Alterations in γ-Actin and Tubulin-Targeted Drug Resistance in Childhood Leukemia

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Background: Proteomic investigations have revealed alterations in cytoskeletal proteins expressed in human acute lymphoblastic leukemia cells that are resistant to microtubule-disrupting agents. We characterized γ-actin expression in antimitotic drug-resistant leukemia and examined the effect of altered γ-actin in resistance of acute lymphoblastic leukemia to antimitotic agents. Methods: Two-dimensional polyacrylamide gel electrophoresis and mass spectrometry were used to identify actin proteins in human acute lymphoblastic leukemia cell lines resistant to vinblastine (CCRF-CEM/VLB100 cells) and desoxyepothilone B (CCRF-CEM/dEpoB140 cells). Fluorescence-based cycle sequencing was used to detect gene mutations. Site-directed mutagenesis was used to generate mutant γ-actin expression plasmids, which were used to transfect mouse NIH/3T3 cells. Clonogenic analysis was used for drug sensitivity studies. A small interfering RNA (siRNA) was used to block γ-actin gene expression in human neuroblastoma SH-EP cells. Expression of γ-actin (normalized to that of β2-microglobulin [β2M]) in primary leukemia cells obtained from patients at diagnosis (n = 44) and relapse (n = 25) was examined using semiquantitative reverse transcription–polymerase chain reaction. Statistical significance of changes in the ratio of γ-actin to β2M expression between diagnosis and relapse samples was determined by two-sided unpaired Student’s t tests.

Results: We identified novel mutant forms of γ-actin and the concomitant loss of wild-type γ-actin in CCRF-CEM/VLB100 cells and CCRF-CEM/dEpoB140 cells. Mouse NIH/3T3 cells that expressed the mutant γ-actin proteins were more resistant to antimicrotubule agents than cells transfected with empty plasmid. Human neuroblastoma SH-EP cells transfected with γ-actin siRNA displayed higher relative resistance to paclitaxel (P < .001), vinblastine (P = .04), and epothilone B (P = .045) than mock-transfected cells. No γ-actin gene mutations were identified in 37 samples of primary leukemia cells (eight from patients at diagnosis, 29 from patients at relapse). γ-Actin gene expression was lower in acute lymphoblastic leukemia samples collected at clinical relapse (n = 25; mean γ-actin/β2M = 0.53) than in samples collected at diagnosis (n = 44; mean γ-actin/β2M = 0.68; difference = 0.15, 95% confidence interval [CI] = 0.04 to 0.27, P = .01). Conclusions: These data provide functional and associative clinical evidence of a novel form of drug resistance that involves interactions between γ-actin and microtubules.

Antimitotic drugs that target tubulin, the main constituent of microtubules, are important in cancer therapy. These agents bind to β-tubulin and act by disrupting microtubule dynamics, which causes mitotic arrest and cell death (1). Antimicrotubule agents are used extensively to treat many types of cancer. For example, an important component of combination cytotoxic chemotherapy for acute lymphoblastic leukemia—the most common childhood cancer, accounting for 30% of all childhood malignancies (2)—is the antimitocable agent, vincristine. More than 97% of patients experience a first complete remission (3), but approximately 20% of patients will relapse, and 15% of children with acute lymphoblastic leukemia will die of their disease (2,3). The major cause of relapse and treatment failure is the development of drug resistance.

It is well established that tubulin is the intracellular target of antimitocable drugs such as taxanes, epothilones, and vinca alkaloids. However, the precise pathways involved in the cytotoxic action of these different agents are poorly defined. Alterations to the drug target, including mutant versions of β-tubulin, and altered expression of microtubule proteins are often associated with cellular resistance to antimitocable drugs (4,5). Emphasis is now starting to focus on identifying the pathways involved in antimitocable drug action and the proteins that contribute to drug resistance (5).

Proteomic studies have identified distinct alterations in cytoskeletal proteins expressed in human leukemia cell lines that are resistant to the microtubule destabilizing agents vincristine and vinblastine (6) or to the microtubule stabilizing agent desoxyepothilone B (7). A number of these proteins are normally...
associated with the actin cytoskeleton, and the alterations are predicted to affect actin microfilament dynamics in cells. Proteomic analysis has also revealed altered expression of actin and actin-associated proteins in in vivo-derived vincristine-resistant human leukemia xenografts (8). Although the actin and tubulin cytoskeletons are often viewed as distinct entities, recent studies have highlighted a number of structural and regulatory interactions shared by their protein constituents [reviewed in (9)]. In addition, pharmacologic studies (10–12) have demonstrated that disruption of one cytoskeletal system can have profound effects on the organization of the other system. For example, cultured cells treated with microtubule-stabilizing agents such as colchicine, vinblastine, or the new microtubule agent combretastatin A4 phosphate display rapid increases in cell contractility and actin stress fiber organization (10,12). In fibroblasts, tubulin-depolymerizing agents stimulate actin polymerization, and the microtubule-stabilizing drug, paclitaxel, inhibits this induced polymerization (13). This indirect evidence suggests that changes in the actin cytoskeleton may occur as a consequence of antimicrotubule drug–induced microtubule disruption. However, there is no direct evidence that the actin cytoskeleton plays a role in microtubule disruption caused by antimicrotubule drugs.

Given the coordinate function of microtubules and microfilaments in many cellular processes, we hypothesized that alterations in the microfilament cytoskeleton that influence microtubule function may be involved in resistance to antimicrotubule agents. We analyzed actin proteins in antimicrotubule drug–resistant leukemia cell lines using two-dimensional (2D) polyacrylamide gel electrophoresis and mass spectrometry and investigated the functional roles of the identified γ-actin changes using protein expression and silencing approaches. We also examined the clinical relevance of mutations in and altered expression of γ-actin by using gene sequencing and reverse transcription–polymerase chain reaction (RT–PCR).

MATERIALS AND METHODS

Cell Culture and Reagents

CCRF-CEM cells [a human T-cell acute lymphoblastic leukemia cell line (14)] and sublines of CCRF-CEM cells that are resistant to vinblastine [CEM/VELB100 cells; (15)] or doxyepothilone B [CEM/dEpoB140 cells; (7)] were maintained as suspension cultures in RPMI-1640 (Gibco, Invitrogen, Mount Waverly, Victoria, Australia) medium containing 10% fetal calf serum (FCS; Gibco, Invitrogen). Human neuroblastoma SH-EP cells (obtained from Dr June Biedler, Fordham University, New York) and mouse fibroblast NIH/3T3 cells (American Type Culture Collection, Manassas, VA) were maintained as adherent cultures in Dulbecco’s modified Eagle Medium (Gibco, Invitrogen) containing 10% FCS. Paclitaxel (Calbiochem, Merck Biosciences, Nottingham, U.K.), doxeyepothilone B (Dr S. Danishefsky, Memorial Sloan Kettering, New York, NY), and epothilone B (Calbiochem) were prepared at concentrations of 2 mM in dimethyl sulfoxide (DMSO). Vinblastine (vinblastine sulfate; David Bull Laboratories, Melbourne, Australia) was prepared as a stock concentration of 1.1 mM in saline (0.9% wt/vol NaCl). Doxorubicin (doxorubicin hydrochloride; Pfizer, Sydney, Australia) was prepared at a stock concentration of 3.45 mM in saline. Etoposide (VP-16; Sigma–Aldrich, Sydney, Australia) was prepared at a stock concentration of 68 mM in DMSO. Drugs diluted in DMSO were stored at −20 °C; those diluted in saline were stored at 4 °C.

2D Polyacrylamide Gel Electrophoresis, Western Blot Analysis, and Mass Spectrometry

Cell pellets (1 × 10⁷ cells total) were resuspended in 500 μl lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% sulfobetaine-3-10, 1% amidosulfobetaine-14, 2 mM tributylphosphine, 65 mM dithiothreitol, 1% carrier ampholyte (pH range 3–10, 0.01% bromphenyl blue) to a final concentration of 1 mg/mL protein as determined by amino acid analysis (6). 2D polyacrylamide gel electrophoresis (PAGE) was performed as previously described (6). Briefly, cells were lysed by pulse sonication twice for 10 seconds on ice. Endonuclease (1 U/μg protein; Sigma–Aldrich) was added, and the lysate was incubated at room temperature for 30 minutes. Protein extracts were centrifuged at 18,000g for 12 minutes, and the supernatant was collected. Protein (100 μg for analytical gels, 500 μg for preparative gels) was cup loaded onto 18-cm immobilized pH gradient strips with a narrow pH range (pH 4.5–5.5; GE Healthcare, Sydney, Australia) that had been rehydrated with 500 μl lysis buffer and subjected to isoelectric focussing for 150,000 Vh on a Multiphor II apparatus (GE Healthcare). 2D sodium dodecyl sulfate (SDS)–PAGE was performed using 8%–18% polyacrylamide gels (6). The gels were stained for total protein with the use of SYPRO Ruby (Bio-Rad, Laboratories, Hercules, CA), or the resolved proteins were transferred to nitrocellulose using standard methods and the membranes were then incubated with a mouse monoclonal antibody against β-actin (clone AC74, 1:3000 dilution, Sigma–Aldrich), a rabbit polyclonal antibody against total actin (1:3000 dilution; Sigma–Aldrich), or a sheep polyclonal antibody against γ-actin (1:2000 dilution; (16)). The membranes were incubated with a species-appropriate horseradish peroxidase–linked immunoglobulin G antibody followed by either Supersignal (Pierce, Rockford, IL) or ECL Plus Western Blotting Detection reagent (GE Healthcare), and densitometric detection of bound antibody was performed with the use of a Typhoon 9410 laser scanner (GE Healthcare). Membranes were stained with Ponceau S for total protein and probed with rabbit polyclonal antibodies against total actin (Sigma–Aldrich) or glyceraldehyde-3-phosphate dehydrogenase (Abcam, Cambridge, U.K.) to control for protein loading.

Proteins displaying altered expression levels among the CCRF-CEM, CEM/VELB100, and CEM/dEpoB cells were excised from the 2D polyacrylamide gels and subjected to matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry as previously described (6,7). Peptides displaying mass differences between the γ-actin proteins from each cell line were selected for amino acid sequencing by electrospray ionization–time of flight tandem mass spectrometry (ESI-TOF/MS/MS) as described (6,7).

γ-Actin Gene Sequencing and Expression in Drug-Resistant Cell Lines and Acute Lymphoblastic Leukemia Samples

We prepared complementary DNA (cDNA), as previously described (7), from RNA that was isolated from CCRF-CEM cells, CEM/VELB100 cells, and CEM/dEpoB140 cells and from
samples of acute lymphoblastic leukemia using standard methods (18). DNA was isolated from samples of acute lymphoblastic leukemia using standard procedures as previously described (19–21). Primary leukemia cells were obtained at initial diagnosis (n = 44) or at bone marrow relapse (n = 25) from children who presented with B- or T-cell acute lymphoblastic leukemia at the Sydney Children’s Hospital (Randwick, NSW, Australia) and were treated on the Australia and New Zealand Children’s Cancer Study Group study V, VI, or VII (19,20) at the Centre for Children’s Cancer and Blood Disorders (Sydney Children’s Hospital). Written informed consent was obtained from a parent or guardian. All patients received vincristine as a component of induction and maintenance therapy for treatment of their disease (19,20). All experimental studies were approved by the Human Research Ethics Committees of the South Eastern Sydney Area Health Service and the University of New South Wales (Approvals: SESAHS 03/286 and HREC 040001). Peripheral blood or bone marrow biopsy specimens were harvested as part of routine diagnosis and treatment protocols. Mononuclear cells were purified on a Ficoll–LyophoPrep density gradient (Nycome, Sydney, Australia), resuspended in 10% DMSO/RPMI 1640, and stored in liquid nitrogen. All cell preparations contained greater than 90% leukemic blasts as determined by morphologic and immunophenotypic analyses.

The cDNAs (50 ng per reaction) were used to sequence the entire γ-actin coding region with the use of the PCR and the following overlapping primer sets: 1) G-Act 1F (5′-ATGGGAAGAAGAGATCGCCGCGCTGGGTCA-3′) and G-Act 654R (5′-TCGGCCGTTGAGGTGAAGAACTGG-3′); 2) G-Act 582F (5′-TGAGGAGGTCCGCAGAAGCTGCA-3′) and G-Act 3′UTR 48R (5′-ATTCTCTAATTACCCACCTCAGCA-3′). Each cDNA amplification reaction contained 1× Pfu amplification buffer (Stratagene, La Jolla, CA), 0.75 mM deoxy-nucleoside triphosphates (dNTPs), 62 ng of each primer, and 0.625 U Pfu Turbo DNA polymerase (Stratagene) in a total volume of 20 μL. The following PCR cycling conditions were used: 96 °C for 1 minute, 35 cycles of 96 °C for 1 minute, 64 °C (G-Act 1F/G-Act 654R) or 60 °C (G-Act 582F/G-Act 3′UTR 48R) for 1 minute, and 72 °C for 45 seconds, followed by 72 °C for 10 minutes. Amplified cDNA was purified and sequenced as previously described (16). The γ-actin nucleotide sequence of the resistant cell lines was compared with that of the parental CCRF-CEM cells and the published sequence (22).

The cDNA from acute lymphoblastic leukemia samples was subjected to semiquantitative RT–PCR to analyze γ-actin gene expression as previously described (8). Briefly, cDNA (50 ng) was amplified in 1× amplification buffer containing 1.5 mM MgCl2, 200 μM of each dNTP, 5 ng of each primer (γ-actin forward and reverse primers: 5′-TGTGTCTTCCTCATGTCG-3′ and 5′-CACGTAGGGAGTCCTTGCAG-3′, respectively; β2-microglobulin (β2M) forward and reverse primers: 5′-ACCCCACCTGAAAAAGATGA-3′ and 5′-ATCTTCAAACCTCCATGATG-3′, respectively), and 1 U Taq Gold polymerase. Reaction conditions were as follows: 94 °C for 4 minutes; 27 cycles of 94 °C for 45 seconds, 55 °C for 45 seconds, and 72 °C for 1 minute 30 seconds; and 72 °C for 5 minutes. PCR products were resolved on 12.5% polyacrylamide gels that were stained with ethidium bromide, visualized by a Gel-Doc UV-transilluminator (Bio-Rad), and subjected to densitometry using the Quantity One software package (Bio-Rad). The relative expression was determined by dividing the densitometric volume of the test γ-actin product by that of the control gene β2M PCR product.

Modeling of γ-Actin Structure

Models of mutant γ-actins were constructed with the use of the DS Modeling 1.0 implementation of the MODELER homology modeling program suite (Accelrys Inc, San Diego, CA). The primary amino acid sequence of γ-actin, in FASTA format, was obtained from the SwissProt database and modified to introduce the mutations identified by cDNA sequence analysis by replacing the required residues with the appropriate one-letter amino acid codes. We used the crystal structure for the complex of β-actin with adenosine diphosphate (23) obtained from the UniProtKB/Swiss-Prot Protein Knowledgebase (accession code: 1j6z) as the template structure for wild-type γ-actin because no crystal structure for γ-actin is available. A block sequence alignment between the primary sequence of mutated γ-actin and the template structure was performed using the 2D Align option in DS Modeling 1.0. We used the Build Models option to build models of the mutant proteins and evaluated the quality of the modeled structures by using the Profiles 3D protocol in the Protein Health module of DS Modeling 1.0.

Construction of Mutant γ-Actin Expression Plasmids

We used the full-length wild-type γ-actin cDNA sequence cloned into the Okayama–Berg vector (22) as template and the GeneEditor in vitro site-directed mutagenesis system (Promega Corporation, Madison, WI) according to the manufacturer’s instructions to generate separate cDNAs coding for each of the four mutant γ-actin proteins identified by mass spectrometry. The γ-actin sequences were then each cloned into the pcDNA 3.1(−) expression vector (Gibco, Invitrogen) using standard procedures.

Stable Transfection of Fibroblasts With γ-Actin Expression Constructs

Subconfluent mouse NIH/3T3 fibroblast cells were transfected by incubation with plasmid DNA from pcDNA 3.1 γ-actin expression vectors (1 μg) and Fugene transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. Stable transfecants were selected by growth for 16 days in medium containing 1 mg/mL G418 (Geneticin, Invitrogen), which selects for cells that express the neomycin resistance gene contained on the expression plasmid. Levels of γ-actin expression in the stable transfecants were determined by western blot analysis as described above. Equal loading was confirmed by Ponceau S staining and quantitation of immunoreactive signals determined as described above.

γ-Actin Gene Silencing by Small Interfering RNA

Human neuroblastoma SH-EP cells were plated onto 24-well plates at 1.5 × 10^4 cells per well and transfected with lipo-fectamine only at (mock) or with small interfering RNAs (siRNAs) targeted against γ-actin (5′-AAGAGATCGCCGCGCTGGAT-3′) or green fluorescent protein as a control (5′-CGCAAGCTGACCCCTGAATTCTG-3′) each at 100 nM (Qiagen, Valencia, CA) using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. The optimum concentration of siRNA (i.e., the concentration that gave the maximal level of gene silencing as determined by treating cells with a range of γ-actin siRNA concentrations [25–120 nM] and performing western
Clonogenic Assay of Drug Resistance

We used clonogenic assays of drug-treated cells to examine the effect of mutant γ-actin expression or siRNA-induced decrease in endogenous γ-actin expression on cellular resistance as previously described (24), with slight modifications. Briefly, stable NIH/3T3 transfectants expressing mutant γ-actin were seeded in replicate wells of a 24-well dish (100 cells per well) in medium containing increasing concentrations of vinblastine (1–50 μg/mL) and incubated for 3 days. The medium was replaced with drug-free medium and the cells were incubated for 4 days, after which the medium was removed. The colonies were stained with 0.5% crystal violet in 50% methanol. We used a Gel Doc imager and Quantity One software to determine the densitometric volume of each well and expressed relative to the respective drug-free control cells. We determined the concentration of vinblastine that killed 50% of the cells (ID50) by using a cubic-spline curve as previously described (6). Relative resistance was determined by dividing the ID50 of each γ-actin transfectant cell line by the ID50 of NIH/3T3 cells transfected with empty vector.

For siRNA-treated SH-EP cells, clonogenic assays were performed essentially as described above with the following minor modifications. SH-EP cells harvested 24 hours after siRNA transfection were plated in six-well plates (150 cells per well) and incubated for 3 days in medium that contained vinblastine (0.025–0.4 nM), paclitaxel (0.4–6.4 nM), epothilone B (0.025–0.4 nM), VP-16 (20–320 nM), or doxorubicin (0.5–8 nM). The medium was then removed and replaced with drug-free medium, and the cells were incubated for an additional 6–11 days until visible colonies formed. Colonies were stained as described above, and individual stained colonies in each well were counted. The surviving fraction was calculated as follows: colony number/ (number of cells seeded × plating efficiency), where plating efficiency equals the colony number divided by the number of cells seeded in drug-free media. Relative resistance was calculated as described for mutant γ-actin expressing NIH/3T3 cells above.

Indirect Immunofluorescence of Drug-Treated Transfectants

Stable NIH/3T3 cell transfectants expressing mutant γ-actin were grown to approximately 80% confluence in four-well chamber slides (Nalge Nunc International, Naperville, IL); treated with either 0.5% DMSO (vehicle control), 10 μM vinblastine, or 10 μM desoxyepothilone B for 30 minutes; and then immunostained for total α-tubulin using standard methods. Briefly, cells were fixed and permeabilized in ice-cold 100% methanol for 10 minutes, washed with phosphate-buffered saline (PBS), incubated in 10% FCS/PBS to block nonspecific binding, and then incubated with primary antibody against α-tubulin (clone DM1A; Sigma–Aldrich) diluted 1:500 in 5% FCS/PBS for 1 hour at 37 °C in a humidified chamber. The cells were washed in PBS and then incubated in 5% bovine serum albumin/PBS containing indocarbocyanine (Cy3)-labeled anti-mouse secondary antibody diluted 1:1000 for 45 minutes at room temperature. After PBS washes, the slides were mounted with DAPI II counterstain mounting medium (Vector Laboratories, Burlingame, CA). Epi-fluorescence microscopy was performed using an Axioplan 2 microscope (Zeiss, Oberkochen, Germany), and images were captured using a Sensicam Charged Coupled Device camera (PCO Imaging, Kelheim, Bavaria, Germany).

Measurement of Drug Accumulation

SH-EP cells plated into a 12-well plate at 3 x 10⁴ cells per well were mock transfected or transfected with control or γ-actin siRNAs as described above and incubated for 48 hours. The medium was removed from the cells and replaced with fresh medium containing either [³H]vincristine sulfate (7.1 Ci/mmol; final concentration of 12.5 nM) (GE Healthcare) or [³H]paclitaxel (14.7 Ci/mmol; final concentration of 50 nM) (Moravek Biochemicals, Brea, CA), and the cells were incubated for 2 hours at 37 °C. Uptake of labeled drug was determined as previously described (7). Briefly, mean incorporation of radiolabelled drug was determined for duplicate samples and expressed as picomoles of drug per milligram of protein. Three independent experiments were performed.

Tubulin Polymerization Assay

SH-EP cells plated onto six-well plates (6 x 10⁴ cells per well) were mock transfected or transfected with either γ-actin or control siRNAs as described above and incubated for 72 hours. The medium was replaced with fresh medium containing paclitaxel (0, 50, or 500 nM), and the cells were incubated for 1 hour at 37 °C. The cells were then washed, lysed in 120 μL hypotonic buffer (1 mM MgCl₂, 2 mM EGTA, 0.5% NP-40, 2 mM phenylmethylsulfonyl fluoride, 10 μL/mL protease inhibitor cocktail [Sigma–Aldrich], 20 mM Tris–HCl [pH 6.8]), incubated in the dark at 37 °C for 10 minutes, and then scraped from the plate and transferred to a microfuge tube. The microfuge tubes were centrifuged at 18000g for 10 minutes, and polymerized tubulin was collected in the pellet. Proteins in the supernatant (soluble fraction) and the pellet (insoluble fraction) were resolved by SDS–PAGE and transferred to membranes, which were probed with a monoclonal antibody to α-tubulin (Sigma–Aldrich). Tubulin levels were determined by densitometry, as described above. The amount of polymerized tubulin was determined by dividing the densitometric value of the polymer (insoluble) fraction by the sum of that for the polymer and soluble fractions for each individual sample, and results were expressed as a percentage (25).
Statistical Analysis

At least three independent experiments were performed for all clonogenic assays. Statistical differences between the ID₉₀ of empty vector versus mutant γ-actin transfectants, or mock- versus siRNA-transfected cells, were determined using two-sided unpaired Student’s t tests. At least three independent semiquantitative RT–PCR reactions were performed for each sample. Statistical differences between the relative expression of γ-actin in diagnosis versus relapse samples was determined by two-sided, unpaired Student’s t tests. Four independent experiments were performed for western blotting in the siRNA-transfected cells. Statistical differences between the relative expression of γ-actin in mock- versus siRNA-transfected cells were determined by two-sided, unpaired Student’s t tests. Six independent experiments were performed for the drug accumulation assays. Statistical differences between the mean incorporation of radiolabeled drug in mock- versus siRNA-transfected cells were also determined by two-sided unpaired Student’s t tests. For statistical analysis, a statistically significant difference was defined as P<.05.

RESULTS

Characterization of γ-Actin Isoforms Expressed by Antimicrotubule Drug–Resistant Leukemia Cells

We first compared expression of cytoskeletal proteins among human leukemia CCRF-CEM cells and two drug-resistant sublines, CEM/VLB100 cells (vinblastine resistant) and CEM/dEpoB140 cells (desoxyepothilone B resistant). 2D PAGE and SYPRO Ruby staining of total cellular protein from the three lines revealed differences in the intensities of protein spots that migrated at positions corresponding to specific actin isoforms. MALDI-TOF mass spectrometry initially identified the extra isoforms as γ-actin (data not shown), and western blot analysis of total cellular protein resolved by 2D PAGE using antibodies against β-, γ-, and total actin confirmed the identities of the proteins (Fig. 1). The ratio of γ-actin protein 1 to β-actin was lower in CEM/VLB100 cells than in CCRF-CEM cells. In addition, CEM/VLB100 cells expressed high levels of a more basic protein (γ-actin 2). The CEM/dEpoB140 cells expressed high levels of two γ-actin proteins (γ-actin 3 and γ-actin 4), γ-actin 4 with a slightly lower molecular weight than γ-actin 3 (Table 1). Analysis of the tryptic peptide masses of the two γ-actin isoforms expressed in the CEM/VLB100 cells revealed a peptide mass shift of 22 Da (Supplementary Fig. 1, available at http://jnccancerspectrum.oxfordjournals.org/jnci/content/vol98/issue19). Sequencing of these peptides using ESI-TOF MS/MS revealed that γ-actin 2 differed from γ-actin 1 by the substitution of a histidine residue for the aspartic acid residue at position 187 (Table 1). This amino acid substitution results in the loss of an isoelectric point (pI 5.46) than wild-type γ-actin (wt-γ-actin)

![Fig. 1. Expression of mutant actin proteins in leukemia cells.](https://example.com/fig1)

### Table 1. Amino acid sequences of peptides excised from two-dimensional gels and DNA sequences of γ-actin gene from drug-resistant leukemia cells

| Cell line/protein     | Peptide mass (Da)* | Peptide sequence* | Amino acid change† | Nucleotide position/change† |
|-----------------------|-------------------|-------------------|--------------------|---------------------------|
| CCRF-CEM/wt-γ-actin   | 1954.1            | VAPEEHP/LLTEAPLNPK|                    |                          |
|                       | 998.5             | DLTDYLMK          |                    |                          |
| CEM/VLB100/γ-actin 1  | 1968.1            | VAPEEHP/LLTEAPLNPK|                    |                          |
|                       | 998.5             | DLTDYLMK          |                    |                          |
| CM/VLB100/γ-actin 2   | 1954.1            | VAPEEHP/LLTEAPLNPK|                    |                          |
|                       | 1020.6            | DLTDYLMK          |                    |                          |
| CCRF-CEM/γ-actin 1    | 3199.6            | TTGIVMDSDGDVTH7VIPYEYALPHAILR |          |                          |
|                       | 1954.1            | VAPEEHPVLLTEAPLNPK|                    |                          |
| CEM/dEpoB 140/γ-actin 3 | 3254.6        | TTGIVMDSDGDVTH7VIPYEYALPHAILR | T162M | 485/aag → atg            |
|                       | 1954.1            | VAPEEHPVLLTEAPLNPK|                    |                          |
| CEM/dEpoB 140/γ-actin 4 | 3199.6        | TTGIVMDSDGDVTH7VIPYEYALPHAILR | T162M | 293/ceg → ctg            |

*Two peptides were sequenced for each protein. Italics represent amino acid positions in wt-γ-actin that were mutated in drug-resistant cells as indicated.
†Amino acid and nucleotide positions in human γ-actin sequence. Changed nucleotides are shown in bold.
(pI 5.31) (Fig. 1). In addition, the CEM/VLB100 γ-actin 1 protein had a peptide mass shift of 14 Da (Supplementary Fig. 1, available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue19) due to a substitution of a valine for a leucine at position 103 (Table 1). This amino acid substitution did not alter the protein charge; thus, the mutant protein migrated to the same position on the 2D gel as did wt-γ-actin in the parental CCRF-CEM cells. Thus, the CEM/VLB100 cells express two mutant γ-actin proteins. Similar analyses revealed that CEM/dEpoB140 cells also express two mutant γ-actin proteins, one in which the threonine at position 162 is changed to a methionine (T162M) and the other in which the proline at position 98 is changed to a leucine (P98L, Table 1). Supplementary Fig. 1, available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue19). Neither CEM/VLB100 cells nor CEM/dEpoB140 cells expressed detectable wt-γ-actin. Confirmation of all four mutations was obtained by cDNA sequencing of the entire γ-actin gene in all cell lines (Table 1). No changes were detected in the β-actin protein in either of the two drug-resistant cell lines.

We constructed a model of γ-actin by using a homology modeling strategy that uses the three-dimensional crystal structure of β-actin as a template (23,26). Such modeling was possible because β-actin differs from γ-actin by only four amino acids near the amino terminus. Modeling of the γ-actin amino acid substitutions onto this structure revealed that the P98L and V103L substitutions both lie in subdomain 1, which occurs within the secondary myosin-binding region of actin (Fig. 2, A). These amino acid substitutions may alter the conformation of the 92–103 loop, which has been implicated in myosin binding (27,28). The T162M substitution of γ-actin resides within a β-sheet located in subdomain 3 (Fig. 2, A). The D187H substitution changes the charge of a surface residue that lies on the face of the protein closest to the filament axis and is in close proximity to the ATP-binding cleft of the protein. By changing the residue at this position from a negative charge to positive charge, this substitution would reduce the hydrophobicity and the number of electrostatic interactions in this region of the molecule.

**Mutant γ-Actin Proteins and Resistance to Antimicrotubule Drugs**

We next examined the functional significance of the amino acid substitutions by transfecting NIH/3T3 mouse fibroblast cells with a single-mutant construct or cotransfecting with a combination of two mutant constructs (P98L with T162M or V103L with D187H, hereafter referred to as “double mutants”) and examining their cellular sensitivity to antimicrotubule drugs. Expression of wt-γ-actin, each of the four single-mutant γ-actins, or either of the two double-mutant γ-actins resulted in only modest increases in the level of total γ-actin compared with that in empty vector-transfected control cells (P98L, V103L, T162M, or D187H γ-actin: 1.3-fold to 1.4-fold increase; wt-γ-actin, 1.2-fold increase) (wt-γ-actin Mean Arbitrary Unit [MAU] = 3372, difference = 607.8, 95% confidence interval [CI] = 51.26 to 1164, P = .04; P98L MAU = 3670, difference = 906.1, 95% CI = –225.6 to 2038, P = .09; V103L MAU = 3970, difference = 1217, 95% CI = –316.7 to 2751, P = .09; D187H MAU = 3569, difference = 805.3, 95% CI = –248.6 to 1859, P = .10; P98L/T162M MAU = 2945, P = .02). Such modeling was possible because the threonine at position 162 is changed to a methionine (T162M) and the other in which the proline at position 98 is changed to a leucine (P98L, Table 1). Supplementary Fig. 1, available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue19). Neither CEM/VLB100 cells nor CEM/dEpoB140 cells expressed detectable wt-γ-actin. Confirmation of all four mutations was obtained by cDNA sequencing of the entire γ-actin gene in all cell lines (Table 1). No changes were detected in the β-actin protein in either of the two drug-resistant cell lines.

We constructed a model of γ-actin by using a homology modeling strategy that uses the three-dimensional crystal structure of β-actin as a template (23,26). Such modeling was possible because β-actin differs from γ-actin by only four amino acids near the amino terminus. Modeling of the γ-actin amino acid substitutions onto this structure revealed that the P98L and V103L substitutions both lie in subdomain 1, which occurs within the secondary myosin-binding region of actin (Fig. 2, A). These amino acid substitutions may alter the conformation of the 92–103 loop, which has been implicated in myosin binding (27,28). The T162M substitution of γ-actin resides within a β-sheet located in subdomain 3 (Fig. 2, A). The D187H substitution changes the charge of a surface residue that lies on the face of the protein closest to the filament axis and is in close proximity to the ATP-binding cleft of the protein. By changing the residue at this position from a negative charge to positive charge, this substitution would reduce the hydrophobicity and the number of electrostatic interactions in this region of the molecule.

**Mutant γ-Actin Proteins and Resistance to Antimicrotubule Drugs**

We next examined the functional significance of the amino acid substitutions by transfecting NIH/3T3 mouse fibroblast cells with a single-mutant construct or cotransfecting with a combination of two mutant constructs (P98L with T162M or V103L with D187H, hereafter referred to as “double mutants”) and examining their cellular sensitivity to antimicrotubule drugs. Expression of wt-γ-actin, each of the four single-mutant γ-actins, or either of the two double-mutant γ-actins resulted in only modest increases in the level of total γ-actin compared with that in empty vector-transfected control cells (P98L, V103L, T162M, or D187H γ-actin: 1.3-fold to 1.4-fold increase; wt-γ-actin, 1.2-fold increase) (wt-γ-actin Mean Arbitrary Unit [MAU] = 3372, difference = 607.8, 95% confidence interval [CI] = 51.26 to 1164, P = .04; P98L MAU = 3670, difference = 906.1, 95% CI = –225.6 to 2038, P = .09; V103L MAU = 3970, difference = 1217, 95% CI = –316.7 to 2751, P = .09; D187H MAU = 3569, difference = 805.3, 95% CI = –248.6 to 1859, P = .10; P98L/T162M MAU = 2945, P = .02).
difference $= 181.2$, $95\%$ CI $= -653.0$ to $1015$, $P = .58$; V103L/D187H MAU $= 3371$, difference $= 607.5$, $95\%$ CI $= 192.3$ to $1407$, $P = .10$). This result was expected because the levels of actin in the cell are subject to autoregulation (29).

We next examined the effect of expression of the mutant actins on drug sensitivity in NIH/3T3 cells. Expression of either subdomain 1 mutant $\gamma$-actin (i.e., P98L or V103L) was sufficient to provide resistance to tubulin-binding agents (Fig. 2, B). Cells transfected with the P98L mutant were 3.8-fold more resistant to vinblastine than cells transfected with empty vector (mean ID$_{50}$ for P98L $= 24.1$ μg/mL and mean ID$_{50}$ for empty vector $= 6.4$ μg/mL; difference $= -17.7$, $95\%$ CI $= -26.9$ to $-8.5$, $P = .003$) (Fig. 2, B); cells transfected with the V103L mutant were 3.6-fold more resistant (mean ID$_{50}$ for V103L $= 23.0$ μg/mL; difference $= -16.6$, $95\%$ CI $= -31.7$ to $-1.5$, $P = .04$). There was no statistically significant difference in sensitivity to vinblastine between cells transfected with empty vector (mean ID$_{50}$ = 6.4) and those transfected with the wt-$\gamma$-actin vector (mean ID$_{50}$ for wt-$\gamma$-actin $= 4.0$ μg/mL; difference $= 2.4$, $95\%$ CI $= -1.5$ to 6.3, $P = .18$). Cells expressing each of the double-mutant $\gamma$-actins (P98L/T162M or V103L/D187H) had ID$_{50}$s for vinblastine that were similar to those of cells expressing either P98L mutant $\gamma$-actin or V103L mutant $\gamma$-actin, suggesting that the double substitutions did not have an additive effect and that the amino acid substitutions in subdomain 1 of $\gamma$-actin played a predominant role in cellular resistance to antimicrotubule drugs. By contrast, cells expressing the T162M or D187H mutant $\gamma$-actins had ID$_{50}$s for vinblastine that were similar to those of control cells (Fig. 2, B). Similar drug-resistance profiles were observed in the $\gamma$-actin, wild-type, mutant, and double-mutant transfected cells treated with either desoxyepothilone B or paclitaxel (data not shown), indicating that the mutant $\gamma$-actin proteins are associated with cellular resistance to a variety of antimicrotubule agents. None of the single- or double-mutant $\gamma$-actin proteins conferred decreased sensitivity to two non-antimicrotubule agents, methotrexate (Fig. 3, A) and cisplatin (data not shown), compared with wt-$\gamma$-actin.

**Effect of Mutant $\gamma$-Actin Expression on Microtubule Disruption**

To examine the mechanism by which the P98L and V103L mutant $\gamma$-actins might have facilitated the survival of cells exposed to antimicrotubule agents, we treated cells expressing empty vector, wt-$\gamma$-actin, or either of the two mutant proteins with vinblastine or desoxyepothilone B and analyzed their microtubules by fluorescence microscopy (Fig. 3). All cells treated with DMSO vehicle displayed a normal microtubule network. However, treatment with vinblastine or desoxyepothilone B caused marked structural changes to cells expressing empty vector or wt-$\gamma$-actin. Specifically, we observed disruption of the cell shape and extensive cell rounding. By contrast, cells expressing either P98L or V103L mutant $\gamma$-actin retained their basic cell shape and substratum adhesion, and their microtubule networks remained largely intact, after exposure to these antimicrotubule drugs (Fig. 3, inset).

Interestingly, cells expressing the T162M or D187H mutant $\gamma$-actins also retained their cell shapes and displayed intact microtubule morphologies when exposed to antimicrotubule drugs (data not shown), even though neither the T162M or D187H $\gamma$-actin mutant–expressing cells displayed statistically significant resistance to these drugs in the clonogenic assay. These results indicate that wt-$\gamma$-actin is required for antimicrotubule drug–induced cell contraction and cell death and suggest that the mutant $\gamma$-actins are able to provide structural support to the microtubules that enables them to resist the impact of antimicrotubule drugs. This finding is the first evidence, to our knowledge, that mutations in actin directly affect the response of cells to antimicrotubule agents.

**Drug Response in Cells with Decreased levels of $\gamma$-Actin**

Because drug-resistant leukemia cells that expressed mutant $\gamma$-actin proteins also expressed no detectable wt-$\gamma$-actin, we investigated the possibility that the drug-resistance phenotype was due to the loss of wt-$\gamma$-actin function by transfecting human neuroblastoma SH-EP cells with siRNAs designed to eliminate wt-$\gamma$-actin expression. We could not use CCRF-CEM cells for these experiments because of their poor transfection efficiency; SH-EP cells were used because they express $\gamma$-actin and are readily transfectable. Transfection of $\gamma$-actin siRNA into SH-EP cells resulted in decreased expression of wt-$\gamma$-actin protein by approximately 50% (mean expression $= 0.66$ arbitrary units [AU]) when assayed 72 hours later, compared with mock-transfected cells (mean expression $= 1.3$ AU; difference $= 0.65$ AU, $95\%$ CI $= 0.39$ to 0.92 AU; $P < .001$) (Fig. 4, A).

This modest, although statistically significant, decrease in $\gamma$-actin expression probably reflects the fact that the $\gamma$-actin gene is an essential gene and cells cannot tolerate excessive silencing (30). Nevertheless, cells transfected with the $\gamma$-actin siRNA displayed several clear phenotypic effects. Compared with mock-transfected

![Fig. 3. Effect of mutant $\gamma$-actin expression on the microtubule network in cells treated with antimicrotubule agents. Mouse NIH/3T3 cells stably transfected with empty vector (EV), vector expressing full-length wild-type $\gamma$-actin (WT), or either of two mutant $\gamma$-actins (P98L or V103L) were treated with dimethyloxide vehicle (control), 10 μM vinblastine (VLB), or 10 μM desoxyepothilone B (dEpoB) for 30 minutes, fixed, stained with an antibody against total α-tubulin, and subjected to immunofluorescence microscopy. Bottom row: The magnified views of cells expressing the V103L mutant $\gamma$-actin demonstrate that the microtubule structures are still intact after drug treatment. Scale bar $= 20 \mu m$.](https://academic.oup.com/jnci/article-abstract/98/19/1363/2521804)
Fig. 4. Effects of small interfering RNA (siRNA) blockade of \( \gamma \)-actin expression on actin structure and cellular resistance to antimicrotubule drugs. (A) Neuroblastoma SH-EP cells were transfected with 100 nM siRNA designed to block \( \gamma \)-actin expression or 100 nM anti–green fluorescent protein siRNA (Control) or transfection reagent only (Mock). The cells were lysed 72 hours later, and equal amounts of each protein lysate were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with a \( \gamma \)-actin–specific antibody and with an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control for loading and transfer. The relative expression of \( \gamma \)-actin was determined by dividing the densitometric value of the \( \gamma \)-actin band by that of the GAPDH band. The graph shows the mean \( \gamma \)-actin/GAPDH relative ratio (bars) and 95% confidence intervals (error bars) from six individual experiments. \( P \) value (two-sided, Student’s \( t \) test) for comparison with mock-transfected cells is represented on graph. (B) F-actin staining. Cells transfected as described above were stained with Alexa Fluor Phalloidin 488 to detect F-actin (green) and with DAPI to detect DNA (blue). Representative images show that SH-EP cells transfected with \( \gamma \)-actin siRNA were larger and had more pronounced stress fibers than mock-transfected or control siRNA-transfected cells. Scale bar = 20 \( \mu \)m. (C) Clonogenic assay in drug-treated cells. Cells transfected as described above were incubated for 24 hours and then seeded into six-well culture dishes and incubated for 3 days in the presence of paclitaxel at the indicated drug concentrations. The cells were then grown for 6–11 days in medium lacking drug until colonies formed that were stained with crystal violet. For each siRNA or control treatment, the surviving fraction at each concentration of paclitaxel was calculated as the number of colonies in the drug-treated wells divided by the number of colonies in wells not treated with paclitaxel. The individual points on the graph correspond to the mean value of the surviving fraction when either \( \gamma \)-actin siRNA or control siRNA was compared to mock-transfected cells from a minimum of six separate experiments; error bars correspond to 95% confidence intervals. Statistically significant \( P \) values for paclitaxel-treated siRNA-transfected cells: \( *P = .02 \) for \( \gamma \)-actin siRNA-transfected versus mock-transfected cells at 0.04 nM paclitaxel; \( \#P = .003 \) for \( \gamma \)-actin siRNA-transfected versus mock-transfected cells at 1.6 nM paclitaxel; \( \#P < .001 \) for \( \gamma \)-actin siRNA-transfected versus mock-transfected cells at 3.2 nM paclitaxel; \( \#P < .001 \) for \( \gamma \)-actin siRNA-transfected versus mock-transfected cells at 6.4 nM paclitaxel; \( \gamma \)-actin siRNA-transfected versus mock-transfected cells at 3.2 nM paclitaxel; \( \gamma \)-actin siRNA-transfected versus mock-transfected cells at 6.4 nM paclitaxel. or control siRNA-transfected cells, \( \gamma \)-actin siRNA-transfected cells were larger and had more pronounced actin stress fiber networks (Fig. 4, B). In addition, compared with mock-transfected cells, SH-EP cells transfected with \( \gamma \)-actin siRNA displayed statistically significantly higher resistance as determined by its higher ID{\textsubscript{50}} to paclitaxel (\( P < .001 \); Fig. 4, C, and Table 2), vinblastine (\( P = .04 \)), and epothilone B (\( P = .045 \), Table 2). By contrast, there was no statistically significant difference in drug sensitivity between control siRNA-treated cells and mock-transfected cells cultured in the presence of these antimicrotubule drugs. Moreover, the effects appeared to be antimicrotubule drug specific because we observed no increase in clonogenic survival among \( \gamma \)-actin siRNA-transfected cells versus mock-transfected cells treated with either of the non-antimicrotubule agents, VP-16 (etoposide) or doxorubicin (Table 2).

To eliminate the possibility that increased survival of the \( \gamma \)-actin siRNA-transfected cells in the presence of antimicrotubule drugs was due to decreased drug accumulation, we performed radiolabeled drug accumulation assays for both vincristine and paclitaxel. We found no statistically significant difference in drug accumulation between the \( \gamma \)-actin siRNA-transfected cells and mock-transfected cells (Table 3). The finding that reduced expression or mutation of \( \gamma \)-actin induces similar drug-resistance phenotypes suggests that it is loss of wt-\( \gamma \)-actin that confers resistance to antimicrotubule drugs.

**Effect of Decreased \( \gamma \)-Actin Expression on Microtubule Polymerization**

The microtubule structure in the NIH/3T3 cell transfectants that expressed mutant \( \gamma \)-actin remained largely intact upon vinblastine or desoxypodophiline B treatment (Fig. 3), suggesting that loss of wt-\( \gamma \)-actin may inhibit an early step in the pathway of antimicrotubule drug action. To address this possibility, we examined the levels of polymerized and unpolymerized tubulin in siRNA-transfected SH-EP cells treated with various concentrations of paclitaxel. Intrinsic levels of polymerized tubulin were not statistically significantly different between the \( \gamma \)-actin siRNA-transfected cells and control siRNA-transfected cells (Fig. 5). Among cells treated with 50 nM or 500 nM paclitaxel, the percentage of polymerized tubulin was statistically significantly lower in cells transfected with \( \gamma \)-actin siRNA (mean percentage of polymerized tubulin in cells treated with 50 nM and 500 nM paclitaxel = 50.7 and 59.8, respectively) than in mock-transfected cells (50 nM paclitaxel: mean percentage of polymerized tubulin = 64.1; difference = 13.4, 95% CI = 5.1 to 21.7; \( P = .008 \); 500 nM paclitaxel: mean percentage of polymerized tubulin = 74.0; difference = 14.2, 95% CI = 11.2 to 17.1; \( P < .001 \)). By contrast, when cells were treated with 50 nM or 500 nM paclitaxel, there was no statistically significant difference in the mean percentage of polymerized tubulin between mock- and control siRNA-transfected cells (control siRNA cells with 50 nM paclitaxel: mean percentage of polymerized tubulin = 60.4; difference = 3.7, 95% CI = −8.5 to 15.9; \( P = .48 \); control siRNA cells with 500 nM paclitaxel: mean percentage of polymerized tubulin = 70.2; difference = 3.9, 95% CI = −1.7 to 9.4; \( P = .14 \)) (Fig. 5). In similar studies in which cells were treated with the microtubule-depolymerizing agent vinblastine, we were unable to quantify the levels of polymerized tubulin because intrinsic levels of polymer are so low (approximately 20% of the total soluble plus polymerized tubulin) that the reduction in polymer levels induced by...
vinblastine produced highly variable and inconsistent results (data not shown). However, the paclitaxel data are consistent with cell imaging data in the mutant γ-actin transfecants (Fig. 3) and suggest that wild-type γ-actin expression is required for disruption of microtubule structures induced by antimicrotubule drugs.

**γ-Actin Status of Samples from Relapsed Leukemia Patients**

To investigate the possible clinical relevance of aberrant forms of γ-actin, we sequenced the γ-actin genes using cDNA or DNA isolated from leukemic blasts obtained from 37 randomly chosen patients (8 patients at diagnosis, 29 patients at relapse) with childhood acute lymphoblastic leukemia who were treated at the Sydney Children’s Hospital. We detected no mutations in the coding region of the γ-actin gene in any of these patient samples, suggesting that disruption of γ-actin protein function via mutations may be a rare event in acute lymphoblastic leukemia. Moreover, our siRNA data demonstrated that loss of wt-γ-actin expression was sufficient to induce resistance to antimicrotubule drugs in human neuroblastoma cells. Therefore, we next examined γ-actin gene expression levels by RT–PCR analysis of 69 acute lymphoblastic leukemia patient samples (44 obtained at diagnosis, 25 obtained at relapse), a subset of which had been used for mutation detection described above. γ-Actin gene expression was statistically significantly lower in acute lymphoblastic leukemia samples collected at clinical relapse (mean expression ratio γ-actin/β2M = 0.53) compared with samples collected at diagnosis (mean expression ratio γ-actin/β2M = 0.68; difference = 0.15, 95% CI = 0.04 to 0.27, P = .01) (Fig. 6, A).

We also examined samples obtained at diagnosis and relapse (two samples were obtained on different days from a continuing relapse and referred to as relapse 1 and relapse 2) from a single acute lymphoblastic leukemia patient and found that the expression of γ-actin was statistically significantly lower in both relapse samples (mean expression ratio γ-actin/β2M at relapse 1 and relapse 2 = 0.17 and 0.16, respectively), than in the sample obtained at diagnosis (mean expression ratio γ-actin/β2M at diagnosis = 0.91, difference (relapse 1) = 0.75, 95% CI = 0.46 to 1.03, P < .001; difference (relapse 2) = 0.75, 95% CI = 0.42 to 1.08, P = .003), with the first relapse sample showing a 5.5-fold reduction in γ-actin expression and the second relapse sample showing a 5.7-fold reduction in γ-actin expression, both compared with the level of γ-actin expression at diagnosis (Fig. 6, B). This patient had aggressive and drug-resistant disease and underwent relapse 6.4 months after diagnosis while still on therapy; there was no remission after the first relapse, and the second relapse thus represents ongoing disease. Together, these data clearly show that a reduction in γ-actin expression is associated with relapsed acute lymphoblastic leukemia in this group of patients.

**DISCUSSION**

Molecular mechanisms of resistance to anticancer agents that target tubulin and microtubules are poorly understood. Herein, we have reported the identification, by high-resolution 2D PAGE and mass spectrometry, of novel mutant forms of γ-actin and the consequent loss of wild-type protein in acute lymphoblastic leukemia cells selected for resistance to microtubule-targeting agents. Mechanistically, loss of wild-type γ-actin via expression of γ-actin mutant proteins or silencing of γ-actin expression decreased the cellular sensitivity to antimicrotubule agents. These findings demonstrate that loss of wt-γ-actin mediates resistance to tubulin-targeted drugs. In addition, we found that γ-actin gene expression was statistically significantly lower in acute lymphoblastic leukemia samples obtained from patients at relapse than in samples obtained from patients at diagnosis. These data indicate that wild-type γ-actin is essential for the efficacy of the following agents:

| Table 3. Drug uptake by γ-actin siRNA–transfected neuroblastoma SH-EP cells* |
|------------------|------------------|------------------|------------------|------------------|
| Transfection     | Mean [3H]vincristine uptake, pmoles/mg of protein (95% CI) | Mean [3H]paclitaxel uptake, pmoles/mg of protein (95% CI) |
|                  | 0 h         | 2 h                  | 0 h         | 2 h                  |
|                  | P†         | P†                  | P†         | P†                  |
| Mock             | 2.91 (1.17 to 4.66) | Referent          | 14.6 (6.15 to 23.09) | Referent          |
| Control siRNA    | 2.80 (0.84 to 4.79) | .88                | 12.7 (9.37 to 15.97) | .41               |
| γ-Actin siRNA    | 2.65 (~1.12 to 6.42) | .80                | 15.2 (10.27 to 20.22) | .80               |

*Time points, 0 and 2 h, refer to the amount of time the mock, control siRNA, and γ-actin siRNA-treated cells were incubated in the presence of radiolabeled drug. Data are from three independent experiments. CI = confidence interval; siRNA = small interfering RNA.

†Student’s t test (two-sided).
Fig. 5. Effect of paclitaxel on tubulin polymerization in γ-actin siRNA–transfected SH-EP cells. Neuroblastoma SH-EP cells were transfected with 100 nM γ-actin siRNA (white bars) or 100 nM anti–green fluorescent protein siRNA (hatched bars), or were mock transfected (black bars), incubated for 72 hours, and then treated with paclitaxel (0, 50, or 500 nM) for 1 hour. The cells were washed; the soluble and polymerized pools of tubulin were separated by detergent solubilization and centrifugation, and the relative amount of each fraction was determined by western blotting with the use of a monoclonal antibody against total α-tubulin. Following detection of anti-tubulin binding with a horseradish peroxidase–conjugated secondary antibody, blots were imaged on a laser scanner and densitometry was performed on the soluble and polymerized tubulin immunoreactive bands. The percentage of polymerized tubulin was calculated by dividing the densitometric value of the polymerized fraction (P) by the densitometric value of the total polymerized and soluble fractions (P + S). Columns represent mean values; error bars correspond to upper 95% confidence intervals for four individual experiments. P values represented on graph refer to mock versus γ-actin siRNA at 50 and 500 nM paclitaxel, respectively.

Fig. 6. Decreased γ-actin gene expression in acute lymphoblastic leukemia patients at relapse. Semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) for γ-actin and β2-microglobulin (β2M) was performed on leukemic blasts isolated from acute lymphoblastic leukemia patients presenting at diagnosis or at clinical relapse. The densitometric volume of the γ-actin RT–PCR product is expressed relative to the densitometric volume of the control gene β2M. (A) γ-Actin gene expression was lower in leukemic blasts isolated from patients at relapse than in blasts isolated from patients at diagnosis (P = .01). Bars correspond to mean values of at least three independent experiments; error bars correspond to 95% confidence intervals. (B) γ-Actin gene expression in leukemic blasts isolated from one individual at diagnosis and from two individual samples obtained at clinical relapse. The γ-actin expression in both relapse samples is statistically significantly reduced (relapse 1, P < .001, and relapse 2, P = .003) compared to the sample isolated at diagnosis. Bars correspond to the mean of at least three independent experiments. Error bars correspond to upper 95% confidence intervals.
Xenopus egg extracts using fluorescence speckle microscopy have highlighted the intricate structural synergy of microtubules and microfilaments (33), and a number of binding proteins have been identified that cross-link the two structures (32). Thus, it seems possible that altered γ-actin expression could affect structural links between the microtubule and actin filament cytoskeletons, resulting in alterations in the cells’ response to antimicrotubule agents. Indeed, we found that primary leukemia cells obtained from acute lymphoblastic leukemia patients at relapse expressed lower levels of γ-actin than cells obtained from patients at diagnosis. This decreased expression of γ-actin in the relapsed acute lymphoblastic leukemia patient samples would presumably render these patients less susceptible to further vincristine treatment.

The following study limitations should be noted. We detected no mutations in the coding region of the γ-actin gene in randomly selected diagnosis and relapse samples from 37 acute lymphoblastic leukemia patients, which raises the possibility that actin mutants do not explain drug resistance in the relapse patients. However, further studies in a larger patient population are required to determine if such mutations are present at low levels in acute lymphoblastic leukemia and in other cancers from patients who have been treated with antimicrotubule drugs. Although we have shown that decreasing wild-type γ-actin levels by approximately 50% confers resistance to antimicrotubule drugs, potentially due to altered cross-talk between microtubules and microfilaments, the precise mechanism by which γ-actin regulates antimicrotubule drug–induced cell death remains to be determined. Further investigation of the signaling pathways involved in microtubule disruption, and actin microfilament regulation, is required to answer this important question. Furthermore, because the actin cytoskeleton is involved in apoptotic signaling (34), we cannot exclude the possibility that loss of wt-γ-actin induces resistance to antimicrotubule drugs by conferring resistance to programmed cell death. However, the cells transfected with mutant γ-actin or siRNA targeted to γ-actin were not resistant to the non-antimicrotubule agents methotrexate, cisplatin, VP16, and doxorubicin. In addition, the vinblastine- and desoxoepothilone B–resistant CEM cells originally harboring these mutations can still undergo apoptosis induced by non-antimicrotubule drugs (data not shown). Further analyses of the γ-actin transfectants with a wider range of cytotoxic agents are required to completely resolve this issue.

In summary, we have identified a novel mechanism of resistance to antimicrotubule drugs in childhood acute lymphoblastic leukemia that highlights a previously unknown but important interaction between microtubules and actin filaments that is mediated by γ-actin. Our data establish γ-actin as an essential factor in the efficacy of antimicrotubule agent therapy and provide, for the first time, functional and associative clinical evidence of a novel form of drug resistance that involves the interaction between γ-actin and microtubules. Directed targeting of the actin cytoskeleton and/or the pathways regulating the interactions between the actin and microtubule systems should be investigated as a possible approach to improving the efficacy of antimicrotubule drugs used in cancer therapy.

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NOTES

N. M. Verrills and S. T. Po’uha contributed equally to this work.

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