ORIGINAL ARTICLE

Topoisomerase II α in Wilms' tumour: gene alterations and immunoexpression

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Background: Topoisomerase II α (topoll α) is an essential enzyme gene in regulating DNA structure and cell proliferation and is encoded by the *TOP2A*. Using cDNA microarray analysis, *TOP2A* has been reported to be one of the top genes overexpressed in Wilms' tumour.

Aim: To evaluate the role of Topoll α in Wilms' tumorigenesis and its prognostic value.

Methods: *TOP2A* gene copy numbers were determined using the fluorescence in situ hybridisation technique, and protein expression levels of Topoll α by immunostaining in 39 samples of primary and 18 samples of metastatic Wilms' tumour.

Results: *TOP2A* gene amplification was detected only in anaplastic Wilms' tumours, and none of the Wilms' tumours showed deletion of the *TOP2A* gene. Topoll α protein overexpression was detected in 97% of Wilms' tumours, and correlated strongly with proliferation, as measured by Ki-67 ($r=0.85$). The high Topoll α expression was associated with the presence of vascular invasion, prominent apoptosis, metastases and adverse clinical outcomes ($p<0.05$).

Conclusions: Our findings suggest that Topoll α overexpression in Wilms' tumours is caused by a change at the transcription level, except for anaplastic Wilms' tumours, in which gene amplification was present. High levels of Topoll α protein are correlated with tumour aggressiveness. The assessment of Topoll α expression in Wilms' tumour may have prognostic value.

Wilms' tumour, also known as nephroblastoma, is one of the most common solid malignant tumours in children, with an incidence of 1/8000.^{1–3} Wilms' tumour is histologically characterised by a triphasic pattern of blastemal, epithelial and stromal components, which have different proliferating potential.¹ Although, with effective treatment, patients with Wilms' tumour have nearly 90% long-term survival for localised disease and >70% for metastatic disease,^{2–4} there is still controversy regarding the best therapeutic strategy and potential molecular targets for antitumour drugs, which can further improve prognosis, reduce treatment-related toxicity and prevent late complications.^{5–8}

In our previous cDNA microarray study, we reported that *TOP2A* had the highest expression change (80.8-fold increase) compared with non-cancerous kidney.⁹ Topoisomerase II α (TopoII α) is an essential nuclear enzyme that regulates DNA topological structure, chromosome condensation and segregation, DNA recombination, transcription and cell cycle progression. Amplification of TopoII α is predominant in actively proliferating cells and has been reported in non-neoplastic kidney lesions and several malignancies, including breast, colon and pancreatic cancer, and meningiomas.^{10–14}

TopoII α inhibitors, antibiotics actinomycin D and anthracycline doxorubicin, along with vincristine, are used as the main chemotherapeutic agents for the treatment of Wilms' tumour.^{6–7} It was suggested that TopoII α gene copy numbers and enzyme expression may correlate with the efficacy of anthracycline treatment in breast cancer.^{10–15–17} However, the mechanisms and molecular pathways of chemotherapy-inhibiting effects are not well understood.

We evaluated *TOP2A* copy numbers and protein expression levels of TopoII α in primary and metastatic Wilms' tumours, and examined their correlation with proliferation indices and various clinicopathological characteristics.

MATERIALS AND METHODS

Case selection

Fifty seven cases of Wilms' tumours (39 primary and 18 metastatic) were retrieved from the files of the Department of Pathology, University of Chicago, Chicago, Illinois, USA, with a protocol approved by the institutional review board. Data on histopathological patterns, presence of anaplasia, vascular invasion, capsule or ink penetration were obtained from pathology reports. Clinical data from 39 patients with primary tumours were obtained from the patients' charts.

Tissue microarray

Paraffin-wax tissue blocks with primary, metastatic and normal adjacent kidneys were subjected to tissue microarray (TMA). A minimum of two tissue cylinders with a diameter of 1.5 mm were arrayed into a recipient block with a manual tissue microarrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA). The recipient TMA block and 21 conventional blocks were cut into 4- μ m-thick sections for analysis by immunohistochemistry (IHC) and fluorescence in situ hybridisation (FISH).

Fluorescence in situ hybridisation

To evaluate the *TOP2A* gene copy number, we carried out FISH using an *LSI TOP2A* probe, with two other probes *HER-2* and *CEP17* as controls in multicolour probe mixture (Vysis/Abbott, Downers Grove, Illinois, USA). *LSI TOP2A* is about 160 kb, a unique sequence probe direct labelled with SpectrumOrange that hybridises to the 17q21–22 region containing *TOP2A*. The *HER-2* probe contains about 190-kb DNA sequences specific for the *HER-2/neu* gene on 17q11.2–q12 and direct labelled with SpectrumGreen. The

Abbreviations: FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry; PFS, progression-free survival; TMA, tissue microarray; topoll α , topoisomerase II α

Figure 1 Representative photomicrographs of (A–C) amplified and (D) non-amplified Wilms' tumour tissue sections after TOP2A/HER-2/CEP17 fluorescence in situ hybridisation (FISH). The TOP2A gene is identified by red fluorescent signals, the HER-2 gene is identified by green fluorescent signals and the chromosome 17 centromere (CEP17) is identified by aqua fluorescent signals. The nuclei were counterstained with 4',6'-diamidino-2-phenylindole-2HCL (blue). (A) TOP2A-amplified metastatic Wilms' tumour tissue with focal anaplasia (TOP2A:CEP17 ratio 2.12). (B,C) Cells from two other TOP2A-amplified anaplastic Wilms' tumour tissues (ratio 2.23). Four signals for TOP2A and HER-2 and two signals for CEP17 are visible. (D) Typical example of non-amplified Wilms' tumour tissue. One signal for each probe can be detected in the monosomic for chromosome 17 cell (arrow). Two signals for each probe can be detected in the disomic for chromosome 17 cell (arrowhead). Original magnification $\times 1250$.

CEP17 probe direct labelled with SpectrumAqua contains α -satellite DNA that hybridises to the centromeric region of chromosome 17 (17p11.1–q11.1). The CEP17 probe was used to distinguish true gene amplification or deletion, and as an internal control for chromosome 17 aneusomy. FISH assay was conducted on TMA samples and conventional sections, according to protocols described previously.¹⁸ In each sample, an average of 82 (range 30–202) well-defined nuclei were scored.^{16, 18} Tumours with TOP2A:CEP17 and HER-2:CEP17 ratios of >2 were considered to be amplified, and tumours with ratios of 1.5:2.0 as indicating the gain of gene copies. TOP2A was considered to be deleted if the ratio was >0.8 .¹⁰

Immunohistochemistry

Antigen retrieval was carried out by heating sections in TRIS–EDTA (pH 9) for 15 min in a microwave oven. Sections were incubated for 1 h at room temperature with mouse monoclonal antibodies against TopoII α (clone 3F6; 1:50; Novocastra, Newcastle, UK) and Ki-67 proliferation marker (clone Ki-S5; 1:50; Dako, Carpinteria, California, USA). This step was followed by 30 min of incubation with goat anti-mouse immunoglobulin G conjugated to a horseradish peroxidase-labelled polymer (EnVision, Dako, Hamburg, Germany). Slides were then developed for 5 min with 3,3'-diaminobenzidine chromogen and counterstained with haematoxylin. For detection of HER-2 protein expression, we used the clinically approved HercepTest kit (Dako) according to the manufacturer's recommendations. Negative controls were prepared by substituting primary antibody with non-immune mouse immunoglobulins.

Evaluation of immunostaining

Nuclear staining of TopoII α and Ki-67 was quantified by using the Chromavision Automated Cellular Imaging System (Clarient Inc, Aliso Viejo, California, USA). The nuclear staining intensity was measured on the basis of three related colour parameters: hue, luminosity and saturation.¹⁹ The Automated Cellular Imaging System software was programmed, by setting colour-specific thresholds, to determine brown (positively stained) and blue (negative) nuclei in the outlined areas of interest, and to calculate the ratio (%) of

positively stained nuclei to all nuclei (nuclear index). For each section, at least 10 representative regions were analysed and roughly 50 000 cells were counted.

Statistical analysis

The association between the continuous expression levels of TopoII α and Ki-67 in the primary tumours ($n = 39$) and the clinicopathological variables was assessed using the t test. Expression levels were dichotomised into low-expression and high-expression groups on the basis of the median expression levels (20% and 25% for TopoII α and Ki-67, respectively).²⁰ Their relationship with clinicopathological variables was assessed using Fisher's exact test. Differences in expression levels between primary, metastatic and normal tissues were assessed with a mixed-effects analysis of variance model. The mixed-effects model was used to account for the within-subject correlation in patients for whom several tissue samples were available. Progression-free survival (PFS), defined as time from surgery to developing metastases, to relapse or death, was estimated by the Kaplan–Meier method. Differences in survival between groups were assessed using the log rank test. Results were considered significant only if $p \leq 0.05$. Data were analysed with the Stata software (College Station, Texas, USA).

RESULTS

Clinicopathological characteristics

Our study included samples of 39 primary and 18 metastatic tumours. Primary tumours were from 39 patients, with one tumour per patient. Of these 39 patients, 8 presented with metastases; however, only 3 metastatic tumour tissue samples from 2 of the 8 patients were available for analysis. The remaining 11 metastatic tissue samples included in the analysis were collected from patients who developed metastases later, and 4 more tissue samples were from 3 patients whose primary tumours were not available for study.

In all, 24 of the 39 patients with primary tumours were African-American and 15 were Caucasian; mean age was 3.4 (standard deviation (SD) 2.36; range 0.5–9) years. The mean primary tumour size was 11.2 (3.2; 0.5–18) cm. The primary

Figure 2 Representative immunostaining for topoisomerase II α (TopoII α) in normal kidney, low-stage and high-stage tumours, metastatic and anaplastic Wilms' tumour. (A) Negative in normal kidney tissue. (B–F) Positive nuclei seen 4.8% in stage I Wilms' tumour; (C) 12.8% in stage II; (D) 28.8% in stage IV; (E) 33.7% in metastatic Wilms' tumour; (F) 38.3% in anaplastic tumour. Original magnification $\times 200$.

cases of Wilms' tumour were classified as stage I ($n = 15$), stage II ($n = 9$), stage III ($n = 11$) and stage IV ($n = 4$). In all, 11 of 20 (55%) male and 4 of 19 female patients (21%) had stage III or stage IV disease. All patients received surgical treatment in combination with pre-surgical (26%) or post-surgical (100%) chemotherapy with either actinomycin D or anthracycline oxorubicin, or both, in combination with vincristine. The median follow-up for all patients was 69.05 (range 3–206) months. Eight patients presented with metastases at the time of diagnosis and 11 patients developed metastases or relapsed 3–76 months later (mean 29.45, median 12 months). Of the 39 patients, 5 (13%) died from

Wilms' tumour, including two of the three patients with unfavourable histology.

We found a significant association between PFS, sex and stage: patients who were female ($p = 0.02$) and had a low tumour stage (I and II; $p = 0.05$) had significantly better prognosis. Worse outcome in male patients may be attributed to uneven stage distribution among male and female patients in our cohort, as confirmed by Fisher's exact test ($p = 0.048$). We found a weak association between longer PFS in patients without lymph node metastases ($p = 0.09$) and renal vein invasion ($p = 0.10$), and in patients treated with TopoII α inhibitors before surgery ($p = 0.08$).

Figure 3 Kaplan-Meier survival curves estimated for patients with (A) low or high levels of topoisomerase II α (TopoII α) expression and (B) low or high Ki-67 levels in primary Wilms' tumour. (A) Difference in progression-free survival (PFS) between groups with low (<20%, dashed line) and high (>20%, solid line) levels of TopoII α protein in primary Wilms' tumour. Censored patients are indicated by marks along the lines. The cut-off value for TopoII α is estimated as 20%. (B) Difference in PFS between patient groups with low (dashed line) and high (solid line) levels of Ki-67 protein in primary Wilms' tumour. The cut off value for Ki-67 is estimated as 25%.

TOP2A gene copies by FISH

Inherent probe hybridisation efficiency was found in 93.75% of Wilms' tumour samples and normal kidney tissues. *TOP2A* amplification or gain was detected in 4 (12%) samples of Wilms' tumour with anaplasia: 2 primary and 2 late metastases from the same patients, which also had a simultaneous gain of *HER-2* gene (fig 1). *TOP2A* was not deleted in all studied tumours. We found a strong positive correlation between the ratios of *TOP2A:CEP17* and *HER-2:CEP17* in samples of Wilms' tumour ($r=0.923$) and in adjacent normal kidney tissues ($r=0.862$). The average percentage of aneusomic (monosomic and polysomic) cells was higher in primary Wilms' tumour than in adjacent normal kidney tissue ($p=0.003$), and higher in metastases than in primary Wilms' tumour ($p=0.032$). We found that a higher percentage of chromosome 17 alterations (above the median value of 45% for monosomy and 48% for aneusomy) was weakly associated with the presence of metastases at initial presentation ($p=0.063$ v $p=0.09$) and tumour progression ($p=0.053$ v $p=0.10$).

TopoII α immunoreactivity

Positive nuclear TopoII α and Ki-67 staining was observed in all 57 samples, whereas staining in adjacent normal kidney tissues was virtually negative (<0.4% positive cells). TopoII α and Ki-67 expression levels were found to be the highest in metastatic or recurrent tumours (22.85% and 31.81%, respectively), high in primary tumours (17.36% and 20.94%) and low in adjacent normal kidney (0.38% and 0.26%). Expression was also low in the two stromal Wilms' tumour (2.9% and 4.85%) samples. Mixed-effects analysis of variation models showed that expression levels of TopoII α and Ki-67 were significantly higher in primary and metastatic tissues than in adjacent normal kidney ($p<0.0001$ for each) and higher in metastatic than in primary tumours ($p=0.05$ and $p=0.02$ for TopoII α and Ki-67, respectively). Figure 2 shows representative images of TopoII α expression in primary and metastatic Wilms' tumours.

We found significant differences between primary tumours expressing high and low levels of TopoII α with respect to the sex of the patients ($p=0.048$), presence of vascular invasion ($p=0.02$), prominent apoptosis ($p=0.02$) and metastases ($p=0.04$). TopoII α levels were higher in patients with stages III and IV, anaplastic tumours and no pre-operative chemotherapy; however, the differences were not significant.

The association of Ki-67 expression levels with clinical and pathological features was similar. The group of patients with low TopoII α and Ki-67 expression levels tended to have longer PFS ($p=0.09$; fig 3).

We found a strong positive correlation between TopoII α and Ki-67 expression levels (fig 4) in 50 000 cells/case, by automated image analysis ($r=0.85$).

Comparison of FISH and IHC

TopoII α nuclear indices were non-significantly higher (27.75% (11.78%)) than the median value of 20% in all four tumour stages with *TOP2A* amplification or gain. We found a weak correlation between *TOP2A:CEP17* ratios and TopoII α expression levels in Wilms' tumour ($r=0.292$).

HER-2 amplification or gain was low, with a mean *HER-2* copy number ranging from 1.5 to 3.71 per cell (*HER-2:CEP17* maximum ratio of 2.23). Immunohistochemistry for the *HER-2* protein was essentially negative in all samples of Wilms' tumour studied.

DISCUSSION

By applying FISH, immunohistochemistry, tissue microarray and automated imaging analysis, we showed that *TOP2A* is amplified in only four samples with anaplastic Wilms' tumour, whereas TopoII α is overexpressed in most samples. This is apparently caused by mechanisms other than gene amplification. Higher TopoII α expression levels strongly correlate with tumour proliferation and progression features, such as vascular invasion, local and distant metastases and marked apoptosis ($p\leq 0.05$). There was no evidence of TopoII α differential expression in predominantly epithelial, blastemal and mixed histological subtypes reported in a semiquantitative study by Granzen and coauthors.²¹ However, we found that two stromal variants of Wilms' tumour showed a sixfold lower TopoII α expression than other variants ($p=0.0031$), and that normal epithelium from adjacent kidney tissues was essentially TopoII α negative.

The results of TopoII α immunohistochemistry strongly correlate with those of our previous study of mRNA levels by cDNA microarrays and real-time polymerase chain reaction⁹ in tissues with Wilms' tumour, which showed marked overexpression (80.8-fold) compared with non-cancerous adjacent tumour kidney tissue. By automated immunostaining, the mean TopoII α protein overexpression in primary tumours was 45-fold that in non-cancerous kidney tissues

Figure 4 Strong correlation ($r=0.85$) between topoisomerase II α (TopoII α) and Ki-67 expression levels in Wilms' tumour tissue, as determined by automated image analysis of immunostained sections.

(17.36% (SD 11.61%) v 0.39% (0.058%); $p < 0.0001$). Our finding of a higher expression of TopoII α protein in high-stage Wilms' tumour is consistent with our previously reported data, an increase of mRNA levels in high-stage Wilms' tumour.⁹

Over the past 25 years, long-term survival rates for children with Wilms' tumour approached 90% for all patients; however, the prognosis for patients who relapse is not as good, with only 30–50% survival expected after retrieval therapy.^{2–7} Therefore, it is important to uncover the mechanisms of drug resistance and its reliable detection to further improve prognosis for children.²² Two main mechanisms have been suggested to explain resistance to TopoII α -inhibiting drugs: deletion and mutation of the *TOP2A* gene,^{23–24} whereas *TOP2A* amplification may cause increased sensitivity to chemotherapy.^{17–25}

In the cases analysed, we found no *TOP2A* deletion, whereas *TOP2A* amplification or gain was detected in only a small subset of four tumours. All tumours with *TOP2A* amplification had unfavourable anaplastic histology and showed simultaneous *HER-2* amplification or gain. Absence of *TOP2A* deletions may explain the overall high sensitivity of patients with Wilms' tumour to anthracyclines and actinomycin D. However, individual drug sensitivity may vary depending on TopoII α protein expression levels.

Our findings suggest that measuring the TopoII α protein expression may have some value in predicting sensitivity to chemotherapy in patients with Wilms' tumour. For instance, patients who underwent pre-operative chemotherapy tended to have lower levels of TopoII α expression and lower proliferation indices (13.26% and 17.54%) than untreated patients (18.99% and 22.02%). Interestingly, a cohort of patients who were treated before the operation and who did not show any sign of tumour progression had the lowest nuclear indices for TopoII α and Ki-67 (7.67% and 11.06%) in tumours that were removed regardless of stage. Our findings coincide with the previous observation of reduced TopoII α expression in Wilms' tumours after chemotherapy.²⁶ Our results also indirectly support in vitro data^{25–27} that the sensitivity of breast cancer cells to TopoII α inhibitors is dependent on the expression levels of TopoII α rather than on the *TOP2A* amplification status.

In contrast with the strong correlation of TopoII α mRNA and protein levels, we found no correlation between *TOP2A* status using FISH and TopoII α using IHC ($r = 0.29$). Mueller *et al*²⁸ obtained similar results in breast tumours, which showed no correlation between *TOP2A* copy number and TopoII α expression levels. These findings suggest that TopoII α expression is highly regulated at the level of transcription and translation.

Ki-67 protein expression in Wilms' tumour tissue was about 25% higher than that of TopoII α expression, but nuclear indices of TopoII α and Ki-67 strongly correlated with each other ($r = 0.85$), and were higher in tissues with metastatic, relapsed and anaplastic Wilms' tumour. High TopoII α expression seems to be a general feature of rapidly proliferating cells, with strong, cell cycle-dependent connection and accumulation of the TopoII α active form of the enzyme during S, G2 and M phases of replicating chromatin.²⁹

In summary, the overexpression of TopoII α in all samples of Wilms' tumour and detection of *TOP2A* amplification in only 12% of those suggest that the abnormality responsible for raised expression of TopoII α mRNA and protein is mainly at the transcription level. To our knowledge, this is the first study to correlate clinical and prognostic features with *TOP2A* status and TopoII α expression levels in tissues with Wilms' tumour. Our results suggest the potential clinical utility of

TopoII α immunostaining in providing guidance for designing treatment for patients with Wilms' tumour.

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