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Allelic Loss on Chromosome 11p is a Less Frequent Event in Bilateral than in Unilateral Wilms' Tumours

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Analyses to detect loss of heterozygosity (LOH) were performed at 11 polymorphic loci on chromosome 11 and, using a polymorphic CA repeat sequence in the WT1 gene, on a series of 39 tumours from 28 unilateral and 10 tumours from 6 bilateral Wilms' tumour (WT) patients. LOH was seen in 13 out of 35 patients including 12 out of 29 unilateral tumours, but only one of 10 bilateral tumours. This suggests that bilateral WT represents a subgroup of WT in which tumour initiating events less frequently involve LOH on chromosome 11 and that either epigenetic events, point mutations or another non-chromosome 11p locus are important in bilateral tumours. The observation of LOH in one WT but not another WT in a bilateral WT patient provides evidence that these tumours arising in the same patient are not monoclonal proliferations and most likely arise via different molecular pathways.
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INTRODUCTION

WILMS' TUMOUR (WT) is a paediatric neoplasm of the kidney which is hypothesised to require at least two mutational steps for tumour development. The first may be prezygotic and acquired from the germinal cells of one parent in hereditary cases of the disease. Thus, some patients will carry this first mutation constitutionally. The second mutation is hypothesised to be postzygotic, resulting in the loss or effective loss of the remaining normal copy of a tumour-suppressor gene. At least one of the genes responsible for WT is known to reside on the short arm of chromosome 11, at 11p13, due to observations of 11p13 deletions in Wilms' tumour/aniridia/genitourinary dysplasia/mental retardation (WAGR) patients and the tumours of sporadic WT patients. Recently WT1, a zinc finger gene, has been cloned from the region of interest at 11p13 [1, 2].

Restriction fragment length polymorphism (RFLP) studies on WT have shown that regions along the short arm of chromosome 11 that are constitutionally heterozygous become homozygous in some tumours [3–6]. This loss of heterozygosity (LOH) for 11p loci corroborated the cytogenetic evidence that

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this region was important in WT aetiology. LOH, occurring via chromosome loss, loss and reduplication of the remaining chromosome or a mitotic recombinational event, was hypothesised to constitute the second mutational event and result in the effective loss of the chromosome carrying the remaining non-mutated WT gene. In tumours not showing LOH, it was assumed that the second event entailed a point mutation or minute deletion within the 11p13 WT locus not revealed by RFLP analysis. However, some WTs demonstrate LOH specific for 11p15 alleles, suggesting the presence of another WT associated gene at 11p15 [7–11].

The possibility of a third WT locus was raised when no linkage was found between 11p loci and WT in two large pedigrees [12, 13]. Recently, a site of non-random LOH has been identified on 16q [14]. Assuming that LOH represents one form of second mutation required for tumour initiation, the patterns of LOH found to occur in WT may be correlated with a distinguishable disease phenotype, as assessed by disease progression or histology, patient sex or age at disease onset. This would support genetic heterogeneity in WT. A difference in the frequency of LOH between unilateral and bilateral patients would also indicate a heterogeneity of disease aetiology. Consequently, this study aimed to perform RFLP analyses on a large group of unilateral and bilateral WTs to define the prevalence of LOH in WT and to investigate the possibility of a correlation between the presence of LOH and tumour progression, tumour laterality, tumour histology or other clinical features.

MATERIALS AND METHODS

A series of 39 primary tumours from 35 WT patients including 29 unilateral primary tumours and 10 primary tumours from 6 bilateral patients were collected at the time of surgical resection. Nine metastatic lesions including two lymph node metastases, four lung metastases and three abdominal recurrences were also collected. Tumours were histologically graded and staged using National Wilms' Tumour Study guidelines [15]. No cases of unfavourable tumour histology were identified. Routine cytogenetic analysis were initially performed by the relevant hospital or private pathology laboratories. 2 patients presenting with WAGR phenotype had consitutional 11p deletions. A third WAGR patient showed constitutional mosaicism for an 11p deletion [NP62; 46,XX/46,XX,del 11(p13)]. One tumour (NP59) showed a small 11p deletion [46,X, −21,+der (21)(p+), del(11)(p13p14), +der(1) t(X;1)(q26;cen)]. This patient's somatic karyotype was normal (46,XX).

DNA extraction

Fresh tumour samples were homogenised using a Kinematica Microdismembranator, speed 10 for 30 sec and lysed in 10 ml of DNA lysis solution [40 mmol/l Tris, pH 8.0, 20 mmol/l EDTA, 0.1 mmol/l NaCl, 0.5% sodium dodecyl sulphate (SDS), 0.1 mg/ml proteinase K], overnight at 37°C. Lysate was extracted with phenol and chloroform and the RNA was removed by incubation with 20 μg/ml final concentration of RNase (37°C, 2 h) before precipitation with ethanol. Samples of either normal kidney from adjacent to the removed tumour or peripheral blood were also collected to represent normal somatic tissue. With peripheral blood samples, DNA was either extracted directly from the whole blood or from Epstein–Barr virus-transformed lymphoblastoid cell lines.

RFLP analysis

10 μg of high molecular weight DNA was digested with the appropriate restriction enzymes in the presence of universal restriction digestion buffer (20 mmol/l Tris–HCl; pH 7.4, 70 mmol/l NaCl, 6 mmol/l $MgCl_2$, 6 mmol/l β-mercaptoethanol) and 4 mmol/l spermidine. Reactions were electrophoresed (0.8–1.4% agarose) in Tris–acetate/EDTA buffer and Southern-blotted into Hybond N nylon membranes. Plasmid DNA was nick translated and prehybridization/hybridization solutions were 6.66 × SSC [16], Denhardt's solution [0.02% (w/v) bovine serum albumin/0.02% (w/v) Ficoll 400, 0.02% (w/v) polyvinyl pyrollidone] × 5, 20 mmol/l sodium phosphate; pH 6.5, 0.1% SDS, 5 mmol/l EDTA; 65°C, 16–24 h. Filters were washed at 65°C with two sequential 30 min washes in (1 × SSC/ 0.1%SDS) followed by (0.5 × SSC/0.1%SDS). Autoradiography was carried out at −70°C for 1–5 days.

Chromosome 11 probes

Probes to 11 RFLPs at 10 separate chromosome 11 loci were used to assay for LOH. Eight of these RFLPs from seven loci resided on the short arm of chromosome 11, these being c-Ha-ras1 (11p15.5-pter), insulin (11p15.5), ribonucleotide reductase M1 subunit (RRM1)(11p15.5), gamma-globin (11p15.5), calcitonin (11p15.4), β-follicle stimulating hormone (βFSH; 11p13) and catalase (11p13), whilst the other three mapped to 11q, these being apolipoprotein AIII, c-ets1 and D11S149. The restriction enzymes used for each probe were MspI/HpaII for probe pEJ6.6 [c-Ha-ras-1; human gene mapping (HGM) 10], RsaI for phins500 (insulin; HGM 10), SacI for phins214 (insulin; HGM 10), HindIII for pHgamma1 (gamma-globin; HGM 10), TaqI for pHTB58 (calcitonin) [17], HindIII for βFSH probe (HGM 10), TaqI for pint800 (catalase; HGM 10), PvuII for pTTH26 (D11S149) [18], SacI for c-ets probe (HGM 10), SacI for pE2-8 (RRM1) [19].

CA repeat PCR and analysis

PCR primers 716 (5′ AATGAGACTTACTGGGTGAGG 3′) and 718 (5′ TTACACAGTAATTTCAAGCAACGG 3′) [20], flank the CA repeat in the 3′ untranslated region of WT1 and result in a PCR fragment of approximately 144 base pairs (bp) [+/− n(2bp)]. For acrylamide–bisacrylamide gel analysis, 150 ng of PCR primer 716 was end-labelled in 1 × polynucleotide kinase (PNK) buffer (50 mmol/l Tris–HCl pH 8.0, 10 mmol/l $MgCl_2$, 10 mmol/l DTT) with 7.4 MBq γ^{32} P-ATP, 11 U T4 PNK; 37°C, 30 min. After phenol/chloroform extraction and ethanol precipitation, the primer was resuspended in 50 μl H_2O. 1 μl of this was used per 20 μl PCR reaction. PCR reactions contained either 200 ng genomic DNA or 1/20th of an RNA reverse transcriptase (RT) reaction in 1 mM dNTPs, 1 μmol/l of each PCR primer, 1 μl of end-labelled PCR primer 716, 1 × Promega Taq buffer and 0.5 U Promega Taq polymerase in a 20 μl volume, 30 cycles of PCR involving 45 sec denaturation at 92°C, 1 min annealing at 58°C and 1 min elongation at 72°C was performed. PCR results were analysed via electrophoresis on either an 8% acrylamide–bisacrylamide vertical gel; 1 × Tris–borate EDTA (TBE) [16] running buffer; or an 8% Nusieve GTG agarose horizontal gel; 1 × TBE/0.5 μg/ml ethidium bromide running buffer. Acrylamide gels were fixed in 10% methanol/10% glacial acetic acid, dried and exposed overnight. Allele sizes were estimated by comparison with a known sequencing reaction. For Nusieve analysis, a radioactive primer was not included as bands were

Table 1. Genetic rearrangements on chromosome 11p in Wilms' tumour samples

Patient	Tumour	Catalase 11p13 pint800	WT1 11p13	β-FSH 11p13	Calcitonin 11p15.4 phTB58	Haemoglobin γA 11p15.5	Haemoglobin γG	RRM1 11p15.5 pE2.8	Insulin 11p15.5 phins 214	Insulin phins 500	c-Ha-ras 11p15.5 pEJ6.6
1	NP2								L		
2	NP5		L	L			L		L		L
3	NP9				M	M	M				
4	NP10	M	M								
5	NP11		L	L	L	L				L	L
6	NP12		L	L			L		L		L
7	NP14								M		M
8	NP16(25)		M		M		M				
9	NP18(21)	M	M	M					M		M
10	NP22*26*(27)	M	M			M	M		M		M
11	NP30(31)					L	L				
12	NP32		M	M							
13	NP33	L				L	L	L			
14	NP34			L	L	L	L				L
15	NP35	L		L		L			L	L	
16	NP36*		M					M		M	
17	NP37		M			M				M	M
18	NP38(39)(68)		M	M			M				M
19	NP40		M	M	M			L			M
20	NP48		M		M	M	M			M	
21	NP50		M		M	M	M	M			
22	NP51		L	L		L			L		L
23	NP52(53)(54)	M	M	M						M	M
24	NP57*	L		L							L
24	NP58*	M	M	M							M
25	NP59(72)	M	L		M	M		M	M		M
26	NP60*61*				M						M
27	NP62*					M			M		M
28	NP63				M						
29	NP64					M			M		
30	NP65			M					M		
31	NP70			M	M	M			M		M
32	NP71	M	M	M		M		M			
33	NP73		L	L		L					L
34	NP74				M						
35	NP75*76*		M	M	M		M		M		

M = Maintenance of heterozygozity; L = loss of heterozygozity; ( ) = metastatic lesion; * = bilateral primary tumour.
In addition, probes D11S325, D11S324, D11S323 and D11S151 (HGM11) were also used to probe 11p13 but did not contribute further information.

visualised with ethidium bromide. 15 μl of each PCR reaction was loaded in bromophenol blue loading buffer (6x : 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll 400) and allele sizes estimated with reference to SpeI cut λ DNA.

RESULTS

RFLP analyses showed LOH in 13 of 39 primary tumours (33%) from 13 of 35 individual patients (37%) (Table 1). Patients NP40 and NP59 showed LOH at 11p15 and 11p13 respectively, exclusively, as had been reported by other researchers [7, 9–11].

Material from all patients displayed constitutional heterozygosity for at least one of the 11p loci, although many showed extensive homozygosity (Table 1). The most informative locus was the c-Ha-ras-1 proto-oncogene (54%), while the least informative probe was catalase (29%). The average level of informity for all 11p probes was 42% with 19 of 35 patients informative for one 11p13 locus and 33 of 35 patients informative for at least one 11p15 marker. 30 of 35 (86%) patients were informative for at least two 11p loci (Table 1). The observed allele frequencies accurately followed expected allele frequencies for these probes and there was no bias towards loss of any particular allele (data not shown).

Of the 29 primary unilateral Wilms' tumours, 12 of 29 (41%) displayed LOH for at least one 11p locus. In contrast, of the 10 primary tumours from 6 bilateral patients, only one tumour displayed LOH (Table 1). In another large study of LOH in WT [21], seven tumours from 5 bilateral patients displayed no evidence for LOH, whereas the same study showed LOH in 11/36 (31%) of unilateral tumours. By combining this data with the data presented here, LOH was found in 23/65 (35%) unilateral patients and 1/17 (6%) bilateral events. Statistical analysis of the prevalence of LOH using combined data shows this difference to be significant (χ^2 4.33; $0.025 < P < 0.05$). Bilateral tumours are uncommon and material for analysis is difficult to obtain, but these data suggest that mutational events

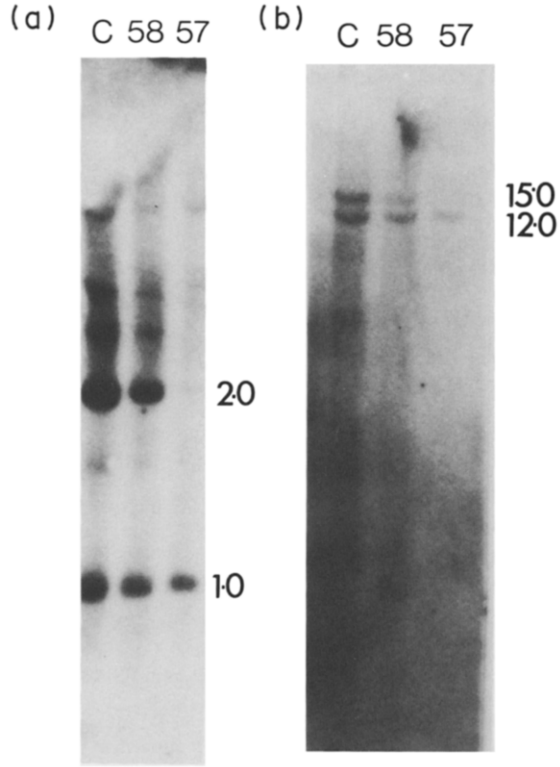

Fig. 1. Autoradiographs showing LOH in one of two tumours in a bilateral Wilms' tumour patient. The samples examined were DNA for (C) leucocytes, (58) left tumour and (57) right tumour of bilateral patient 24 (see Table 1). Panel A shows hybridisation with a c-Ha-ras1 probe (pEJ6.6) while Panel B shows hybridisation with a BFSH probe.

involving chromosome 11, such as would be detected by this technology, are less frequent in bilateral Wilms' tumours than in unilateral Wilms' tumours.

For some bilateral WT patients, independent WTs were sampled in the same patient. For 1 patient, the tumour from the left kidney (NP58) showed the same allelic pattern as the peripheral blood. However, the tumour from the right kidney (NP57) demonstrated LOH for all of the informative loci (Table 1, Fig. 1). It therefore appears that LOH for the short arm of chromosome 11, including the 11p13 region, has occurred only in the right kidney tumour, but that this LOH event was not involved in the tumour initiation process in the left kidney. This provides the first evidence in WT that bilateral lesions within the one individual are not monoclonal proliferations but may arise via distinct molecular genetic pathways. If all bilateral WT lesions are polyclonal in nature, then each tumour can be regarded as arising as a result of a separate 'tumour-initiating event'.

Examination of the clinical features of this study group established that it represented a typical population of WT patients with respect to distribution of sex, clinical stage of tumour progression, tumour histology, tumour laterality and age at diagnosis. To investigate the possibility that the presence of LOH is correlated with adverse tumour progression, the frequency of LOH was correlated with (i) advanced degree of progression at presentation, as assessed by clinical stage (clinical stages I and II, early stages of progression; stages III and IV, advanced stages of progression involving non-haematogenous and haematogenous metastasis respectively); (ii) adverse progression during follow-up as assessed by presentation of metastases in early stage patients after initial diagnosis; and (iii) tumour histology using the classification of Beckwith and Palmer [15]. As seen in other studies, all metastatic lesions displayed the same allelic pattern as the primary lesion from which they evolved (data not shown), thus verifying their clonality [22]. There was no correlation between (i) LOH and advanced stage at presentation (stages III and IV); (ii) LOH and all patients with adverse tumour progression (stages III and IV, plus those with early stage disease who developed metastases later); (iii) LOH and tumour histology. Although all metastatic samples displayed the same RFLP pattern as their matching primary tumours, these did not always display an identical histology, again supporting a lack of correlation between LOH and tumour histology.

In this study, parental origin of the alleles lost was ascertained for five of the tumours showing LOH. In all cases the allele lost was of maternal origin. This strengthens the data reported by other groups [6, 7]. There was only one familial tumour in this study (NP2). The half-brother of this patient also developed a WT. These children had different fathers but shared a common mother, indicating that the mother was the obligate carrier. Tumour NP2 displayed maternal allele loss for the 11p15 at insulin locus region, indicating that the inherited mutation was either not on chromosome 11 or is proximal to the insulin locus. A similar situation was reported by Grundy *et al.* [13] and provides evidence against the hypothesis that maternal specific LOH results from a requirement for an initial mutation in a paternal WT gene which is revealed only due to the loss of a normal maternal homologue.

DISCUSSION

This study has shown that LOH is observed in 37% of WT in this series with one tumour showing LOH which only encompasses 11p15 and not extending to 11p13 and another tumour showing the reverse. This is in keeping with the findings of others [7, 9–11]. This provides further evidence that independent loci at 11p13 and 11p15.5 may be involved in WT development. Overall, we found no relationship between the presence of LOH and tumour progression, the various sub types of favourable tumour histology, patient sex or age at diagnosis. If LOH merely represents a lesion related to tumour progression rather than initiation, it should have been more prevalent in advanced stages of WT or more frequently seen in metastatic lesions. This suggests that in the bilateral tumours either point mutations or small deletions occur within critical 11p WT genes or, alternatively, critical mutational events may have occurred on another chromosome.

Recently, a zinc finger gene, WT1, which has been implicated in the aetiology of Wilms' tumour, has been cloned from the area of interest within 11p13 [1, 2]. WT1 is involved in genitourinary development [23, 24]. Deletions and rearrangements within WT1 are associated with the development of Wilms' Tumour [20, 25, 26]. More recently, germ line mutations in WT1 have been identified in association with abnormal urogenital development (Denys-Drash Syndrome) [27, 28]. More recently, point mutations in the zinc finger region of WT1 have been identified in some Wilms' tumours from patients who were phenotypically normal [29]. Some, but not all, of the patients reported in these studies had bilateral tumours [26–29]. Thus, it is possible that small mutations on a scale not detectable in a LOH study might contribute to development of WT in patients with bilateral tumours.

Alternatively, aetiology of bilateral WT may involve a non-

chromosome 11 locus. Evidence for the existence of this is derived from linkage studies [12, 13] on familial Wilms' Tumour cases which demonstrate lack of linkage to chromosome 11. More recently, a site of non-random LOH has been identified on chromosome 16q in a large series of WT [14]. This study presents further indirect evidence for the possibility of a non-chromosome 11 locus. Further detailed studies involving mutational analysis within genes known to be important in WT such as WT1 and studies of LOH at other putative loci such as the chromosome 16q locus need to be performed in bilateral tumours to further eliminate this.

One bilateral WT patient studied in this series showed discordance for LOH in tumours taken from both kidneys. Our results suggest tumour initiation in this patient involved different mutational events. Dao *et al.* [22] have previously reported a bilateral patient in which LOH was observed in only one tumour. However, both of these tumours were removed from the left kidney, one from the upper pole and the other from the lower pole. The authors expressed caution as the patient had presented with nephroblastomatosis and perhaps one of the tumours was actually a portion of this rather than true WT. In our patient 24 (NP57, NP58) there was no evidence of nephroblastomatosis. Both tumours were large and it was unlikely that either can be regarded as contaminating somatic tissue.

In summary, this study presents confirmatory evidence that there are independent 11p13 and 11p15 loci on chromosome 11p which are important in development of WT. It also shows that the tumour initiating events in the WT of bilateral patients less frequently involve LOH suggesting that WT in these individuals involves another non-chromosome 11 gene or small deletions or point mutations within 11p WT genes rather than extensive LOH.

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