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Original Paper

Gain of Chromosome Arm 17q Predicts Unfavourable Outcome in Neuroblastoma Patients

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Gain of chromosome arm 17q has recently been reported in neuroblastoma tumours. We analysed 17q status in relation to other known prognostic features and clinical outcome in a series of 45 tumours. Chromosome 17 status was detected by cytogenetic analysis, fluorescence *in situ* hybridisation (FISH) and comparative genomic hybridisation (CGH) and correlated with other clinical and genetic factors. Survival analysis was calculated by the Kaplan–Meier estimation. Twenty-eight out of 45 tumours showed 17q gain, and this was associated with established indicators of poor prognosis; stage 4 disease ($P<0.001$), age above 1 year at diagnosis ($P<0.001$), 1p deletion ($P<0.01$), *MYCN* amplification ($P=0.03$) and diploidy/tetraploidy ($P=0.04$). 17q gain was associated with poor outcome: 3-year survival was 13.5% compared with 100% for tumours without 17q gain ($P=0.0001$); and progression-free survival (PFS) was 8.1% after 3 years compared with 83% for 17q normal tumours ($P=0.0001$). PFS in 28 *MYCN* non-amplified patients indicated that 17q status has discriminatory power within this group: PFS 0% for 17q gain ($n=14$) versus 100% for normal 17q ($n=14$) ($P=0.0001$). This study indicates that 17q changes have prognostic significance in neuroblastoma and should be a target for molecular cytogenetic detection at diagnosis. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

NEUROBLASTOMA IS one of the most common extracranial solid tumours of children. Despite advances in the treatment of childhood malignant diseases, metastatic neuroblastoma in children over the age of 1 year continues to have a poor outcome. However, the clinical behaviour of neuroblastoma varies greatly from spontaneous regression to malignant progression showing resistance to intensive therapeutic regimens. In view of this variation in clinical behaviour, there is a need to select appropriate therapy. At present, therapeutic characterisation is based upon patient's age and stage of disease. However, there are a number of molecular pathological features which are known to be associated with prognosis. In view of this, the current international recommendation is that data on these features should be collected from all patients with neuroblastoma so that new therapeutic groups can be defined [1]. By this means it is hoped in the future that high- and low-risk patients can be identified more accurately. Currently, the main genetic feature which influences the therapeutic approach in individual cases is amplification of the *MYCN* oncogene. However, despite the currently recognised prognostic features in neuroblastoma, the low-risk group includes a subset of patients who respond poorly, while some patients with poor prognostic features are long-term survivors [2, 3]. Thus, there remains a significant gap in understanding the changes leading to malignant transformation and progression of this disease.

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In searching for clinically useful prognostic markers, we have focused on the involvement of chromosome 17 in neuroblastoma. Unbalanced translocation resulting in loss of distal 1p and gain of 17q has previously been identified by fluorescence *in situ* hybridisation (FISH) in primary tumours [4, 5]. Also, Caron has demonstrated gain of 17q in 38% of neuroblastoma tumours by a Southern blot technique, and showed that this feature had a negative prognostic impact [6]. More recently, two comparative genomic hybridisation (CGH) studies have shown that over-representation of chromosome 17 material occurs in approximately 75% of primary tumours either as gain of the whole chromosome or as a gain of part of the q arm only [7, 8]. We were also able to confirm by FISH that 17q can translocate on to a number of chromosome regions other than 1p, and that such re-arrangements result in gain of 17q material [9]. Meddeb and associates [10] reported very similar findings of multiple unbalanced translocations involving 17q in neuroblastoma. Moreover, we noted that both in their series and in our own, patients with 17q gain also showed poor prognostic features. Therefore, in order to investigate the possible role of chromosome 17q re-arrangements as a prognostic factor in neuroblastoma, we analysed 45 neuroblastoma tumours and related the results to clinical outcome and other well-established prognostic features.

PATIENTS AND METHODS

Patients

Primary tumour material from 45 patients with neuroblastoma was studied prior to therapy. The patients' ages ranged from 1 month to 20 years, and 18 were female. They presented between October 1987 and April 1996. The sole criterion for inclusion in the series was technical success in establishing chromosome 17 status. The diagnosis of neuroblastoma and staging was according to the International Neuroblastoma Staging System (INSS) [1]. The distribution of stages was: stage 1 (2 patients), stage 2 (3 patients), stage 3 (10 patients), stage 4 (25 patients) and stage 4s (5 patients). The histological diagnosis of neuroblastoma was reviewed centrally by a panel of United Kingdom Children's Cancer Study Group (UKCCSG) pathologists. Therapy was administered according to current national and international protocols (the UKCCSG, European Neuroblastoma Study Group and Localised Neuroblastoma European Study Group). Briefly, patients with stage 1 and 2 neuroblastoma were observed following surgical resection; patients with stage 3 and infants with stage 4 received conventional platinum-containing chemotherapy, followed by surgical excision of the residual tumour. Patients with stage 4 neuroblastoma over the age of 1 year were randomised to either a rapid schedule of intensive chemotherapy, or to conventional therapy followed by surgical excision of the residual tumour and high-dose melphalan with autologous bone marrow rescue. Patients with stage 4s were merely observed unless their disease caused life-threatening symptoms.

Detection of chomosome 17 status

Cytogenetics. Tumour tissues or infiltrated bone marrows were obtained from patients prior to therapy. Samples were processed for cytogenetics according to published protocols [11]. Karyotypes were described according to ISCN (1995) [12].

FISH. The probes used were FITC-labelled chromosome 17 paint (Cambio) and indirectly digoxigenin-labelled Oncor probes: myeloperoxidase (*MPO*) gene from locus 17q21.3–q23 and chromosome 17q telomeric probe. For metaphase FISH, chromosome 17 paint was used in simultaneous combination with 17q probe in order to confirm the number of copies of 17q material. For interphase FISH, digoxigenin-labelled Oncor probe TP53 from locus 17p13 and biotin-labelled MPO probe were used. The probes cohybridised to chromosomes or nuclei at 37°C overnight, and slides were then washed in 50% formamide/2xSSC for 15 min, followed by 2xSSC for 3 × 5 min. Indirectly labelled probes were detected by rhodamine-conjugated antidigoxigenin (Boehringer, Mannheim, Germany) or fluorescein-avidin (Vector Lab., Burlingame, California, U.S.A.). Slides were counterstained with DAPI in an antifade solution. Images were captured by CCD camera and examined using a Smart Capture FISH station (Digital Scientific, Cambridge, U.K.).

CGH. The CGH procedure was modified from that of Kallioniemi and associates [13] and described elsewhere [8]. Briefly, target metaphases were obtained from phytohaemagglutinin-stimulated blood lymphocytes and treated with RNAse and pepsin. Tumour DNA was directly labelled by nick-translation with fluorescein-12-dUTP, and reference placental DNA was labelled by tetramethylrhodamine-6-dUTP (Boehringer). Equal amounts (200–400 ng) of tumour and reference DNA samples were mixed and precipitated with 30 μg of unlabelled Cot-1 DNA. DNA samples were dissolved in hybridisation mixture (50% formamide/2xSSC at pH 7.0) and denatured. Metaphase slides were denatured in 70% formamide/2xSSC at pH 7.0 and dehydrated through an ethanol series.

Hybridisation of DNAs to chromosomes was for 2–3 days at 37°C. After hybridisation, slides were washed and counterstained with DAPI in an antifade solution. The hybridised metaphase cells were examined using a Smart Capture FISH station (Digital Scientific) at three fluorescence excitation frequencies (360, 490 and 570 nm) [14]. Briefly, images were captured, normalised linearly and automatically synthesised to a 24-bit colour image. Digital processing was carried out in several steps; background removal, banding enhancement, thresholding and fluorescence equalisation. Fluorescence ratio (FR) analysis was performed as the ratio of total green to total red at each position across the width and orthogonal to the chromosome axis. Six to ten homologues of each chromosome were analysed and the mean FR values were computed. Interpretation of CGH results was based on comparison of tumour data with the control experiment; this established the green:red fluorescence ratio along the length of each chromosome which resulted from simultaneous hybridisation of two differentially labelled DNA extracts from normal placenta. The ratio fluctuations in this experiment were within the range of 0.9–1.1. FR values outside the range 0.85–1.15 were considered to represent abnormalities—values above 1.15 are termed 'chromosomal gain' and values below 0.85 'chromosomal loses'.

Detection of chromosome arm 1p deletion and oncogene MYCN amplification by FISH

Double-colour FISH was undertaken to detect 1p deletion in tumour interphase nuclei. The probes used were Oncor cosmid D1Z2 (1p36.3) labelled by biotin, and pericentromeric digoxigenin-labelled PUC1.77 probe. For detection of *MYCN* amplification, Oncor digoxigenin-labelled *MYCN* DNA probe was applied. Hybridisation and washing conditions were performed as described above. Deletion of 1p36

was recognised by comparison of the numbers of green and red signals on tumour nuclei, and *MYCN* amplification by the presence of multiple red signals per cell.

Southern blot analysis

MYCN amplification was detected by a standard Southern blot hybridisation method described elsewhere [8].

Statistical analysis

Survival and progression-free survival (PFS) were calculated using Kaplan–Meier estimation [15] and group comparisons were done by the log-rank test. The χ^2 test with Yate's correction for continuity was used to evaluate the relationship between 17q status and other clinical and biological features. Two multivariate analyses were used to assess the independent influence of 17q status on PFS, firstly with 1p status and stage (stage 4 versus other stages combined) and secondly with age group [16]. The scope for multivariate analysis was limited because of the small sample size; stage and age group were not included in the same model because the numbers of stage 4 patients under 1 year of age and low-stage patients over the age of 1 year were very limited. Two toxic deaths were censored in the analysis.

RESULTS

Detection of chromosome 17 abnormalities

17q gain was defined as a structural rearrangement of chromosome 17 which resulted in partial gain of 17q relative to 17p. This almost invariably involved a breakpoint in 17q12–21, with gain of the distal 17q arm. We detected such abnormalities in 28 out of 45 neuroblastoma tumours analysed (62%), and thus we divided patients into two groups: (1) with 17q gain ($n=28$, cases 1–28), and (2) without gain of 17q ($n=17$, cases 29–45) (Table 1).

The status of chromosome 17 in neuroblastoma cells was detected by tumour chromosome analysis in 27 patients, either in classical cytogenetic studies or by metaphase FISH. In the remaining 18 patients, chromosomes were not available, so tumour DNA was analysed by CGH. In 5 of the latter cases, interphase FISH was performed and corroborated the CGH results. Overall, chromosome 17 status was successfully analysed by two different approaches in 27 patients (60%) and no discrepancies were found in the results from various methods.

17q gain and other prognostic factors

Clinical factors. 17q gain was found more frequently in patients with stage 4 (22 out of 25 patients) compared with other stages combined (6 out of 20) ($\chi^2=13.5$, $P<0.001$) (Table 2). There was also a higher proportion of patients with 17q gain in the group older than 1 year (25 out of 31) compared with those aged under 1 year at diagnosis (3 of 14), ($\chi^2=11.9$, $P<0.001$).

Genetic factors. Deletion of chromosome arm 1p was detected in 23 patients, either by interphase FISH or CGH, and among them 20 had 17q gain. Among 15 children without evidence of 1p deletion, 5 had 17q gain. This indicates an association between 1p deletion and gain of 17q ($\chi^2=9.3$, $P<0.01$). *MYCN* amplification was found in 13 patients, either by interphase FISH or Southern blot analysis or both, and 12 of them had gain of 17q. Among 28 children with single copy of *MYCN*, 17q gain was detected in 14 cases ($\chi^2=5.1$, $P=0.03$). We also found a higher proportion of patients with 17q gain in the group with diploid/tetraploid number of chromosomes (19 of 24) versus near-triploid number (2 of 7) ($\chi^2=4.2$, $P=0.04$).

17q gain and survival

The overall 3-year survival for all patients in our series was 41.6% (95% CI 22–61%), and PFS, 35.2% (95% CI 16–54%). The median follow-up of the surviving patients was 20 months (range 4–96 months). One of the 2 toxic deaths, which were censored from analysis, had gain of 17q.

In univariate analysis, partial gain of chromosome arm 17q was significantly associated with poor outcome. Three-year survival was 13.5% for those with 17q gain compared with 100% for those without 17q gain ($P=0.0001$; Figure 1) and 3-year PFS was 8.1% (95% CI 0–23) for those with 17q gain compared with 83% (95% CI 54–100) for those with normal 17q ($P=0.0001$; Figure 2). The only child without 17q gain who relapsed is still alive and disease free 7 years after diagnosis. All 3 patients with stage 4 disease and without 17q gain remain alive and disease-free.

For comparison, we carried out univariate analysis of other prognostic factors in neuroblastoma. Stage of disease was significantly associated with prognosis in our patients; with only 10% PFS for stage 4, versus 62% for stage 3, and 100% for stages 1, 2 and 4s ($P=0.02$). Patient age had a similar

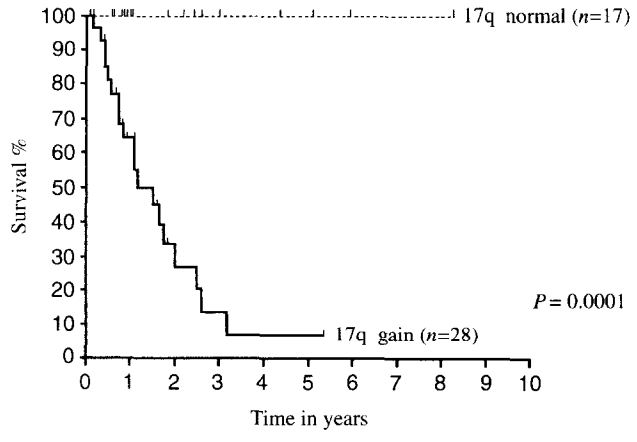

Figure 1. The overall survival in two groups of patients with 17q gain versus 17q normal.

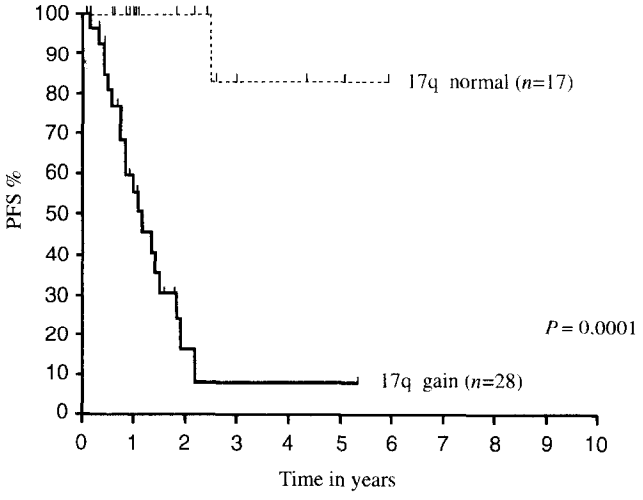

Figure 2. Progression-free survival in patients with 17q gain versus 17q normal.

prognostic impact; those aged under 1 year had 85% PFS compared with 10% for older children ($P < 0.001$). The effect of three genetic features on survival was also studied. The presence of chromosome arm 1p deletion reduced PFS to 20% compared with 66% without deletion ($P = 0.21$).

MYCN amplification was not important for prognosis in this series: after 2.5 years of observation, PFS for children with amplified *MYCN* was 35% versus 43% without amplification ($P = 0.56$). Ploidy status was tested in 24 children with diploid/tetraploid number of chromosomes, and in 7 with near-triploid number. Although the first group had lower survival (10%) than the latter (62%), the results were not statistically significant ($P = 0.16$).

Thus, 17q gain appears to be the strongest genetic indicator of survival in our series of patients and at least as important as well-known clinical factors.

Table 1. *17q status in* 45 *neuroblastoma tumours detected by classical cytogenetics, metaphase and interphase FISH or CGH, presented with patients' age, stage of neuroblastoma,* 1*p and* MYCN *status. 'Unclear'—complex rearrangements, chromosome* 17 *status not possible to determine*

					Cytogenetics			FISH metaphase	FISH interphase	CGH
Case	Stage	Age (years)	1p del	*MYCN* amplification	No of chromosomes or ploidy	Chromosome 17 rearrangements	No of normal chromosome 17	No of 17q signals	No of *TP*53/MPO	gain
1.	3	< 1	del	–	46	der(1)t(1;17)(p36;q11–12)	2	3		
2.	4	3	del		45	der(1)t(1;17)(p36;q11–12)	2	3		
3.	3	1.5	del	+	90	der(1)t(1;17)(p3?2;q12)	4			17q
4.	4	< 1	del	+	46	der(1)t(1;17)(p32;q11.2)	2	3		
5.	4	2.5	del	+	46	der(1)t(1;17)(p22;q21)	2	3		17q
6.	4	4	del	–	53	der(1)t(1;17)(p1?;q11–12)	3	4		
7.	4	2.5	del	+	45	der(9)t(9;17)(p1?3;q21)	2	3		
8.	4	2	del	–	47	der(10)t(10;17)(q22;q21)	2	3		
9.	4	3	del	–	46	der(11)t(11;17)(p15;q21) der(11)t(11;17)(p15;q21) der(11)(q24)	2	4		
10.	4	7.5	del	+	47	der(14;17)(q10;q10)	2	3		
11.	3	2	del	+	46	der(14)t(14;17)(q32.3;q21)				
12.	4	4	del		49–50	der(16)t(16;17)(q?11;q?21) add(17)(q23)	2	4		
13.	3	3.5	–	–	47	trp(17)(q21q25)	1	4		
14.	4	2	–	+	45	der(17)i(17)(q10)hsr(17)(q2?)	2	4		
15.	4	3		–	82–87	i(17)(q10)	4	6		
16.	4	7.5	–	–	47	unclear	2	4	2/4	
17.	4	4		–	40–41	unclear		3		
18.	4s	<1	–	–	44–47 87–92	unclear	2 or 4	3 or 6		
19.	4	3	del	+	46	unclear	2	3	2/3	
20.	4	3.5	del	–	1.6				2/5	17q
21.	4	18	del	–	1.64				3/5	17q
22.	3	5	del	+					2/3	17q
23.	4	1	del	–	98–106					17q
24.	4	14	del	+						17q
25.	4	2	del	+						17q
26.	4	7.5	del	+						17q
27.	4	13	–	–						17q
28.	4	2								17q
29.	3	< 1	–	–	55–56	not found	3			
30.	3	< 1	–	–	63	not found	4			17
31.	1	< 1	–	–	71–78	not found	4			normal
32.	3	< 1			55	not found	4			
33.	4	3	del	–	46	not found	2	2		
34.	2	< 1			54–61	not found	4	4		17
35.	4	1	del	–	46	not found	2	2		
36.	4	20	–	–	46	t(16;17)(p13;q21)	1	2		normal
37.	1	< 1	–	–					3/3	17
38.	3	2	–	–					4/4	17
39.	2	3	–	–						17
40.	4s	< 1	–	–						17
41.	4s	< 1	–	–						17
42.	4s	< 1	del	+	46					17
43.	4s	< 1	–	–	60–64					17
44.	2	< 1		–	64					17
45.	3	2		–	69–71					17

Table 2. 17q status by stage of neuroblastoma

17q status	Stage of neuroblastoma 1	2	3	4	4s	Total
17q normal	2	3	5	3	4	17
17q gain	0	0	5	22	1	28
Total	2	3	10	25	5	45

We also performed some further limited analysis concerning the independent influence of 17q status on PFS. Multivariate analysis showed that 17q status has a significant influence on PFS after chromosome 1p status and stage (stage 4 versus other stages combined) were taken into account (hazard ratio 38.5, $P=0.004$). Likewise, 17q status was significant after age group was accounted for (hazard ratio 61.5, $P=0.003$). Univariate analysis of PFS in 28 *MYCN* non-amplified patients indicated that, of the 14 with 17q gain, 11 relapsed or died of disease, while all 14 patients who were both *MYCN* non-amplified and 17q normal are alive and disease free at last follow-up ($P=0.0001$; Figure 3). In addition, we found that for *MYCN* non-amplified patients, there was a higher proportion with stage 4 in the 17q gain group (11/14) compared with 17q normal (2/14) ($\chi^2=9.2$, $P<0.01$).

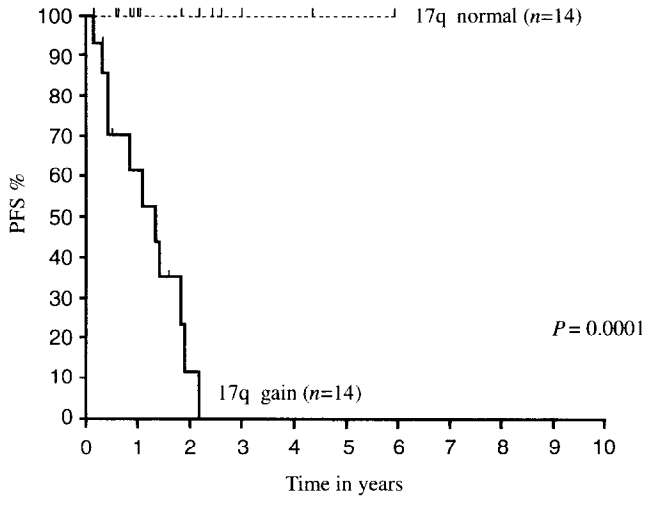

Figure 3. Progression-free survival according to 17q status in patients with single copy of *MYCN*.

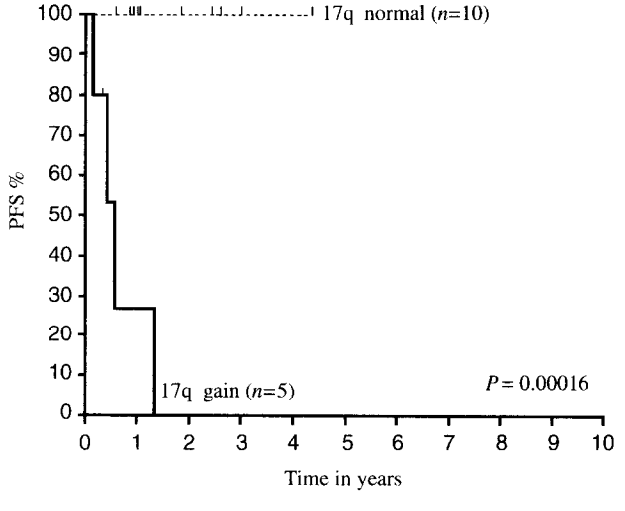

Figure 4. Progression-free survival according to 17q status in patients with no loss of chromosome arm 1p.

It is likely that 17q status also discriminates within the 1p normal group (PFS 0% for 17q gain, $n=5$, versus 100% for normal 17q, $n=10$, $P=0.0016$), although the number of patients is very limited (Figure 4).

DISCUSSION

Initial cytogenetic studies by Gilbert and associates in 1984 suggested that 17q is involved in more than 20% of neuroblastoma tumours [17]. This was not confirmed by subsequent reports, although it is significant that neuroblastoma karyotypes frequently include many marker chromosomes and unidentified products of unbalanced translocations, especially in advanced stages of disease. With the advent of molecular cytogenetic techniques (FISH, CGH), the presence of 17q re-arrangements in neuroblastoma is being detected more and more frequently [7, 8, 18, 19], raising the possibility that this is a feature of considerable biological significance.

To date, only a single study has addressed the prognostic implications of 17q gain, based on survival analysis. Caron [6] found that patients with extra copies of 17q (termed 'add.17q') had 18% 2-year event-free survival compared with 72% without add.17q. In contrast to our results, in that series both 1p and *MYCN* had a more powerful prognostic influence than 17q. This discrepancy is very likely due to different criteria in identification of 17q gain; in Caron's study this was detected by Southern blot analysis using 17q markers only; hence this group may include patients with gains of whole chromosome 17. In our series, based on chromosome analysis, FISH or CGH, all 17q gain cases involved a structural abnormality resulting in imbalance between 17p and q arm.

We found a clear relationship between 17q gain and other poor prognostic genetic and clinical factors. In common with the CGH study of Plantaz and colleagues [7], we found a correlation between 17q gain and deletion of chromosome arm 1p. This association is not surprising since a physical connection between these chromosomes is recurrently seen in the form of unbalanced translocation der(1)t(1p;17q); this results in loss of 1p and gain of 17q. The frequency of such re-arrangements still needs to be determined in primary neuroblastoma tumours, but based on our findings and literature review [9] we estimate that in nearly 40–50% of 17q translocations the partner site is chromosome arm 1p. The relationship between *MYCN* amplification and gain of 17q is more obscure. We are aware of cases where *MYCN* homogenously staining regions (hsr) are flanked by 17q material in both cell lines (IMR32, Kelly) [5] and in a primary tumour (case 14). Such re-arrangements do not generally seem to occur in primary tumours, where *MYCN* amplifies mainly in the form of double-minute chromosomes (dmin). However, there is a documented association between *MYCN* amplification and LOH on chromosome arm 1p [20, 21]. Taking into account the strong correlation between 1p deletion, *MYCN* amplification and 17q gain, it is possible that all three events co-exist in number of cases and are functionally or structurally related. Nevertheless, since in our series the number of 17q gains ($n=28$) exceeded the number of 1p deletions ($n=23$) and the number of *MYCN* amplifications ($n=13$), it appears that the latter two features are subsets of the 17q gain

group. It is unclear whether 17q gain is significantly connected with diploid/tetraploid number of chromosomes ($P=0.04$); since we could analyse only 7 near-triploid tumours, further investigation is necessary.

Our findings seem to be supported by the recently published results of 18 primary neuroblastoma tumours by Meddeb and associates [10]. The presence of 17q gain was more frequently detected than chromosome arm 1p deletion and *MYCN* amplification. Moreover, the authors also suggest that gain of 17q is associated with age over one year at diagnosis, advanced stage of disease, presence of 1p deletion and diploid/tetraploid number of chromosomes. Nevertheless, with the exception of ploidy, the results were not statistically significant, presumably due to the small number of patients.

We found that 17q gain is the most powerful genetic prognostic indicator of poor prognosis in our series of patients. In addition, 17q has discriminatory power in cases with single-copy *MYCN* ($P=0.0001$) and also within the group of cases who show no loss of 1p ($P=0.0016$). The latter is in agreement with Caron's [6] observation that in patients without LOH on 1p, gain of 17q might be of additional prognostic value.

In addition to the clinical importance of 17q status in prognosis, which is apparent from our results, it seems likely that this region includes a gene or genes of great biological importance in tumour progression or in resistance to therapy; in this respect we note the prevalence of 17q gain in stage 4 disease. The identification of genes involved in the breakpoint region at 17q12–21 should be a research priority. Possible candidate genes include *THRA*1, *RARA*, *BRCA*1, *NGFR* or the familial Wilms' tumour gene *FWT*1, whose location in this region has recently been postulated [22].

The possible role of the gene dosage effect distal to 17q12–21 also needs to be elucidated. In particular, localisation of the *nm23-H1* gene at 17q21.3–22 has been reported [23], and overexpression of this gene has been linked to aggressive tumour behaviour in neuroblastoma [24]. We suggest that it is important to clarify the relationship between 17q gain at the cytogenetic level and amplification/overexpression of this gene.

Gain of chromosome arm 17q has been reported in a wide variety of both solid and haematological cancers, this gain predominantly taking the form of isochromosome 17q (reviewed in [25]). Different structural mechanisms are involved in i(17q) formation and in the unbalanced 17q translocations seen in neuroblastoma, but it is particularly interesting that i(17q) has been associated with tumour aggressiveness and poor outcome in several malignancies, for example, childhood acute lymphoblastic leukaemia [26].

In summary, this preliminary study indicates that 17q changes have clear prognostic significance in neuroblastoma and should be a target for molecular cytogenetic detection in tumours at diagnosis. Prospective evaluation of 17q status in a large number of neuroblastoma tumours is necessary both to confirm its prognostic significance and to allow for a full multivariate statistical analysis. Further investigation is also necessary to elucidate the role of this chromosomal region in tumour biology.

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