

Novel Regulation of Nuclear Factor-YB by *miR-485-3p* Affects the Expression of DNA Topoisomerase II α and Drug Responsiveness^[S]

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ABSTRACT

Nuclear factor (NF)-YB, a subunit of the transcription factor nuclear factor Y (NF-Y) complex, binds and activates CCAAT-containing promoters. Our previous work suggested that NF-YB may be a mediator of topoisomerase II α (Top2 α), working through the Top2 α promoter. DNA topoisomerase II (Top2) is an essential nuclear enzyme and the primary target for several clinically important anticancer drugs. Our teniposide-resistant human lymphoblastic leukemia CEM cells (CEM/VM-1-5) express reduced Top2 α protein compared with parental CEM cells. To study the regulation of Top2 α during the development of drug resistance, we found that NF-YB protein expression is increased in CEM/VM-1-5 cells compared with parental CEM cells. This further suggests that increased NF-YB may be a negative regulator of Top2 α in CEM/VM-1-5 cells. We asked what causes the up-regulation of NF-YB in CEM/VM-1-5 cells.

We found by microRNA profiling that *hsa-miR-485-3p* is lower in CEM/VM-1-5 cells compared with CEM cells. MicroRNA target prediction programs revealed that the 3'-untranslated region (3'-UTR) of NF-YB harbors a putative *hsa-miR-485-3p* binding site. We thus hypothesized that *hsa-miR-485-3p* mediates drug responsiveness by decreasing NF-YB expression, which in turn negatively regulates Top2 α expression. To test this, we overexpressed *miR-485-3p* in CEM/VM-1-5 cells and found that this led to reduced expression of NF-YB, a corresponding up-regulation of Top2 α , and increased sensitivity to the Top2 inhibitors. Results in CEM cells were replicated in drug-sensitive and -resistant human rhabdomyosarcoma Rh30 cells, suggesting that our findings represent a general phenomenon. Ours is the first study to show that *miR-485-3p* mediates Top2 α down-regulation in part by altered regulation of NF-YB.

Introduction

Nuclear factor-Y (NF-Y) is a conserved transcription factor that consists of three subunits—NF-YA, NF-YB, and NF-

YC—and binds specifically to the CCAAT elements in promoters (Ronchi et al., 1995). Our previous work suggested that one of these subunits, NF-YB, may mediate Top2 α expression, working through inverted CCAAT element 3 (ICE3) in the Top2 α promoter (Morgan and Beck, 2001). DNA topoisomerase II (Top2) is an essential nuclear enzyme involved in many cellular processes (Champoux, 1990; Wang, 2002; Nitiss, 2009a) and mammalian cells have two isoforms of the type II enzymes: Top2 α (170 kDa) (Tsai-Pflugfelder et al., 1988), and Top2 β (180 kDa) (Chung et al., 1989). Top2-DNA covalent complexes serve as the cytotoxic target for many anticancer drugs such as doxorubicin, etoposide, teniposide, and amsacrine (Liu, 1989; Beck, 1996; Pommier et al., 2010). However, tumors frequently become refractory to treatments with Top2 inhibitors because of the emergence of drug resis-

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ABBREVIATIONS: NF-Y, nuclear factor Y; 3'-UTR, 3'-untranslated region; *hsa-miR-485-3p*, human microRNA-485-3p; ICE, inverted CCAAT element; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; RT, reverse transcriptase; Top2, DNA topoisomerase II; Top2 α , topoisomerase II α ; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; C_t, cycle threshold.

tance (Nitiss and Beck, 1996; Nitiss, 2009b). Our previous work suggested that the transcription factor NF-YB is a mediator of Top2 α , working through the Top2 α promoter (Morgan et al., 2000). Our current experiments revealed an inverse relationship between the expression of Top2 α protein and NF-YB protein in drug-sensitive CEM cells compared with the teniposide-resistant CEM/VM-1-5 cells (Danks et al., 1988) and in drug-sensitive human rhabdomyosarcoma Rh30 cells and the etoposide-resistant Rh30/v1 cells (Bhat et al., 1999). We asked in the present study what causes the up-regulation of NF-YB in the CEM/VM-1-5 and Rh30/v1 cells that express decreased levels of Top2 α .

There are reports that microRNAs, a group of nonprotein coding, single-stranded RNAs of 20 to 22 nucleotides often aberrantly expressed or mutated in cancer (Calin and Croce, 2006), may play roles as either oncogenes or tumor suppressors (Esquela-Kerscher and Slack, 2006), and may mediate drug-responsiveness (Mishra et al., 2007; To et al., 2008). Accordingly, we asked whether microRNAs might be involved in the regulation of NF-YB, which further mediates Top2 α expression and drug-responsiveness in CEM and CEM/VM-1-5 cells and in Rh30 and Rh30/v1 cells. MicroRNAs function through perfect or near-perfect base pairing with protein coding mRNA 3'-untranslated regions (3'-UTRs) for mRNA degradation or translational repression (Bartel, 2004). We have found by microRNA profiling that a particular microRNA, *hsa-miR-485-3p*, may target the 3'-UTR of NF-YB to affect the expression of Top2 α .

Materials and Methods

Cell Lines and Culture Conditions. The human lymphoblastic leukemia CEM cell line and its teniposide-resistant subline, CEM/VM-1-5, developed in our laboratory (Danks et al., 1988), were cultured in suspension in RPMI 1640 medium. The human rhabdomyosarcoma Rh30 cell line and its etoposide-resistant subline, Rh30/v1 (Bhat et al., 1999), were grown as monolayers and also cultured in RPMI 1640. HEK293T cells were cultured in DMEM. Both RPMI 1640 and DMEM (Lonza Walkersville, Inc., Walkersville, MD) were supplemented with 10% fetal bovine serum (Gemini, Irvine, CA), and 2 mM L-glutamine (Lonza Walkersville, Inc.). All cell lines were subcultured twice per week in either RPMI 1640 or DMEM and were incubated at 37°C in a humidified chamber containing 5% CO₂/95% air.

Western Blot Analysis. Nuclear protein extracts were prepared from logarithmically growing cells as described previously (Morgan and Beck, 2001). Protein concentration was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein samples (~15–30 μ g) were separated on a 4 to 12% Bis-Tris gradient gel (Invitrogen, Carlsbad, CA), electrophoretically transferred onto nitrocellulose, and incubated with either purified mouse anti-Top2 α monoclonal antibody (BD Biosciences, San Jose, CA), rabbit anti-NF-YB polyclonal antibody, or mouse anti-NF-YA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibody was detected using the enhanced chemiluminescence detection method (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) according to the manufacturer's instructions. Chemiluminescence signals were quantified by densitometric scanning using an Imaging Densitometer (Scion Corporation, Frederick, MD). Equal loading of nuclear protein was determined by blotting the membrane with anti-proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology).

Isolation of Total RNA and Reverse Transcriptase-PCR. Total RNAs were extracted from CEM and CEM/VM-1-5 and Rh30 and Rh30/v1 cells with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Single-stranded cDNA was synthesized

by using the ThermoScript reverse transcriptase (RT)-PCR System (Invitrogen). The PCRs were carried out for each transcript under the following conditions: initial denaturation at 94°C for 5 min, and then 30 to 40 cycles at 94°C for 30 s, 50 to 60°C for 60 s, and 72°C for 90 s, and 1 cycle of 72°C for 10 min. The 3'-UTR sequences of NF-YB containing the putative *mir-485-3p* binding site were amplified using the following primers: NF-YB 3'-UTR: sense, 5'-TCT AGA AAG CAA GTG AAA GGT GCC AT-3'; and antisense, 5'-TCT AGA ATC ATG AAT TAA CCC AGC CG-3'. To delete the putative *mir-485-3p* binding site, we used the following primers: sense, 5'-TCT AGA AAG CAA GTG AAA GGT GCC AT-3'; and antisense, 5'-TCT AGA CCT GAT GCT TGA CTA ATT GAG G-3', and the sequences were defined as NF-YB 3'-UTR-d.

Construction of Expression Vectors. The *miR-Ctrl* (pCDH-empty vector) and *miR-485-3p* expression vectors (pCDH-*miR-485-3p*) were packaged into lentiviruses by cotransfection of HEK293T cells with three plasmids: a lentiviral expression vector plus pMD2.G and psPAX2. (The latter two plasmids were generous gifts of Dr. Didier Trono, Department of Genetics and Microbiology, University of Geneva, Geneva, Switzerland.) CEM/VM-1-5 cells transduced with *miR-Ctrl* or *miR-485-3p* expression vectors are defined as CEM/VM-1-5 *miR-Ctrl* or CEM/VM-1-5 *miR-485-3p*, respectively (Same as Rh30/v1 cells). The NF-YB 3'-UTR containing the putative *mir-485-3p* binding site and NF-YB 3'-UTR-d without the putative *mir-485-3p* binding site were amplified by PCR and then cloned into the pGL3-thymidine kinase vector (generous gift of Dr. Hyun-Young Jeong, Department of Biopharmaceutical Sciences, University of Illinois at Chicago, Chicago, IL). The luciferase reporters containing the NF-YB 3'-UTR with or without the putative *mir-485-3p* binding site are defined as pGL3-NF-YB-3'-UTR or the pGL3-NF-YB-3'-UTR-d, respectively.

Cytotoxicity Assay. Drug-induced cytotoxicity was measured by the MTT assay (Mosmann, 1983). Exponentially growing cells were plated at 4000 to 5000 cells/well in 96-well microtiter plates (100 μ l/well) in triplicate. Etoposide, doxorubicin, or vinblastine (all from Sigma-Aldrich, St. Louis, MO) was added to the cells at various concentrations in a final volume of 200 μ l/well, and the cells were incubated at 37°C for 72 h. After drug exposure, 20 μ l of MTT compound (5 mg/ml in phosphate-buffered saline; Sigma-Aldrich) was added to each well, and the cells were incubated at 37°C for 3 h. The plates were centrifuged in a swinging bucket rotor (3000 rpm, 20 min, 4°C), and the medium was removed. After 200 μ l of dimethyl sulfoxide was added to each well, and the plates were incubated for 10 min at 37°C, the metabolic activity of the cells was measured by quantifying the conversion of the yellow MTT to a purple metabolite, MTT-formazan. Absorbance was read at 560 nm using a microplate reader. Experimental samples were measured for each drug concentration, and the experiments were replicated at least three times. The IC₅₀ value (the concentration of drug that killed 50% of the cells) was calculated by Prism software (GraphPad Software Inc., San Diego, CA).

Luciferase Assay. Luciferase assays were carried out in HEK293T cells. Cells were seeded in 24-well plates in triplicate and transfected with appropriate plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 24 h of transfection, cells were lysed and treated with Dual-Light assay system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Relative luciferase activities were measured and normalized against β -galactosidase activity. All of the experiments were replicated at least three times.

Results

Inverse Expression of Top2 α and NF-YB Proteins in CEM and CEM/VM-1-5 Cells and Rh30 and Rh30/v1 Cells. Previous work from our laboratory suggested that the transcription factor NF-YB is a mediator of Top2 α , working

through the ICE3 in the Top2 α promoter (Morgan and Beck, 2001). We found that NF-YB protein levels were 3-fold higher in CEM/VM-1-5 cells compared with CEM cells (Fig. 1, A and B), and there is an inverse relation between the protein levels of Top2 α and NF-YB in CEM and CEM/VM-1-5 cells by Western blots (Fig. 1A). To determine whether this is a cell line-specific phenomenon, we examined the human rhabdomyosarcoma Rh30 cell line and compared it with its etoposide-resistant subline, Rh30/v1 (Bhat et al., 1999). We observed a similar result: Top2 α is down-regulated in the etoposide-resistant Rh30/v1 cell line, and there is an inverse relation between the protein levels of Top2 α and NF-YB (Fig. 1, D and E). In addition, the expression of NF-YA protein is similar between drug-sensitive and -resistant cells (Supplemental Fig. S1). These data suggest that increased NF-YB may be either related to or the cause of reduced Top2 α in CEM/VM-1-5 and Rh30/v1 cells. Furthermore, we found that NF-YB mRNA levels were similar in CEM, CEM/VM-1-5 and Rh30, Rh30/v1 cells (Fig. 1, C and F), indicating that the observed differences in NF-YB protein levels are probably a consequence of regulation at the posttranscriptional level. Post-transcriptional repression is a major mechanism by which microRNAs regulate gene expression (Carrington and Ambros, 2003; Bartel, 2004). Accordingly, we then asked whether microRNAs are involved in the regulation of NF-YB protein during the development of drug resistance.

Expression of Micro-RNAs in Human Lymphoblastic Leukemia CEM and CEM/VM-1-5 Cells. We used microRNA profiling by the Applied Biosystems real-time PCR array (TaqMan Human MicroRNA Array v1.0) to analyze microRNA expression in CEM and CEM/VM-1-5 cells. The array carries unique hairpin-loop RT primer sets that allow for detection of 365 mature human microRNAs in the cells. Compared with CEM cells, microRNA profiling revealed that CEM/VM-1-5 cells with acquired resistance to teniposide

demonstrated substantial changes in microRNA expression. Compared with parental controls, 32 microRNAs were consistently either up- or down-regulated in CEM/VM-1-5 cells, as determined by C_t (cycle threshold) values from three separate microRNA profiling experiments (T.-T. Ho, A. D. Arslan, Y.-Y. Mo, X. He, and W. T. Beck, unpublished observations). Because higher C_t values indicate lower expression levels, one of the microRNAs, *hsa-miR-485-3p*, was found to be consistently substantially lower in CEM/VM-1-5 cells compared with CEM cells after conversion (Wu et al., 2009) to expression level (Fig. 2, A and B). It is noteworthy that the decreased expression of *miR-485-3p* in CEM/VM-1-5 cells is inversely related to the overexpression of NF-YB protein (Fig. 1, A and B).

Using miRanda (<http://www.microrna.org/microrna/home.do>), Target Scan (<http://www.targetscan.org/>), and MicroCosm (<http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl>) prediction programs, we found that the 3'-UTR of NF-YB harbors a putative *hsa-miR-485-3p* binding site (Fig. 2C), which is conserved in human, Rhesus monkeys, chimpanzees, and dogs. Moreover, we found by sequencing that there is no mutation in the region of NF-YB 3'-UTR, to which *miR-485-3p* binds in either CEM or CEM/VM-1-5 cells (data not shown), suggesting that the binding of microRNAs to the NF-YB 3'-UTRs is unlikely to be altered.

NF-YB Is a Direct Target for *miR-485-3p*. To validate that NF-YB is a direct target of *miR-485-3p*, we constructed a luciferase reporter (pGL3-thymidine kinase) carrying the NF-YB 3'-UTR with the putative *miR-485-3p* binding site. We cotransfected *miR-485-3p* expression vector and luciferase reporter into HEK293T cells. Relative luciferase activity of the NF-YB 3'-UTR with *miR-485-3p* binding site, which was cotransfected with the *miR-485-3p* expression vector (pCDH-*miR-485-3p*), was significantly lower (~46%)

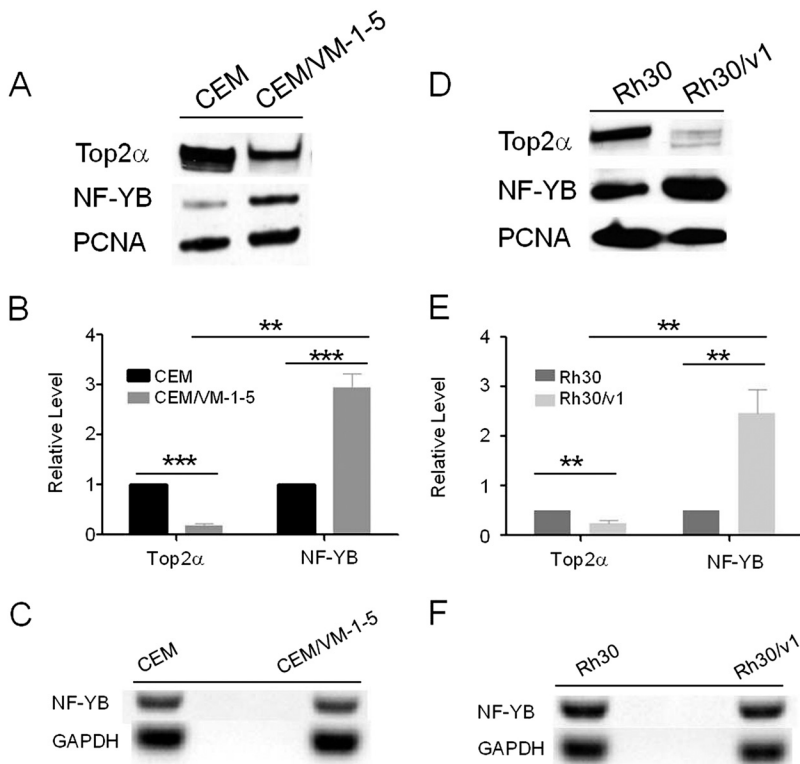


Fig. 1. Inverse relationship between the protein levels of Top2 α and NF-YB in CEM and CEM/VM-1-5 cells and Rh30 and Rh30/v1 cells. Western blots of nuclear Top2 α and NF-YB expression in CEM and CEM/VM-1-5 cells (A) and Rh30 and Rh30/v1 cells (D). PCNA served as loading control for nuclear proteins. Average of Top2 α and NF-YB levels from three independent experiments \pm S.D. are shown, determined by densitometric scanning on Western blots and normalized to PCNA signal; either CEM (B) or Rh30 (E) was assigned a value of 1 for comparison. **, $p < 0.005$; ***, $p < 0.0001$. C and F, NF-YB mRNA expression was analyzed by semiquantitative RT-PCR in CEM and CEM/VM-1-5 cells (C) and Rh30 and Rh30/v1 cells (F).

than that of the *miR-Ctrl* (pCDH-empty vector) cotransfected cells (Fig. 3A). Moreover, the relative luciferase activity of the pGL3-NFYB-3'-UTR with the *miR-485-3p* binding site was also lower (~45%) than that of the pGL3-NFYB-3'-UTR-d (pGL3-NFYB 3'-UTR without *miR-485-3p* binding site), which was carried out in HEK293T cells cotransfected with the *miR-485-3p* expression vector (Fig. 3B).

miR-485-3p Modulates the Sensitivity of CEM/VM-1-5 Cells to Top2 Inhibitors. To ascertain whether *miR-485-3p* regulates NF-YB, we examined the effects of overexpressing *miR-485-3p* on NF-YB expression in CEM/VM-1-5 cells, which have reduced endogenous *miR-485-3p* expression (Fig. 2A). Thus, we transduced CEM/VM-1-5 cells with either the pCDH-*miR-485-3p* or the *miR-Ctrl* (pCDH-empty vector) virus particles. We validated *miR-485-3p* expression in CEM/VM-1-5 *miR-485-3p* cells by Taqman real-time PCR (data not shown). Western blot analysis revealed decreased NF-YB protein levels in pCDH-*miR-485-3p* virus stably transduced CEM/VM-1-5 cells, compared with the *miR-Ctrl* transduced CEM/VM-1-5 cells and no difference in NF-YA protein levels was seen in response to *miR-485-3p* overexpression cells (Fig. 4A).

To determine whether this was a cell line-specific phenomenon, we examined the etoposide-resistant human rhabdo-

myosarcoma Rh30/v1 cells. Introduction of *miR-485-3p* into these cells also decreased their NF-YB protein levels in Rh30/v1 cells (Fig. 4B), indicating that the *miR-485-3p*-mediated regulation of NF-YB is not cell line-specific.

Because *miR-485-3p* targets NF-YB and NF-YB may negatively regulate the expression of Top2 α , the above findings suggest an interesting relationship between NF-YB, *miR-485-3p*, and Top2 α . Accordingly, we analyzed the levels of Top2 α in CEM/VM-1-5 *miR-485-3p* cells (Fig. 4A). It is noteworthy that we found that the level of Top2 α protein is up-regulated, whereas the level of NF-YB is down-regulated compared with CEM/VM-1-5 *miR-Ctrl* cells, suggesting that NF-YB regulates Top2 α expression via *miR-485-3p*. A similar effect was observed in the etoposide-resistant Rh30 subline Rh30/v1 (Fig. 4B). We thus hypothesized that *hsa-miR-485-3p* may regulate drug-responsiveness by increasing NF-YB expression, which in turn negatively regulates Top2 α expression. To test this, we performed the following experiments.

We examined the effects of *miR-485-3p* on the drug sensitivity of CEM/VM-1-5 cells. MTT assays revealed that CEM/VM-1-5 *miR-485-3p* cells exhibited enhanced sensitivity, 2-fold to etoposide, compared with CEM/VM-1-5 *miR-Ctrl* cells, as indicated by decreased IC₅₀ values (Fig. 4C). Similar

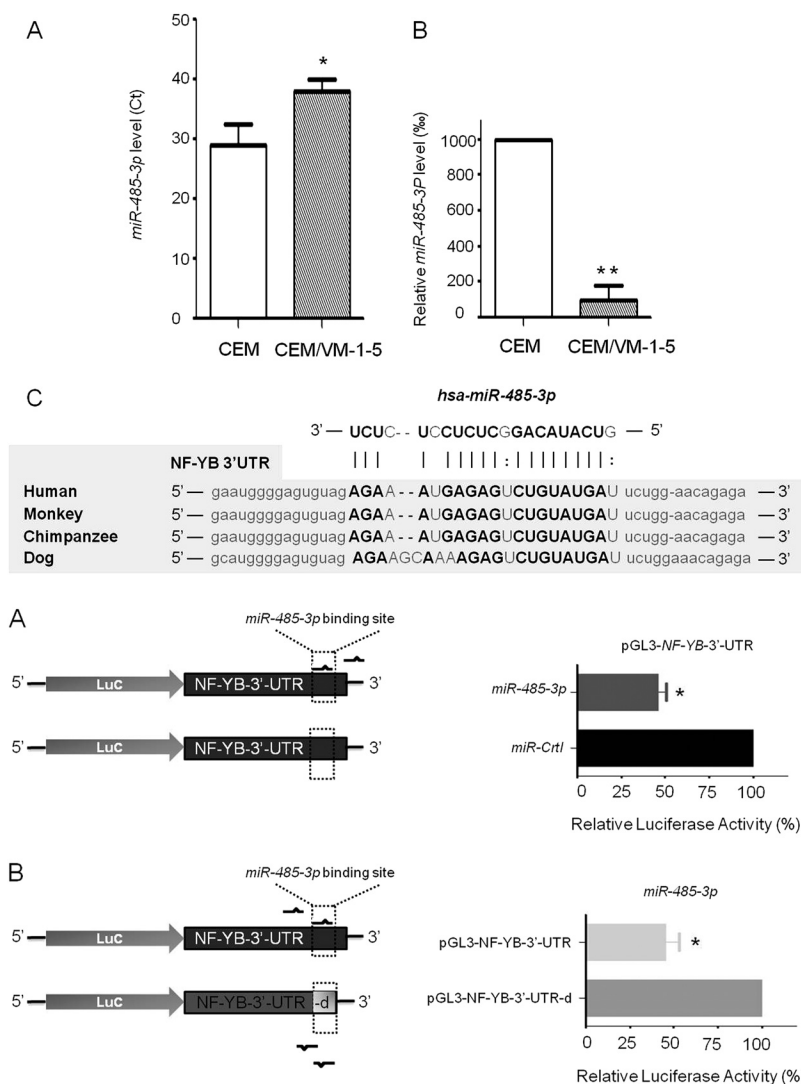


Fig. 2. NF-YB is a putative target for *hsa-miR-485-3p*. Expression of *hsa-miR-485-3p* in CEM and CEM/VM-1-5 cells was determined by Applied Biosystems real-time PCR array (TaqMan Human MicroRNA Array version 1.0) according to the manufacturer's instructions. Ct values (A) or relative expression levels of *miR-485-3p* (B) are means of three pairs of microRNA profile results from CEM and CEM/VM-1-5 cells \pm S.D. *, $p < 0.05$; **, $p < 0.01$. C, sequence alignment of putative *hsa-miR-485-3p* binding sites in 3'-UTR of NF-YB gene of four species. The base pairing nucleotides are in boldface type.

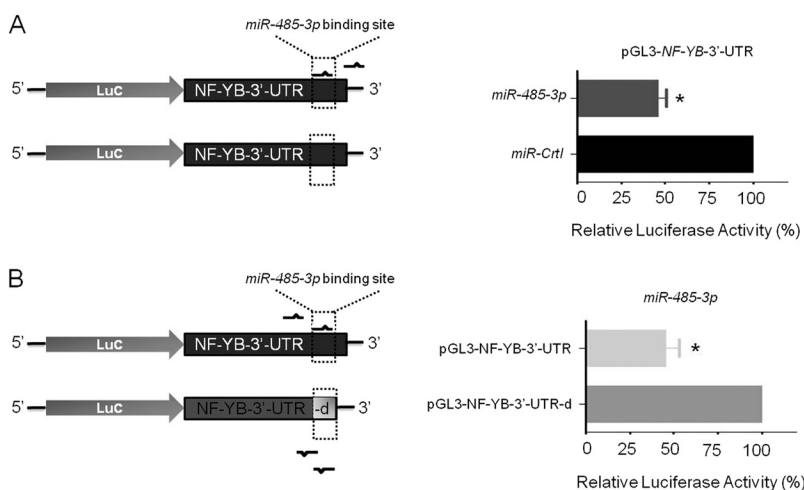


Fig. 3. *MiR-485-3p* targets NF-YB 3'-UTR. A, luciferase reporter containing a putative *miR-485-3p* binding site, NF-YB-3'-UTR (pGL3-NFYB-3'-UTR), was cotransfected with either *miR-Ctrl* (pCDH-empty vector) or the *miR-485-3p* expression vector (pCDH-*miR-485-3p*) with schematic diagram on the left. B, *MiR-485-3p* expression vector (pCDH-*miR-485-3p*) was cotransfected with either luciferase reporter containing NF-YB-3'-UTR with or without putative *miR-485-3p* binding site, which was defined as pGL3-NFYB-3'-UTR or pGL3-NFYB-3'-UTR-d, respectively, with schematic diagram (left). Relative luciferase activities were measured and normalized against β -galactosidase activity. Values are average of three separate experiments done in triplicate \pm S.E.; *, $p < 0.05$.

results were seen in doxorubicin-treated cells (data not shown). However, no significant difference in responsiveness to vinblastine, a microtubule inhibitor, was found in *miR-485-3p* overexpression CEM/VM-1-5 cells (Fig. 4D). Taken together, *miR-485-3p*-mediated down-regulation of NF-YB in CEM/VM-1-5 cells was accompanied by increased sensitivity of CEM/VM-1-5 cells to Top2 inhibitors.

Discussion

In this study, we confirmed our previous observation that Top2 α was down-regulated in drug-resistant CEM/VM-1-5 and Rh30/v1 cells. Moreover, we found that Top2 α down-regulation was accompanied by up-regulation of transcription factor NF-YB in resistant cells compared with parental drug-sensitive cells. Recent studies indicate that microRNAs are involved in mediating drug sensitivity and resistance. Therefore, based on microRNA profiling results and computer-based microRNA target prediction programs, we studied the role of *miR-485-3p* that is differentially expressed in drug-sensitive and -resistant cells and has a putative target to the 3'-UTR of NF-YB. We first examined the repression effect of *miR-485-3p* to its target, NF-YB, by overexpressing *miR-485-3p* in the cells. We also confirmed, by luciferase assay, the binding of *miR-485-3p* to NF-YB 3'-UTR. Overexpressing *miR-485-3p* in CEM/VM-1-5 cells led to reduced expression of NF-YB, a corresponding up-regulation of Top2 α , and increased sensitivity to the Top2 inhibitors.

Cell cycle-dependent regulation of Top2 α expression is mediated in part by the transcription factor NF-Y (Magan et al., 2003), a conserved transcription factor that consists of three different subunits, NF-YA, NF-YB, and NF-YC, and binds specifically to the CCAAT elements in the promoter (Ronchi et al., 1995). NF-Y has been reported to regulate the expression of several key cell cycle regulators, such as cyclin B1/B2 and *cdc25 C* (Manni et al., 2001). Some studies show that

knockout of NF-YB causes early mouse embryo lethality (Bhattacharya et al., 2003) and overexpressing a dominant-negative mutant of NF-YA, which contains amino acid substitutions within the DNA-binding domain, also inhibits cell proliferation and growth (Hu and Maity, 2000). Because we showed earlier (Morgan and Beck, 2001) that NF-YB may be a mediator of Top2 α at ICE3 in the Top2 α promoter, and other studies also suggested the regulatory role for NF-Y in the Top2 α promoter (Magan et al., 2003), we further examined the possibility that NF-YB causes down-regulation of Top2 α in the drug-resistant cells. We have now shown that the NF-YB protein is up-regulated in the CEM/VM-1-5 cells that have decreased levels of Top2 α compared with the drug-sensitive CEM cells. We observed a similar result in the human rhabdomyosarcoma Rh30 cell line and its etoposide-resistant subline, Rh30/v1, which also has down-regulated Top2 α protein compared with the drug-sensitive parents. This strongly suggests that NF-YB is a negative regulator of Top2 α . However, examination of Top2 α and NF-YB gene expression levels in the National Cancer Institute (NCI)-60 panel of human tumor cancer cell lines (<http://discover.nci.nih.gov/cellminer/>) (Shankavaram et al., 2009) suggests that there is no significant overall correlation, negative or positive, between Top2 α and NF-YB at the mRNA level (Fig. 5). Some cell lines show a positive relationship between the two genes, others show a negative relationship, and yet others show an inverse relationship, as we have demonstrated here. The CEM cell line in this figure shows that both genes are overexpressed. One explanation for this apparent contradiction with our present results is that CEM cell line in the NCI-60 panel is diploid (Roschke et al., 2003) instead of near tetraploid as are our CEM and CEM/VM-1-5 cell lines (Kusumoto et al., 1996). By contrast, some of the cell lines showing an inverse relationship between Top2 α and NF-YB are derived from solid tumors, and, of possible relevance to our

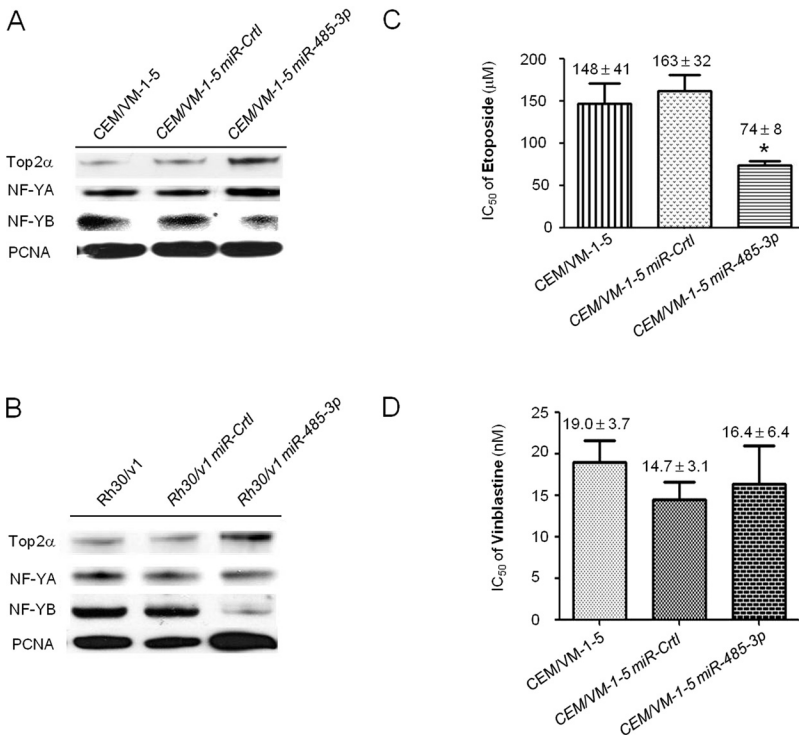


Fig. 4. *MiR-485-3p* inhibition on NF-YB and mediation on drug responsiveness. Western blots of nuclear Top2 α , NF-YB, and NF-YA expression in CEM/VM-1-5 (A) and Rh30/v1 cells (B). CEM/VM-1-5 and Rh30/v1 cells were transduced with either *miR-485-3p* expression virus or control virus (*miR-Ctrl*). PCNA served as loading control for nuclear protein. IC₅₀ values of cells exposed to etoposide (C) or vinblastine (D) at various concentrations were calculated from the percentage of viable cells after exposure to treatment obtained from MTT assay. Values are average of three independent experiments done in triplicate \pm S.E. *, $p < 0.05$.

results, one is in the drug-resistant NCI/ADR-RES cell line. Moreover, examination of the genes of cell lines in which both genes are inversely expressed (Fig. 5, middle group), the ratio of *Top2α* to *NF-YB*, and the differential (opposite) expression of both genes tends to be higher than in the rest of the cells (i.e., the top and bottom groups in Fig. 5, in which genes are expressed in the same direction). We can speculate that the inverse relationship that we have observed herein and in those NCI-60 cells with inverse expression of these genes may reflect aspects of the biology of drug resistance. Clearly, examination of the expression of these genes in more pairs of drug-sensitive and drug-resistant cell lines is warranted.

The mechanism underlying the differential expression of *miR-485-3p* in drug-resistant and -sensitive cells is not clear. Some studies suggest that epigenetic alterations (Grady et

al., 2008), deregulation of microRNA processing factors (Melo et al., 2009), and chromosomal abnormalities (Zhang et al., 2006) can contribute to down-regulation or up-regulation of microRNAs. *MiR-485-3p* resides on chromosome 14q32.31, a region that includes *miR-127* (Saito et al., 2006), and *miR-370* (Meng et al., 2008), which has been suggested to be epigenetically regulated. Furthermore, chromosome 14q32.31 is a region in which allelic deletions (Suzuki et al., 1989) and translocations (Avet-Loiseau et al., 2002) are frequently identified. Because microRNAs are expressed differently in drug-sensitive versus drug-resistant cells, and they have been shown to mediate drug response (Mishra et al., 2007; To et al., 2008; Bourguignon et al., 2009), studying the regulation of microRNAs during the emergence of drug resistance may reveal new therapeutic opportunities.

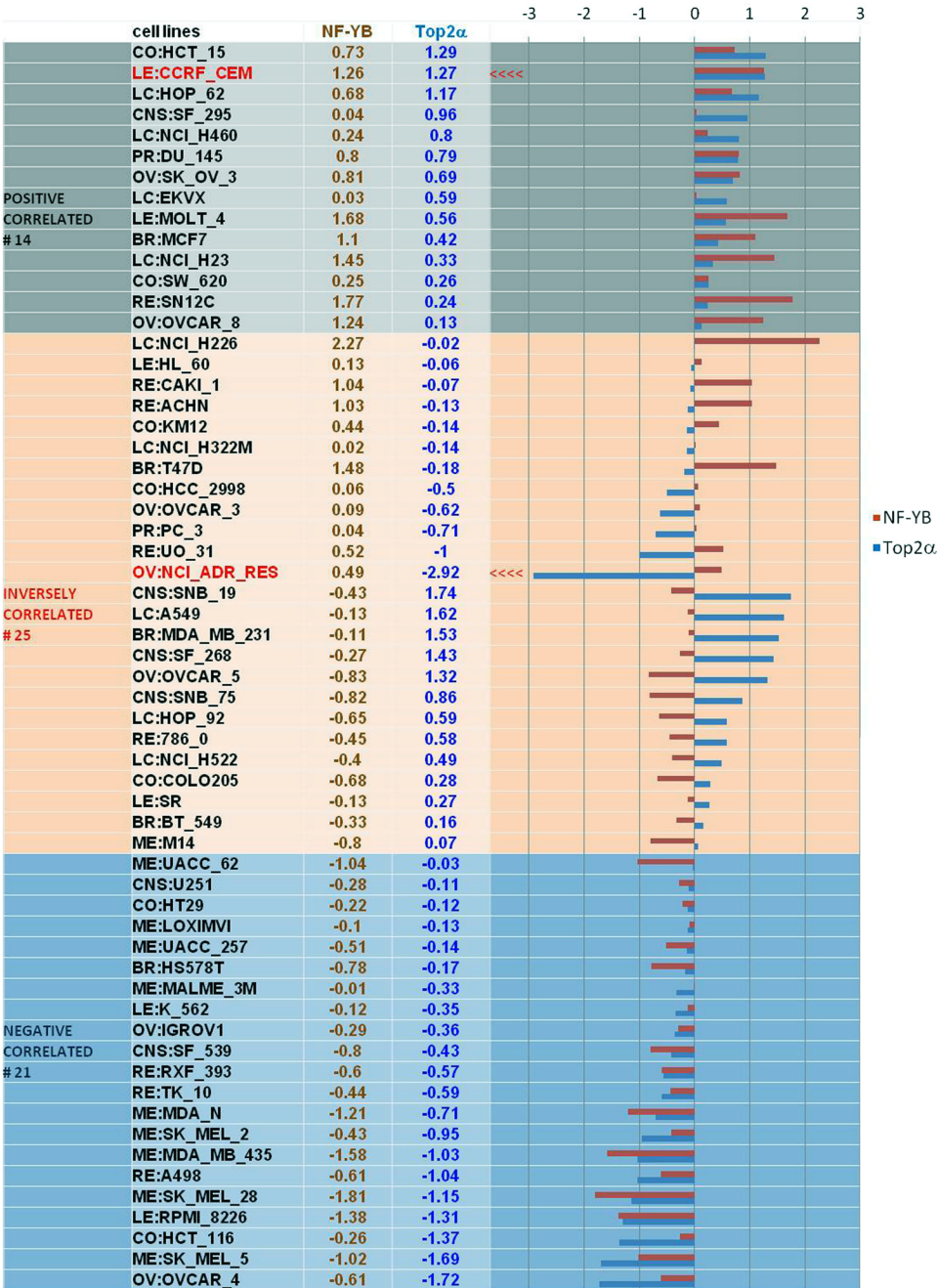


Fig. 5. NF-YB and *Top2α* mRNA expression levels in the NCI-60 panel of tumor cell lines. Shown is a graphical summary of NF-YB and *Top2α* mRNA levels in the form of a mean graph of the NCI-60 cells. Expression above the mean levels is drawn to the right of the centerline, and below the mean is drawn to the left.

In conclusion, we believe that ours is the first study to demonstrate that *miR-485-3p* expression can mediate etoposide sensitivity indirectly by fine-tuning Top2 α expression through the modification of NF-YB expression. Accordingly, *miR-485-3p* can be a putative therapeutic target to modulate etoposide resistance in tumor cells.

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Authorship Contributions

Participated in research design: Chen, He, and Beck.

Conducted experiments: Chen.

Contributed new reagents or analytic tools: Mo.

Performed data analysis: Chen, He, Arslan, Mo, Reinhold, Pommier, and Beck.

Wrote or contributed to the writing of the manuscript: Chen and Beck.

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