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FAS-antisense 1 lncRNA and production of soluble versus membrane Fas in B-cell lymphoma

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Abstract

Impaired Fas-mediated apoptosis is associated with poor clinical outcomes and cancer chemoresistance. Soluble Fas receptor (sFas), produced by skipping of exon 6, inhibits apoptosis by sequestering Fas ligand. Serum sFas is associated with poor prognosis of non-Hodgkin's lymphomas.

We found that the alternative splicing of Fas in lymphomas is tightly regulated by a lncRNA corresponding to an antisense transcript of Fas (FAS-AS1). Levels of FAS-AS1 correlate inversely with production of sFas and FAS-AS1 binding to the RBM5 inhibits RBM5-mediated exon 6 skipping. EZH2, often mutated or overexpressed in lymphomas, hyper-methylates the FAS-AS1 promoter and represses the FAS-AS1 expression. EZH2-mediated repression of FAS-AS1 promoter can be released by DZNeP or overcome by ectopic expression of FAS-AS1, both of which increase levels of FAS-AS1 and correspondingly decrease expression of sFas. Treatment with Bruton's tyrosine kinase (BTK) inhibitor or EZH2 knockdown decreases the levels of EZH2, RBM5 and sFas thereby enhances Fas-mediated apoptosis.

This is the first report showing functional regulation of Fas repression by its antisense RNA. Our results reveal new therapeutic targets in lymphomas and provide a rationale for the use of EZH2 inhibitors or ibrutinib in combination with chemotherapeutic agents that recruit Fas for effective cell killing.

Keywords

Fas; FAS-AS1 lncRNA; RBM5; EZH2; DZNeP; Ibrutinib

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

Authorship and contributions

LS designed, performed the majority of the experiments, and analyzed the data. RM, FKB, and JW performed some of the experiments. FS, SN & LWK reviewed work, provided critical suggestions and tumor specimens. LS, ZB and FS contributed to the research design, data analysis and writing of the manuscript.

Supplementary Material and Methods

Supplementary information is available at *Leukemia's* website.

INTRODUCTION

Fas (APO-1, CD95, and TNFRSF6) is a member of the tumor necrosis factor receptor superfamily that plays a major role in the extrinsic pathway of apoptosis. Once Fas is activated by its ligand (FasL), a cascade of events leads to apoptosis.¹ Fas receptor is expressed by B and T-cells, as well as numerous tumor cells and some normal human tissues, yet sensitivity to Fas-mediated apoptosis does not always correlate with Fas expression levels.² Defects of the Fas-mediated apoptotic pathway are often associated with lympho-proliferative disorders and autoimmune diseases.^{3–5}

Overall survival of individuals with non-Hodgkin's lymphoma (NHL) has improved in recent years with advancements in chemotherapy regimens.⁶ However, NHL still demonstrates frequent relapses and a high mortality rate of nearly 30%.⁶ One of the likely mechanisms for NHL relapse is the survival and expansion of cells that are resistant to Fas-mediated apoptosis. It has been shown that the cells lacking Fas or cells with defective Fas-signaling are resistant to conventional doses of chemotherapy and radiation.^{7–10} Fas levels and its signaling are thus important determinants of the effectiveness of chemotherapy.

Tumor cells can evade apoptosis by several mechanisms; one of them is the release of soluble decoy receptors. The Fas mRNA can be alternatively spliced during mRNA maturation to either include or exclude exon 6 that encodes the trans-membrane domain of Fas receptor.^{11–14} The serum levels of the soluble isoform of the Fas receptor (sFas) are elevated in patients with malignant lymphoma and chronic lymphocytic leukemia (CLL).^{15–18} Soluble Fas blocks apoptosis induced by FasL in vitro, suggesting that production of sFas in lymphoma might be protective¹⁹ and consistent with poor clinical outcomes.¹⁸ The overall survival and disease-free survival rates are significantly lower in lymphoma patients if they express elevated serum sFas levels.¹⁸

The current challenge is to understand regulation of Fas signaling in lymphoma in order to overcome resistance to FasL and chemotherapy. In this study, we investigated the regulation of alternative splicing of Fas pre-mRNA that produces soluble decoy Fas receptor known to be up-regulated in lymphoma and a likely source of chemoresistance.

MATERIALS AND METHODS

Cells

Granta-519 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Remaining cell lines were obtained from the American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 (Hyclone) with 10% fetal bovine serum (Atlanta Biologicals) (RPMI-1640/10% FBS) in 5% CO₂ atmosphere at 37°C. Cell lines were authenticated by STR analysis (MD Anderson Cancer Center Characterized Cell Line Core) and regularly tested for mycoplasma (Lonza).

Peripheral blood B-lymphocytes were isolated from healthy donors' blood, obtained from Gulf Coast Blood Center (Houston, TX), with CD19-positive magnetic beads and released with the competitive CD19 DETACHaBEAD (Invitrogen).

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted with RNeasy Mini Kit according to the manufacturer's instructions (Qiagen Sciences). The first-strand cDNA was synthesized using a Superscript™ II reverse transcriptase kit (Invitrogen) according to the manufacturer's protocol. Samples were analyzed on 96-well microtiter plates using the StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with primers listed in the Supplementary Table 1 using 40 cycles of 95°C for 15 seconds and 60°C for 1 minute and by SYBR green method for detection. qRT-PCR data were analyzed by the Step-One software version 2.1.

RNA Immunoprecipitation (IP) and immunoblotting (IB)

For precipitation of RNA-protein complexes, 1×10^7 cells were homogenized using EZ Magna RIP kit per manufacturer's protocol (Millipore). Supernatants were incubated with 1–2 µg of anti-RBM5 (Active motif) or mouse IgG (Invitrogen) for 1 hour at 4°C followed by precipitation with protein A/G agarose (Pierce).

IB was performed according to standard protocols as described previously.^{20,21} Proteins were detected by IB with anti-RBM5, anti-EZH2, anti-histone-3 (Santa Cruz Biotechnology), anti-PARP, anti-cleaved caspase-3, and anti-caspase-8 (Cell Signaling Technologies) primary antibodies (1:1000 dilution in 5% nonfat milk), followed by the corresponding HRP-conjugated secondary goat anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch). Equal loading was verified by blotting with anti-β-actin-HRP antibody (1:10,000; Sigma Aldrich). Visualization was achieved by Supersignal West Pico chemiluminescent substrate (Pierce).

Chromatin Immunoprecipitation (ChIP)

Cells were crosslinked by incubation with 1% formaldehyde (Sigma Aldrich) at 37°C for 15 minutes. The reaction was stopped by addition of glycine (0.125 M final concentration; Sigma Aldrich) and processed for chromatin isolation as described in supplementary methods.

Enzyme-linked immunosorbent assay (ELISA)

Cells (0.1×10^6 /mL) were grown for 24 hours as described above before incubating with 1 µM DZNeP (Cayman Chemical). Cell culture supernatants were collected 24 hours later and analyzed for sFas levels by ELISA (R&D Systems).

Apoptosis induction and flow cytometry analysis

Cells were incubated with DZNeP or ibrutinib (PCI-32765; Selleckchem) in 1mL of RPMI-1640/10% FBS for 24 hours. Cell death was induced by incubation with the indicated dose of super FasL (Enzo) in RPMI-1640/10% FBS for 18 hours at 37°C. Apoptosis was analyzed by flow cytometry using PI apoptosis kit (BD Bioscience) according to manufacturer's recommendations.

For detection of surface proteins and ligand binding, cells were washed in 1% FBS/PBS and incubated with 0.25 µg/mL of mouse IgG blocking reagent (Invitrogen). Cells were then incubated with either PE-conjugated anti-Fas antibody (UB2; Beckman Coulter), isotype

control mouse IgG₁-PE, or 3µg of FLAG-tagged FasL (Enzo) at 4°C for 20 minutes in the dark. Phycolink anti-FLAG-RPE (Prozyme) was used for detection of bound FasL. Flow cytometry was performed on a LSR Fortessa flow cytometer with Diva software (BD Bioscience). Data were analyzed with FlowJo software (Tree Star).

Plasmids and transfections

The pcDNA3 encoding FAS-AS1 (Saf gene) and empty vector were a gift from Dr. Suan Chuang, Taiwan.²² The pLKO.1 vector control and EZH2 shRNA expressing plasmids were a gift from Dr. Kunal Rai, USA. Plasmid encoding T7-RBM5, and Fas-WT minigene were a gift from Juan Valcarcel, Spain.¹² The transfection of the plasmids was performed using Lipofectamine 2000 (Invitrogen) per the manufacturer's protocol.

Statistical analysis

Experimental data are reported as means ± SEM from three independent experiments, unless otherwise indicated. Differences between groups were calculated using the two-tailed Student's *t*-test (GraphPad Prism, La Jolla, CA). A P-value less than .05 was considered statistically significant.

RESULTS

It has been reported previously that the serum levels of sFas are elevated and often associated with the poor outcome of NHLs thereby suggesting that the level of sFas might be important for the evasion of apoptosis and chemoresistance.¹⁸ A qRT-PCR analysis using specific primers to quantify total Fas mRNA and alternatively spliced Fas mRNA encoding sFas revealed that alternative splicing of Fas pre-mRNA to sFas isoform (skipping of exon 6) is highly up regulated in lymphoma-derived cell lines and tissues (Figure 1A and Supplementary Figures 1A–B).

An antisense RNA transcribed from the opposite strand of the intron 1 of the human Fas gene, FAS-AS1, was identified just recently.²² To test the hypothesis that FAS-AS1 might regulate exon 6 skipping, we determined the expression level of FAS-AS1 lncRNA in lymphoma cell lines and tissue samples by qRT-PCR. The levels of FAS-AS1 lncRNA were substantially reduced in primary lymphomas and lymphoma cell lines when compared to healthy donor CD19⁺ B lymphocytes (Figure 1B and Supplementary Figure 1C). We plotted the levels of sFas mRNA and FAS-AS1 lncRNA in lymphoma and other cell lines on X-Y graph (Figure 1C) and calculated Pearson correlation coefficient ($r^2 = -0.58$) that suggested their inverse correlation and thus, a possible involvement of FAS-AS1 in regulation of alternative isoform expression as observed in other pairs of gene and anti-sense mRNA regulatory models.²³ We screened GEO Database Profiles (<http://www.ncbi.nlm.nih.gov/geo/>), and confirmed the abundance of FAS-AS1 lncRNA in normal tissues.

The gene expression is regulated by various genetic and epigenetic factors such as hypermethylation of promoter-associated histone H3.²⁴ To elucidate the methylation status of the FAS-AS1 promoter, we performed the ChIP-qPCR for trimethylation of lysine 27 of histone H3 (H3K27me3) using the primers spanning the promoter of FAS-AS1 (90752786–90753636) in Z-138 cells, which exhibited clear inverse correlation of sFas and FAS-AS1.

The positions of the designed primers were determined from the transcription start site (TSS, A; -400, B; -900). The specific primer from the FAS-AS1 lncRNA (region C, +700) served as the negative control, and the qPCR for α -satellite DNA served as a positive control for the H3K27me3 ChIP. The promoter region of FAS-AS1 was hyper-methylated as compared to the respective IgG control (Figure 1D). The H3K27me3 was not observed within the non-coding region of FAS-AS1 lncRNA (region C; +700), serving as a negative control. However the positive control, α -satellite DNA showed tri-methylation confirming the sensitivity of the ChIP experiment. This result suggested that the expression of FAS-AS1 in lymphoma is repressed by promoter hyper-methylation.

The H3K27me3 is carried out by the methyl transferase EZH2.²⁵ We hypothesized that elevated levels and/or increased activity of EZH2 in lymphoma cells increase H3K27me3 at FAS-AS1 promoter and thus decrease FAS-AS1 expression. We first analyzed gene expression data from 89 lymphoma tissue samples and 34 lymphoma cell lines in the ONCOMINE (cancer microarray database and integrated data-mining platform).²⁶ We compared mRNA levels of EZH2 in lymphomas to healthy donor's B-lymphocytes from Basso's Lymphoma dataset²⁷ and Baretinna Cancer Cell Line Encyclopedia dataset²⁸ (Figures 1E-F and Supplementary Figure 1D). This analysis showed that EZH2 mRNA is highly expressed in lymphomas (Burkitt's [BL], diffuse large B-cell [DLBCL], follicular [FL], and mantle cell [MCL]) compared to normal lymph nodes (Supplementary Figure 2A; www.proteinatlas.org). The micro RNA analysis (August 2010 release of microRNA.org) also revealed significantly lower levels of miR-26a (Supplementary Figures 2B-C), which is known to negatively regulate the expression of EZH2.²⁹ These results suggested that the EZH2 expression is substantially up regulated in B-cell lymphomas and may contribute to the hyper methylation of FAS-AS1 promoter.

DZNeP (3-Deazaneplanocin A) is a methyl transferase inhibitor of H3K27me3 and thus it can de-repress silenced genes.³⁰ We treated cells with DZNeP to analyze its effect on the levels of EZH2, and total H3K27me3 by IB. DZNeP reduced the levels of H3K27me3 without changing EZH2 levels (Figure 2A). To test whether DZNeP inhibits the activity of EZH2, we performed a direct EZH2 activity assay. The transfer of methyl group to the H3 peptide was significantly reduced in the presence of DZNeP (Figure 2B), confirming that DZNeP effectively ablates EZH2 enzymatic activity. Using ChIP validated antibody specific to H3K27me3, we confirmed decreased FAS-AS1 promoter methylation in cells treated with DZNeP (Figures 2C-D).

Our data suggested that FAS-AS1 expression can be modulated by DZNeP. To determine whether the production of sFas is altered by treatment with DZNeP, we analyzed sFas mRNA levels in DZNeP-treated Z-138 cells and the levels of sFas in the media from lymphoma cell lines. Consistent with the results above, treatment with DZNeP showed a profound increase in levels of FAS-AS1 lncRNA (Figure 2E) and modest but significant decrease in sFas mRNA (Figure 2F) in Z-138 cells. A more detailed analysis of DZNeP-treated cells showed decreased production of sFas as determined by ELISA (Figures 2G). We concluded that DZNeP inhibits histone methylation of the FAS-AS1 promoter, leading to altered Fas mRNA processing responsible for the decreased levels of sFas.

We hypothesized that decreased levels of sFas mRNA should translate into elevated levels of membrane-bound Fas (mFas) mRNA and thus mFas protein levels. We analyzed DZNeP- and buffer-treated lymphoma cell lines for surface mFas expression by flow cytometry and confocal microscopy.²⁰ Both methods showed consistently elevated mFas levels in DZNeP-treated cells (Figure 3A and Supplementary Figure 2D, respectively). These results suggested that the inhibition of EZH2 can indirectly influence the expression of mFas.

To test whether elevated mFas conferred enhanced binding of FasL, we used flow cytometry to evaluate the ability of DZNeP- and buffer-treated lymphoma cell lines to bind FasL. All tested lymphoma cell lines bound significantly higher levels of FasL upon DZNeP treatment (Figure 3B). Pre-treatment with DZNeP also enhanced the levels of FasL-induced apoptosis (Figure 3C) in proportion to enhanced surface expression of mFas (Figure 3A).

An IB analysis of the Fas signaling cascade in the Z-138 cells, which were sensitized to FasL by DZNeP, showed increased processing of pro-caspase-8 in DZNeP pre-treated cells compared to buffer-treated controls (Figure 3D), confirming enhanced proximal Fas signaling at the death-inducing signaling complex.

Thus far, we determined that DZNeP-induced increase in expression of FAS-AS1 shows a reciprocal reduction of sFas and increased mFas expression. In the next step, we tested if the ectopic expression of FAS-AS1 could regulate skipping of exon 6.

A transient expression of FAS-AS1 in Z-138 cells and HeLa cells (Figure 4A and Supplementary Figure 3A, respectively) showed decreased exon 6 skipping (Figure 4B, right panel) that translated into decreased sFas/mFas mRNA ratio (Figure 4B and Supplementary Figure 3B). The FAS-AS1 lncRNA expression levels served as positive control for the ectopic expression (Figure 4A and Supplementary Figure 3A). The FAS-AS1 expressing vector was co-transfected with a plasmid encoding GFP and cells were sorted based on the GFP expression to select FAS-AS1 expressing cells for further analysis. The expression of FAS-AS1 was accompanied by increased expression of mFas and enhanced rates of Fas-mediated apoptosis compared to vector transfected cells (Figures 4C–D). This result suggested that FAS-AS1 lncRNA may stimulate the expression of cell surface mFas and thus also FasL-induced apoptosis.

If FAS-AS1 mediates the apoptosis sensitizing actions of DZNeP, then the cells with knocked-down Fas-AS1 will show attenuated Fas-mediated apoptosis with DZNeP. To test this hypothesis, we used siRNA to knock-down FAS-AS1 and tested the effects of DZNeP on responses to FasL. As predicted, cells transfected with FAS-AS1 siRNA showed more than 50% reduction of sFas induced apoptosis compared to vector control (Figure 4E). As predicted, cells transfected with FAS-AS1 siRNA remained resistant to Fas-mediated apoptosis when treated with DZNeP (Figure 4F) confirming that FAS-AS1 mediated, the apoptosis effects of DZNeP (Figures 4F–G).

To confirm whether the DZNeP-mediated effects are specific to methyl transferase EZH2, we used the shRNAs to knockdown EZH2 in Z-138 cells. The levels of EZH2 were reduced by one of the three tested shRNA clones (Figure 5A–B). Analysis RNA confirmed that the reduction in the levels of EZH2 was accompanied by increase of FAS-AS1 lncRNA and

decrease of sFas. This result suggested that EZH2 may regulate the expression levels of FAS-AS1 in Z-138 cells.

We hypothesized that decreased levels of EZH2 should translate into elevated levels of membrane-bound Fas (mFas) mRNA and thus mFas protein levels. We analyzed EZH2 shRNA and control shRNA transfected Z-138 cells for surface mFas expression by flow cytometry. The surface mFas levels were elevated in Z-138 cells expressing EZH2 shRNA when compared to vector controls (Figure 5C). These results confirmed that the inhibition of EZH2 may regulate the levels of FAS-AS1 lncRNA and influence mFas expression. The increased expression of mFas was accompanied by correspondingly enhanced rates of FasL-induced apoptosis in Z-138 cells expressing EZH2 shRNA as compared to vector transfected cells (Figures 5D). These results suggested that EZH2 knockdown in cells may stimulate the expression of FAS-AS1 and in turn regulate the cell surface mFas levels and FasL-induced apoptosis.

RBM5 (RNA binding motif protein 5) contains structural elements shared by factors involved in pre-mRNA splicing and other aspects of RNA regulation: two RNA recognition motifs (RRM), two zinc fingers, one arginine-serine rich domain, and one glycine patch.³¹ Because RBM5 was shown to promote exon 6 skipping,¹² we hypothesized that FAS-AS1 lncRNA might bind directly to RBM5 and alter skipping of exon 6.

We first queried the FAS-AS1 and RBM5 sequences through *catRAPID* algorithm to estimate their binding propensity.³² This algorithm predicted that FAS-AS1 lncRNA could bind to two RRM (RRM1 and RRM2) of RBM5 as depicted in the schematic representation (Figure 6A). To test whether FAS-AS1 lncRNA indeed binds RBM5, we treated Z-138 cells with DZNeP and subjected them to IP using RBM5 antibody followed by RNA extraction and qRT-PCR with FAS-AS1-specific primers. This analysis revealed that RBM5 binding to FAS-AS1 lncRNA is enhanced in DZNeP-treated cells (Figures 6B–C). This enhanced association of FAS-AS1 with RBM5 was accompanied by decreased association of RBM5 with Fas pre-mRNA (Figure 6D), suggesting a direct involvement of FAS-AS1 in regulation of exon 6 skipping. Notably, DZNeP did not change the expression levels of either EZH2 or RBM5 (Figure 6B, right panel). As a specificity control of FAS-AS1 binding to RBM5, we assayed a possible interaction of other known Fas mRNA splicing protein, HuR, with FAS-AS1 lncRNA by RNA-IP. As predicted, HuR did not interact with FAS-AS1 (Supplementary Figure 3H).

Taken together, our data suggested that DZNeP increases FAS-AS1 lncRNA, which sequesters RBM5 away from Fas pre-mRNA, and thereby suppresses its alternative splicing into soluble form of Fas (sFas).

To confirm that FAS-AS1 regulate RBM5-mediated splicing of Fas pre-mRNA, we co-transfected HeLa cells with plasmids encoding Fas minigene, FAS-AS1, or RBM5 and evaluated the expression of sFas and mFas mRNAs (Supplementary Figure 3C). Expression of RBM5 promoted exon skipping and increased the sFas/mFas mRNA ratio. On the other hand, co-transfection with FAS-AS1 significantly decreased this ratio, showing that FAS-

AS1 has the dominant effect and confirmed FAS-AS1 interference with RBM5-mediated exon 6 skipping.

We concluded that inhibition of EZH2 activity by DZNeP elevates FAS-AS1 lncRNA, which then sequesters RBM5 from Fas pre-mRNA and thus inhibits skipping of exon 6 and lowers the expression of sFas.

Ibrutinib (PCI-32765) is a selective inhibitor of the Bruton's tyrosine kinase (BTK),³³ the major mediator of B-cell receptor signaling³⁴ and thus crucial for B-cell development.³⁵ Ibrutinib was shown to downregulate c-Myc,³⁶ which inhibits expression of miR-26a, a known negative regulator of EZH2 expression.²⁹ Thus, we tested whether this BTK-targeting drug affects expression of EZH2 and subsequently interaction of FAS-AS1 with RBM5 and expression of sFas/mFas.

We analyzed total cellular RNA from Z-138 cells treated with ibrutinib or buffer for 40 hours by qRT-PCR. The predominant effect was an increased expression of FAS-AS1 lncRNAs followed by upregulation of mFas mRNA and downregulation of sFas. As expected, mRNA levels of both EZH2 and RBM5 were reduced in ibrutinib-treated cells (Figure 7A). These changes were accompanied by elevated expression of mFas on the surface of ibrutinib-treated cells and corresponding enhancement of Fas-mediated apoptosis (Figures 7B–C).

Our results thus suggest that lymphoma cells can be sensitized to Fas-mediated apoptosis through decreasing the expression of EZH2 and RBM5 using BTK inhibitor ibrutinib or by directly targeting the activity of EZH2 by DZNeP.

DISCUSSION

We report here that the long-noncoding antisense RNA to Fas (FAS-AS1) acts as a novel modulator of Fas resistance. Primary B-cell lymphomas and lymphoma-derived cell lines showed elevated expression of sFas relative to healthy B-lymphocytes and concomitantly low expression of FAS-AS1 lncRNA. Our data revealed that the expression of FAS-AS1 is repressed by the EZH2-mediated tri-methylation of H3K27 at its promoter. This repression is released by H3K27me3 inhibitor DZNeP and by selectively downregulating the expression of EZH2. RNA binding protein RBM5 is known to enhance the skipping of exon 6 during Fas pre-mRNA processing. We found that the FAS-AS1 lncRNA binds to RBM5 and interferes with RBM5-mediated skipping of exon 6. Thus, our data show that RBM5 and EZH2 coordinate the skipping of exon 6 resulting in the production of sFas. BTK inhibitor ibrutinib decreased the expression of EZH2, RBM5, and subsequently sFas levels and increased the expression of FAS-AS1 and membrane-bound Fas. This two prong process sensitized lymphoma cells to Fas-mediated apoptosis (Figure 8). However, we cannot fully exclude additional effects of ibrutinib on apoptotic pathways.

NHL includes lymphoid malignancies ranging from slowly growing indolent lymphomas to aggressive lymphomas such as BL, DLBCL, and MCL, each of them characterized by a distinct histopathology and cytogenetic abnormalities. Yet, for over a decade, the standard of care for intermediate grade NHLs has been CHOP therapy regimen (cyclophosphamide,

doxorubicin, vincristine, and prednisone) with a 5-year survival for patients just above 60%. Recent advancement in targeted therapy with the addition of anti-CD20 antibody rituximab to the CHOP regimen, has improved remission rates, but mortality rate still remains relatively high, around 30%.⁶ In the current year, 213 companies are developing over 300 new lymphoma drugs (Lymphoma Drug Pipeline Update 2013). Targeting overexpressed, histology-specific molecules contributing to lymphoma development or lymphoma cell survival should further improve treatment outcomes.

EZH2, the catalytic subunit of the Polycomb Repressor Complex 2 involved in H3K27 methylation responsible for epigenetic regulation of gene expression, is overexpressed in NHLs and is often associated with disease progression and poor prognosis.^{37,38} EZH2 is required for germinal center formation and its mutants promote lymphoid transformation.³⁹ Somatic mutations in EZH2 are present in 22% of germinal-center DLBCL and 17% of follicular lymphomas, which together with the common overexpression of EZH2 in lymphomas make EZH2 a prominent defect in NHL.^{40,41} EZH2 mutants shift its functionality from mono-methylation of H3K27 towards tri-methylation, resembling the effect of EZH2 overexpression, leading to enhanced repression of EZH2 targets.^{42,43} Inhibitors of EZH2 and global H3K27me3 (GSK126, EPZ005687, and DZNep) were successfully tested in tissue culture and animal models. GSK126 and EPZ005687 effectively inhibited wild-type and mutant EZH2 but had the most potent growth suppressive effect on EZH2 mutant DLBCL cell lines.^{44,45} DZNep showed promising results in preclinical models of MCL.⁴⁶ Inhibitor of histone methyltransferase EZH2, EPZ-6438 (Epizyme), is being tested in phase I/II clinical trial for DLBCL and FL.

DLBCL, MCL, and CLL depend on BCR signaling for their survival.^{47–49} BTK is the main mediator of B-cell receptor signaling crucial for the proliferation and survival of B-cells and was proposed as a suitable target for lymphoma therapy.^{50,51} The BTK inhibitor ibrutinib (Janssen Biotech) is being tested in phase III clinical trials for CLL (fast track) and MCL, phase II trial for DLBCL and multiple myeloma, and in phase I trial for FL. Another two BTK inhibitors, CC-292 (Celgene) and ONO4059 (Ono Pharma USA), are being tested in phase I trials for lymphomas and the former also as a maintenance therapy for acute myelogenous leukemia.

Fas death receptor stimulation by FasL-bearing cells is important for the elimination of unwanted or damaged cells, particularly in the lymphoid system.^{52,53} Fas-disabling mutations lead to autoimmune disorders and lymphoma because of the failure to eliminate autoreactive lymphocytes.^{54,55} Fas has evolved to be a key component of responses to chemotherapy, especially genotoxic agents, that elevate expression of Fas and/or FasL to effectively eliminate tumor cells.^{56,57} Cells with defective Fas or sub optimal Fas expression are resistant to regular doses of chemotherapy and radiation.^{7–10} One of the mechanisms of Fas resistance is a production of soluble Fas decoy (sFas), which is able to sequester FasL away from membrane-bound Fas receptor and thus protects lymphoma cells from Fas-mediated apoptosis.¹⁹ The serum levels of sFas are elevated in patients with malignant lymphoma and CLL.^{15–18} The overall survival and the disease-free survival rates are significantly lower in patients with aggressive NHL and elevated serum sFas levels.¹⁸

Although it was known that sFas is produced by an alternative splicing of Fas pre-mRNA (skipping of exon 6) that involves RBM5, mechanisms regulating this process in cancer cells remained largely unknown.¹² This study reveals cooperation between EZH2-mediated suppression of FAS-AS1 lncRNA and RBM5-mediated exon 6 skipping leading to production of sFas, which is a candidate mechanism for resistance to Fas-mediated apoptosis. Targeting of this mechanism by DZNeP and/or ibrutinib results in the coordinated suppression of sFas and expression mFas to overcome Fas resistance as illustrated in Figure 8. Although we cannot exclude a possibility that FAS-AS1 and RBM5 affect expression of other molecules involved in Fas signaling and its regulation, such as cFLIP,¹² our data clearly show that FAS-AS1 sensitizes cells to Fas-mediated apoptosis at least in part by regulation of Fas splicing through sequestration of RBM5. RBM5 is considered a tumor suppressor that is down regulated in a majority of solid tumors. In contrast, expression of RBM5 RNA was reportedly upregulated in 5-fluorouracil-resistant colorectal and breast cancer cells,⁵⁸ and breast and ovarian cancers.⁵⁹ In other study reported,⁵⁹ RBM5 RNA expression was positively correlated with expression of the HER2 oncogene.

We found that the basal level of RBM5 is very low in healthy donor B-cells (also reported in NCBI database) and it is significantly increased in B-cell lymphomas (Supplementary Figure 3D–G). This suggests that RBM5 may not act as tumor suppressor in B-cell lymphomas and that the regulation of RBM5 may be cell type specific. We found that a majority of the lymphoma-derived cell lines tested showed increased sensitivity to Fas-mediated apoptosis upon treatment with DZNeP (Figure 3C). However, JVM2 (MFI; 3800) and Granta-519 (MFI; 2600) cells, which express high levels of Fas on their surface were not sensitized to FasL-mediated apoptosis by DZNeP treatment (Figure 3C). Clearly, Fas signaling can be subjected to regulation through additional mechanisms.⁵³ Recent reports have revealed that cell surface molecules nucleolin, human herpesvirus-8 K1, and CD44v6/v9, which have their own signaling functions, can also bind and block membrane Fas by interfering with binding of FasL.^{60–62} Though DZNeP may potentially have targets other than EZH2, our analysis using shRNA-mediated downregulation of EZH2 suggests that most of the DZNeP effects on cell death in our experiments are mediated through inhibition of EZH2 (Figure 5). Thus, combining EZH2 inhibitors or BTK inhibitors can prime cells to effectively mediate Fas apoptosis triggered by conventional chemotherapeutic agents.

We conclude that FAS-AS1 lncRNA, expression of which is controlled by EZH2-mediated histone 3methylation, is a novel modulator of the sFas expression. Our finding that FAS-AS1 lncRNA regulates skipping of exon 6 through direct interaction with RBM5 reveals a novel layer of modulation of the Fas death receptor signaling in human lymphomas. Given the altered surface expression of Fas and the often impaired Fas signaling in chemoresistance, we anticipate that addition of EZH2 or BTK inhibitors might reduce chemoresistance by the coordinated suppression of sFas and upregulation of membrane Fas production. Therefore, we look with enthusiasm toward pre-clinical studies and clinical trials, which should determine whether EZH2 and/or BTK-targeting therapeutics can restore Fas-signaling and chemotherapy responses in lymphoma and other malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of FAS-AS1 lncRNA inversely correlates with the levels of sFas mRNA in lymphomas

(A–B) The total cellular RNA isolated from healthy donors (n=4), primary lymphoma tissues (n=6), and B-cell lymphoma-derived cell lines (n=11) was analyzed for (A) the average sFas/total Fas RNA levels normalized to 1 in the healthy donors, and (B) FAS-AS1 lncRNA by qRT-PCR normalized to GAPDH. The inset image shows the expression of FAS-AS1 as determined by the RT-PCR from the total cellular RNA isolated from healthy donor (HD 35), primary lymphoma patient samples (P559; BL, P393; DLBCL, P225; MCL, P227; MCL). (C) The levels of FAS-AS1 lncRNA plotted against soluble Fas mRNA levels, to illustrate their relationship (n=10) in various cell lines with Pearson correlation coefficient

of $r^2 = -0.58$. (D) ChIP analysis of H3K27me3 on the promoter regions of FAS-AS1 using specific antibodies that recognize H3K27me3 and control IgG. The locations of primers used for the ChIP assay are indicated by arrows. The specific primer from the FAS-AS1 lncRNA (region C, +700) serves as the negative control, and the qPCR for α -satellite DNA served as a positive control for the H3K27me3 ChIP. Statistical significance was calculated by student's T test (α satellite: $P < 0.01$, A: $P < 0.01$, B: $P < 0.01$, C: $P < 0.11$) (E–F) EZH2 expression levels in (E) 89 primary B cell lymphoma tissues and (F) 34 lymphoma-derived cell lines (Oncomine database; www.oncomine.org). Data sets in a single panel were from the same study. Gene expression profile of EZH2 was log transformed and normalized in different histopathological types of lymphomas.

Figure 2. High EZH2 expression and hyper-methylation of the FAS AS1 promoter is reversed upon treatment with DZNep

(A) SP53 cells were treated with methyltransferase inhibitor DZNep in different concentration in μM for 24 hours and analyzed for the levels of EZH2, H3K27me3, and Actin by IB (B) DZNep 1 μM was tested in vitro for the inhibition of EZH2-mediated methylation using microtiter plate precoated with histone H3 peptide substrate and H3K27me3-specific antibody. ($P < 0.01$) (C–D) ChIP assays were performed to determine the extent of H3K27me3 on FAS-AS1 promoter in the presence or absence of the 1 μM DZNep. The positions for the various primers are determined from the transcription start site (TSS, A; –400, B; –900) and the various control primers in Z-138 cells. The specific primer from the FAS-AS1 lncRNA (region C, +700) serves as the negative control, and the qPCR for α -satellite DNA served as a positive control for the H3K27me3 ChIP. The levels of methylation levels are reduced in b and c regions of FAS-AS1 lncRNA promoter. (E & F) Z-138 cells were treated with 1 μM DZNep for 24 hours and total cellular RNA was isolated and subjected to qRT-PCR with primers specific for FAS-AS1 lncRNA (E) ($P < 0.01$) and sFas mRNA (F) ($P < 0.01$). (G) Lymphoma derived cell lines were incubated with and

without 1 μ M DZNeP for 24 hours and concentrations of sFas in the media were determined by ELISA (* denotes $P < 0.01$).

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Figure 3. DZNeP stimulates the expression of lymphoma cell surface Fas and increases their sensitivity to FasL

(A) Surface levels of Fas in control and DZNeP-treated cells were analyzed by flow cytometry after staining of cells with anti-Fas antibody UB2 conjugated with PE. Representative results from one of least three independent experiments are shown (* denotes $P < 0.01$, ** denotes $P < 0.01$). (B) DZNeP-treated cells were incubated with FLAG-tagged FasL (100ng) for 20 minutes and the levels of bound ligand were analyzed by flow cytometry after staining with an anti-FLAG-PE secondary antibody or isotype control IgG-PE antibody. Representative data of 3 independent experiments are shown. The vertical dotted line intersecting the graph shows the increase in the Fas ligand binding to cells. (C) Cells were treated with DZNeP for 24hrs followed by incubation with FasL (100ng/mL) for 12 hours. Cells were analyzed by PI staining and flow cytometry for sub-G1 fraction. Mean value and SEM from at least 3 independent experiments with 3 replicates in each are shown. (D) The cell extracts from Z-138 cells treated with DZNeP, FasL or their combination as in (C) were analyzed by IB analysis for activation of indicated Fas signaling proteins.

Figure 4. Ectopic expression of FAS-AS1 inhibits alternative splicing of Fas and sensitizes lymphoma cells to FasL

(A–B) Z-138 cells were transfected with pcDNA3 expressing FAS-AS1 IncRNA or empty vector. The total cellular RNA was isolated 48 hrs post transfection and subjected to qRT-PCR with (A) FAS-AS1 to determine the efficiency of transfection ($P < 0.01$) or (B) sFas-specific primers to determine the sFas/mFas isoform ratio ($P < 0.01$). (C) Empty vector or pcDNA3-FAS-AS1 were co-transfected with pEGFP-N1. Surface levels of mFas in control and FAS-AS1 transfected cells (sorted based on GFP signal) were analyzed by flow cytometry after staining of cells with anti-Fas antibody UB2 conjugated with PE. Representative results from one of three independent experiments are shown. (D) Sorted

cells from (C) were challenged with FasL (100ng/mL) for 12 hours and analyzed after PI staining and flow cytometry (Vector; $P < 0.09$, FAS-AS1; $P < 0.01$). (E) Z-138 cells were transfected with increasing concentrations of FAS-AS siRNA and the level of FAS-AS1 lncRNA was analyzed by RT-PCR. (F & G) Z-138 cells transfected with 25nM of scrambled- or FAS-AS1-specific siRNA were pre-treated with DZNeP, challenged with FasL (100ng/mL) for 12 hours, stained with PI, and analyzed for apoptosis by flow cytometry (DZNeP+sFas: Vector; $P < 0.09$, FAS-AS1 siRNA; $P < 0.01$).

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Figure 5. Knockdown of EZH2 inhibits alternative splicing of Fas and sensitizes lymphoma cells to FasL

(A) Z-138 cells were transfected with either pLKO.1 vector control or pLKO.1 expressing shRNA targeting EZH2. 60 hrs post transfection the protein lysates were blotted with EZH2 antibody. Actin served as loading control. (B) The total cellular RNA was isolated 60 hrs post transfection and subjected to qRT-PCR with primers specific to EZH2, FAS-AS1 and sFas (vector control vs. shRNA EZH2.1; $P < 0.01$). (C) Empty vector- or pLKO.1 carrying shRNA to EZH2 transfected cells were analyzed for the level of membrane Fas by flow cytometry. Representative results of three replicates are shown (vector control vs. shRNA EZH2.1; $P < 0.01$). (D) Empty vector- pLKO.1 carrying shRNA to EZH2 transfected cells were challenged with FasL (100ng/mL) for 12 hours and analyzed after PI staining and flow cytometry (vector control vs. shRNA EZH2.1; $P < 0.001$).

Figure 6. FAS-AS1 binds RBM5 and interferes with RBM5-mediated exon 6 skipping of Fas pre-mRNA

(A) Prediction of the interaction map of FAS-AS1 lncRNA and RBM5 generated by use of the *catRapid* program (http://service.tartagialab.com/page/catrapid_group). Interaction propensity is measured in procedure defined units (p.d.u.). The positive interaction propensity is shown for binding of FAS-AS1 lncRNA to the RNA Recognition Motifs in RBM5 [RRM1 98–178, RRM2 231–315]. A schematic representation of RBM5 protein is shown with location of RRM1 and RRM2 domains. (B) Whole-cell lysates from control or 1 μ M DZNeP-treated Z-138 cells were immunoprecipitated with anti-RBM5 antibody or control IgG. Precipitates were analyzed by IB analysis for RBM5 and by qRT-PCR for the levels of co-precipitated FAS-AS1 lncRNA. The whole cell extracts from B were analyzed in immunoblots for EZH2, RBM5, H3K27me3, and H3 levels. (C–D) Real time-PCR analysis using primers for FAS-AS1 (RBM5 DZNeP, $p < 0.01$) (C) and Fas pre-mRNA (RBM5 DZNeP, $P < 0.01$) (D) in immunoprecipitants of control antibody and RBM5 antibody on extracts of Z-138 cells treated with DZNeP.

Figure 7. Ibrutinib inhibits the expression of EZH2 and RBM5 and sensitizes lymphoma cells to Fas-mediated apoptosis

(A) Z-138 cells were treated with 10 μ M ibrutinib (PCI) for 48 hours. Total cellular RNA was extracted and analyzed by qRT-PCR for the expression of mFas, sFas, EZH2, and RBM5 mRNAs and FAS-AS1 lncRNA. ($P < 0.001$) (B) Cell surface levels of Fas in control and ibrutinib-treated cells were stained with the PE-conjugated anti-Fas antibody UB2 and analyzed by flow cytometry. Representative results from at least three independent replicates are shown. ($P < 0.01$) (C) BJAB, SU-DHL-9 and Z-138 cells were treated with 10 μ M Ibrutinib (24hrs) and then challenged with FasL (100ng/mL) for 12 hours, stained with PI and analyzed by flow cytometry. Representative results from at least three independent replicates are shown ($P < 0.001$).

Figure 8. Regulation of sFas expression and sensitization of lymphoma cells to Fas-mediated apoptosis by DZNeP and ibrutinib

(A) BTK promotes the expression of RBM5 and EZH2 in lymphoma cells. RBM5 promotes skipping of exon 6 from Fas pre-mRNA leading to production of soluble decoy sFas. EZH2 tri-methylates the FAS-AS1 promoter and represses transcription of FAS-AS1 lncRNA. (B) In DZNeP-treated cells, EZH2 activity is compromised, which promotes the expression of FAS-AS1 lncRNA. FAS-AS1 lncRNA binds and sequesters RBM5 protein preventing RBM5-mediated skipping of exon 6 and ultimately lower sFas, this leads to sensitization to Fas-mediated apoptosis. Because DZNeP affects EZH2, but not RBM5, the sFas production because of RBM5 still remains and the effect depends on the residual levels of RBM5 expression. (C) Ibrutinib treatment decreases expression of both EZH2 and RBM5 combined with enhanced expression of FAS-AS1 further which results in more pronounced decrease in the levels of sFas corresponding to more efficient interference with crosstalk between EZH2

and RBM5-mediated skipping of exon 6 and thus production of sFas. This translates into sensitization to Fas-mediated apoptosis.

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