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Original Paper

Sensitive and Reliable Detection of Genomic Imbalances in Human Neuroblastomas using Comparative Genomic Hybridisation Analysis

M. Van Gele,[1] N. Van Roy,[1] A. Jauch,[8] G. Laureys,[2] Y. Benoit,[2] V. Schelfhout[3] C.R. De Potter,[3] P. Brock,[4] A. Uyttebroeck,[4] R. Sciot,[5] E. Schuuring,[6] R. Versteeg[7] and F. Speleman[1]

[1]Department of Medical Genetics; [2]Department of Paediatric Haemato-Oncology; [3]N. Goormaghtigh Institute for Pathology, University Hospital, De Pinelaan 185, B-900 Ghent; [4]Paediatric Oncology, University Hospital; [5]Department of Pathology, University Hospital, Leuven, Belgium; [6]Department of Pathology, University of Leiden, Leiden; [7]Institute of Human Genetics, Academic Medical Center University of Amsterdam, The Netherlands; and [8]Institute for Human Genetics and Anthropology, Ruprecht-Karls-University of Heidelberg, Heidelberg, Germany

Deletions of the short arm of chromosome 1, extra copies of chromosome 17q and *MYCN* amplification are the most frequently encountered genetic changes in neuroblastomas. Standard techniques for detection of one or more of these genetic changes are karyotyping, FISH analysis and LOH analysis by Southern blot or PCR. Each of these techniques has its own particular limitations. More recently, comparative genomic hybridisation (CGH) was introduced for detection of genomic imbalances including deletions, duplications and gene amplification. We evaluated the sensitivity and reliability of CGH for detection of the most frequently encountered genetic changes in neuroblastoma. For this purpose a panel of well-characterised neuroblastoma cell lines as well as a series of 11 primary neuroblastomas was analysed. Our results show that CGH is a valuable tool for the genetic characterisation of neuroblastomas, both for the detection of frequently occurring genomic imbalances and for the identification of previously unnoticed genetic changes. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

A NUMBER of frequently occurring genetic changes have been described in neuroblastoma. These include partial losses of chromosome arms 1p, 11q and 14q [1–3], gains of material from the long arm of chromosome 17 [4–6] and *MYCN* amplification [7]. Classical approaches for the detection of these genetic changes are karyotyping, FISH (fluorescence *in situ* hybridisation) and LOH (loss of heterozygosity) analysis by Southern blot or PCR (polymerase chain reaction). Karyotyping allows the analysis of the entire genome, but is time-consuming and requires short-term *in vitro* cell culture. FISH and LOH analysis are fast, but these techniques are restricted to the analysis of a limited number of small DNA targets. Recently, a new method for the detection of gains and losses of chromosomal regions, termed comparative genomic hybridisation (CGH), has been described [8, 9]. One of the major advantages of CGH is that it allows, like karyotyping, the entire genome to be studied in one single experiment. CGH can be performed on uncultured primary fresh or frozen tumour biopsies or even on paraffin-embedded tumour material [10]. In order to assess the sensitivity and reliability of CGH for the genetic analysis of neuroblastoma, we analysed a panel of neuroblastoma cell lines with well-characterised 1p deletions and 17q duplications. In addition, CGH was performed on a series of 11 primary neuroblastomas.

Correspondence to F. Speleman.

MATERIALS AND METHODS

Neuroblastoma cell lines and primary neuroblastomas

The following neuroblastoma cell lines were analysed: IMR32 [11], SK-N-AS [12], GI-ME-N [13], NGP [14], TR14 [15], SMS-KCNR [16] and UHG-NP [6]. Detailed

karyotypic information and references for karyotypes of these cell lines have been previously described by Van Roy and associates [5]. All cell lines carry 1p deletions except NGP. This cell line has no 1p deletion, but carries a reciprocal translocation t(1;15)(p36.2;q24) [6, 17, 18]. Cell line UHG-NP was established in our laboratory from a primary tumour of a 3-year-old boy. Clinical details of this patient have been reported [5]. Eleven primary neuroblastoma tumours (two stage 1, one stage 2, one stage 3, six stage 4 and one stage 4s) were analysed. Detailed biological and clinical data of these tumours will be reported elsewhere.

CGH analysis

Metaphase spreads were prepared from phytohemagglutinin-stimulated lymphocytes from healthy individuals according to standard procedures. Slides were stored in plastic boxes with silica gel at −20°C or in ethanol (70%) at 4°C before use. DNA was extracted from neuroblastoma cell lines and peripheral blood from a healthy male individual as previously described [19]. Primary tumour DNA was extracted from 50 µm cryosections from biopsies frozen at −80°C. Evaluation of the tumour cell percentage was done on 5 µm sections. Tumour samples containing less than 60% of tumour cells were excluded from further analysis. Labelling of DNAs, *in situ* hybridisation, fluorescence microscopy, digital image acquisition and processing were essentially done according to du Manoir and associates [9, 20]. For the analysis of primary tumour DNAs, images were recorded using a Leitz DM microscope, a black and white CCD camera (Sony IMAC-CCD S30) and dedicated software (ISIS, MetaSystems, Germany). Further processing of these images was done using the ISIS-CGH software (MetaSystems, Germany).

Southern blot analysis

Southern blot analysis was performed as described previously [21]. To determine DNA amplification of the chromosome 11q13 region, two 11q13 probes, *EMS1/cortactin* (probe U21C8) and *cyclin D1* (probe U21B31A) were used [22]. As a control for DNA loading and polyploidy, two probes, *SEA* on chromosome 11q14 and thyroglobulin on chromosome 8, were used. *EMS1/cyclin D1* were considered amplified when the intensity of the signals was increased at least 2-fold (in three different digests) relative to both the thyroglobulin and *SEA* signal. Digested DNA from placenta and cell line UMSCC2 were used as control for normal and eight extra copies of the 11q13 region, respectively.

RESULTS

Figure 1 summarises all chromosomal imbalances detected by CGH. Vertical lines on the left and right of the chromosome ideograms show under- and over-represented regions, respectively. Amplifications are indicated by bold lines.

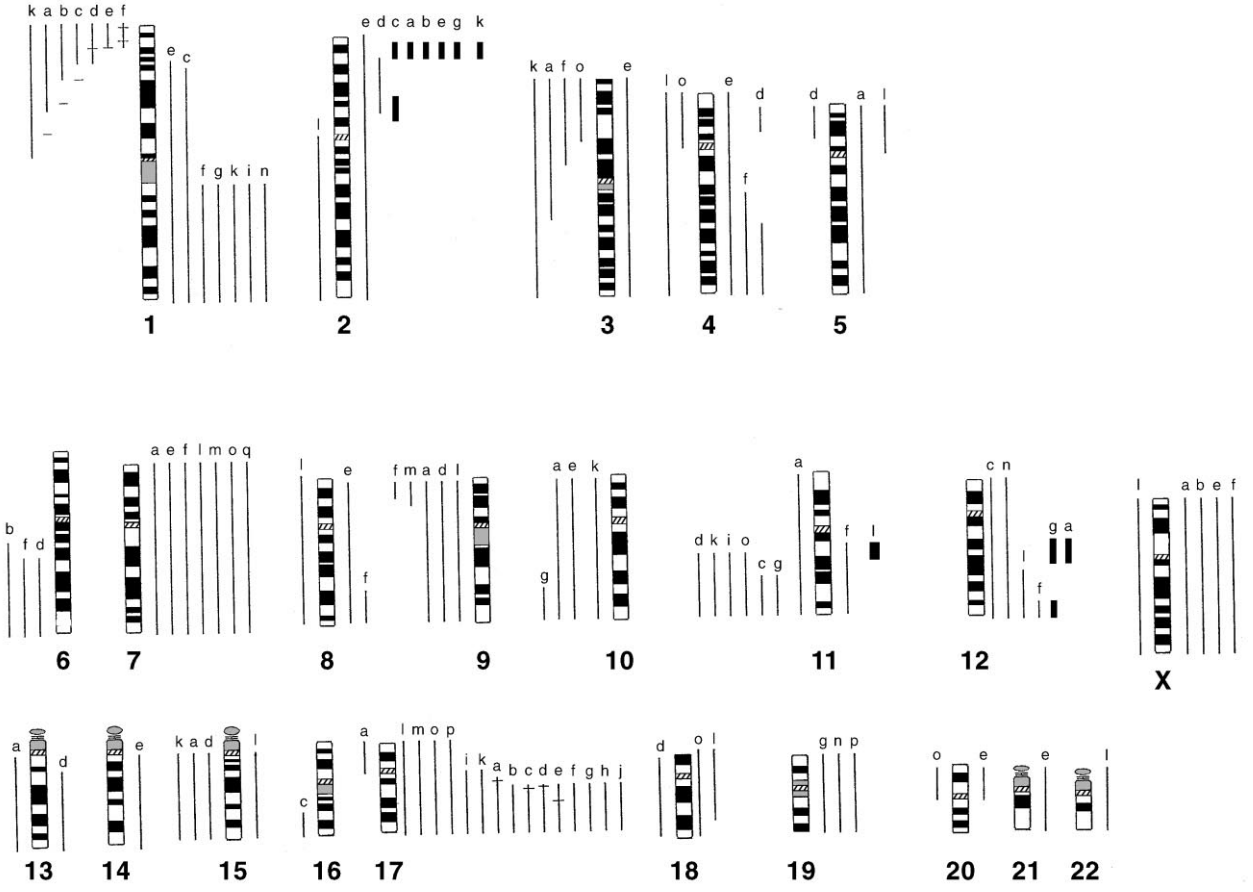

Figure 1. Summary of all chromosomal imbalances detected by CGH in neuroblastoma cell lines TR14 (a), SMS-KCNR (b), IMR32 (c), GI-ME-N (d), UHG-NP (e), SK-N-AS (f) and NGP (g) and primary tumours VDK (h), AH (i), VHM (j), RM (k), TD (l), SJ (m), BFA (n), AY (o), FA (p), YH (q). The vertical lines on the left side of the chromosome ideograms indicate under-representations, those on the right side over-representations of the corresponding chromosomal regions. Amplifications are indicated by solid bars. The position of 1p breakpoints and 17q breakpoints as determined by FISH with region-specific probes in neuroblastoma cell line [5, 6] (Van Roy and associates, pp. 1974–1978) are indicated by horizontal lines.

Chromosome 1p deletions

As shown in Figure 1, CGH allowed detection of all 1p deletions, including the smallest 1p deletion in SK-N-AS (labelled f). Localisation of breakpoint of 1p deletions in these cell lines was previously determined by LOH and FISH analysis [5, 23]. The localisation of these breakpoints as determined by CGH did not correspond exactly with the molecular analyses but nevertheless were fairly accurate (Figure 1).

Gene amplification

All previously reported gene amplifications or amplified regions (*MYC*N, 2p24.3; 2p23; *MDM2* and *SAS*, 12q13–15; *RSN*, 12q24) were accurately detected. In one stage 4 tumour, amplification at 11q13 was observed in the absence of *MYC*N amplification. Southern blot analysis using probes for the *EMS1/cortactin* and *cyclin D1* genes located at 11q13 showed significant amplification for both genes (approximately 10-fold), thus confirming the CGH observation. Amplification of both these genes has been reported frequently (13–30% of the cases) in carcinomas of the breast, head and neck, bladder and lung [24]. This is the first finding of involvement of these 11q13 genes in neuroblastoma.

17*q over-representation*

Extra copies of 17q were found in all cell lines which is in agreement with previous FISH analyses. The breakpoint position as determined by the CGH ratio profiles appears to be more distally located as compared to the position determined by molecular cytogenetic analysis (Figure 1). Gain of chromosome 17q material or extra copies of chromosome 17 were found in all cell lines and in 8 out of 10 primary tumours with chromosomal imbalances.

Abnormalities involving chromosome 17 are therefore the most frequently observed genetic aberration in this series. Interestingly, chromosome 17 imbalances were not restricted to stage 3 and 4 tumours, but were also found in one stage 1 and one stage 2 tumour. This observation provides further support for a role of one or more genes located on 17q in the development of both localised and disseminated neuroblastomas.

Other genomic regions

Detailed molecular cytogenetic analysis of previously unnoticed or partially characterised imbalances in neuroblastoma cell lines will be described elsewhere. Chromosomal imbalances (also including 1p deletions, 17q over-representation and gene amplification) were found in all neuroblastoma cell lines and in 10 out of 11 primary neuroblastomas. The average number of imbalances in the cell lines was 11, whereas an average of 3.9 imbalances was found in the primary tumours. However, in primary stage 4 tumours this average was higher (5.5). The higher number of imbalances in cell lines may be explained by the fact that they represent a selected population of highly aggressive tumour cells which grow particularly well *in vitro*.

DISCUSSION

We conclude that CGH is a reliable and sensitive method for the genetic analysis of neuroblastoma. The method can be used, preferably in combination with a second independent method, in order to determine the most important genetic parameters (*MYC*N amplification and 1p deletion). In addition, this approach also offers the possibility of identifying other genomic regions which may play a role in certain neuroblastoma subsets. We have previously shown that genes other than *MYC*N can be amplified in neuroblastoma [25]. CGH analysis of a first series of primary neuroblastomas revealed that 11q13 amplifications which occur frequently in some carcinomas may also occur in neuroblastoma. Moreover, this analysis also confirmed the frequent occurrence of chromosome 17 or 17q over-representation in all stages of disease. Analysis of a large series of neuroblastomas belonging to different risk groups and correlation with other biological as well as clinical parameters is warranted in order to obtain a better insight into the genetic mechanisms which lead to the currently observed variable biological behaviour of this tumour.

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