

Role of DNA Content Analysis and Immunohistochemistry in the Evaluation of the Risk of Unfavourable Outcome in Wilms' Tumours

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Abstract. *Background:* Wilms' tumour (WT) is the most common solid tumour affecting young children. Its histological diversity leads to difficulties in predicting the outcome. *Materials and Methods:* Image analysis cytometry and immunohistochemistry with a selected panel of antibodies were performed in 23 cases of WT considered of intermediate risk according to the revised International Society of Pediatric Oncology (SIOP) working classification of renal tumours of childhood. In this series, a tumour was considered aggressive according to its propensity for metastases or its recurrence. *Results:* Out of the 14 non-aggressive WT, 4 were found to be diploid and 10 were aneuploid including 6 that were heterogeneous for DNA-ploidy. All the tumours presented a low proliferative index and were negative for p53 and p57^{kip2} immunostaining. Out of the 9 aggressive tumours, all were aneuploid and 4 were found to be heterogeneous for DNA-ploidy. They all presented a high degree of cell proliferation and 7 were positive for p53 immunostaining. Only two were positive for the p57^{kip2} marker. The only fatal case revealed an aneuploid-homogeneous DNA-ploidy analysis, was p53 and p57^{kip2} positive and presented a high cell proliferation index. *Conclusion:* A significant correlation between the presence of focal DNA-aneuploidy in Wilms' tumours and adverse prognosis is not established, but some immunohistochemical markers may be useful for the clinical evaluation of these tumours and to help in predicting the risk of an unfavourable outcome.

Wilms' tumour (WT) is the most common renal malignancy affecting children under the age of one year. It represents 6-

8% of all pediatric solid carcinomas and occurs most commonly in children between the age of 1 and 5.

WT is a complex embryonal neoplasm arising from metanephric blastema which typically exhibits triphasic epithelial, blastemal and stromal differentiation (1). It is found in a few syndromes which exhibit cytogenetic and molecular defects and the studies related to these have led to a greater understanding of the mechanisms involved in the development of WT (2, 3). New classifications based on favourable and unfavourable histology groups have been shown to be useful for the choice of treatment (4, 5).

Tremendous histological diversity exists in WT, as well as a large number of pathological associations, all of which lead to difficulty in determining a prognosis.

An important goal in the understanding of human neoplasia is to assess the aggressivity of the tumour. The rate of cellular proliferation is one predictive feature and the DNA content can be estimated or measured by a variety of techniques including image analysis and flow cytometry.

In contrast to medulloblastoma and neuroblastoma, an aneuploid DNA content in WT seems to be associated with unfavourable histology (anaplasia) and a poor clinical outcome (6-9). But some studies have demonstrated that the DNA content in cells of WT is not uniform, complicating the interpretation of the histogram obtained (10). Furthermore, image analysis measuring DNA content has shown aneuploidy in tumors which was not detected by flow cytometry (11, 12). These factors suggest that aneuploidy may be more commonly revealed, especially if more sensitive techniques are employed. In an effort to determine the effective role of nuclear DNA content in the evaluation of tumour prognosis, the DNA-ploidy status in Wilm's tumours was analysed and whether or not heterogeneity exists and what are its clinical implications.

In previous studies on gastric carcinomas, we have tested variations of DNA-content using image analysis cytometry and analysed comparative expression of some immunohistochemical markers (13-15). Using a similar approach to

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Key Words: Image DNA analysis, immunohistochemistry, Wilms' tumour, p53, p57^{kip2}, Ki-67.

characterize the DNA-ploidy heterogeneity profile of WT, 23 WT were analysed and p53, p57^{kip2} and Ki-67 were studied immunohistochemically.

Materials and Methods

Materials. Twenty-three cases of Wilms' tumour diagnosed between 1986 and 2004 were analysed. In the 12 female and 11 male patients the average age at surgery was 3.3 years (3 months-9 years). For all the cases, the clinical data were obtained from the referring clinicians' reports and their follow-up. All the cases were considered to be intermediate risk tumours according to the revised International Society of Pediatric Oncology (SIOP) Working Classification of renal tumours in childhood (16) and most of the tumours were stage I or II excepted in 4 cases. Aggressiveness in these tumours was assessed according to their recurrence or propensity for metastases. Detailed histological analysis of all the tumours was obtained by systematic cartographic tissue sampling. The number of blocks for the analysis was calculated as a function of the maximal area of the tumour (one block per 1.5-2 cm²) depending on the number of available blocks. For each tumour a mean of 4-5 blocks was analysed by DNA-ploidy cytometry. Any non-tumoral tissue from these blocks was removed by microdissection in order to obtain pure tumoral samples for the DNA-ploidy analysis. In order to analyse the tumour cell population only, image analysis and not flow cytometry was used. Image analysis permits more sensitive detection of aneuploid tumour populations than flow cytometry when a small number of tumour cells are present in the specimen or these cells are intermixed with non-tumour cells.

DNA-ploidy analysis. For image DNA-ploidy analysis (static cytometry), all the tissue samples were processed following the technique of Hedley (17), which provided a cytospin of each sample. For Feulgen staining, the cytopspins were first hydrolysed in 5N HCl at 25°C for 45 min. The hydrolysis was stopped by rinsing in distilled water. The specimens were then stained with Schiff reagent (Schiff reagent for microscopy, No 1.0933, Merck, KGaA, Darmstadt, Germany) for 60 min, then rinsed with sulphite solution. After dehydration in a graded series of alcohols and xylene the cytopspins were coverslipped using Eukitt mounting medium (O. Kindler GmbH, Ziegelhofstrasse 214, D-79110, Freiburg). The DNA analysis was performed on Feulgen-stained cytopspins using an Autocyte Quic DNA image analysis system, version 1.1, (Autocyte Inc, Burlington, NC, USA). A minimum of 200 nuclei was measured per cytospin (300 nuclei for most of the cases). Only well preserved nuclei were selected by the operator. As controls 30 to 50 human lymphocytes from a thymus specimen prepared on the same slide beside the analysis sample were used (external reference). At least 6 tissue polymorphonuclear leucocytes were used as the internal reference. The coefficient of variation of the DNA index thus measured was below 5%. The histograms were interpreted according to the European Society for Analytical Cellular Pathology (ESACP) recommendations (18) which allow DNA stemlines to be identified as abnormal (or aneuploid) if they deviate by more than 10% from the diploid (2c) or tetraploid regions (4c). Rare events in the DNA histograms often called 5c or 9c exceeding events because they represent non-proliferating abnormal cells with different chromosomal aneuploidies were also considered as abnormal cells. Considering that in normal tissues and most low-grade or slowly proliferating neoplasms, approximately 85% of the cell population constitute the G0/1 peak and 15% of

the cells are in the S-phase and G2/M phases, only the samples in which more than 15% of the cells had a DNA-index in excess of 1.1, were considered as aneuploid.

For statistical purposes the tumours were classified into two groups, diploid and non-diploid (aneuploid). A tumour was considered to be diploid when all the samples were diploid. A tumour was considered to be aneuploid when at least one sample was aneuploid. A tumour was considered DNA-ploidy heterogeneous when at least one sample had a ploidy pattern different from the others (diploid vs. aneuploid). This resulted in different patterns, diploid, aneuploid heterogeneous and aneuploid homogeneous.

Immunohistochemistry. The immunohistochemical analysis was performed on parallel tissue sections from the same paraffin blocks processed for cytometry, using the Mib-1 antibody (1:50) for Ki-67 Antigen, clone Mib-1, M7240 (DakoCytomation, Denmark A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark) and the D0-7 antibody (1:400) for p53 protein, M7001 (DakoCytomation, Denmark A/S, DK-2600 Glostrup, Denmark).

After deparaffinization and rehydration, the 4 µm sections were treated with 0.5% H₂O₂ in methanol for 10 min to inhibit endogenous peroxidase. Antigen retrieval was performed according to the antibody and the sections were preincubated for 15 min in phosphate-buffered saline (PBS) containing 5% bovine serum albumen (BSA) and then incubated with the relevant antibody at room temperature (RT) for 60 min, washed with PBS and subsequently incubated with the second biotinylated antibody at RT for 30 min. Antibody binding was revealed using the streptavidin-biotin-complex-peroxidase (streptABCComplex Duet Reagent Set, K0492; DakoCytomation, Denmark A/S, DK-2600 Glostrup, Denmark) for 30 min at RT. The sections were then reacted for 10 min in DAB solution, washed in running water, counterstained with hemalun and mounted. The negative control consisted of omission of the primary antibody.

The proliferative activity was established through the Ki-67 (Mib-1) labelling index and computer analysis (Immunoquic system, Autocyte Inc., Burlington, NC, USA). Multiple microscopic fields (200x) of each tumour were analysed. The count from the field with the highest proliferative activity was used for the study. Less than 50% of cells positive for Ki-67 was considered a low proliferative index and more than 50% of Ki-67 positive cells a high proliferative index.

The p53 staining results were read semiquantitatively by two independent observers. The samples were classified into two categories according to a distribution proposed by Symmans *et al.* (19). The first group included the cases without or with rare p53 positive cells (negative), and those with a low proportion of positive staining nuclei (less than 10% positive cells) and the second group corresponded to the cases displaying a high proportion of positively stained nuclei (more than 10% of positive cells).

For the samples submitted to p57^{kip2} immunohistochemistry, four- mm thick tissue sections were mounted on aminopropylmethoxysilane-coated glass slides, deparaffinized in xylol, taken through to absolute alcohol, blocked for endogenous peroxidase with 0.1% hydrogen peroxide in methanol (45 min) and rehydrated through graded alcohols. They were boiled for 15 min in 10 mM citrate buffer, pH 6.0 (microwave oven) and rinsed in Tris-buffered saline (TBS; Tris 0.05 M, NaCl 0.9%, pH7.6). To reduce nonspecific binding, they were incubated for 10 min in normal horse serum (Pel-Freez Biologicals, Rogers, AK, USA) 1:30 in TBS. After 40min incubation with a prediluted anti-p57^{kip2} mouse monoclonal

Table I. Pathological data, DNA-ploidy and immunohistochemistry patterns in Wilms' tumours.

No	Gender*	Age (yrs)	Area (cm ²)	Stage	Blocks	DNA-ploidy**	Heterogeneity	p57***	Ki67****	p53***	Aggressiveness
1	F	1	12.0	I	6	A	yes	L	10-40%	L	yes
2	F	3	14.0	I	7	A	no	L	50-90%	L	yes
3	M	2	8.0	III	5	A	no	L	60-70%	H	yes
4	F	3	15.0	IV	7	A	no	L	50-60%	H	yes
5	F	9 mths	9.0	III	5	A	yes	L	20-30%	L	no
6	M	5	15.0	I	8	A	no	L	30%	L	no
7	F	3	7.0	I	3	A	yes	L	40%	H	no
8	F	8	18.0	II	9	A	no	H	10-50%	L	yes
9	F	2	4.0	I	2	A	yes	L	10-50%	L	no
10	M	1	6.5	II	3	A	no	L	10-50%	L	no
11	F	4	9.0	I	4	A	yes	L	50%	H	yes
12	M	2	4.0	I	2	D	no	L	50%	L	no
13	F	1	11.0	II	5	A	yes	L	30-90%	L	yes
14	F	7	6.5	II	3	A	yes	L	80%	H	no
15	M	3 mths	3.0	I	2	A	no	H	80%	L	no
16	F	4	11.5	I	6	A	yes	L	50%	H	yes
17	F	1	1.6	I	2	A	yes	H	60-70%	L	no
18	F	6	10.5	III	5	A	no	H	80%	H	yes
19	M	6	4.5	I	2	D	no	L	40%	L	no
20	M	9	8.0	I	4	A	no	L	5-10%	L	no
21	M	1	11.0	I	4	D	no	L	5-10%	L	no
22	M	2	21.0	I	10	D	no	L	10-50%	L	no
23	M	5	8.0	II	4	A	yes	L	10-20%	L	yes

*Gender: F, female; M, male. **DNA-ploidy: D, diploid; A, aneuploid. ***p53 and p57 expression: H (high), more than 50% positive cells; L (low), less than 50% positive cells. ****Ki-67: per case field with minimal and maximal proliferative activity.

antibody (Clone 57P06, NeoMarkers, Fremont, CA, USA), the sections were incubated for 30 min with biotinylated horse antimouse immunoglobulins (Vector, Burlingame, CA, USA) diluted 1:400 in TBS containing 2% bovine serum albumin, then for 30 min with Vectastain ABC Kit (PK4000, Vector Laboratories Inc., Burlingame, CA 94010, USA) prepared according to the manufacturer's instructions. The peroxidase activity was revealed with 5-5'-diaminobenzidine as chromogen and the sections were counterstained in Mayer's acid-free haematoxylin. As negative control, the primary antibody was replaced by a mouse hybridoma supernatant of similar isotype (IgG2b). The samples were classified into two categories according to the same criteria as for p53.

Statistical analysis. Possible correlations between DNA-ploidy, intratumoral DNA-ploidy heterogeneity, proliferative activity, p53 and p57^{kip2} overexpression were analysed by means of the Fischer's exact test. A *p*-value of less than 0.05 was considered statistically significant.

Consent and approval. The analysis was performed on retrospectively selected material from the tissue bank according to the rules of our institution.

Results

Out of the 23 tumours analysed, 19 were aneuploid for DNA-ploidy (82%), of these 9 were homogeneous for DNA-ploidy (41%) and the remaining 10 tumours showed heterogeneity for DNA-ploidy analysis (59%).

Out of the 23 tumours, 9 were considered aggressive according to their recurrence or their propensity for metastases, but only one patient died from his tumour. Up to now, the remaining 14 patients have been free of tumour for 2 to 13 years after treatment (Table I).

Tumours considered non-aggressive. DNA-ploidy. Four cases were found to be diploid and no diploid WT showed recurrence or metastasis. All the diploid cases presented at stage I.

Out of the non-aggressive, but aneuploid tumours (10 cases) six were heterogeneous and four homogeneous for DNA-ploidy.

Immunohistochemistry. The diploid cases presented less than 50% nuclear positivity for the cell proliferation marker Ki-67 and were all negative for p53 and p57^{kip2} immunostaining.

All the aneuploid tumours showed a low proportion of p53 positive nuclei and only two cases presented a noticeable positivity for p57^{kip2} staining. Seven tumours showed a low rate of cell proliferation (less than 50% of the cells positive for Ki-67 immunostaining).

Tumours considered aggressive. DNA-ploidy. Out of the nine tumours considered to be aggressive, four were found to be

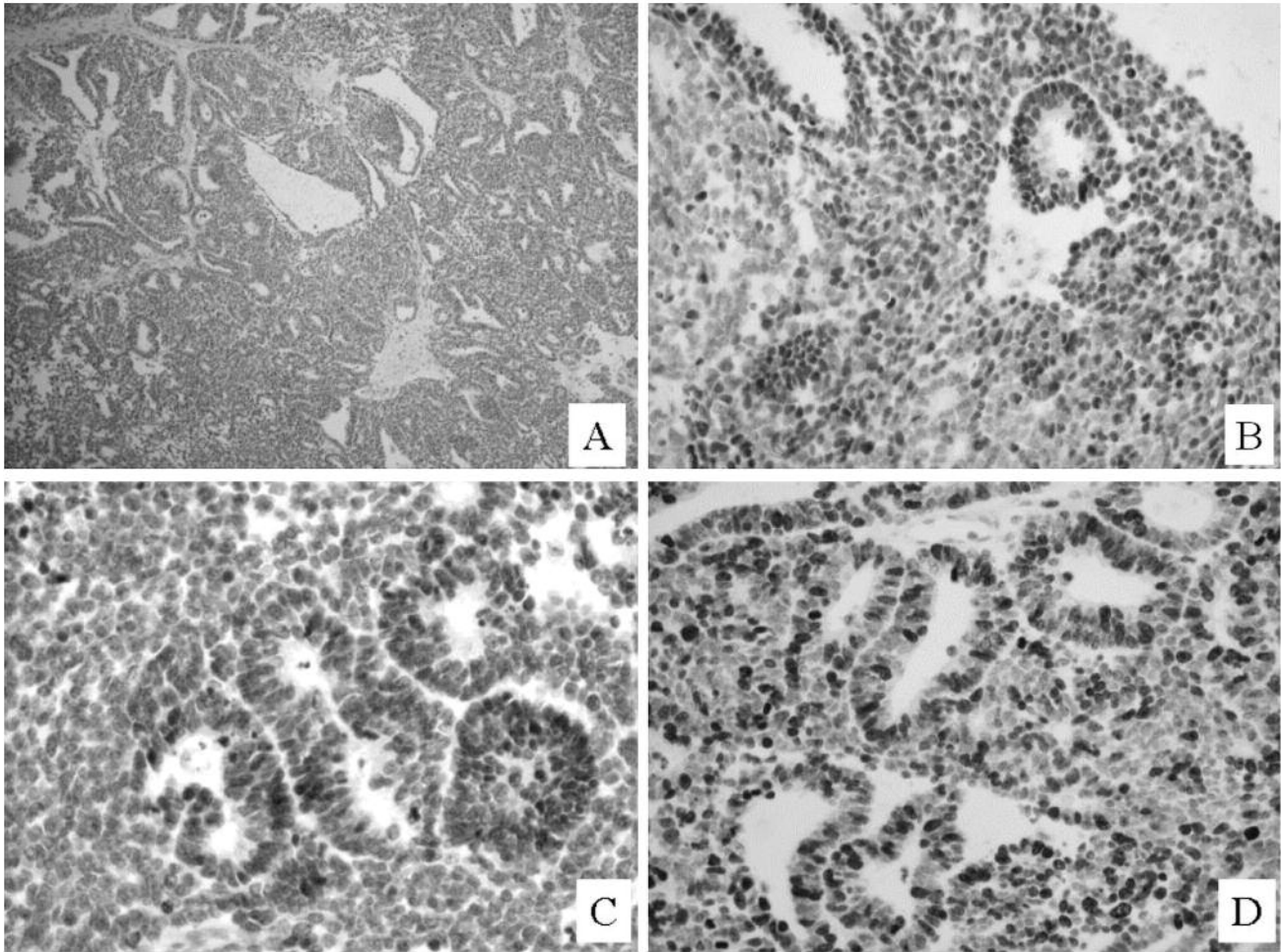


Figure 1. Case 18: (A) Histological view, HE staining, original magnification 4x; (B) p53 positive staining, original magnification 40x; (C) p57^{kip2} positive staining, original magnification 40x; (D) Ki-67 positive staining, original magnification 40x.

heterogeneous and five tumours were homogeneous for DNA-ploidy. However, there was no statistically significant correlation between the aggressive tumours and their DNA heterogeneity (p -value 0.653).

A significant correlation between good prognosis and diploid tumours was demonstrated but DNA-ploidy analysis is not able to discriminate which tumour will display an unfavourable outcome between the aneuploid tumours (homogeneous or heterogeneous).

Immunohistochemistry. All these tumours presented a high degree of cell proliferation (more than 50% of the cells were positive for Ki-67 immunostaining) which was significantly different from the diploid tumours (p -value 0.0007). Seven out of the group of aggressive tumours, homogeneous or heterogeneous for DNA-ploidy, presented a high proportion of positive staining for p53 nuclei which was also significantly different from the diploid tumours (p -value

0.0001). p57^{kip2} was positive in only two aggressive tumours which were positive for p53 and aneuploid-homogeneous for DNA-ploidy.

The only fatal case was aneuploid-homogeneous, positive for p53 and p57^{kip2} immunostaining and presented a high cell proliferation index (Figure 1).

Discussion

Several studies have been performed to evaluate the usefulness of DNA ploidy analysis for identifying high risk patients.

In a study by Yildiz *et al.* (10) of paraffin-embedded tissue from 44 tumours, an aneuploid DNA content was found in 45% with 30% of the tumours demonstrating heterogeneity among the studied blocks. This DNA content heterogeneity was the only correlate to unfavourable histology and survival. The high incidence of aneuploidy in

that study contrasted with the lower incidence of anaplasia found in other studies (5-6% of WT specimens) (6-9, 20) this feature being proposed as the most relevant criterion of histological unfavourable prognosis (21). In the present study, of cases considered as intermediate risk most of the tumours displayed a stage I or II at surgery without anaplasia. Using image analysis cytometry, a higher rate of aneuploidy was found in our collective (82%) than in the studies performed by flow cytometry (40-48%) and DNA content heterogeneity was evaluated at 59% of the aneuploid tumours. Considering that the data from the literature describe a mean of 40% of DNA aneuploidy in WT (6, 9, 10), these values may correspond to the rate of DNA homogeneous aneuploidy in the present series. Our results confirmed the suggestion that aneuploidy is more common if more sensitive techniques are employed. Furthermore, DNA-ploidy heterogeneity implies the analysis of many different regions from tumours in order to reveal DNA-aneuploidy. The 59% of heterogeneous-aneuploid tumours in the present study was higher than the heterogeneity described in Wilms' tumours by the study from Yildiz *et al.*, but similar to the values reported in gastric or oesophageal carcinomas (15, 22-25).

Despite no significant correlation between DNA heterogeneity and tumour aggressiveness being found, the absence of aggressive transformation of the diploid cases should be taken in consideration.

Furthermore, the significantly lower proportion of p53 positive nuclei and rate of cell proliferation should be noted in all diploid cases than in the aggressive cases.

From the immunohistochemical analysis of p57^{kip2} which encodes a cyclin-cdk inhibitor located near the 11p15.5 imprinted domain implicated in the inactivation of H19 gene in WTs, only 4 positive cases were found, all of which were aneuploid, but only two were from the aggressive group. These data did not reach significance probably due to the limited number of cases, but the presence of an association between DNA-aneuploidy, p53 and p57^{kip2} positivity in the patient who died from the tumour must suggest the presence of tumoral progression occurring by way of different gene alterations, which may not be part of the same pathways, but contribute to increase the aggressivity of the tumour.

Nowadays, new molecular studies exploring gene expression profiles of WT (26-29) could reflect the behaviour of these tumours and explain the overgrowth of some DNA-aneuploid cell populations.

Tumour prognosis actually is not only dependent on tumour aggressivity alone, but must be considered also to be related to the degree of resistance to adjuvant therapy.

The introduction of new adjuvant therapeutic protocols must be taken into consideration when follow-up of our patients is analysed. The high survival rate of the patients with WT implies a new interpretation of the aggressivity of the tumours.

This is the reason why, in the present study which included only one patient who died from his tumour, all the cases with recurrence or metastasis were designated as aggressive tumours, and the survival rate was not considered alone.

In conclusion, an increased number of WT are aneuploid and a high percentage of heterogeneity for DNA-ploidy is confirmed. It has also been demonstrated a significant correlation between good prognosis and diploid tumours but reveals that DNA-ploidy analysis is not able to discriminate which tumour will display an unfavourable outcome between the aneuploid tumours (homogeneous or heterogeneous). Immunohistochemical markers, such as p53, Ki-67 could be useful for tumour evaluation and for predicting the risk of an unfavourable outcome.

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Received November 28, 2007

Revised January 17, 2008

Accepted February 4, 2008