Title:

NEPHROGENIC RESTS IN WILMS TUMORS TREATED WITH PREOPERATIVE CHEMOTHERAPY: THE UK SIOP WILMS TUMOUR 2001 TRIAL EXPERIENCE

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Abbreviations Table

NR -	nephrogenic rest
WT -	Wilms tumor
SIOP -	International Society of Pediatric Oncology
PLNR -	perilobar nephrogenic rest
ILNR -	intralobar nephrogenic rest
NWTS -	National Wilms Tumor Study
NBL -	nephroblastomatosis
DHPLN -	diffuse hyperplastic perilobar nephroblastomatosis
SGB -	Simpson Golabi Behmel
BWS -	Beckwith-Wiedemann syndrome
HH -	hemihypertrophy

ABSTRACT

Background. Nephrogenic rests (NRs) are abnormally persistent foci of embryonal cells, thought to be the precursor lesion of Wilms tumors (WTs). To date their presence has not been systematically examined in WTs treated with preoperative chemotherapy. **Methods.** A systematic analysis of data on NRs in WTs treated with pre-operative chemotherapyobtained from the United Kingdom (UK) cohort of the International Society of Pediatric Oncology (SIOP) WT 2001 trial. The study was based on central pathology review of full sets of slides from pathological specimens, with a median of 28 slides reviewed per case. **Results.** NRs were identified in 40% of unilateral WTs including 25% perilobar nephrogenic rest (PLNR), 9% intralobar nephrogenic rest (ILNR), 5% both PLNR and ILNR, and 1% nephroblastomatosis, and in 93% of cases with bilateral lesions. ILNRs were associated with stromal histology, a younger age at diagnosis and found frequently in patients with congenital anomaliesassociated with*WT1* mutation. PLNRs were found frequently in patients with overgrowth syndromes. **Conclusions.** The prevalence of NRs in WTs after preoperative chemotherapy observed in SIOP UK WT 2001 Study is similar to the previously published data on NRs not treated with preoperative chemotherapy. Their epidemiology supports at least two pathways to Wilms tumorigenesis.

INTRODUCTION

Nephrogenic rests(NRs) are regarded as precursor lesions of Wilms tumor (WT) and have been defined as 'foci of abnormally persistent nephrogenic cells, retaining cells that can be induced to form a Wilms tumor'[1,2].Rests are subdivided into two main types, perilobar nephrogenic rest (PLNR), confined to the periphery of the renal lobe, and intralobar nephrogenic rest (ILNR), found anywhere within the renal lobe[2,3]. Nephroblastomatosis is defined as the 'diffuse or multifocal presence of nephrogenic rests or their recognised derivatives'[2]. They can be further sub-classified as incipient or dormant, regressing or sclerosing, obsolescent, and hyperplastic NRs[2].

Improved understanding of the development and fates of NRsas precursors of WTs will help understanding of their clinical significance, and the developmental biological relationship to WT. Several studies have investigated the progression of genetic changes in NRs and their associated WTs, either focussing on specific genes or using genome wide approaches[4-6]. These have identified common genetic changes in both NRs and WTs, assumed to occur at an early stage, with later changes found only in tumors. Such studies are ongoing and aim to help identify key WT driver genes in a manner analogous to that of Vogelstein and Fearon's model for colorectal cancer[7].

To date, almost all studies of NRs come from the North American National Wilms Tumor Study (NWTS) group. The largest of these, studying 5,954 patients from NWTS3, 4 and 5 studies identified NRs in 42% of patients with WTs (20% PLNR, 18% ILNR, 4% PLNR+ILNR)[3].

In the NWTS study NRs were found in 28% of patients with unifocal tumors and in over 90% with multifocal disease[3]. The almost universal presence of NRs in bilateral WT disease is thought to reflect germline changes resulting in predisposition to WTdevelopment[4,8]. Approximately 5-10% of children with WT have disease affecting

bothkidneys[9]. This may be either bilateral WT, bilateral NRs or unilateral WT with contralateral NR(s). The distribution of NRs varies by ethnic group. Fukuzawa *et al.* reported PLNR rates of 8% in Asian-Americans and only 2% in Japanese patients, compared to 24% in white Americans[10]. In a series of 127 Wilms tumors from India, no cases of PLNR were observed, whereas 45% had associated ILNR[11].

In contrast to the NWTS trials, the patients in International Society of Paediatric Oncology (SIOP) trials undergo pre-operative chemotherapy prior to nephrectomy (2 drugs for 4 weeks for localized, and 3 drugs for 6 weeks for metastatic tumours at presentation)[12]. This paper summarised the patterns of NRs in the UK SIOP WT 2001 Trial.

MATERIALS AND METHODS

Patients

The SIOP WT 2001 trial was an 11 year international multicentre study that recruited 4,763 patients with renal tumours of childhood. All paediatric patients at registered institutions with a renal tumor were eligible for registration onto this study. The UK study centre registered patients from across all paediatric cancer primary treatment centres in the UK and Ireland (841 cases), and in addition a few patients from centres in New Zealand (6 cases) and Australia (2 cases). Full informed consent was given for all participants.

Inclusion Criteria

This study included the patientswho fulfilled the following criteria: a) were diagnosed preoperatively as having intrarenal WT; b) planned to receive preoperative chemotherapy according to the SIOP WT 2001 protocol; c) had total and/or partial nephrectomy; and d) tumors werereviewed by the UKand SIOP Renal Tumor Pathology Panel.

Patients were classified as having unilateral or bilateral disease at initial presentation. Bilateral disease was defined as the presence of WTsand/or NRs in both kidneys. Tumors were further sub-classified and staged on the basis ofpatient's registration details and post-nephrectomy pathological investigations. In a small number of cases contralateral tumors developed at a later date and these were classified as metachronous bilateral tumors.

The presence of congenital anomalies was recorded on the case report form at registration. These data were later reviewed, any missing data or contradictions discussed with local centres and the study database updated.

Histological Review

As part of the study, all registered cases were sampled according to the SIOP 2001 Trial Pathology protocol[13]and submitted for central pathology review for diagnosis,classification and staging[12]. For all patients registered by the UK study centre this was performed by a UKRenal Tumour Pathology Panel (chaired by one of the authors, GMV) to whom full sets of all histological slides were submitted directly by each centre. Cases were also later reviewed by the SIOP Renal Tumour Pathology Panel. The number of slides submitted varied from 6 to 78 (median 28), with only 3% of cases having fewer than 10 slides. In these cases it was clearly stated that tumors were small and virtually completely sampled. WTs treated with preoperative chemotherapy were classified as low risk (completely necrotic tumors), intermediate risk (comprising epithelial, stromal, mixed, regressive, focal anaplasia types) or high risk (blastemaltype or diffuse anaplasia type on any cellular background) as per the SIOP WT 2001 Classification[14].Where NRs were present they were classified as PLNR, ILNR, both/combined (PLNR and ILNR), andnephroblastomatosis (NBL), which includedmultiple/multifocal NRs and diffuse hyperplastic perilobar nephroblastomatosis (DHPLN).

Histological features of NRs in cases treated with preoperative chemotherapy will be described in a separate paper (*in preparation*).

Statistical methods

Summary statistics were used to describe the data. A Chi squared test was used for comparison of proportions. A Wilcoxon-Mann-Whitney test was used to compare distributions of continuous variables. Frequency distributions for age at diagnosis were inspected using histograms with Gaussian kernel smoothing. Results were considered statistically significant at the five per cent with no adjustment for multiple testing. All

analyses presented are univariable i.e. not adjusted for other factors. The analysis was performed in Stata v12[15].

RESULTS

Patients

There were 849 patients registered in SIOP WT 2001 Trial in the UK.This study involved 705/849 (83%) patients who fulfilled the criteria for this analysis including 636/705 (90%)with unilateral and 69/705(10%)with bilateral WTs (Figure 1).

Frequency of Nephrogenic Rest

NRs were identified in 318/705 (45%) of all cases including 253/636 (40%) with unilateral and 65/69 (94%) with bilateral disease.

Unilateral Disease

Out of 636 patients with unilateral WTs, 159 patients (25%) had PLNR, 58(9%) ILNR, 30 (5%) combined PLNR and ILNR, and 6 (1%) nephroblastomatosis (Table 1).

A significant association was found with $p<0.001$ when comparing ILNR presence or not with presence of stromal type WT (i.e. - 24/88 (27%) casesand 33/549 (6%) is stromal versus all other WT types, respectively).No other WT type showed a significant association with any type of NRs (Table 1).

344/636 (54%) of patients with unilateral WT were female, of whom 138/344(40%) had NRs including 91/344 (26%) with PLNR alone and29/344 (8%) with ILNR only.Of male patients, 115/292(39%) had NRsincluding 68/292 (23%) with PLNR,and29/292 (10%) with ILNR only. There were no statistically significant difference betweenNR types and gender (Table 2).

The distribution of age at diagnosis of patients with unilateral WTs by gender is shown in Table 2 and Figure 2. Male patients had a younger age at diagnosis in all rest groups except diffuse nephroblastomatosis, where numbers were very small. Patients with

ILNR had a younger median age at diagnosis of 1.9 years compared to patients with no ILNR with median age 3.4 years ($p < 0.001$).

There is a suggestion of a bimodal distribution of age at diagnosis of unilateral WTs in females, peaking at 2 and 4 years. Whilst these peaks may be attributed to those tumors containing ILNR and PLNR, such a pattern is also seen in patients with no NRs (Figure 2).

Bilateral Disease

In 65/69 (93%) patients who presented with bilateral disease NRs were identified in one or both of the kidneys (Figure 3).

Although there was a more noticeable excess of females (65%, 45/69) in patients with bilateral disease, it was not significantly different when compared to unilateral disease ($p=0.052$). Patients with bilateral disease were of younger age at diagnosis compared to patients with unilateral WT alone ($p < 0.001$). Median age at diagnosis of patients with bilateral disease was 1.8 years, ranging from 2 months to 11 years (patients under 6 months of age were not initially treated with pre-operative chemotherapy).

There were 35 histologically confirmed bilateral WTs, with a median age at diagnosis of 1.8 years. Of these, 86% (30/35) had NRs identified in one or both kidneys including 43% (15/35) with PLNR, 20% (7/35) with ILNR, and 23% (8/35) with combined ILNR and PLNR.

There were 26 cases in which there were documented contralateral lesions on imagining studies with a histologically confirmed WT in the ipsilateral kidney. In 14 of the cases there was histological confirmation of at least one of these lesions as NRs. Four of these patients later developed a metachronous WT on the side of the rest at 0.6, 1.0, 1.7 and 1.9 years, respectively. In three of these cases ILNRs were present (two also had PLNRs) and in the fourth case there was a hyperplastic PLNR. In six cases it was not possible to confirm

histologically on the material available whether the lesion in one kidney was NR or WT. In all of these cases NRs were identified in the kidney with the tumor.In the remaining six cases no biopsy of the lesions in the contralateral kidney was done. In each of these cases rests were identified on the side of the confirmed tumor (three PLNR, two nephroblastomatosis, one PLNR and ILNR).

Of the eightpatients who presented with bilateral NRs, one was a 2 month old baby with Beckwith-Wiedemann syndrome who was diagnosed clinically with bilateral nephroblastomatosis. The remaining seven cases (median age at diagnosis of 1.4 years, range 0.17-2.4 years) had biopsy on at least one side. In one case there was progression to histologically confirmed anaplastic WT 13 months after diagnosis. Full details of this case have been described by Popov *et al.*[16].

Congenital Anomalies and Nephrogenic Rest

Congenital anomalies were present in 12% (81/705) of patients, including 9% (58/636) of patients with unilateral WT and 33% (23/69) of patients with bilateral disease. The presence and type of NRsin 49 (60%) patients with specific anomalies associated with WTs are presented in Table 3. There were a further 32patients with a range of other abnormalities, 18 (56%) of whom had NRs (39% PLNR, 9% ILNR, 3%, both PLNR and ILNR, and 3% nephroblastomatosis).

ILNRs were identified in 6/7 cases of aniridia, and all of them were associated with stromal type WT. There was one case of Denys-Drash syndrome who had bilateral stromal type WTs with ILNRs at 4 months of age. In a further 20 cases (14 girls and 6 boys) other urogenital abnormalities were registered, and five of them had bilateral disease. Eight of these cases had NRs including five with ILNR only, and three with both PLNR and ILNR. Ten of these WTs were stromal type.

There were three cases of Simpson Golabi Behmel syndrome; two had bilateral nephroblatomatosis (aged 2 and 5 months) and a third a mixed type WT and PLNR (aged 2.1 years).

Eight patients had Beckwith Wiedemann syndrome. The median age at diagnosis was 1.63 years (range 2 months – 6.24 years). One case was excluded from the main analyses as there was no further data available after registration. Of the remaining seven cases, three had bilateral disease (two bilateral tumors, one bilateral diffuse perilobar nephroblastomatosis). There was evidence of rest in 5/7 cases, (one diffuse perilobar nephroblastomatosis, three PLNR only, one case of PLNR and ILNR). There was no association with a specific histological subtype(s).

There were 12 further patients with hemihypertrophy, including two cases of bilateral disease, one with diffuse bilateral nephroblastomatosis, the other with bilateral WT with PLNR bilaterally. Of the 10 unilateral cases four had PLNR, one ILNR and one nephroblastomatosis.

DISCUSSION

We presentedthe first analysis of NRs in patients with WTs treated with preoperative chemotherapy. The studyof such detailed data was dependent on the centralised pathology review process built in to the SIOP WT 2001 protocol and the high proportion of all nationally registered cases. The review of the complete sets of slides prepared according to the protocol in each case was in contrast to review of only a limited number of 'representative' slides in previous studies[17]. This was the likely reason why NRs were found in only around 15% of cases in the previous SIOP and UK Trials (*author's, GMV, personal observation, unpublished data*).

The overall frequency of NRs identified in post chemotherapy specimens in this study was similar to that found in a large study of nearly 6,000 cases of primarily operated WTs in the US (45% *vs*. 42%)[3]. In contrast to our studywhereWTs were classified as unilateral and bilateral, Breslow *et al.* have analysed the occurrence of NRs in unifocal and multifocal disease, the latter category comprising unilateral/multifocal, bilateral-at-onset, and late (metachronous) bilateral tumors[3]. Therefore, it was impossible to compare the results of these two studiesdirectly. When comparedSIOP unilateral *versus*NWTS unifocaltumors, there was a higher prevalence of PLNR in our study (25% *vs*. 14%) (Table 4). However, it was virtually identical to another study on white American children only(24%)[10]. Ethnicity was not collected as part of the data set of SIOP WT 2001 and so it was not possible to investigate this aspect in the current study.The prevalence of ILNR in our group was lowerthan in the NWTS group(9% vs 17%, respectively). This lower proportion may reflect difficulties in identifying definite ILNR in tumors with extensive chemotherapy induced changes. Finally, the prevalence of combined PLNR and ILNR was higher in our study (5% *vs*.2%) but the numbers were too small for meaningful comparison.

Preoperative chemotherapy significantly alters histological features of WT and, therefore, histological criteria for classifying WTs differs between the SIOP and NWTS[13], making, again, a direct comparison of occurrence of NRs in different histological types of WT unfeasible. Anaplastic type WT, which is the only type classified on the basis of the same criteria in the SIOP andNWTS, showed a very similar prevalence of NRs in both groups (45% in our and 41% in the NWTSseries). Still, as in the NWTS study[3], our analysis found that there was asignificant association of ILNRs both with stromal type WT and a younger age at diagnosis; however, analysis was unadjusted for other factors.The suggested earlier origin of ILNR may be reflected in the earlier age at diagnosis[18]. ILNRs were identified in all but one case of WT associated with aniridia and 8/20 cases associated with urogenital anomalies, further supporting *WT1*mutations in their pathogenesis. It has been previously shown that constitutional *WT1* mutation syndromes (e.g. WAGR syndrome and Denys-Drash syndrome) are associated with ILNR,whereas 11p15 epimutations, such as in Beckwith Wiedemann syndrome, with PLNR[3]. However germ-line genetic data was not analysed as part of this work and so it is not possible to confirm the presence of WT1 gene mutations/deletions in all of these cases.

As observed previously[3], we also found that both unilateral and bilateral tumors were more common in girls than boys, and boys had a younger age distribution at diagnosis. But, unlike the US cohort, the current study found no excess of PLNR or deficit of ILNR among girls and the bimodal age distribution of cases among females was present even in the absence of rests[3]. This distribution may be a reflection of the two separate molecular pathways occurring even in the absence of morphologically recognisable rests[3].

In contrast to patients with ILNRs, patients with PLNRs were older at diagnosis. Furthermore, PLNRs were seen in the majority of patients with Beckwith-Wiedemann syndrome and/or hemihypertrophy, as observed in the US cohorts[1,3]. Together, this data

support previous suggestions of at least two pathways to WT development, so called 'type I', characterised by a young age at diagnosis, presence of ILNRs and stromal histology, likely involving *WT1* mutations, and 'type 2', associated with an older age at diagnosis, PLNR and non-stromal histology, likely involving deregulation of IGF2[3].

The younger age of onset in males in our and other studies also suggests that there may also be differences in pathological mechanisms between genders, perhaps either related to critical genes being on the sex chromosomes themselves or a differing impact of mutations in genes determining urogenital development according to the embryonic environment in the two sexes[3].

NRs were present in 94% of cases of bilateral disease consistent with Beckwith's observation of the presence of rests in almost all cases of bilateral disease[1].In the NWTS study, NRs were found in 90% of multifocal WTs[3]. This observation supports the concept that in bilateral disease a mutation or epimutation in the germline, or very early in development, results in an underlying predisposition to rest and consequently to tumor development.

The difficulty in histologically definitively distinguishingNRs from WT in some cases, even by highly experienced specialist review pathologists, as seen in one unilateral and six bilateral lesions, was also highlighted in this series. This mayrepresent a therapeutic challenge since the management of NRs and WTs differs. Further understanding the molecular progression of rest to tumor may help to define future biomarkers to aid such clinical decision making.

In this cohort, 29% (4/14) of cases in which there was histological confirmation of tumor on one side and only NR contralaterally at the time of initial surgery developed a metachronous contralateral WT, despite chemotherapy for the original tumor. Metachronous tumors have previously been observed to occur in only 1.2% of patients with unilateral WT,

and the presence of NR is recognised as a risk factor for this[19]. Both ILNR and PLNR, particularly when hyperplastic, have been linked with the development of metachronous tumors, but numbers in this series were small and so no further correlation is possible[1,19-21]. Development of metachronous tumors after chemotherapy and/or surgical removal of NRs in the remaining kidney is well-recognised[21] but better understanding of the molecular pathology underlying the fates of rest may help in further understanding the progression.

CONCLUSION

Our study showed no observed difference on the overall prevalence of NRs(318/705 = 45%) compared the NWTS study (2,494/5,954 = 42%), except for ILNRs which were not found as frequently as in the NWTS study.We cannot conclude that the difference is due to preoperative chemotherapy due to potential difference in patients characteristics. ILNRs were associated with stromal type WT and younger age, offering further support to the beliefthat there are at least two separate pathways of Wilms tumorigensis, whereby *WT1* pathway mutations lead to ILNR and stromal type WTs, and IGF2 pathway changes result in PLNR and other non-stromal histological subtypes[3,22,23].Congenital anomalies were present in 12% of patients including 9% of patients with unilateral WT and 33% of patients with bilateral disease. In patients with congenital anomalies, NRs were found in 60% of cases.

Further integration of detailed histopathological information with molecular and genetic information of patient's tumors, rests, and germline and the radiological images of tumors and NRs has the potential to support some of the difficult clinical decisions, particularly regarding management of bilateral disease.

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CONFLICT OF INTEREST

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Ethics – Trial was approved by Trent MREC (MREC/01/4/086). Consent was taken from all patients, or those with parental responsibility.

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Figure Legends:

Fig. 1. Distribution of patients in the SIOP WT 2001 Study (UK data)

Fig. 2. Frequency distributions of age at diagnosis by gender and by rest type for patients with unilateral Wilms tumor

Fig. 3. Distribution of bilateral disease