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RESPONSE TO EPIRUBICIN IN PATIENTS WITH SUPERFICIAL BLADDER CANCER AND EXPRESSION OF THE TOPOISOMERASE II α AND β GENES

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Biopsies of superficial bladder cancer were analysed to study the relationship between response to epirubicin and the expression of the human topoisomerase II α and β genes. Tissue samples were obtained prior to treatment and a marker tumour was left in the bladder. Transcript levels of both genes were generally lower in biopsies taken following treatment failure. Levels of topoisomerase II mRNA were uniformly lower in tumour tissue than in biopsies of normal tissue.

The majority of patients with transitional cell cancer of the bladder present with superficial tumours restricted to the urothelium (pTa) or lamina propria (pT1). These cancers are managed surgically using transurethral resection, but approximately 70% of patients develop recurrent disease within 2 years (Fleischmann and Goldberg, 1993). Intravesical chemotherapy is administered to reduce the recurrence rate and prolong the disease-free interval. This form of treatment is unlike systemic chemotherapy in that the tumour is directly exposed to a single agent (such as epirubicin) for a predetermined period.

The type II topoisomerases (referred to as topo II) are the major intracellular target for many antineoplastic agents, including epirubicin. These drugs stabilise a normally transient reaction intermediate called the cleavable complex, consisting of a topo II protomer bound covalently to the 5′ end of each cut DNA strand via a phosphotyrosyl bond (Zhang *et al.,* 1990). These protein-associated DNA strand breaks are not necessarily cytotoxic *per se,* but are probably converted to a lethal lesion following interaction with the translocating replication machinery (Tsao *et al.,* 1993).

In vitro studies using cell lines have indicated that a correlation exists between expression of topo II and sensitivity to topo II-targeting drugs (Davies *et al.,* 1988; Fry *et al.,* 1991; Long *et al.,* 1991; Webb *et al.,* 1991; Nitiss *et al.,* 1993). Cells containing low levels of topo II form fewer strand breaks and are less sensitive to drugs interacting with topo II. However, clinical evidence for a role for topo II in response to chemotherapy has been difficult to acquire, primarily because biopsies from solid tumours are rarely available immediately before and after chemotherapy. Moreover, many of the studies were performed before the existence of 2 closely related isoforms of topoisomerase II were identified. The topoisomerase II α and β isozymes are 170 and 180 kDa, respectively, and are the products of distinct genes on different chromosomes (Chung *et al.,* 1989; Drake *et al.,* 1989; Jenkins *et al.,* 1992; Tan *et al.,* 1992; Austin *et al.,* 1993). There is some evidence from *in vitro* studies that the α isozyme is more susceptible to inhibition by antineoplastic drugs than is the β isozyme (Drake *et al.,* 1989).

The aim of this study was to investigate the role of the topoisomerase II α and β isozymes in the response of superficial bladder cancers to epirubicin. As part of a clinical trial, a marker tumour was left in the bladder following surgery and then removed approximately 3 months later if the tumour did not respond to epirubicin (Popert *et al.,* 1994). The biopsies from these tumours enabled us to compare topoisomerase II mRNA levels in the same human bladder cancer before and after a single exposure to a topo II inhibitor and to determine whether topo II expression correlated with response to therapy.

MATERIAL AND METHODS

RNA extraction

Tumour samples were obtained during routine cystoscopy, immediately frozen in liquid nitrogen and stored at −80°C. Normal bladder tissue was obtained during donor nephroureterectomy and frozen and stored in the same way as the tumour samples. To extract RNA, the samples were processed as described by Chomczynski and Sacchi (1987). One microliter of RNA guard (Pharmacia, Milton Keynes, UK) was added to minimise degradation during storage at −70°C. RNA concentration was quantified spectrophotometrically and its integrity assessed by agarose gel electrophoresis in TBE buffer.

Tumour response

The samples were obtained from patients with histopathologically confirmed pTa or pT1 transitional cell cancer of the bladder, entered in a clinical trial assessing tumour response to 2 doses of epirubicin. At cystoscopy, the tumour was resected except for an approximately 0.5 cm marker area if unifocal, or a solitary marker tumour if multifocal. Patients with recurrent disease were eligible provided i.v. urography demonstrated normal upper tracts and no intravesical chemotherapy had been received during the year prior to entry. Exclusion criteria included other malignancies, pelvic radiotherapy, WHO status greater than 2 and untreated urinary tract infection. The trial was approved by the hospital ethical committees, and all patients gave written informed consent prior to study entry (Popert *et al.,* 1994).

When histological confirmation of bladder cancer and its grade and stage had been obtained, the patient was randomised by the method of sealed envelopes to receive either 1 (50 mg in 50 ml saline) or 2 (100 mg in 50 ml saline) mg ml^{-1} epirubicin. The bladder was drained by urethral catheterisation and the drug administered using a Uromatic Cysto-Plus device (Baxter Healthcare, Elgham, UK). After 1 hr, the bladder contents were drained. The treatment was given between 10 and 21 days after cystoscopy. Approximately 3 months after the initial operation the cystoscopy was repeated. The response was classified either as complete (defined as no visible or microscopic evidence of bladder cancer), or as no response (defined as persistence of lesion at the site of the marker with histopathological confirmation). In the case of persisting marker tumour at the 3 month cystoscopy, all tumour was resected and approximately half sent for histopathology and half frozen for the biochemical studies.

RNase protection assay

The topoisomerase α and β probes were prepared as described by Jenkins *et al.* (1992) and Davies *et al.* (1993), respectively. All radiolabelled antisense transcripts were synthesised *in vitro* using T_3 RNA polymerase and [α-^{32}P]-CTP by the method outlined in Ausubel *et al.* (1989). The topo II α

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TABLE I – CLINICAL DETAILS OF PATIENTS FROM WHOM BIOPSIES WERE OBTAINED

Biopsy code	Sex	Age	Primary or recurrent	Stage	Grade	Dose (mg ml^{-1})
Responders						
1C	M	64	primary	pT1	G1	2
1I	F	64	primary	pTa	G2	1
2I	F	69	primary	pTa	G1	1
2J	M	86	primary	pT1	G2	2
3E	M	78	primary	pTa	G2	1
3G	M	80	primary	pT1	G2	2
Non-responders						
1G and 1H	F	63	primary	pTa	G1	2
1J and 1K	M	73	recurrent	pTa	G1	1
5B and 5C	F	69	primary	pTa	G2	2
5D and 5E	F	77	recurrent	pT1	G2	1
5F and 5G	M	68	recurrent	pTa	G1	2
5H and 5I	F	54	recurrent	pTa	G1	1

plasmid was linearised with EcoRI prior to antisense transcript synthesis and produced a 215 bp protected fragment. The topo II β plasmid was linearised with BamHI and produced 2 protected fragments of 228 and 296 bp (Davies *et al.*, 1993). In each reaction, an internal loading control of an antisense transcript to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. This probe was digested with HindIII and produced a 120 bp protected fragment. Each gel lane was loaded with 5 μg of total RNA. The conditions for annealing and digesting the RNA-RNA hybrids were as described by Jenkins *et al.* (1992).

RESULTS

Topo II α and β mRNA levels were measured in biopsies from 12 patients with superficial bladder cancer, 6 of whom responded to epirubicin. Responders and non-responders were similar in terms of sex, age, grade, stage and dose of epirubicin. However, all the responders had primary tumours, whereas 4/6 of the non-responders had recurrent disease (Table I).

The levels of the topo II α and β transcripts are shown in Tables II–IV. Figure 1 shows representative RNase protection assays quantified by reference to an internal loading control. The mean levels of the 3 transcripts taken from the 12 cancer biopsies before treatment were 2.04 ± 0.21 for topo II α, 14.81 ± 3.03 for topo II β-1 and 0.75 ± 0.12 for topo II β-2. A comparison of the mRNA levels between responders and non-responders in the biopsies taken before treatment showed that the responders had lower levels of all 3 transcripts, achieving statistical significance for topo II β-1 ($p = 0.04$, Welch's *t*-test). Comparing mRNA levels in non-responders before and after treatment, the mean post-treatment levels were lower for all 3 transcripts, but this did not achieve statistical significance.

A comparison of topo II α and β mRNA levels in the 12 pre-treatment cancer biopsies with those in normal bladder (Table IV) showed that levels were uniformly lower in the cancer biopsies for all 3 transcripts ($p < 0.007$ for all 3 using Welch's *t*-test).

Response to epirubicin did not appear to be related to topo II mRNA levels, although, unexpectedly, mean levels of topo II β-1 mRNA were higher in the biopsies from non-responders. However, failure to respond to epirubicin was associated with a downregulation of mRNA expression in the majority of the cancer biopsies taken after treatment (Table II).

TABLE II – EXPRESSION OF TOPO II mRNA IN BLADDER CANCER BIOPSIES BEFORE AND AFTER TREATMENT WITH EPIRUBICIN, FROM PATIENTS WHO FAILED TO RESPOND TO INTRAVESICAL CHEMOTHERAPY[1]

Biopsy	mRNA level		
	Topo II α	Topo II β-1	Topo II β-2
Before (1G)	1.58	12.80	0.39
After (1H)	1.41	10.37	0.22
Before (1J)	1.56	8.19	0.16
After (1K)	2.19	11.70	0.47
Before (5B)	3.62	32.92	1.21
After (5C)	3.28	26.62	1.09
Before (5D)	2.90	20.46	1.08
After (5E)	1.36	8.36	0.50
Before (5F)	2.08	16.70	0.57
After (5G)	1.45	14.17	0.44
Before (5H)	1.95	36.65	1.43
After (5I)	1.38	9.54	1.00

[1]Topo II mRNA levels are expressed in terms of integrated optical density values from scanned autoradiographs, equalised in terms of a GAPDH loading control.

TABLE III – EXPRESSION of TOPO II mRNA IN BIOPSIES TAKEN BEFORE INTRAVESICAL CHEMOTHERAPY FROM PATIENTS WHOSE TUMOURS RESPONDED TO EPIRUBICIN[1]

Biopsy	mRNA level		
	Topo II α	Topo II β-1	Topo II β-2
1C	0.85	6.19	0.46
1I	2.53	13.89	1.11
2I	1.43	10.25	1.07
2J	1.82	6.09	0.45
3E	1.71	10.24	0.82
3G	2.43	3.30	0.22

[1]Topo II mRNA levels are expressed in terms of integrated optical density values from scanned autoradiographs, equalised in terms of a GAPDH loading control.

TABLE IV – EXPRESSION OF TOPO II mRNA IN BIOPSIES OF NORMAL BLADDER[1]

Topo II α mRNA level	Topo II β-1 mRNA level	Topo II β-2 mRNA level
9.6	14.5	2.7
2.4	34.0	1.5
11.2	53.7	7.8
5.9	54.1	4.9
5.5	41.1	5.4
6.7	48.7	8.7
6.2	38.7	2.8
6.8 ± 1.1	40.7 ± 5.2	4.8 ± 1.0

[1]Topo II mRNA levels are expressed in terms of integrated optical density values from scanned autoradiographs, equalised in terms of a GAPDH loading control. Last line gives mean ± SD.

DISCUSSION

There have been previous attempts to correlate expression of the topoisomerase II isozymes and clinical responses to chemotherapy with topo II poisons. However, in general, these studies have been performed on biopsies from leukaemic patients because of the relative ease with which leukaemic blasts can be obtained (Kaufmann *et al.*, 1994). The study described here provided the opportunity to study solid tumour material before and after therapy with a single agent targetting topoisomerase II.

We have measured the levels of the topo II α and β mRNAs in bladder cancer biopsies resected from patients before and

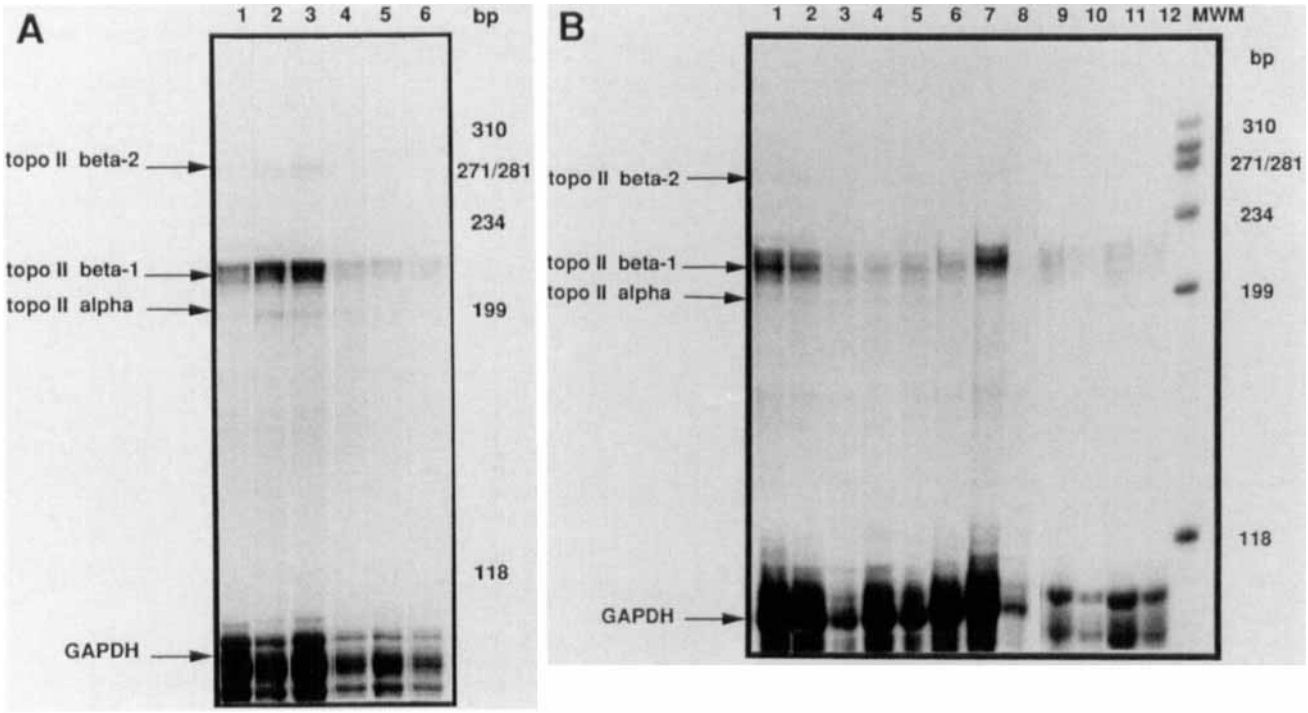

FIGURE 1 – *(a)* RNase protection analysis of topoisomerase IIα and β mRNA levels in 5 μg total RNA from biopsies of human bladder tumours which responded to epirubicin. Lanes: 1, biopsy 1C; 2, biopsy 1I; 3, biopsy 2I; 4, biopsy 2J; 5, biopsy 3E; 6, biopsy 3G. The positions of the protected fragments are indicated on the left. The sizes of the m.w. standards (bp) are shown on the right. Densitometric scanning of autoradiograms to quantify expression levels was performed when each signal was within the linear range. *(b)* RNase protection analysis of topoisomerase IIα and β mRNA levels in 5 μg total RNA from biopsies of human bladder tumours which failed to respond to epirubicin (odd numbered lanes, before treatment; even numbered lanes, after treatment). Lanes: 1, biopsy 1G; 2, biopsy 1H; 3, biopsy 1J; 4, biopsy 1K; 5, biopsy 5B, 6, biopsy 5C; 7, biopsy 5D; 8, biopsy 5E; 9, biopsy 5F; 10, biopsy 5G; 11, biopsy 5H; 12, biopsy 5I.

after treatment with a single dose of epirubicin. This therapy caused the disappearance of a marker tumour in over 40% of patients, indicating that it is an effective treatment for some superficial bladder cancers (Popert *et al.,* 1994). Expression of topo II mRNA was downregulated in the resistant cancers, but there was no apparent relationship between these mRNA levels and initial response to epirubicin. A consistent finding was that the tumour biopsies had a high topo II β to topo II α mRNA ratio, possibly indicating a low S-phase fraction in these tumours, since it has been shown previously that topo II α (but not β) is a proliferation marker in cultured cell lines.

Levels of all 3 topo II transcripts were lower in cancer biopsies than in normal bladder. This downregulation of topo II expression in the cancers was further reduced following exposure to epirubicin and is consistent with previous reports indicating that a reduction in the amount of the target protein is a common reason for the development of resistance to agents that kill cells via an interaction with topoisomerase II (reviewed by Beck and Danks, 1991; Pommier, 1993).

In an extensive study of adult acute myelogeneous leukaemia (AML) patients, Kaufmann *et al.* (1994) found wide variation in sensitivity to topo II inhibitors and topo II α and β protein levels among blasts from different patients. However, no correlation between topoisomerase expression and drug sensitivity *in vitro* or *in vivo* was apparent. Other studies have suggested that topo II mRNA levels are higher in tumour than in adjacent normal tissues. For example, Hasegawa *et al.* (1993) showed that topo II mRNA was undetectable in normal lung tissue but detectable in lung cancer biopsies. The general lack of studies on human solid tumour material precludes conclusions about the relationship between topo II expression and *in vivo* responses to chemotherapy. However, at this stage it would appear that there is no obvious general relationship between expression of either isozyme and drug sensitivity. Our results suggest the possibility that upon relapse bladder cancer cells may acquire resistance at least in part via downregulation of the expression of both topo II genes. Moreover, the downregulation of β gene expression may be more significant than downregulation of the α gene. However, the tumour cells may be heterogeneous in their expression of topo II, and there are other factors that can influence response to epirubicin, such as the expression of *mdr*-1 (Simon and Schindler, 1994).

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