR downstream a luciferase reporter (pSGG-SMO-3′-UTR). Notably, co-transfection of these cells with a vector expressing miR-193b (miR-Vec-193b) caused a significant reduction of luciferase activity, unlike what resulted from the co-transfection with miR-30a (miR-Vec-30a), irrelevant for SMO expression. This indicates that miR-193b may influence SMO expression levels through specific binding to its 3′-UTR (Figure 2A). Moreover, the reduction in luciferase activity induced by hsa-miR-193b may be reversed after mutation of four positions in the seed sequence of miR-193b target site (Figure 2B and C). We analyzed the expression levels of SMO in JURKAT cells after transfection of hsa-miR-193b and hsa-miR-30a, confirming that hsa-miR-193b was able to induce a significant reduction in the expression levels of SMO (Figure 2D). Such reduction was observed both through qRT–PCR (Figure 2D) and western blot (Figure 2E), thus validating that SMO is actually a target for these miRNAs. To verify that Smoothened regulation by this miRNA has an effect in the GLI/Hh signalling pathway, we studied the expression levels of human GLI1 after exogenous expression of hsa-miR-193b (Figure 2F). Our results indicate a significant down-regulation of GLI1.

SMO abrogation reduces cell survival and proliferation

To assess the cellular effects of inhibiting GLI/Hh signalling through SMO abrogation, we performed in vitro studies of cell viability and proliferation. The T-cell lymphoblastic leukaemia/lymphoma–derived JURKAT cell line was infected with retroviral particles expressing a pool of SMO-specific shRNAs. We checked that interference with shRNAs elicited a dramatic reduction of 98% in SMO mRNA levels (Figure 3C). Our results showed that previously selected SMO-deficient cells exhibited a significant reduction in their viability (Figure 3A) and proliferation (Figure 3B) of JURKAT cells. In order to overcome the specificity question regarding the use of a pool of shRNAs, these results were corroborated in JURKAT cells after specific neutralization of SMO activity by treatment with cyclopamine (Figure 3D and E), unlike what resulted from the treatment with the control alkaloid tomatidine. The effect on the GLI/Hh signalling was assessed by determining GLI1 significant down-regulation (Figure 3F).
Mechanisms whereby SMO up-regulation may elicit the tumorigenic effects

Concerning the mechanisms underlying the tumorigenic effects of SMO over-expression, we found that SMO abrogation in JURKAT cells using specific shRNAs or cyclopamine induced significant reductions of BCL-2 and CCND3 expression levels (Figure 4A and B). The treatment with tomatidine does not have any negative effect on the normal viability and cell cycle processes.

Discussion

We report here the first evidence, to our knowledge, for up-regulation of wild-type Smoothened in mouse and human T-LBL samples. This event entails activation of the pathway, as shown by up-regulation of Gli1/GLI1 in the T-LBL samples. Interestingly, the activation of the GLI/Hh pathway in T-LBL may promote alterations in cell survival and proliferation. This suggests an oncogenic role for SMO up-regulation in these lymphomas.

Previous evidence has shown that an abnormal activation of the GLI/Hh pathway may contribute to accelerate either the onset of tumorigenesis or the rate of tumour growth (30). The activation of the pathway in haematological tumours does not seem to occur through mutations in GLI/Hh signalling members. Current data suggest that in most haematopoietic neoplasms, the GLI/Hh signalling does not play an independent role in tumour initiation but instead contributes to tumour maintenance, growth and resistance to chemotherapy. Activation of GLI/Hh signalling through SMO up-regulation has been reported in BCR-ABL1-positive leukaemia stem cells (31). High expression of SMO has been detected in tumour samples and cell lines from DLBCL (24,32), as well as in MCL, an aggressive subtype of B-cell lymphoma (18). Moreover, abnormal expression of SMO has been reported as an early event in the formation of human pancreatic tumours (33).

Increasing evidence in the literature has shown that miRNAs play a pivotal role in haematological malignancies (34), and several miRNAs (miR-324-5p, miR-125b and miR-326) have been identified as suppressors of Smoothened and GLI/Hh signalling during medulloblastoma formation (35). It has also been shown that the microRNA-17/92 cluster synergizes with GLI/Hh signalling in cerebellar granule cell precursors and in medulloblastoma (36). However, specific miRNAs...
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had not been involved previously in the activation of GLI/Hh signaling in malignant lymphomas. Interestingly, we show here that up-regulation of Smoothened in T-LBLs correlates with down-regulation of specific miRNAs. In particular, two distinct miRNAs, mmu-miR-30a and mmu-miR-141, may contribute to the reduction of Smo expression in mouse T-LBLs. The down-regulation of hsa-miR-193b seems to exhibit a similar effect on the orthologous human SMO gene in human T-cell lymphoblastic leukaemia/lymphoma cells. Thus, loss of function of specific miRNAs would be a mechanism by which Smoothened is unrestrained in these tumours. The magnitudes of the down-regulations induced by specific miRNAs on Smoothened and GLI1/GLI1 exhibit differences with those induced by the treatment with shRNAs against SMO and/or cyclopamine. This can be attributed to the expected subtle effect of a single miRNA on its target. However, the biological consequences of the altered regulation of SMO and the GLI/Hh pathway become evident by the SMO abrogation experiments, thus indicating that sustained up-regulation of wild-type Smoothened and the concomitant activation of the GLI/Hh signalling pathway may contribute to the progression of a subset of T-cell lymphomas.

The fact that inappropriate activation of GLI/Hh signalling promotes cell survival and proliferation has been widely demonstrated in haematological malignancies, as shown in chronic lymphocytic leukaemia (19), DLBCL (24), MCL (18), ALK-positive ALCCL (37), CML (31, 38), AML (39) and ALLs (20, 21). In these tumours, inhibition of abnormal GLI/Hh signalling resulted in a reduction of tumour cell number. With particular regard to the mechanisms by which over-expression of Smo may enhance cell viability and proliferation, it has been reported that the GLI/Hh signalling regulates proliferation and survival by activation of cyclins (D and E) and cyclin-dependent kinases (40) as well as pro-survival factors such as Bcl-2 (17, 41). Several works have reported over-expression of CCND3 in this type of lymphomas (42, 43). Moreover, deregulation of CCND3 has been reported in one case of T-ALL showing a relocation of the CCND3 locus to the immunoglobulin heavy locus (44). However, there is currently no indication of the connection between the over-expression of cyclins and specific oncogenic pathways in patients with T-LBLs. In contrast to B-cell lymphomas, little evidence on the expression of BCL-2 and other family members is available in T-cell lymphomas. Alterations of BCL-2 expression patterns have been related to apoptosis and proliferation in peripheral T-cell lymphomas (45). More recently, Feng et al. (46) reported high levels of BCL-2 expression in T-lymphoblastic lymphoma cells. Our data suggest, for the first time, that up-regulation of SMO may be a mechanism whereby T-cell lymphoblastic lymphoma cells elicit the up-regulation of CCND3 and BCL-2, thus contributing to their survival and proliferation.

In conclusion, our observations suggest a model according to which miRNA-based up-regulation of Smoothened may contribute to T-cell lymphomagenesis, a process known to involve other genetic abnormalities and the deregulation of other relevant signalling pathways such as Notch signalling (3–6). Although GLI/Hh signalling pathway blockade alone might be insufficient for the treatment of lymphomas or leukaemias, these data provide a rationale for using a new therapeutic approach to treat this type of lymphomas, based on controlling Smoothened expression or the GLI/Hh signalling pathway activity, at least as an adjuvant to increase the susceptibility to current chemo-therapeutic agents or to potentiate other targeted therapies for haematological neoplasms.

Supplementary Material

Supplementary Tables 1–3 can be found at http://carcin.oxfordjournals.org/.

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