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Original Paper

Molecular Analysis of the Region of Distal 1p Commonly Deleted in Neuroblastoma

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Cellular, cytogenetic, and molecular evidence indicates that chromosome band 1p36 is often deleted in neuroblastoma cell lines and tumours, suggesting the presence of one or more tumour suppressor genes in this region. We used a multifaceted approach to analyse the commonly deleted region. 28 distal 1p-specific polymorphic loci were used to detect loss of heterozygosity (LOH) in a panel of primary neuroblastoma tumours. Thirty-two of 122 tumours (26%) demonstrated LOH at three or more loci. In addition, a patient with a constitutional deletion of 1p36.2–.3 and two neuroblastoma cell lines with 1p36 abnormalities were characterised by FISH. When combined with the LOH data, a single consensus region of deletion was defined proximally by *PLOD* and distally by *D1S80*, a region spanning approximately five megabases. Several proposed candidate tumour suppressor genes, including *ID3*, *CDC2L1*, *DAN*, *PAX7*, *E2F2*, *TNFR2* and *TCEB3*, map outside of this region; however, the transcription factor *HKR3* cannot be excluded. LOH for 1p is correlated with adverse clinical and biological features and a poor prognosis, but 1p LOH is not an independent predictor of overall survival. To identify additional candidate genes, an integrated physical map of 1p35–36 is being constructed. The current map includes 445 polymerase chain reaction (PCR)-formatted markers and 608 YACs. This map will help identify region-specific transcripts by direct selection and sequencing. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

Deletion of the distal short arm of chromosome 1 is the most common cytogenetic chromosomal abnormality observed in neuroblastomas [1]. Previous cytogenetic and molecular analyses of neuroblastoma tumours have identified a shared region of deletion at 1p36 in 25–30% of primary tumours [2–7]. Two reports of constitutional abnormalities involving 1p36 in infants diagnosed with neuroblastoma have been described [8, 9], as have 1p36-specific abnormalities in several neuroblastoma cell lines [10, 11]. Along with functional studies of tumour suppression by chromosome transfer experiments [12], these studies suggest that one or more tumour suppressor genes map to 1p36, and that disruption of this gene plays a role in the onset or progression of neuroblastoma.

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We undertook a multifaceted approach to characterise further the presence and significance of 1p36 deletions in neuroblastomas. We performed loss of heterozygosity (LOH) and fluorescence *in situ* hybridisation (FISH) studies to narrow the defined consensus region of deletion within 1p36. Eight candidate neuroblastoma suppressor genes have been mapped relative to this region. Also, we determined if correlations exist between the presence of 1p deletion and other clinical and biological parameters. By defining a new consensus region of deletion within 1p36, we mapped the positions of several candidate suppressor genes relative to this region. Finally, we are constructing an integrated physical map of 1p36 to allow for rapid mapping of genes and to serve as a basis for sequencing this region.

MATERIALS AND METHODS

Loss of heterozygosity and FISH studies were performed as described previously [7]. All polymorphisms have been

described and are available through the Genome database (GDB). Correlations between 1p LOH and prognostic variables were calculated as described previously [13].

Primers specific to the *E2F2* (GDB #636071) and *TCEB3* (5′ AAGCCTTATTTCTTTAGAAAAACG 3′ and 5′ CGTTCCACAGGAACCAGCTTCT 3′, provided by R. Conaway) genes were used to identify yeast artificial chromosomes (YACs) for each locus. YACs 777G2 and 965B7 were identified for *E2F2*, and YACs 777G2, 850H7 and 984G1 were identified for *TCEB3*. Further sequence-tagged site (STS) content mapping established that both *E2F2* and *TCEB3* mapped in a contig shared with the *FUCA1* and *ID3* genes. FISH probes for *PLOD* and *TCEB3* were provided by R. Myllyla and R. Conaway, respectively. A P1 clone for *HKR3* was obtained from Genome Systems as described [14].

A framework genetic map of chromsome 1 was constructed using markers from the Généthon version 3 linkage map [15]. The most informative polymorphic marker among those haplotyped was chosen, and all other chromosome 1 genetic markers in the CEPH Genotype database were mapped in relation to our established framework map using Multimap [16]. STSs were identified through the GDB, the Whitehead Institute Center for Genome Research, the Cooperative Human Linkage Center and Généthon [15, 17, 18]. Additional STSs were created from the 3′-untranslated regions of genes mapping to 1p36, from subcloned YAC insert end sequences, and from genomic sequences of genetic marker and FISH probes. Primer sequences were created using Primer2.2 (MIT). YAC libraries were screened as described [19]. YAC DNA was isolated using PureGene Gentra Systems PCR was performed as described previously [7]. Genomic information was stored in a 4th-dimension (ACI/US) database that was custom designed for 1p36. Graphical representation of the integrated map was created by Sigma (Los Alamos, New Mexico, U.S.A.).

RESULTS

Previously, we and others had defined a consensus region of 1p36, which contains approximately 30 megabases of DNA, that is deleted in 25–30% of primary neuroblastomas [2–7]. To narrow this region further, we collected a series of 28 highly polymorphic DNA markers, the majority of which are microsatellite markers that can be assayed by PCR. These polymorphisms included all 1p36 dinucleotide repeat markers present in the Généthon version 2 linkage map [20], which allowed us to correlate deletion breakpoints with the chromosome 1 genetic map. 24 of the 28 markers mapped within 1p36, giving us nearly 1 megabase resolution of deletion breakpoints. These polymorphisms were used to perform LOH studies on 122 tumour and corresponding constitutional DNAs from primary neuroblastoma patients representative of stage, age and *MYC*N amplification status. We also included DNA from a neuroblastoma patient with a constitutional deletion of 1p36 and the corresponding parental DNAs [9].

LOH was detected at three or more loci in 32 of the 122 neuroblastomas (26%). The majority of tumours with LOH had deletions that included all of distal 1p; however, several cases demonstrated LOH for only a portion of 1p36. Two cases appeared to have interstitial deletions, as did the constitutional deletion case. When combined, the tumours defined a single consensus region of LOH within 1p36 (Figure 1). This region was marked distally by the subtelomeric marker *D1S80* and proximally by the dinucleotide repeat *D1S228* within 1p36.2. The distal boundary was defined by the constitutional deletion case, which retained two copies of *D1S80* while losing heterozygosity for several proximal markers. Similarly, the proximal boundary was defined by tumour 705, which retained heterozygosity for *D1S228* while deleting all informative loci distal to this marker. 25 of the 32 tumors with 1p LOH also had amplification of the *MYC*N oncogene. A replication error phenotype was only rarely seen (< 1%) at microsatellite loci.

LOH for 1p has been correlated with *MYC*N amplification and a poor prognosis, but it is unclear whether 1p LOH is an independent predictor of prognosis [21–24]. To obtain greater statistical power, we expanded the number of cases to 156 and performed LOH analysis on the additional tumours. The expanded set of tumours was representative for disease stage, age and *MYC*N amplification. LOH for 1p was compared to several clinical and biological features of neuroblastoma, including age, stage, primary site, serum LDH, *MYC*N status and ploidy. In univariate analysis, 1p LOH was found to be significantly correlated with age at diagnosis greater than one year ($P < 0.001$), *MYC*N amplification ($P < 0.001$), elevated serum LDH ($P < 0.001$) and near diploidy ($P = 0.035$). In addition, 1p LOH also correlated with

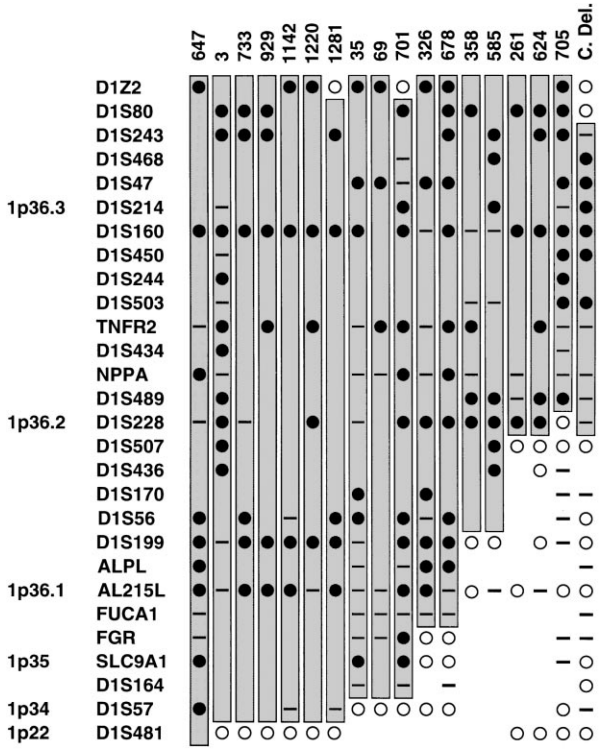

Figure 1. Summary of 1p36 LOH in neuroblastomas and a constitutional deletion patient. Polymorphisms used are listed on the left, in order from distal (top) to proximal, with approximate cytogenetic positions listed. Sixteen tumours with partial 1p LOH and the constitutional patient (C. Del.) are listed at the top of the figure. Tumour 647, with LOH for all informative 1p loci, is also shown; this tumour is representative of an additional 15 tumours with similar results (not shown). Shaded bars show the maximum region of deletion for each tumour. Filled circles indicate LOH; open circles, retention of heterozygosity; dashes, not informative; no symbol, not determined. The order of loci is a best approximation based upon available genetic and physical mapping data [15, 18].

decreased survival ($P < 0.001$) as reported previously. The four-year survival for tumours with 1p LOH was 32%, in contrast to 67% in tumours without this abnormality.

To determine whether 1p LOH had prognostic significance independent of other prognostic variables, multivariate analysis was performed. By a log-rank test, 1p LOH did not have predictive significance of survival when stratified for *MYC*N amplification ($P = 0.16$). To confirm these results, we constructed a forward stepwise Cox model with 1p LOH, *MYC*N amplification, ploidy (diploid versus hyperdiploid), serum LDH (>/< 1500 units/l) and age at diagnosis (>/< 1 year) as independent variables. *MYC*N amplification, near diploidy and age at diagnosis over 1 year were all independent predictors of a poor prognosis ($P < 0.05$). However, 1p LOH was not an independent predictor of prognosis ($P = 0.72$). Interestingly, 8 of 12 patients (67%) with 1p LOH and single-copy *MYC*N were long-term survivors, but only 2 of 8 patients (25%) with *MYC*N amplification and no 1p deletion survived up to 3 years.

Next, we wished to define further the consensus region of deletion within 1p36 by analysing neuroblastomas with reported 1p36 abnormalities. These included an EBV-transformed lymphoblastoid cell line from a neuroblastoma patient with a constitutional deletion of 1p36, and two neuroblastoma cell lines: NGP, reported to contain a balanced translocation t(1;15)(p36;q24), and SK-N-AS, reported to contain a deletion within 1p36 [8–10]. The cell lines were hybridised in series with a panel of 1p36-specific FISH probes. Interestingly, NGP appeared to contain a duplication within 1p36.2 (Figure 2). Markers between and including *NPPA* and *D1S160* demonstrated three signals, present on the normal and derivative chromosomes 1 and also on the derivative chromosome 15. Markers proximal to *NPPA* were present on the normal and derivative chromosomes 1, while markers distal to *D1S160* were present only on the normal chromosome 1 and derivative 15, which contains the translocated 1p36.3 material. As the distal breakpoint defined by NGP remains within the consensus region, it is possible that this breakpoint disrupts a suppressor locus.

As demonstrated by the LOH studies, FISH analysis of the constitutional case indicated a deletion extending to, but not including, *D1Z2* distally and *D1S228* proximally (Figure 2). SK-N-AS demonstrated a smaller, overlapping deletion extending from *CDC2L1* (p58) at 1p36.3 to the *PLOD* (lysyl hydroxylase) gene in distal 1p36.2. Inclusion of these two deletions with the LOH studies allowed the definition of a new, smaller consensus region. This region is marked proximally by *PLOD*, as defined by SK-N-AS, and distally by *D1S80*, which is retained in the constitutional deletion case. Using fractional length estimates by FISH and radiation hybrid mapping information (data not shown), we estimate that the consensus region is five megabases in length.

A number of genes mapping to distal 1p have been suggested as potential neuroblastoma candidate genes [7, 14, 25]. These include the *CDK2* homologue *CDC2L1* (p58); the zinc finger-containing transcription factors *HKR3* and *DAN*; the transcription factors *PAX7*, *ID3* and *E2F2*; the transcription elongation factor *TCEB3* (Elongin A) and tumour necrosis factor receptor 2 (*TNFR2*). To determine whether these genes could be considered actual candidates, we mapped each relative to our consensus region of deletion. In each case, the genes were mapped by FISH to normal human metaphase chromosomes as well as to SK-N-AS, NGP and the constitutional deletion case. As shown in Figure 2, *CDC2L1* mapped distal to the consensus region of deletion, while *PAX7*, *DAN*, *ID3*, *TCEB3* and *E2F2* mapped proximal to the region. We found that *TNFR2* was deleted in all informative tumours with 1p36 LOH, but it was not deleted in SK-N-AS. However, *HKR3* was deleted in all informative tumours, the constitutional case, and SK-N-AS. Thus, *HKR3* remains a viable candidate suppressor gene while *TNFR2* can be provisionally eliminated by the SK-N-AS results. Further characterisation of *HKR3* is presented elsewhere in this volume (pp. 1991–1996).

In a comprehensive effort to identify the suppressor gene, and as an aid in further characterisation of the region, we are constructing an integrated physical map of 1p36. STSs have been constructed or collected for virtually all loci mapping to 1p36, including genetic markers, genes, expressed sequence tags (ESTs), random STSs and FISH probes. A total of 445 STSs have been identified, including 139 specific for genetic markers and 110 for expressed sequences. This collection of STSs was used to screen YAC libraries, which identified 608 YACs that could be individually confirmed for STS content. We have assembled these markers and clones relative to a framework genetic map of 1p36 consisting of 24 PCR-formatted markers arrayed in a linear order with 1000:1 likelihood. Additional ordering of clones and markers was

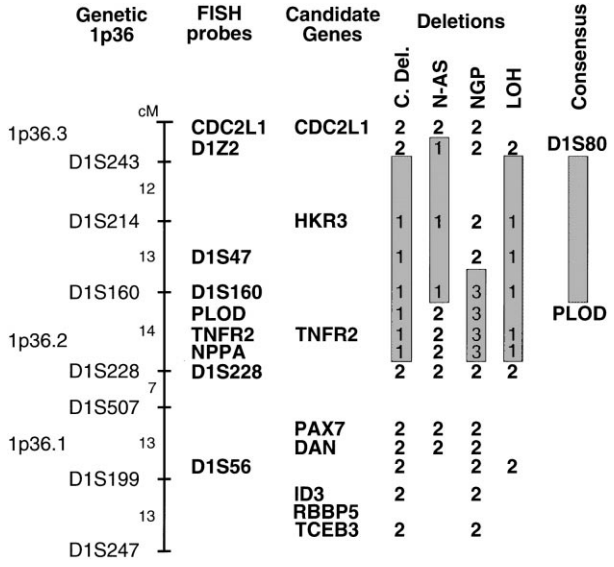

Figure 2. Summary of FISH and candidate gene mapping. On the left is a genetic map of 1p36, with genetic distances in centimorgans displayed to the left of the vertical line. Approximate cytogenetic band locations are shown to the left of the genetic map. The location of probes used for FISH are shown in relation to the genetic map to the right of the vertical line. To the right of the FISH probes are shown the mapping positions of the putative neuroblastoma candidate genes. The precise locations of *DAN* and *PAX7* are not certain but are known to lie proximal to *TNFR2* and distal to *ID3* from radiation hybrid analysis (data not shown). To the right of the FISH markers and candidate genes is shown the extent of rearrangement in the constitutional deletion case, neuroblastoma cell lines SK-N-AS and NGP and the consensus region of LOH. On the far right is the consensus region of deletion defined by the FISH and LOH results. Shaded bars indicate regions of deletion (duplication in the case of NGP). The observed number of copies of each FISH marker and candidate gene is shown below each cell line. Probes suitable for FISH were not available for all candidate genes; these genes were mapped by a variety of techniques (see Materials and Methods).

performed by STS content mapping, somatic hybrid analysis, FISH and radiation hybrid mapping. Together, this combines existing genetic, radiation hybrid and FISH markers into a single integrated map that reflects physical distance. The average insert size of the identified YACs is 840 kilobases, which predicts that the map contains 5.9 genome equivalents for 1p36. Future mapping efforts will be directed at STS ordering by radiation hybrid mapping, creation of a sequence-ready map in PAC clones and directed sequencing of the consensus region of deletion.

DISCUSSION

Our LOH studies on primary tumours and FISH results on neuroblastoma and constitutional cell lines have identified a consensus region of allelic loss within 1p36.2–.3. This region is defined distally by the subtelomeric marker *D1S80* and proximally by the *PLOD* gene. Based upon cytogenetic and radiation hybrid maps, we estimate that the consensus region contains approximately 5 megabases of DNA, which corresponds to 34 centimorgans of genetic distance [26]. This defined region compares favourably with other studies. Martinsson and associates defined two tumours with LOH distal only to *D1S244*; the location of *PLOD* relative to *D1S244* is uncertain but is thought to be in the same vicinity [6]. All of our tumours with LOH had only a single identified region of loss on 1p. A previous study identified three tumours with proximal 1p deletions that did not extend to 1p36 [3]. Although we could not confirm these findings, the majority of our polymorphisms mapped distal to this region. Other studies have postulated the presence of one or more additional 1p36 suppressor genes based upon differences of *MYC*N amplification and imprinting status in tumours with small or large 1p36 deletions [4, 5]. While our tumours with smaller 1p36 deletions often had a normal *MYC*N copy number, the correlation between *MYC*N amplification and small deletions was not significant [13].

In our series, 1p LOH was correlated with a poor prognosis in univariate analysis but was not an independent prognostic indicator in multivariate analysis. This finding is in agreement with several previous studies [21–23]. However, Caron and associates found that 1p LOH was the most significant prognostic indicator in multivariate analysis in their series [24]. There are several possibilities for these disparate results, including differences in patient populations, different treatment regimens, variant criteria for LOH analysis and variations in multivariate analysis models. The majority of tumours with 1p LOH are also amplified for *MYC*N, and tumours exhibiting only one of these abnormalities are infrequent, making assessment of prognostic independence for *MYC*N amplification and 1p LOH difficult. A multicentre study that includes biological and clinical characteristics of several hundred tumours is probably required to fully address this issue.

Our analysis of cell lines by FISH further narrowed the region of LOH defined by the primary tumours. Analysis of the constitutional deletion case and SK-N-AS supported earlier findings of interstitial deletions within 1p36 in these cell lines [9–11]. In addition to the constitutional deletion case, another constitutional abnormality involving an apparent balanced 1;17 translocation in a patient with neuroblastoma has been reported [8]. However, our consensus region of LOH, as well as the two cell line deletions, are distal to the breakpoint defined by this case [27]. It remains possible that an additional gene involved in tumorigenesis is disrupted in the translocation case. Interestingly, the NGP cell line contained a duplication of several megabases. While the proximal 1p36 breakpoint defined by NGP is proximal to our consensus region, the distal breakpoint lies within this region. Further analysis of NGP is warranted to determine whether a gene is disrupted by either duplication breakpoint.

We have mapped eight potential candidate suppressor genes relative to our defined region of deletion. Six of these genes (*CDC2L1*, *PAX7*, *DAN*, *E2F2*, *ID3* and *TCEB3*) mapped outside of the region. *TNFR2* was deleted in all tumours but was retained in SK-N-AS, while *HKR3* was deleted in all tumours and cell lines. *HKR3* maps distal to the distal NGP breakpoint, although it remains possible that *HKR3* expression is altered in this cell line. Our continuing efforts to physically map 1p36 will undoubtedly identify additional genes and ESTs within the deleted region. Characterisation of these genes, as has been performed for *HKR3* and *TNFR2* [14, 28], will determine whether they remain viable candidates. As subtle disruptions of 1p36.2–.3 appear to be rare in neuroblastomas, identification of the suppressor gene in this region will likely require the analysis of many additional tumours, as well as large-scale physical mapping, genomic sequencing and high throughput mutational analysis of identified transcripts.

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