DNA Topoisomerase IIα in Multiple Myeloma: A Marker of Cell Proliferation and Not Drug Resistance

Carla S. Wilson, M.D., Ph.D., L. Jeffrey Medeiros, M.D., Raymond Lai, M.D., Ph.D., Anthony W. Butch, Ph.D., Althea McCourty, B.S., Kathy Kelly, Ph.D., Russell K. Brynes, M.D.

Department of Pathology, University of Arkansas for Medical Sciences (CSW), Little Rock, Arkansas; Division of Pathology, The University of Texas M.D. Anderson Cancer Center (LJM, RL), Houston, Texas; Department of Pathology, UCLA Medical Center (AWB, KK), Los Angeles, California; Department of Pathology and Laboratory Medicine, University of Southern California (AM, RKB), Los Angeles, California

DNA topoisomerase IIα (topo IIα) is the target for a number of antineoplastic agents. Down-regulation of this enzyme is one form of drug resistance. Topo IIα is also involved in DNA replication and transcription and serves as an indicator of proliferation rate in many human malignancies. This study examines whether topo IIα is one of the mechanisms of chemoresistance commonly observed in multiple myeloma (MM) or alternatively, whether topo IIα is associated with tumor cell proliferation. Bone marrow (BM) biopsy sections from 72 cases of MM, stratified according to proliferative activity (bromodeoxyuridine uptake), were immunostained for topo IIα. Immunoreactivity with an additional marker of drug resistance, glutathione-*S*-transferase π, and the proliferation marker Ki-67 were also examined. Topo IIα was expressed in 26 (36%) cases and correlated strongly with proliferative activity ($P < .001$). A role for drug resistance could not be supported, given this strong relationship with proliferation and the finding that glutathione-*S*-transferase π expression in 57 (78%) cases was independent of topo IIα immunoreactivity. Topo IIα was identified in 91 to 100% of highly proliferative tumors, as evaluated by bromodeoxyuridine uptake or Ki-67 reactivity, respectively. Proliferation also correlated with the histologic grade of the MM. Therefore, topo IIα immunoreactivity is primarily a marker of cell proliferation in MM and as such is likely to have prognostic significance. Highly proliferative tumors are most likely to be sensitive to chemotherapeutic protocols using anti–topo IIα agents.

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Address reprint requests to: Carla S. Wilson, M.D., Ph.D., University of New Mexico, Department of Pathology, 915 Camino de Salud NE, Albuquerque, NM 87131; e-mail: cswilson@salud.unm.edu; fax: 505-272-5186.

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Multiple myeloma (MM) is an incurable disease, in large part because of drug resistance. Multiple mechanisms of drug resistance are likely to play a role. The classical mechanism of multidrug resistance (MDR) is increased expression of the *mdr*1 gene, which encodes for the membrane P-glycoprotein (p-170) (1). Many cases of MM have P-glycoprotein–positive plasma cells (2–4). Another mechanism of drug resistance in MM is glutathione-*S*-transferase π (GST) expression, whereby chemical insults induce gene upregulation (5). Increased GST leads to more rapid detoxification of drugs of the nitrogen mustard category of alkylating agents through alteration of intracellular drug metabolism (6). Melphalan, included in this category, is one of the most frequently used chemotherapeutic agents in the initial treatment of MM. The mRNA levels for the genes GST-2 and *mdr*1 significantly correlate in MM, supporting the theory that resistant tumors have several concurrent mechanisms of chemoresistance (7).

A special form of drug resistance, referred to as atypical MDR, is associated with quantitative and qualitative alterations in DNA topoisomerase IIα (topo IIα) (8). The α isoform of topo II is a major target for antineoplastic drugs that act to stabilize topo IIα-DNA complexes and interfere with DNA replication and transcription. These drugs include numerous chemotherapeutic agents: epipodophyllotoxins (etoposide), teniposide, aminoacridines, anthracyclines, and anthracenediones (9). The myeloma cell lines RPMI 8226 and Dox1V exhibit drug resistance to these agents that is associated with decreased topo IIα protein, mRNA, and transcription activity (10, 11). The few reported studies using patient specimens suggest that topo IIα inhibitors may not be clinically useful in MM because of either the absence of topo IIα in a majority of cases

(12) or the movement of topo IIα from the nucleus into the cytoplasm, away from its site of drug interaction (13).

Topo IIα creates double-stranded breaks to catalyze the unknotting or decatenation of DNA in mammalian cells (14). Intranuclear levels are therefore cell-cycle and proliferation dependent, with overexpression reported in a number of human malignancies (15–25). Tissue sections from a number of these tumors show parallel immunoreactivity for topo IIα and the proliferation marker Ki-67. Although this has not been examined in MM, the reportedly low incidence of topo IIα in MM may reflect the high percentage of cells often arrested in the nonproliferative G_0/G_1 phase of the cell cycle. The fraction of proliferating plasma cells in MM is most commonly assessed by bromodeoxyuridine labeling and is expressed as a labeling index (BrdU LI) (26, 27).

The purpose of this study is to assess topo IIα expression in MM to determine whether paucity of topo IIα, as a drug target, is a mechanism of MDR or alternatively, whether topo IIα is associated with proliferation. Immunoreactivity for topo IIα in trephine biopsy sections was compared with the drug resistance protein GST and to the proliferation markers Ki-67 and BrdU LI.

MATERIALS AND METHODS

Case Selection

The study population included all MM patients with bone marrow (BM) specimens submitted for analysis of proliferative activity using the BrdU LI technique, between May 1996 and December 1997 at the University of Arkansas for Medical Sciences. Case selection was based on the BrdU LI of the tumors. From 364 cases of MM with a positive BrdU LI (expressed as a percentage of labeled cells) of ≥0.2, five cases were selected from each of 15 different levels of BrdU LI using 0.2 intervals from BrdU LI of 0.2 to 2 and using 1.0 intervals from 2.0 to 6.0 and then >7.0, to encompass a wide spectrum of proliferative activity. The first five cases in each designated BrdU LI interval, with >30% BM plasma cells and sufficient material remaining in paraffin blocks, were chosen for examination. At BrdU LI of 5.0 to 5.9 and 6.0 to 6.9, only three and four cases, respectively, fulfilled these criteria. Therefore, the final sample size for this study was 72 cases.

Bromodeoxyuridine (BrdU) Labeling Index

Mononuclear cells were isolated from BM aspirate specimens by Ficoll-Hypaque (Sigma, St. Louis, MO) gradient separation and incubated for 1 hour at 37° C in media containing 10% fetal bovine serum, 40 uM 5-bromo-2'-deoxyuridine, and 5-fluoro-2'-deoxyuridine (Sigma). After culture, cells were extensively washed, cytocentrifuged onto glass slides, fixed in 70% ethanol, and treated with 4 N HCl for DNA strand separation. Cells were stained with rhodamine-conjugated rabbit anti-human kappa and lambda antibodies (DAKO, Carpinteria, CA). BrdU uptake was identified using a mouse anti-BrdU monoclonal antibody (IgG1, Becton-Dickinson, San Jose, CA) followed by fluorescein-conjugated goat anti-mouse IgG1-specific antibodies (Southern Biotechnology Associates, Birmingham, AL). Five hundred kappa- or lambda-positive plasma cells were counted by fluorescent microscopy, and the number of BrdU-positive plasma cells was determined. The BrdU labeling index (BrdU-LI) was defined as the number of kappa- or lambda-positive plasma cells that were BrdU positive divided by the number of kappa- or lambda-positive plasma cells, expressed as a percentage of labeled cells (28). As previously reported, only small numbers of MM plasma cells have *in vitro* proliferative activity and are capable of incorporating BrdU during a 1-hour culture period (28, 29). Proliferative activity was considered to be low, intermediate, or high based on a BrdU LI of <1.0, 1.0 to 2.8, or ≥3.0, respectively (29).

Immunohistochemical Staining

Zenker-fixed BM biopsy sections were stained with a TechMate 1000 automated immunostainer (Ventana Biotek Medical Systems, Tucson, AZ), using monoclonal antibodies to topo IIα (clone JH2, 1:40, Signet, Dedham, MA), Ki-67 (MIB-1, 1:100, Immunotech, Westbrook, ME.), and GST (clone 350-10, 1:30, DAKO). All sections were placed in 0.01 mol/L citrate buffer at pH 6.0 (HIER Buffer, Ventana Biotek) and heated twice in a microwave (5 min/cycle) for epitope retrieval before staining. Reactivity was detected with an avidin-biotin immunoperoxidase detection system employing 3', 3'-diaminobenzidine-tetrahydrochloride dihydrate (Ventana Biotek) as the chromogen. Ki-67 positivity was graded as the percentage of plasma cells with positive nuclear staining: <10%, 10 to 20%, or >20% plasma cells, at 400× magnification in any area of the tumor. As previously described (30), these percentages correspond to low, intermediate, and high proliferative rates. Therefore, Ki-67 was considered positive in the biopsy sections if 10 to 20% of plasma cells stained and strongly positive if >20% of plasma cells stained as described. Topo IIα and GST were similarly considered positive or strongly positive if 10 to 20% or >20% of plasma cells had nuclear staining, respectively. Evaluation of cytoplasmic staining for topo IIα was also attempted, but results were inconsistent and varied

with background staining. All cases were evaluated independently by three authors (CSW, RL, RKB), without knowledge of the BrdU LI or patient clinical status. Cases with discrepant interpretations were jointly reviewed by the three observers at a multiheaded microscope and reconciled.

Morphologic Criteria

Hematoxylin and eosin–stained trephine biopsy sections were reviewed and the cases of MM were graded using the Bartl *et al.* (31) classification as follows: Grade I—low grade (Marschalko and small cell types), Grade II—intermediate grade (cleaved, polymorphous, and asynchronous types), and Grade III—high grade (blastic type).

Statistics

Statistical analysis was performed using SigmaStat for Windows 2.0 software (Jandel Corp., San Rafael, CA). Comparison for variables involving nonparametric data was determined using the Spearman correlation test. Fisher's exact test was used to compare differences in a variable between groups. Differences were considered significant at $P < .05$.

RESULTS

Patient Characteristics

The median age of the 72 patients was 57 years (range, 27 to 77 y). One patient had a nonsecretory MM. The remaining MM cases synthesized the following paraproteins: 32 IgG kappa, 11 IgG lambda, 11 IgA kappa, 7 IgA lambda, 7 kappa light chain, and 3 lambda light chain.

Correlation of Topo IIα with BrdU-LI and Ki-67

Topo IIα immunoreactivity was detected in 26 of 72 (36%) MM. Topo IIα expression strongly correlated with proliferative activity of MM, as measured by BrdU LI (% labeled cells; $P < .001$) and Ki-67 ($P < .0001$; Figs. 1, 2). The two measurements of proliferative activity also strongly correlated with one another ($P < .001$). Among highly proliferative MMs, 91% evaluated by BrdU LI and 100% evaluated by Ki-67 showed topo IIα immunoreactivity. Table 1 shows the percentage of cases having topo IIα–positive or Ki-67–positive MM cells when grouped by the BrdU LI. One case with a low BrdU LI showed strong positivity for both Ki-67 and topo IIα. Review of the BM biopsy sections from this case showed an interstitial infiltrate of mixed Marshalko type (low grade) and asynchronous type (intermediate grade) MM cells. However, focal nodules of entirely asynchronous type MM cells were also present, embedded in fibrotic stroma. These cells had increased mitoses and were strongly positive for Ki-67 and topo IIα, suggesting focal clonal transformation of low-grade MM to higher grade MM. It is likely that because of fibrosis, these cells were not represented in the aspirate material assessed for the BrdU LI. Review of the aspirate smears showed numerous Marschalko-type plasma cells with only a few intermingled asynchronous forms. In general, Ki-67 reactivity was more predictive of topo IIα positivity than was the BrdU LI. Among the Ki-67–positive cases, 70% (19/27) were strongly positive. Topo IIα was strongly positive in 38% (10/26) of cases with topo IIα expression.

Relationship of Topo IIα with GST

GST was expressed in 56 of 72 (78%) MM and the majority showed strong positivity (48/56) (Fig. 1). No correlation with BrdU LI, Ki-67, or topo IIα immunoreactivity was identified. GST positivity was found in 88% (23/26) of topo IIα–positive MM and 72% (33/46) of topo IIα–negative MM. GST reactivity could not be predicted by knowledge of histologic grade of MM, percentage plasma cell involvement, or paraprotein isotype.

Correlation of Proliferation Markers with Histologic Grade

Only two cases had high-grade, blastic-type morphology (Bartl Grade III) so they were combined with the intermediate grade tumors (Bartl Grade II) for statistical evaluation. Proliferative activity correlated with the histologic grade of MM. No cases (0/19) of low histologic grade MM (Bartl Grade I) had high proliferative activity as assessed by Ki-67 evaluation. In contrast, low-grade histology was seen in 49% (26/53) of low to intermediately proliferative MM ($P < .001$). Similarly, low-grade histology was found in 48% (24/50) of low to intermediately proliferative MM *versus* 9% (2/23) of highly proliferative tumors based on BrdU LI ($P = .001$).

DISCUSSION

Topo IIα is associated with the active proliferation of mammalian cells. Previous studies have not specifically examined topo IIα in relationship to proliferation in MM. The most commonly used indicator of proliferative activity in MM is the BrdU LI, which is performed on cultured BM aspirate material. The BrdU LI measures the percentage of plasma cells in the S phase of the cell cycle. Using this technique, the proliferative activity of myeloma cells has important prognostic implications, with high proliferative activity correlating with shortened patient survival (32, 33). An alternative mea-

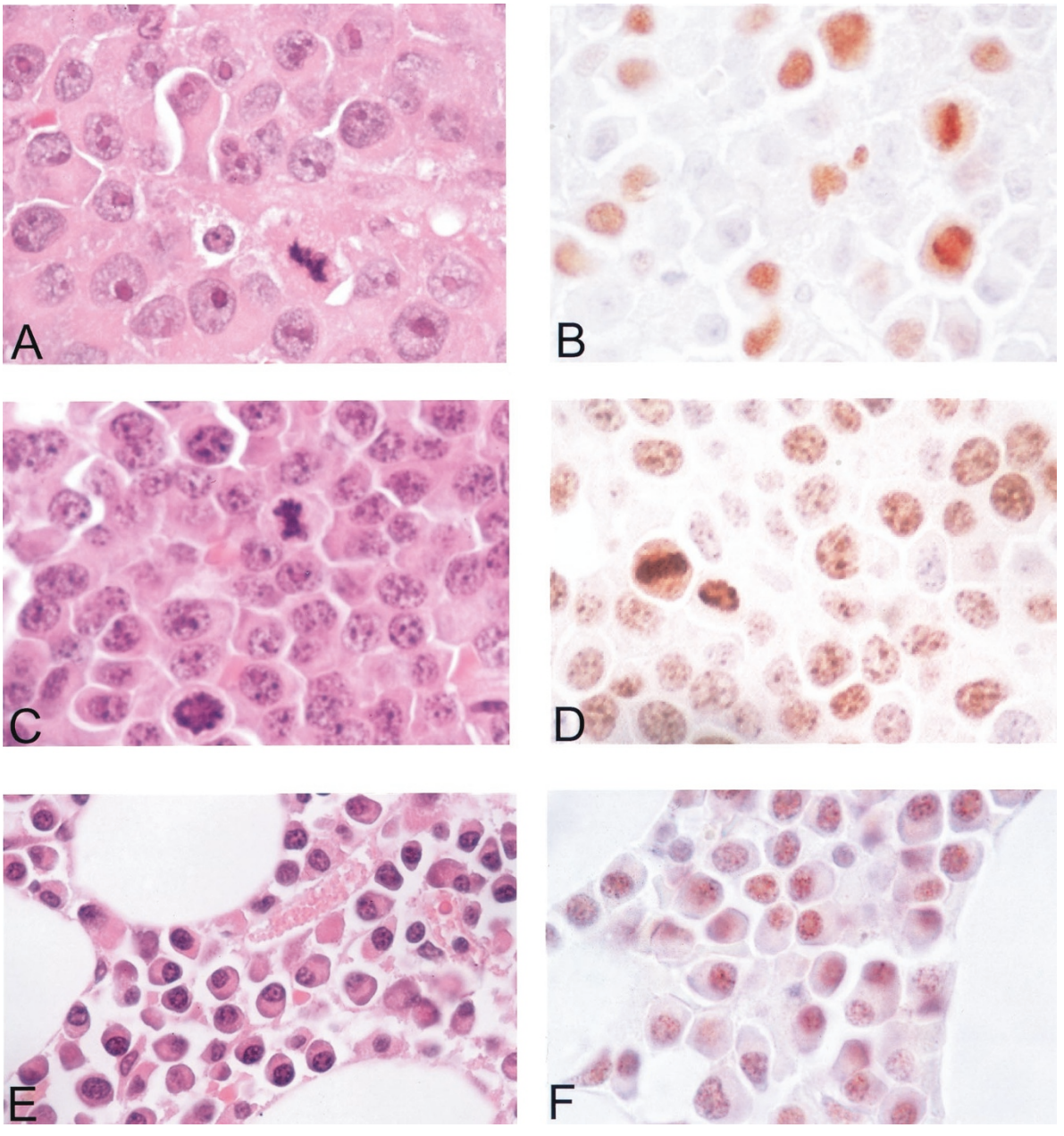

FIGURE 1. High-grade myeloma (**A,** Bartl Grade III, hematoxylin and eosin stain) with nuclear positivity for DNA topoisomerase IIα in >20% of plasma cells (**B,** immunoperoxidase stain). Intermediate-grade myeloma (**C,** Bartl Grade II, hematoxylin and eosin stain) with nuclear staining for Ki-67 in >20% of plasma cells (**D,** immunoperoxidase stain). Low-grade myeloma (**E,** Bartl Grade I, hematoxylin and eosin stain) showing glutathione-*S*-transferase π expression in a majority of plasma cells (**F,** immunoperoxidase stain). Original magnification, 500x (**A–F**).

sure of assessing proliferation in tumor sections is Ki-67 expression, evaluated by immunostaining with the MIB-1 antibody. Ki-67 discriminates between cycling (G_1, S, and G_2+M) and resting (G_0) cells. In this study, Ki-67 immunoreactivity strongly correlated with the BrdU LI. A significant correlation was also found between topo IIα immunoreactivity and these two measures of proliferation. This finding strongly supports a role for topo IIα as a proliferation marker in MM.

Topo IIα was expressed in 36 % of MM cases in this study and, depending on the measurement used, in either 91% (BrdU LI) or 100% (Ki-67) of MM with high proliferative activity. Ki-67 immunoreactivity best predicted for topo IIα positivity. Flow cytometric analyses of ovarian carcinomas and cultured human glioma cells have shown that topo IIα–positive cells are mainly in the S to G_2+M phases of the cell cycle, analogous to Ki-67 (22). Our rare cases with discrepant Ki-67 and BrdU LI results most likely reflect different MM subpopulations in the aspirate and biopsy specimens from a given individual. In addition, the incidence of topo IIα positivity was relatively high because of the study design. Our selection of cases based on different BrdU LI levels allowed for a greater proportion of

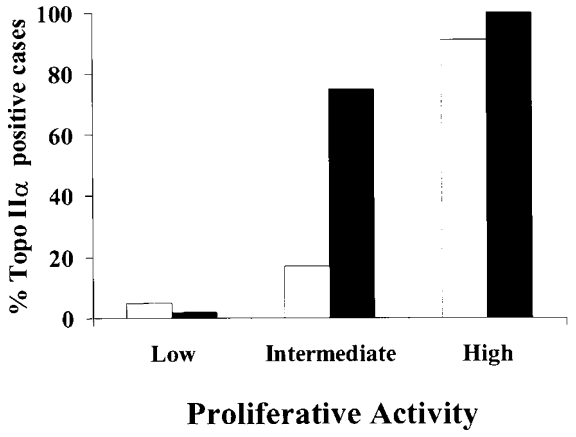

FIGURE 2. Percentage of MM cases with DNA topoisomerase IIα expression based on proliferative activity of the plasma cells. White bars represent low (<1.0), intermediate (1.0 to 2.8), and high (>3.0) BrdU LI (percentage of labeled cells). Black bars represent low (<10%), intermediate (10 to 20%), and high (>20%) Ki-67 expression.

TABLE 1. Number of Cases Having Topoisomerase IIα– and Ki-67–Positive Myeloma Cells When Grouped by BrdU Labeling Index

	BrdU Labeling Index		
	Low	Intermediate	High
Topo IIα positive (%)	1/20 (5)	5/30 (17)	20/22 (91)
Ki-67 positive (%)	1/20 (5)	6/30 (20)	20/22 (91)

BrdU, bromodeoxyuridine.

MM with higher proliferative activity than is normally found. Increasing proliferative activity, by all measures, correlated with higher histologic grade of the MM.

Down-regulation of topo IIα is also a mechanism of drug resistance. The few previous studies of topo IIα expression in MM have suggested that resistance of myeloma cells to topo IIα inhibitors may be due to a paucity of drug target in the nucleus of malignant cells. Topo IIα mRNA was weakly detected in only one of 16 specimens from patients with MM and is decreased in a doxorubicin-resistant variant of the human myeloma cell line RPMI 8226 (11, 12). Decreased topo IIα and alterations in expression of GST are also found in RPMI 8226 cells, without a direct relationship between the two (10). Finally, a recent study suggested that movement of topo IIα from the nucleus to the cytoplasm in RPMI 8226 cells and patient MM cells contributes to drug resistance, with shuttling of the protein being triggered by increased cell-cell contact (13). However, immunofluorescent labeling of 10 patient specimens in this study showed nuclear staining in 10 to 15% of BM cells. These cells likely represent the proliferative fraction of the tumors, given our findings. Although shuttling of topo IIα from the nuclear compartment may play a role in MDR, our finding of a strong association between nuclear topo IIα staining and proliferation confirms that this is the primary role for topo IIα.

GST immunoreactivity was present in 78% of MM in this study and was not related to topo IIα reactivity. A high incidence of GST expression in MM has been previously documented (2, 12). GST was coexpressed with another marker of drug resistance, p-170, in 77% of BM specimens from MM patients, with no relationship to tumor stage or myeloma isotype (2). Relatively high levels of GST mRNA were also found in 76% of MM, possibly reflecting an oncogenic change rather than chemotherapeutic drug–induced expression (12). The comparably high incidence of GST expression in MM at all levels of proliferative activity in our study supports the possibility that GST expression is an intrinsic property of many MM. Whether or not increased GST expression leads to more rapid detoxification of chemotherapeutic agents in MM remains to be clarified.

Overexpression of topo IIα in human malignancies is due to either a dysregulated or qualitative alteration of topo IIα in the cell cycle and is associated with more aggressive biological behavior in many tumor types (16, 23, 24). *In vitro* studies suggest that tumors with high topo IIα nuclear expression may have greater sensitivity to chemotherapeutic regimens (34, 35). However, one study of topo IIα immunoreactivity in breast cancer failed to show such a relationship (25).

In summary, we found that topo IIα expression can be identified in MM cells by immunohistochemical staining of paraffin-embedded BM sections. This enzyme is closely associated with cell proliferation in MM and represents a useful alternative to evaluation by BrdU LI or Ki-67. Topo IIα immunoreactivity, as a gauge of proliferation, may be a prognostic indicator of patient survival. Future clinical studies will be required to determine whether topo IIα immunoreactivity is also an indicator of chemotherapeutic sensitivity.

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