DNA topoisomerase I and II expression in drug resistant
germ cell tumours

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A small number of testicular germ cell tumours are refractory to current chemotherapy regimens. DNA topoisomerase I is the target for several new drugs and a potential candidate treatment for chemorefractory germ cell tumours. DNA topoisomerase IIα is the target for etoposide, which is currently used regularly in germ cell tumour treatment. The expression of DNA topoisomerase I and II were therefore assessed immunohistochemically in a range of testicular tumours, especially those with persistent malignant elements on retroperitoneal lymph node dissection. Pre-chemotherapy orchidectomy specimens were matched with post-chemotherapy retroperitoneal lymph node dissections to examine changes in expression. There was considerable variation in the expression of topoisomerase I in different tumour types. Both yolk sac tumours and teratoma, mature showed universal expression of topoisomerase I, while 38% of seminomas and 30% of embryonal carcinomas were positive. Strong topoisomerase IIα expression was found in embryonal carcinoma. There was a negative correlation between topoisomerase I and II expression (P=0.004) and downregulation of topoisomerase IIα after chemotherapy (P=0.02). Topoisomerase I expression appears to increase in those cases with residual teratoma, mature, but is largely unchanged in those cases remaining as embryonal carcinoma. These results suggest that topoisomerase I inhibitors may be useful in chemorefractory germ cell tumours, especially yolk sac tumours and where there are unresectable residual teratoma, mature deposits.

Keywords: topoisomerase; germ cell tumour; testis; embryonal carcinoma; seminoma; Ki-67

The treatment of metastatic non-seminomatous germ cell tumours consists of orchidectomy plus ’BEP’ combination chemotherapy – bleomycin, etoposide and cisplatin. First line chemotherapy gives a 5-year survival rate for patients with metastatic germ cell tumours (GCT) of over 80% (International Germ Cell Cancer Collaborative Group, 1997). Despite the high success rate of this treatment, the BEP regimen has serious side effects and there is a 1% incidence of drug fatality. Residual masses may remain and it has become common practise to excise these surgically (Einhorn et al, 1981). Histology from these masses has a complex range of appearances and the complete resection of embryonal carcinoma (EC) or other malignant elements is important (Fox et al, 1993; Fizazi et al, 1999; Albers et al, 2000). Residual teratoma, mature (TM) is also resected where possible because of the danger of dedifferentiation, or transformation to a variety of other tumour types (Hull et al, 1994; Little et al, 1994; Michael et al, 2000).

Controversy exists regarding the use of further adjuvant chemotherapy (salvage chemotherapy) in these patients, as most are cured by surgery alone (Tait et al, 1984; Williams et al, 1997). It is clear however that effective salvage chemotherapy does exist for those patients who do relapse (Loehrer et al, 1998; Shamash et al, 1999). The omission of chemotherapy in these patients would be considered as undertreatment. Patients whose germ cell tumours recur at greater than 2 years from original resection (late recurrences) have a generally poor chemo-responsiveness to current therapies (Michael et al, 2000). There remains therefore, a small subgroup of patients who are resistant to current modes of therapy.

The human DNA topoisomerases are members of the gyrase family of enzymes. They are nuclear enzymes, which transiently break and unwind DNA at times when the cell requires access to the genetic material for replication and transcription (Wang, 1996). They are also involved in many cellular activities including chromosome condensation and recombination, DNA repair and segregation during mitosis (Husain et al, 1994).

Genes coding for the topoisomerase (topo) enzymes are highly conserved across species. Topo I is targeted by the camptothecins (Takimoto et al, 1998) and has been shown to be elevated in many human malignancies (Husain et al, 1994; Mcleod et al, 1994). Topo II isoenzymes are the targets for the epipodophyllotoxins and for DNA intercalators such as the anthracyclines (Sano and Shuhin, 1995).

Typically, these drugs do not inhibit the free enzyme but stabilise the covalently bound topoisomerase-DNA complex (Rubin, 2000), thus preventing the rejoining of the broken DNA fragments. They act not so much as inhibitors, but as ‘poisons’. The sensitivity of cells to the topoisomerase-targeted drugs is therefore related to the levels of topoisomerase in the nucleus, making immunohistochemical assessment a putative measure of likely cell sensitivity to chemotherapy (Madden and Champoux, 1992). The camptothecins have been investigated recently as possible agents for testicular GCT’s (Sano and Shuhin, 1995; Miki et al, 1997; Coleman et al, 2000).
The aims of this project are to identify the presence of the topo I and IIz in different testicular tumour types, especially those resistant to current therapeutic regimens, to aid the identification of potential targets for salvage chemotherapy using the topoisomerase inhibitors.

MATERIALS AND METHODS

Cases were identified from the Barts and The London NHS Trust medical oncology database. These included 14 matched cases of orchidectomy plus retroperitoneal lymph node dissection from patients who had residual tumour following their initial course of therapy. Some of the matched pre-chemotherapy orchidectomy tumour blocks were requested from other hospitals, which were largely those participating in the East Anglia germ cell tumour group. Thirteen cases were also identified of retroperitoneal lymph node dissection following an initial course of therapy but where the original orchidectomy was not available. Nearly all these cases had residual malignant or immature elements and therefore represented a selection of the cases which had not responded to conventional therapy. Twelve orchidectomies were also identified from patients who had been diagnosed with seminomas for comparison with the teratoma group. All cases were anonymised.

The cases were all reviewed and the different elements within them identified assisted by immunohistochemistry where appropriate. Embryonal carcinoma was identified by the immunohistochemical presence of CD30 in the primary tumours, or by histological appearances in the post-chemotherapy cases (Berney et al, 2001a). 3 μm formalin fixed, paraffin embedded tissue sections were cut from the selected tissue blocks. Antigen retrieval was used for all of the antibodies used. The antibodies used were topo I (mouse monoclonal antibody Clone ID6, Novacastra), topo II (mouse monoclonal antibody Code 3F6, Novacastra), topo IIz (rabbit polyclonal antibody, Novacastra) and Ki-67 (rabbit polyclonal Code AO47, Dako). Ki-67 was included to compare the proliferation index with the topo II enzymes. Normal tonsil was used for a positive control and a negative control was included for each case.

Protocols for immunohistochemistry were followed according to those used for the topoisomerases in previous studies (Monnin et al, 1999). All the antibodies used show nuclear staining. Cytoplasmic staining was disregarded. Protocols for immunohistochemical detection of topo I followed a previous study (Holden et al, 1997) and graded the expression of the enzyme in the tissue samples on a subjective scale of 0 to 3+. Staining 2+ or 3+ was counted as positive, and anything less as negative. The expression of the topo II enzyme and Ki-67 was assessed by quantitatively calculating the number of positive cells out of 500 (expressed as a percentage). Fifty cells were counted in 10 random fields within the tumour area. Five hundred cells were counted within each case.

RESULTS

The expression of topo I in the different tumour types is represented in Table 1. Tonsillar tissue, used as a positive control, showed strong staining in lymphoid follicles and in squamous epithelium. Macrophages, which are seen in all normal follicles ingesting apoptotic debris, were noted to be negative (Figure 1). Background fibrous tissue was also negative for all the tested antibodies. Positive internal controls included lymphocytes especially in the seminomas. Negative internal controls were areas of normal spermatogenesis in the testes where the spermatids, as expected, were seen to be negative (Figure 1).

Universal strong expression was shown in TM (Figure 3) and also in yolk sac (Figure 4). Pre chemotherapy, both EC and seminoma showed similar percentages of cases to be positive (five out of 12 and five out of 13 respectively). After chemotherapy however, only two out of 11 cases of EC showed nuclear positivity. Prominent cytoplasmic staining was noted in the EC group in many cases but this was not counted as positive. Seminomas were chosen as the control group to attempt to determine statistically which of the groups of tumour may respond well to anti-topo I therapy. TM was universally positive for topo I and is significantly different from the seminoma group (P<0.003, Fisher’s exact test). Yolk sac (YS) was also significantly different from seminoma (P<0.018). The changes in expression of topo I after chemotherapy showed that both increases and decreases in expression occurred. Increases occurred in cases which differentiated from EC to TM or YS. Cases with persistent EC either became negative or remained unchanged.

Twenty-eight of the total number of specimens were stained with both the polyclonal and monoclonal version of the topo IIz antibody. The tonsil used for control purposes showed very strong nuclear staining in follicles and in basal epithelium. Similar to topo I, spermatids were negative where present in primary orchidectomy cases. The correlation between both antibodies against topo II was strong (R=0.908; P<0.001) after testing 28 of the cases and therefore the remaining cases were tested with the monoclonal antibody only. The frequency distribution of this

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**Table 1** Topoisomerase I and IIz positivity in selected tumour types

| Primary | Primary | Post- | Yolk | Post |
|---------|---------|-------|------|-------|
| seminoma| EC      | chemotherapy| sac   | chemotherapy| TM |
| Topo I  |         |       |      |       |      |
| Positive| 5       | 5     | 2    | 6     | 10   |
| Negative| 8       | 7     | 9    | 0     | 0    |
| Topo II |         |       |      |       |      |
| Positive| 6       | 8     | 5    | 1     | 0    |
| Negative| 7       | 4     | 6    | 5     | 10   |

EC=Embryonal carcinoma, TM=Teratoma, mature.
antibody was bimodal with 24% used as the cut off between positive and negative cases (Figure 5). Topo II positivity is compared with the tumour type in Table 1. The highest topo II index was seen in cases of EC. Lower rates were seen in seminomas (Figure 6), YS and TM. The TM group showed a significantly lower topo IIa index than seminoma (P=0.019, Fisher’s exact test). Topo I was also compared with topo II expression (Table 2). There was a strong inverse correlation between the two proteins (P=0.004, Fisher’s exact test), however a few tumours showed dual high expression. Ki-67 showed a positive correlation (r=0.69, P<0.01) with topo II expression (Figure 7), demonstrating that topo IIa expression is a reasonable measure of proliferating cells. This has been reported in many previous studies (Davidson et al, 2000; Gupta et al, 2000; Morisaki et al, 2000). There was a significant reduction (P=0.02, Wilcoxon signed ranks matched pairs test) in topo II positivity after chemotherapy (Figure 8). It was noted that the pre-treatment samples were normally distributed (P=0.188, Anderson-Darling test) while the post treatment samples were not normally distributed (P<0.001, Anderson-Darling test).

**DISCUSSION**

DNA Topoisomerase I has been identified as a molecular target for the plant alkaloid Camptothecin (Husain et al, 1994; Holden et al, 1997). This is a derivative of *Camptotheca acuminata*...
Table 2 Comparison of topoisomerase I and II

| Topoisomerase II Positive | Topoisomerase II Negative |
|---------------------------|--------------------------|
| Topoisomerase I Positive  | 6                        | 23                      |
| Topoisomerase I Negative  | 15                       | 9                       |

There is a negative correlation between topo I and II ($P=0.004$, Fisher’s exact test).

The finding of most interest was the tumour specificity of high topo I expression. Both the TM group, and the yolk sac group show universal strong expression making them potential candidates for treatment with topotecan or irinotecan. The presence of high levels of topo I in TM was unexpected. TM comprises only a small minority of primary GCT’s, and these are treated by surgery followed by surveillance. However, after the administration of chemotherapy, the tumours frequently change their morphology and approximately 40% of cases show pure TM. These are routinely resected, though in cases with widespread metastases these foci may be inaccessible to surgery. Despite their quiescent nature, there is a risk of progression and presentation as late recurrences (Michael et al., 2000). Thus, if topo I has been upregulated in these foci, administration of an inhibitor may represent an adjunct or alternative to surgical removal in specific cases. Yolk sac tumour is more aggressive than TM but showed similar strong positive alternative to surgical removal in specific cases. Yolk sac tumour is more aggressive than TM but showed similar strong positive expression for topo I. Therefore, in those cases resistant to standard regimens, administration of a topo I inhibitor may be efficacious.

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Topoisomerase I Positive 6 23
Topoisomerase I Negative 15 9

Figure 7 Comparison of the percentage of Ki-67 positive cells with the percentage of topoisomerase II positive cells ($r=0.69$, $P<0.01$).

Figure 8 Comparison of topoisomerase II expression in matched samples before and after chemotherapy ($P=0.02$, Wilcoxon signed ranks matched pairs test).

Topoisomerase I has been measured previously in a variety of other malignancies and normal tissues, by immunohistochemistry, RT-PCR, enzymatic activity and Western blotting. Increased expression has been shown in ovarian epithelial carcinomas (Codegoni et al., 1998), transitional cell carcinomas of the bladder (Momn et al., 1999), renal cell carcinomas (Gupta et al., 2000) and lung tumours (Mirski et al., 2000). Normal topo I levels were seen in the background tissue of the above studies.

Two isoforms of the topo II enzyme exist – alpha and beta. The alpha form is the more important. Its activity is highest during S, G2 and M phases, with low activity during G1 phase. Both the monoclonal and polyclonal antibodies to topo IIa used in this study are specific for the carboxy-terminal of the molecule and do not cross-react with topo IIβ. The activity of topo IIa can be up-regulated by the inhibition of topo I (Whitacre et al., 1997). This has an important clinical relevance since, it is conceivable that topo I inhibitors could be used to up-regulate topo IIa, thus making cells more sensitive to topo IIa inhibitors. The results supported an inverse relationship between topo I and topo IIa (Table 2).

Many drugs inhibit topo IIa by stabilising the topo II-DNA cleavable complex (Houlbrook et al., 1995). Some topo IIa inhibitors are able to intercalate part of their structure between adjacent DNA bases, e.g. the anthracyclines and anthracene diones which are effectively trapped in the nucleus at a high concentration. They can therefore be administered by a single injection, and will be held in the cell until S phase when topo IIa activity peaks and they are able exert their effect. Other inhibitors, such as etoposide, do not intercalate and require prolonged administration to increase the chance of exposure to cells during S phase. Seminomas are well known to be sensitive to etoposide (Mencel et al., 1994). Evidence suggests that the cellular level of topo IIa determines the degree of drug toxicity (Houlbrook et al., 1995). The decreased expression of topo IIa has been shown to be associated with resistance to chemotherapy (Koshiyama et al., 2001).

The primary embryonal carcinomas were the group with the highest expression of topo IIa (8 out of 12) while TM had the lowest (0 out of 10). On comparison with the seminoma group, TM had a significantly lower topo IIa ($P=0.019$). The significant reduction in topo IIa after chemotherapy in matched cases is explained by the transformation to TM from EC. The lack of a normal distribution in the post-chemotherapy cases highlights the variable response to primary chemotherapy.
Ki-67 has been shown to be a useful marker in assessment of likelihood of relapse in metastatic germ cell tumours (Berney et al., 2001b). Comparison of Ki-67 with topo I levels shows a good correlation, indicating that topo I levels are a fair indicator of proliferating cells. Topo I is thought to be most active in cells with a high S phase fraction as DNA replication forks collide with the stabilised topo I-DNA complex (D’Arpa et al., 1990). However, non-replicating cells have been shown to be sensitive to topo I, possibly because of collisions with transcriptional complexes (Morris and Geller, 1996; Wu and Liu, 1997). Therefore in resistant cases, topo I inhibitors may be of great utility.

It should be recognised that upstream and downstream variables may affect the sensitivity of the tumour to these drugs. The transport proteins Mrp2/Moat (Allen et al., 1999) and Bcrp/Mxr (Koike et al., 1997) have been implicated in the efflux of topo I inhibitors and etoposide is a substrate for the cellular efflux protein Mdr1 (Rubin, 2000). However, clinical trials on tumours resistant to conventional chemotherapy and in cases not amenable to surgery are necessary to evaluate the response of these specific types of tumour to the camptothecins.

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REFERENCES

Albers P, Ganz A, Hannig E, Miersch WE, Muller SC (2000) Salvage surgery of chemorefractory germ cell tumours with elevated tumour markers. J Urol 164: 381 – 384
Allen JD, Brinkhuis RF, Wijnholds J, Schinkel AH (1999) The mouse Bcrp1/mxr/abcp gene: Amplification and overexpression in cell lines selected for resistance to Topotecan, mitoxantrone, or doxorubicin. Cancer Res 59: 4237–4241
Berney DM, Shamash J, Pieronni K, Oliver RTD (2001a) Loss of CD30 expression, indicating that topo IIα may be of great utility.
Berney DM, Shamash J, Hendry WF, Arora A, Jordan S, Oliver RTD (2001b) Prediction of Ki-67 with topo IIα and downstream variables. Histopathology 39: 382 – 385
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Berney DM, Shamash J, Hendry WF, Arora A, Jordan S, Oliver RTD (2001b) Loss of CD30 expression in metastatic embryonal carcinoma: an effect of chemotherapy?. Histopathology 39: 382 – 385
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Monnin KA, Bronstein IB, Gaffney DK, Holden JA (1999) Elevations of DNA Topoisomerase I in transitional cell carcinoma of the urinary bladder: correlation with DNA topoisomerase II-alpha and p53 expression. *Human Pathol* 30: 384–391

Morris EJ, Geller HM (1996) Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase I: evidence for cell cycle-independent toxicity. *J Cell Biol* 134: 757–770

Morisaki K, Kuroda S, Matsumoto Y, Kunihiro K, Nagao S (2000) Expression of DNA topoisomerase I, IIalpha, and IIbeta in human brain tumors. *Brain Tumor Pathol* 17: 7–13

Rubin EH (2000) DNA topoisomerase expression in tumours – a novel target for chemotherapy. *Human Pathol* 31: 631–632

Sano K, Shuhin T (1995) A study of topoisomerase activity in human testicular cancers. *Anticancer Res* 15: 2117–2120

Shamash J, Oliver RTD, Ong J, Raja M, Edmonds P, Gallagher CJ, Ostrowski J, LeVay J, Williams M (1999) 60% salvage rate for germ cell tumours using sequential m-BOP, surgery and ifosfamide based chemotherapy. *Annals of Oncology* 10: 683–692

Tait D, Peckham MJ, Hendry WF, Goldstraw P (1984) Post-chemotherapy surgery in advanced non-seminomatous germ cell testicular tumours: the significance of histology with particular reference to differentiated (mature) teratoma. *Br J Cancer* 50: 601–609

Takimoto CH, Wright J, Arbuck SG (1998) Clinical applications of the camptothecins. *Biochim Biophys Acta* 1400: 107–119

Wang JC (1996) DNA topoisomerases. *Annu Rev Biochem* 65: 635–692

Whitacre CM, Zborowska E, Gordon NH, Mackay W, Berger NA (1997) Topotecan increases topoisomerase II alpha levels and sensitivity of treatment with etoposide in schedule-dependent process. *Cancer Res* 15: 1425–1428

Williams SD, Stablein DM, Einhorn LH, Muggia FM, Weiss RB, Donohue JP, Paulson DF, Brunner KW, Jacobs EM, Spaulding JT, DeWys WD, Crawford ED (1997) Immediate adjuvant chemotherapy versus observation with treatment at relapse in pathological stage II testicular cancer. *New Engl J Med* 317: 1433–1438

Wu J, Liu LF (1997) Processing of topoisomerase I cleavable complexes into DNA damage by transcription. *Nucl Acids Res* 25: 4181–4186