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# DNA quantitation of Wilms' tumour (nephroblastoma) using flow cytometry and image analysis

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## Abstract

***Aims*** **To compare flow cytometry (FCM) with image analysis (IA) in the DNA quantitation of Wilms' tumour (WT) and to correlate data so obtained with recognised clinical and pathological prognostic parameters.**

***Methods*** **Thirty six patients with histologically proved WT diagnosed between 1980–89 were investigated. Fifteen patients had stage I disease, 10 stage II, six stage III, two stage IV and three stage V. Suspension of nuclei obtained by pepsin digestion of paraffin wax embedded tumour tissue was analysed using a FACScan flow cytometer, and a CAS-100 image analyser.**

***Results*** **Tumours were concordant in most instances, however, IA identified aneuploidy in two tumour samples which were diploid by FCM. Aneuploidy was detected in 5/33 tumours with favourable histology and 3/3 with unfavourable histology. Three of 28 patients with Stage I, II and V disease and 5/8 patients with stage III and IV had aneuploid tumours. All patients with unfavourable histology died of disease. In the group with favourable histology, 4/5 patients with aneuploid tumours developed recurrent disease compared with 1/27 diploid tumours ($p < 0.0001$).**

***Conclusions*** **Ploidy may be a useful additional prognostic indicator in Wilms' tumour with favourable histology. Larger scale studies are needed to confirm the relation of ploidy to survival in early stage WT.**



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Wilms' tumour (nephroblastoma, WT), one of the commonest tumours of childhood, is among the most eminently curable of all childhood cancers. Previous studies have identified the importance of routine histopathological assessment and staging in assessing prognosis.[1–4] Current treatment schedules have further improved survival with up to 73% of high risk patients achieving long term disease free survival.[4] It has been noted, however, that around 10% of stage I and II patients with favourable histopathological criteria relapse,[4] highlighting the need for additional prognostic indicators in this disease.

DNA analysis, by flow cytometry (FCM) and image analysis (IA), has been a useful adjunct in assessing the behaviour of many malignant tumours.[5–7] This study was undertaken (a) to compare FCM with IA in the DNA assessment of WT, and (b) to correlate the data obtained with established clinical and pathological prognostic criteria.

## Methods

From a total of 48 consecutive patients presenting with WT to the Oncology Department, Our Lady's Hospital For Sick Children, Dublin between 1980–89, enough diagnostic tumour material was available from only 36 patients. There were 15 males and 21 females; median age at diagnosis was 2·86 years (range 0·25 to 10·2 years). All sections were reviewed by a histopathologist with a special interest in WT (Professor HB Marsden, Manchester, England) for categorisation as either favourable or unfavourable histology and patients were treated as per the United Kingom Childrens' Cancer Study Group (UKCCSG) WT-1[8] and WT-2 protocols.

Sections of 4 $\mu$m were initially stained with haematoxylin and eosin to establish the presence of non-necrotic tumour in the samples. Two 50 $\mu$m paraffin wax sections of the tumour were dewaxed and pepsin digested according to the method described by Hedley *et al*[9] to obtain a suspension of nuclei. This was split into two samples for FCM and IA.

### FLOW CYTOMETRIC ANALYSIS

The suspension of nuclei was washed in 0·15 M phosphate buffer, stained with propidium iodide (Sigma, St Louis, MO) and analysed by flow cytometry (FACScan, Becton Dickinson), using a 15 mW air cooled argon ion laser with an excitation wavelength of 488 nm: $2 \times 10^4$ nuclei were analysed using CellFIT software (Becton Dickinson). Instrument alignment was checked prior to analysing samples using peripheral blood mononuclear cells which invariably yielded coefficients of variation (CV) of less than 3%. The DNA index (DI) was calculated as the ratio of the modal channel number of $G_0/G_1$ phase of the second peak to the $G_0/G_1$ phase of the first peak. Samples with only one $G_0/G_1$ peak were considered diploid. Aneuploidy was defined by the presence of a second $G_0/G_1$ peak with a DI of 1·15–1·8 or more than 2·2. Tetraploidy was defined by the presence of more than 10% of cells in the $G_2M$ phase of the cell cycle.

Cell cycle statistics were calculated on diploid and tetraploid tumours only. Proliferative index (PI) was calculated by adding the percentage of cells in the S and $G_2M$ phases.

IMAGE ANALYSIS

A volume of 60 μl of the suspension of nuclei was fixed in an equal volume of 10% formalin for at least 30 minutes and cytospun on to slides. Feulgen staining was performed using a CAS quantitative DNA staining kit (Becton Dickinson). Two calibration slides of rat hepatocyte nuclei (supplied with the kit) were stained simultaneously with each batch of test slides. The stained slides were analysed on a CAS 100 image analyser (Becton Dickinson),[10] using the QDA program. In the test sample, 40 spindle shaped nuclei (fibroblasts and endothelial cells) selected from the whole sample were analysed to obtain DNA content of normal control cells, followed by 200 tumour nuclei. Histograms were automatically generated and DI calculated by the computer. The quality of the histograms was checked using the CV of peak obtained from the control nuclei. Data obtained from histograms included ploidy (diploid, aneuploid, or tetraploid), 2·5C exceeding rate and 5C exceeding rate.

STATISTICS

Correlation of ploidy with single parameters was performed using the Fisher exact test and significance in difference between the 2·5C exceeding rate of diploid and aneuploid tumours determined using the Mann Whitney U test. Actuarial survival curves were obtained with the Kaplan-Meier product limit method and statistical comparison between two survival curves was made with the log rank test.

## Results

Evaluable DNA patterns using both FCM and IA were obtained from tumour samples of 35 patients. For technical reasons (insufficient material in block), IA was not possible on a tumour sample from one patient with stage V disease at diagnosis which was diploid on FCM. Thirty three of 35 samples were concordant on FCM and IA; of these 26 were diploid samples, six aneuploid and there was one tetraploid sample (table 1). Image analysis also identified two aneuploid stem lines in the tumour samples of cases 3 and 9, both of which were diploid on FCM (table 1), and in addition, two aneuploid stem lines were observed in tumour samples from cases 1, 2, and 4, which had shown only a single aneuploid peak on FCM.

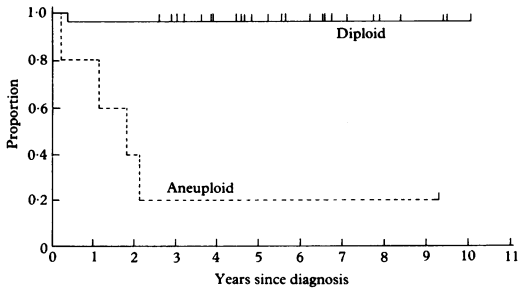
The mean CV of the diploid $G_0G_1$ peak on FCM was 4·93 (SD 1·67) (range 1·2–9·5%). The percentage of cells in S phase was 3·4–35·3% (mean 14·3%) and in the $G_2M$ phase was 1·3–5·7% (mean 3·1%) for the diploid tumours; the corresponding values for the tetraploid tumour were 16·3 and 34% respectively. The proliferation index was 4·9–36% (mean 18·1%). Mean CV of peak obtained from 40 control nuclei using IA was 4·89% (SD 1·14) (range 1·8–7·8%). Diploid tumours (n = 26) had a 2·5C exceeding rate of 10·3–53·8% (mean 31·1%) and a 5C exceeding rate of 0–1·9% (mean 0·14%); aneuploid tumours (n = 8) had a 2·5C exceeding rate of 44.1–99% (mean 62·6%) and a 5C exceeding rate of 4·24–19 (mean 9·01%). These differences were highly significant by the Mann Whitney U test at a p value of $<0·001$ for the 2·5C exceeding rate and $<0·0001$ for the 5C exceeding rate.

There was no correlation between proliferation index as measured by FCM and survival. However, using an arbitrary cut-off level of 40% for the 2·5C exceeding rate, it was found

*Clinical features, histology, ploidy status, and outcome in 36 patients with Wilms' tumour*

| Case No | Age (years) and sex | Histology | Stage | DNA index | | Follow up (months) |
|---|---|---|---|---|---|---|
| | | | | FCM | IA | |
| 1 | 4·39 / M | FH | IV | 1·29 | 1·38, 1·6* | R (24) |
| 2 | 4·73 / F | UH (Anap.) | III | 1·47 | 1·44, 1·78* | R (5) |
| 3 | 1·66 / F | FH | I | 1·12 | 1·31, 1·59* | R (21) |
| 4 | 5·57 / F | UH (Anap.) | III | 1·75 | 1·61, 1·75* | R (8) |
| 5 | 10·2 / F | FH | III | 1·55 | 1·47* | R (1) |
| 6 | 4·74 / F | FH | I | 1·34 | 1·41* | R (13) |
| 7 | 2·72 / M | FH | I | 1·00, 2·0 | 1·07, 2+ | A/W (34) |
| 8 | 2·04 / M | FH | II | 1·31 | 1·62* | A/W (109) |
| 9 | 6·19 / M | UH (Anap.) | IV | 1·00 | 1·56, 1·7* | R (25) |
| 10 | 2·83 / M | FH | II | 1·00 | 0·99 | R (4)/A/W (73) |
| 11 | 1·73 / M | FH | I | 1·00 | 1·07 | A/W (44) |
| 12 | 6·21 / F | FH | I | 1·00 | 1·02 | A/W (84) |
| 13 | 3·12 / M | FH | V | 1·00 | 1·02 | A/W (33) |
| 14 | 1·31 / M | FH | I | 1·00 | 0·99 | A/W (65) |
| 15 | 5·86 / F | FH | I | 1·00 | 1·03 | A/W (51) |
| 16 | 1·72 / F | FH | I | 1·00 | 1·05 | A/W (54) |
| 17 | 1·09 / M | FH | I | 1·00 | 0·94 | A/W (43) |
| 18 | 5·6 / F | FH | I | 1·00 | 0·95 | A/W (53) |
| 19 | 1·2 / F | FH | II | 1·00 | 1·04 | A/W (36) |
| 20 | 6·38 / M | FH | II | 1·00 | 1·04 | A/W (44) |
| 21 | 3·71 / M | FH | I | 1·00 | 1·02 | A/W (56) |
| 22 | 5·72 / F | FH | II | 1·00 | 0·99 | A/W (77) |
| 23 | 1·91 / F | FH | V | 1·00 | N/A | A/W (28) |
| 24 | 3·78 / F | FH | I | 1·00 | 1·00 | A/W (60) |
| 25 | 4·4 / M | FH | I | 1·00 | 1·03 | A/W (92) |
| 26 | 3·16 / M | FH | III | 1·00 | 1·03 | A/W (77) |
| 27 | 3·36 / M | FH | II | 1·00 | 1·06 | A/W (32) |
| 28 | 2·04 / F | FH | III | 1·00 | 0·82 | A/W (33) |
| 29 | 0·45 / M | FH | V | 1·00 | 0·92 | A/W (73) |
| 30 | 2·43 / F | FH | II | 1·00 | 0·93 | A/W (111) |
| 31 | 2·23 / F | FH | II | 1·00 | 0·99 | A/W (93) |
| 32 | 2·64 / F | FH | II | 1·00 | 1·00 | A/W (118) |
| 33 | 0·25 / F | FH | I | 1·00 | 0·95 | A/W (77) |
| 34 | 5·21 / F | FH | I | 1·00 | 0·97 | A/W (79) |
| 35 | 5·13 / M | FH | I | 1·00 | 1·04 | A/W (99) |
| 36 | 1·99 / F | FH | III | 1·00 | 0·99 | A/W (66) |

R = relapse (time from diagnosis to relapse); A/W = alive and well (time from diagnosis till date); * = aneuploid; + = tetraploid; N/A = not analysed; FH = favourable histology; UH = unfavourable histology.




*Relapse free survival curves of patients with FH diploid (n = 27) and FH aneuploid (n = 5) tumours, (p < 0·0001).*

that 7/14 tumours with a 2·5C exceeding rate of >40% suffered relapse compared with only 3/23 tumours with a value of <40%. (p = 0·012, Fisher's exact test). Seven of eight patients with a 5C exceeding rate of >5% suffered disease relapse compared with 3/28 patients with a value <5% (p = 0·0001).

PLOIDY VS OTHER PROGNOSTIC PARAMETERS

Eight out of 36 patients developed recurrent disease; these included 3/28 stage I, II and V patients (diploid one, aneuploid two) and 5/8 stage III and IV patients (all aneuploid). Seven of eight patients with aneuploid tumours and 1/27 patients with diploid tumours have had relapse of disease (p value = 0·0001) (see table 1); all cases of aneuploid relapses have died of disease. The single relapse among the diploid group was a local recurrence 4 months from diagnosis which responded to radiotherapy and alternative chemotherapy. The patient whose tumour showed a tetraploid DNA pattern has had DFS of more than 2 years. In the UH group, all three patients had diffuse anaplastic tumours which were aneuploid on FCM and IA, and all patients died of disease. In the FH group, 4/5 patients with aneuploid tumours compared with only 1/27 patients with diploid tumours have developed recurrent disease (figure).

## Discussion

Based on experience gathered from adult tumours in which aneuploidy has been shown to confer an adverse prognosis,[5-7] we were prompted to investigate ploidy as an additional prognostic parameter in WT. Previous studies on DNA analysis of WT[11-15] have been performed using FCM or IA. The advantages with FCM are its wide availability and speed of analysis; it is, however, unselective and can miss small populations of aneuploid cells within the same sample[16] as was demonstrated in this study. IA, on the other hand, is time consuming, but because of observer interaction, more selective. Also, in contrast to FCM, IA can deal with small tumour samples (touch preparations, needle aspirates) which are frequently the only material available. Correlation between the two methods is usually good,[16] though Koss *et al* noted that 20% of diploid urothelial tumours analysed by FCM were aneuploid by IA.[17] One of the aims of this study was to compare data obtained using both methods; similar results were obtained in all but two patients. IA in this study identified more than one aneuploid stem line in three patients, the significance of which is unclear.

Fifteen per cent of patients with FH had aneuploid tumours; this is in contrast to Kumar *et al* who reported only 4·5% of FH tumours in their series to be aneuploid.[14] The incidence of recurrent disease was significantly higher in these patients; two of the patients from this group had stage I disease and developed lung metastases within 2 years of diagnosis which were resistant to both chemotherapy and radiotherapy. This is again in contrast with Rainwater *et al* who noted that >97% of patients with lower stage disease were cured irrespective of ploidy status.[13] All patients with UH in this study had diffuse anaplasia and were aneuploid on DNA analysis. It was interesting to note that two other renal tumours, a bone metastasising renal tumour and a malignant rhabdoid tumour which were processed at the same time, were diploid on both FCM and IA; this observation is in agreement with others.[11-14] Clearly alternative and as yet undefined tumour characteristics are operative in these latter histological variants which only a molecular biological approach can hope to dissect.

Calculation of proliferative index (PI) from S and $G_2M$ fractions of the cell cycle was done only for diploid tumours as aneuploid tumours present more difficulties for S phase fraction analysis. The variation in PI between tumour samples was considerable (4·9–36%) and did not correlate with rate of relapse. This is in agreement with the study by Kumar *et al*,[14] although other reports, particularly in adult lymphomas, have shown a significant association between PI and rate of recurrent disease.[18] It is possible that an artificially high S phase fraction in some patients in this study could have been due to debris, or nuclear clumping.[19]

The 2·5C and 5C exceeding rates as measured by IA were useful predictors of relapse. These values represent percentage of cells with DNA content over 2·5 and five times the haploid DNA value respectively. Some of these authors, in a previous study,[20] noted that the 5C exceeding rate of benign smooth muscle tumours never exceeded 1% whilst the value in their malignant counterparts was invariably over 5%. In this study, the 2·5C and 5C exceeding rates served to differentiate the less aggressive from the more aggressive tumours.

In conclusion this study has demonstrated the usefulness of ploidy as an additional prognostic parameter in WT. There was good correlation between FCM and IA; the speed of the former makes it the method of choice although IA can identify aneuploidy in some tumours found to be diploid by FCM. Additional information obtained by 2·5C and 5C exceeding rates using IA may discriminate the more aggressively behaving tumours. Larger scale studies are needed to confirm the relationship of ploidy to survival in WT, particularly for early stage disease.

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