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Incidence of Mutation and Deletion in Topoisomerase IIα mRNA of Etoposide and mAMSA-resistant Cell Lines

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The efficacy of all chemotherapeutic agents is limited by the occurrence of drug resistance. To further understand resistance to topoisomerase II inhibitors, 50 sublines were isolated as single clones from parental cells by exposure to VP-16 (etoposide) or mAMSA (*m*-amsacrine). Subsequently, a population of cells from each subline was exposed to three-fold higher drug concentrations allowing 16 stable sublines to be established at higher extracellular drug concentration. Finally, 66 sublines were picked up. The frequency and nature of mutations in the *topoisomerase II* gene in the drug-selected cell lines were evaluated. In order to screen a large number of cell lines, an RNAse protection assay was developed and mismatches were observed in 13.6% of resistant cell lines (12% of resistant cell lines exposed to lower drug concentrations and 18.8% of resistant cell lines exposed to higher drug concentrations). Some of these mutations are located in vital regions of topoisomerase II (phosphorylation sites in the C-terminal or N-terminal, and nuclear localizing signal of topoisomerase II). Our findings suggest that mutations of *topoisomerase II* gene are an important and frequent mechanism of resistance to topoisomerase II inhibitors.

Key words: Topoisomerase II — Mutation — Drug resistance — Multidrug resistance — Chemotherapy

Human topoisomerase IIα is a ubiquitous, ATP-dependent, nuclear enzyme that belongs to the family of type II DNA topoisomerases, which are found in all living organisms. While type II topoisomerases are essential for viability, interest has been stimulated by the demonstration that these enzymes are targets for a diverse group of antineoplastic agents, including the epipodophyllotoxins, the anthracyclines, the acridines and others. Exposure of cells to increasing concentrations of topoisomerase poisons remains a strategy for identification of areas of topoisomerase II that are important for function. Using this approach, point mutations in the topoisomerase IIα cDNA have been demonstrated clustered in the highly conserved AB domain, which is thought to mediate the breakage-reunion action of topoisomerase, or near tyrosine 805, the active site for covalent binding to DNA.[1, 2] More recently, deletions in the C-terminal region have been reported.[3]

MATERIALS AND METHODS

Cell lines and cell cultures The cell lines described in the present report were initially isolated as single clones; and the clones were then exposed to increasing concentrations of drug. Four parental breast cancer cell lines, ZR-75B, MCF7, MDA-MB-231 and T47D cells, were used. The initial selections were performed at a different drug concentration for each cell line (ZR-75B: 300 n*M* VP-16 (etoposide), 30 n*M* mAMSA (*m*-amsacrine); MCF7: 500 n*M* VP-16, 50 n*M* mAMSA; MDA-MB-231: 1000 n*M* VP-16, 100 n*M* mAMSA; T47D: 300 n*M* VP-16, 30 n*M* mAMSA). These concentrations were those that were toxic to most cells, and resulted in the survival of only a few cells that grew as isolated colonies. Clones were picked up from the highest drug concentration in which single colonies could be isolated, and this varied among the three cell lines. The initial isolates (50 cell lines) were designated ZR-VPs (300), MCF7-VPs (500), MDA-VPs (1000), T47D-VPs (300), ZR-ms (30), MCF-ms (50), and MDA-ms (100), and T47D-ms (30), because they were isolated from plates containing 300, 500, 1000, and 300 n*M* VP-16 and 30, 50, 100, 30 n*M* mAMSA, respectively (Table I). Subsequently, a population of cells from each of these initial isolates, which did not have mutation, was exposed to a 3-fold higher concentration of drug and sixteen additional sublines were established: ZR-VPs (900), MCF7-VPs (1500), MDA-VPs (3000), T47D-VPs (300), ZR-ms (90), MCF7-ms (150), MDA-ms (300), and T47D-ms (90) (Table I).

The parental cells and the resistant sublines were grown in monolayer in minimum essential medium (MEM) containing 10% fetal bovine serum, 2 m*M* L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37°C. The resistant sublines have been maintained continuously in VP-16 at the concentrations indicated in parentheses.

RNAse protection assay Total RNA (20 μg) was hybrid-

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ized with 2×10^5 cpm of antisense RNA probes synthesized using a modification of the method of Melton *et al*.[4] RNAse protection analysis was performed on these hybrids as described.[5] Following heat denaturation of the RNA probe (Fig. 1) and sample RNA at 85°C for 5 min, and overnight hybridization at 45°C, the samples were digested with an RNAse mixture at 30°C for 1 h before treatment with a freshly prepared mixture of Proteinase K and 10% SDS for 15 min at 37°C. The samples were extracted with phenol and chloroform, ethanol-precipitated, lyophilized, and resuspended in gel loading buffer for separation on a 6% polyacrylamide gel. Samples were electrophoresed at 55 mA for 2–3 h, followed by autoradiography for 1–3 days.

Sequencing The T7 and SP6 Sequenase Version 2.0 kits from Amersham (Buckinghamshire, UK) were used for cycle sequencing. Cycle sequencing reactions were analyzed on the Visible Genetic Open Gene automated sequencing system. All sequences were confirmed in both directions.

RESULTS AND DISCUSSION

The principal change observed initially was a decrease in the expression and activity of topoisomerase IIα, with increased multidrug resistance protein (MRP) levels in some cell lines. We found decreased expression of topoisomerase IIα (less than 50% of parental cells) in 39 of 50 initial isolates, suggesting that reduced expression is a common adaptation. Reduction of topoisomerase IIα levels effectively diminishes the main intracellular target of VP-16, mAMSA, and other topoisomerase II poisons, and can confer broad cross-resistance to these agents.[6]

To identify acquired mutations in 66 cell lines, an RNAse protection assay was developed. RNAse protection assay is a novel method to detect mismatches in RNA. When mRNA of a cell line has acquired mutations, extra bands will appear. Fragments covering the entire coding sequence of topoisomerase IIα as well as the 91 and 48 residues at the 5′ and 3′ ends, respectively, were isolated after PCR amplification and subcloned in pGEM3Z (Fig. 1). Using this approach, nine mismatches have been identified in eight cell lines (Figs. 1, 2). In these cell lines, mutations were determined by sequencing (Fig. 1).

The frequency and nature of mutations in topoisomerase IIα in the drug-selected cell lines were evaluated. In order to screen a large number of cell lines, an RNAse protection assay was developed. Nine mismatches have been identified in eight cell lines. Mutations confirmed by sequencing were observed in 13.6% of resistant cell lines [12% (6/50) of resistant cell lines exposed to lower drug concentrations and 18.8% (3/16) of resistant cell lines exposed to higher drug concentrations]. Our findings suggest that mutations of *topoisomerase IIα* gene are an important and frequent mechanism of resistance to topoisomerase II inhibitors, but are independent of the degree of resistance.

Table I. Fold Resistance Relative to Parental Cells

Cell lines	VP	AM	Cell lines	VP	AM	Cell lines	VP	AM	Cell lines	VP	AM
ZR-75B	1	1	MDA-MB-231	1	1	MCF7	1	1	T47D	1	1
ZR-VP2 (300)	134	45	MDA-VP4 (1000)	16	6	MCF7-VP3 (500)	23	12	T47D-VP1 (300)	56	23
ZR-VP3 (300)	77	24	MDA-VP7 (1000)	20	10	MCF7-VP6 (500)	22	11	T47D-VP4 (300)	65	32
ZR-VP11 (300)	137	15	MDA-VP11 (1000)	18	7	MCF7-VP7 (500)	23	7	T47D-VP5 (300)	76	23
ZR-VP13 (300)	317	70	MDA-VP12 (1000)	35	11	MCF7-VP8 (500)	49	6	T47D-VP7 (300)	44	15
ZR-VP16 (300)	172	47	MDA-VP13 (1000)	18	7	MCF7-VP10 (500)	47	5	T47D-VP10 (300)	93	36
ZR-VP11 (900)	647	25	MDA-VP20 (1000)	12	6	MCF7-VP13 (500)	37	10	T47D-VP15 (300)	55	2340
ZR-VP13 (900)	810	111	MDA-VP7 (3000)	227	22	MCF7-VP15 (500)	25	18	T47D-VP7 (900)	120	40
ZR-m1 (30)	84	6	MDA-VP13 (3000)	35	11	MCF7-VP17 (500)	17	10	T47D-VP10 (900)	312	39
ZR-m3 (30)	45	12	MDA-m4 (100)	44	22	MCF7-VP6 (1500)	61	11	T47D-m2 (50)	43	23
ZR-m4 (30)	79	14	MDA-m5 (100)	64	32	MCF7-VP17 (1500)	49	6	T47D-m6 (50)	47	48
ZR-m6 (30)	56	12	MDA-m6 (100)	33	24	MCF7-m1 (50)	12	27	T47D-m7 (50)	56	71
ZR-m7 (30)	67	16	MDA-m14 (100)	44	22	MCF7-m2 (50)	15	29	T47D-m9 (50)	56	32
ZR-m8 (30)	78	23	MDA-m15 (300)	51	32	MCF7-m3 (50)	12	14	T47D-m15 (50)	47	48
ZR-m9 (30)	56	14	MDA-m14 (100)	168	135	MCF7-m4 (50)	15	16	T47D-m19 (50)	89	69
ZR-m1 (90)	320	48	MDA-m15 (300)	187	138	MCF7-m6 (50)	15	11	T47D-m6 (150)	85	92
ZR-m4 (90)	255	33				MCF7-m10 (50)	16	15	T47D-m7 (150)	85	135
						MCF7-m11 (50)	16	6			
						MCF7-m1 (150)	20	28			
						MCF7-m2 (150)	23	37			

VP, etoposide; AM, *m*-amsacrine.

Partial characterization of these mutations to determine their significance yielded the following results.

(A) T47D-VP1 (300) T47D-VP1 was isolated following exposure to 300 n*M* VP-16. Cytotoxity studies confirmed resistance to VP-16 and other topoisomerase II agents. RNAse protection analysis identified a deletion of 200 base pairs, confirmed by sequence analysis to comprise residues 4265 to 4464. Comparison with the genomic DNA sequence of parental cells confirmed this to represent loss of a complete exon. Genomic DNA clones from T47D-VP1 were either wild type or lacked 26 nucleotides spanning the 3′ exon/intron junction of the deleted exon. The latter is a novel mechanism of acquired resistance: acquired aberrant splicing. Immunoblotting identified a 159 kDa protein predicted from the 200 bp deletion and an early termination. More importantly, the 159 kDa protein was localized principally, but not exclusively in the cytoplasm, suggesting that nuclear translocation was impaired (data not shown).[7)]

(B) MCF7-VP8 (500) MCF7-VP8 was isolated following exposure to 500 n*M* VP-16. Cytotoxicity studies confirmed resistance to etoposide and other topoisomerase II poisons. RNAse protection analysis identified a deletion of 200 base pairs and 'AA insertion,' confirmed by sequence analysis to comprise residues 4458 to 4459 of the topoisomerase IIα cDNA in MCF-7-VP8. Reduced topoi-

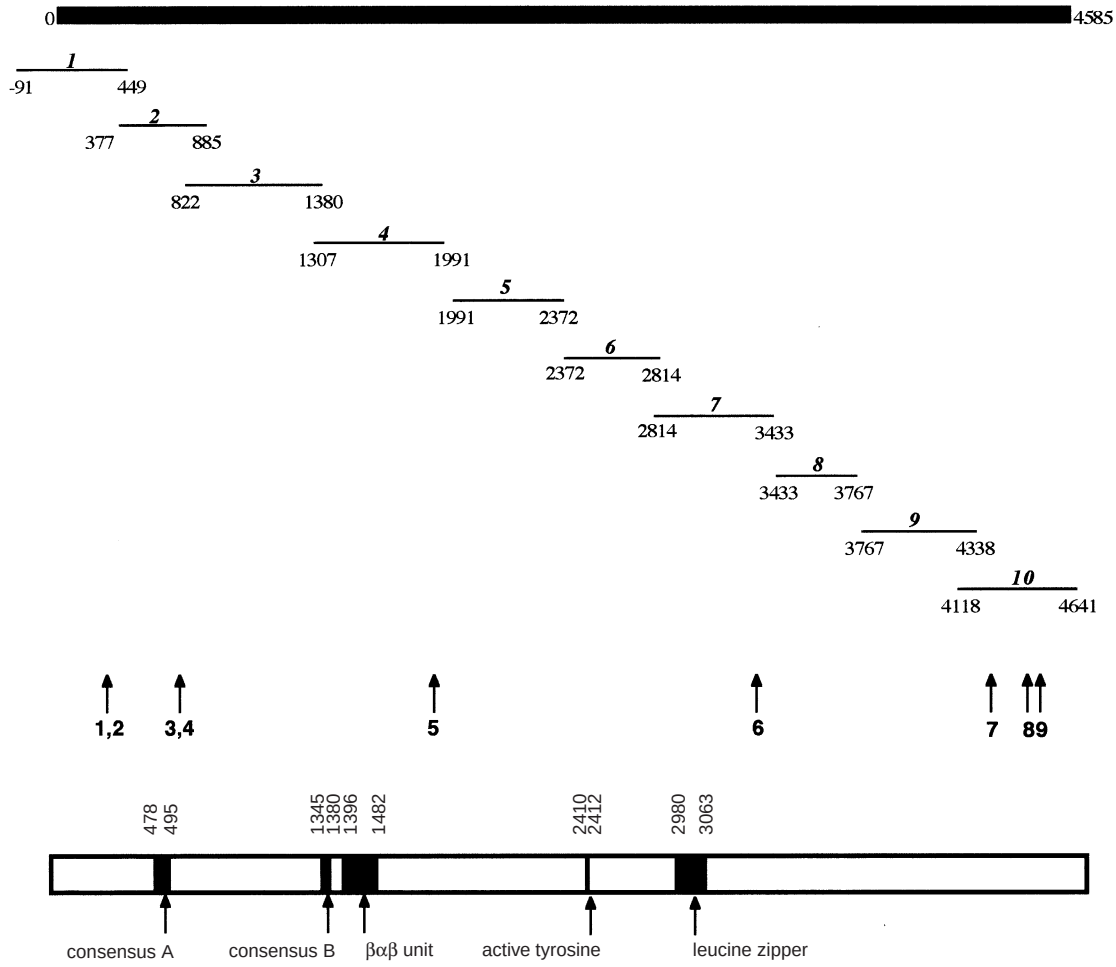

Fig. 1. Templates for RNAse protection assay probe and acquired mutations in topoisomerase IIα. Topoisomerase II c-DNA was divided into 10 areas. Riboprobes for RNAse protection assay were prepared with each fragment. 1, ZR-mAMSA1 (90), 18 bp deletion (344–361); 2, ZR-mAMSA4 (90), 18 bp deletion (344–361); 3, MDA-mAMSA6 (250), 3 bp deletion (641–643, AAG, Lys); 4, MDA-mAMSA14 (250), 3 bp deletion (641–643, AAG, Lys); 5, T47D-VP5 (300), 1959 C⇒A, Ser⇒Arg; 6, MDA-VP7 (3000), 615 deletion (3193–3807); 7, T47D-VP1 (300), 200 bp deletion (4265–4464); 8, MCF7-VP8 (500), "AA" insertion (4458–4459); 9, T47D-VP5 (300), 4483 G⇒A, Asp⇒Asn.

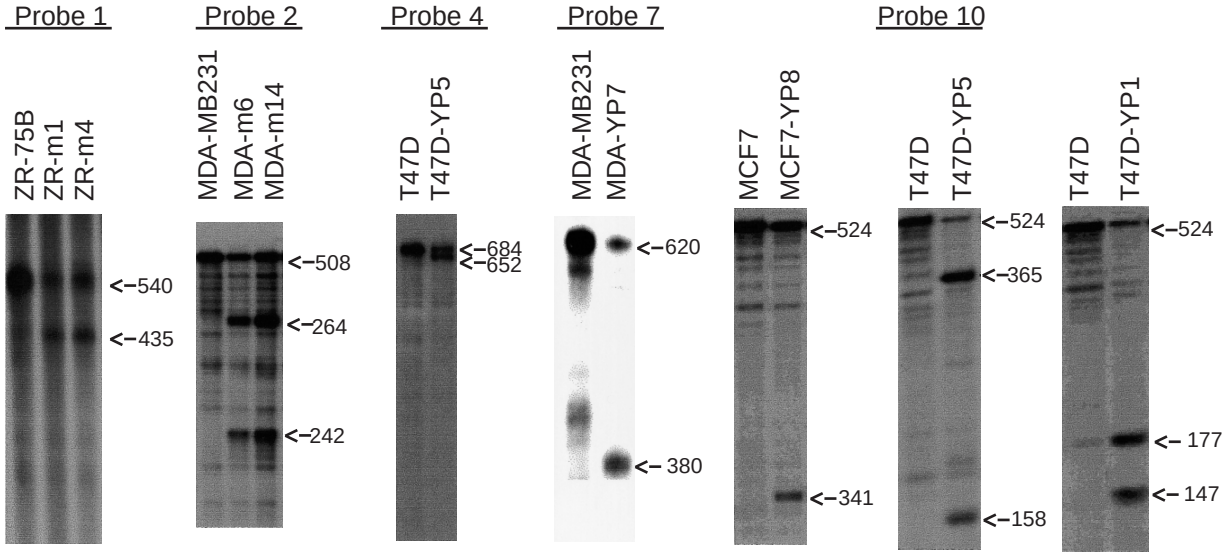

Fig. 2. Results of RNAse protection assay. Mutations and deletions were found by RNAse protection assay. Extra bands: 435 bp in probe 1; 264 bp and 242 bp in probe 2; 652 bp in probe 4; 380 bp in probe 7; 341 bp, 365 bp, 158 bp, 177 bp, and 147 bp in probe 10.

somerase IIα mRNA and protein levels were observed in the cell line. It was surprising to find that nuclear extracts from the cell line had comparable topoisomerase II activity to that of parental cells. Analysis of the extent of phosphorylation demonstrated that topoisomerase IIα from the resistant cells was relatively hypophosphorylated compared to that of parental cells. This was not unexpected, given the available evidence which suggests that the C-terminal region is the principal area for this modification, and the fact that the truncation resulted in a loss of 17 serines and 7 threonines that are supposed to be phosphorylation sites.[3, 8] The results demonstrate unequivocally that phosphorylation sites exist elsewhere, since the protein was clearly phosphorylated. While a conclusive explanation for the relatively high activity of the truncated topoisomerase cannot be provided, two possibilities can be considered: (1) phosphorylation sites outside of the C-terminal domain can bring about a greater degree of activation; or (2) the C-terminal region has a negative effect on the activity which can be relieved by phosphorylation, or better still by truncation, a hypothesis that has been previously suggested,[9] and argued against.[10] In this cell line, hypophosphorylation secondary to loss of a proportion of the C-terminal domain of topoisomerase IIα mediated the restored activity despite a fall in topoisomerase IIα mRNA and protein, which resulted in cross resistance to topoisomerase II poisons.[7]

(C) MDA-VP7 (3000) MDA-VP7 (3000) was originally isolated from parental MDA-MB231 cells by exposure to 1000 n*M* VP-16. Subsequent to this, a population was gradually advanced and maintained in 3000 n*M* VP-16. In the initial isolate, reduced topoisomerase II levels and activity were observed. Subsequent to this, topoisomerase II activity returned to normal, although the levels of protein, while higher than those in the initial selection, remained lower than normal. The latter appears to have resulted from the acquisition of a mutation in one of the two topoisomerase IIα alleles, characterized by the in-frame deletion of 615 residues, including residues 3193 to 3807. Originally identified by RNAse protection analysis, the genomic organization surrounding this deletion is currently being characterized. The deletion appears to render the protein product unstable, since the expected 140 kDa product is not detectable by immunoblotting or immunoprecipitation with a polyclonal antibody. Increased activity was observed in nuclear extracts from cells metabolically labeled with orthophosphate.[11] This resulted in the restoration of topoisomerase activity, while maintaining reduced protein levels, allowing the growth rate to normalize while maintaining a reduced susceptibility to the formation of cleavable complexes.[12, 13]

(D) ZR-m1 (90) and ZR-m4 (90) These were originally isolated from parental ZR-75B cells by exposure to 30 n*M* mAMSA. Subsequent to this, a population was gradually advanced and maintained in 90 n*M* mAMSA. Both have 18 bp deletions and the decatenation activity of nuclear extracts is insensitive to VP-16. The deletion includes a serine that could be a potential phosphorylation site.[14]

(E) Other mutations To search for other mutations, functional expression of wild-type and mutant human topoi-

somerases in yeast is planned. TOP2 yeast mutants with temperature-conditional expression of topoisomerase can be complemented by wild-type human topoisomerase II. For individual mutations, sensitivity to etoposide or mAMSA of yeast harboring mutant human *topoisomerase II* genes can be examined.[15)]

It has been thought that modulation of the expression and cellular accumulation of topoisomerase II inhibitors are likely to more important clinically than acquired mutations as a mechanism of resistance to topoisomerase II inhibitors. Though we need to consider whether the drug concentration used for isolating resistant cell lines was adequate as a clinical model of drug resistance, acquired mutations were actually observed in 13.6% of resistant cell lines in present studies. If our *in-vitro* findings reflect the situation in clinical samples, acquired mutations of the *topoisomerase IIα* gene must be an important and frequent mechanism of resistance to topoisomerase II inhibitors.

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