Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 520–523, 1995

0959-8049/95 $9.50+0.00

0959-8049(95)00031-3

Cytogenetic Evolution of *MYCN* and *MDM2* Amplification in the Neuroblastoma LS Tumour and its Cell Line

R. Corvi, L. Savelyeva, L. Amler, R. Handgretinger and M. Schwab

Amplification of the *MYCN* gene is frequently seen either in extrachromosomal double minutes (DMs) or in homogeneously staining regions (HSRs) of aggressively growing neuroblastomas. Total genomic DNA from cell line LS, from early passages of the same line and from original tumour material was biotinylated and hybridised to metaphase chromosomes of normal human lymphocytes. The reverse genomic hybridisation revealed the amplified DNA to be derived both from chromosome 2p23-24, which is the position of *MYCN*, and from chromosome 12 band q13-14. The *MDM2* gene, located at 12q13-14, was found amplified both in early and late passages of LS, in addition to amplified *MYCN*. Amplification units of *MYCN* and *MDM2* appear first to develop within DMs, which then integrate into different chromosomes to develop to HSRs.

Key words: oncogenes, reverse genomic hybridisation, tumorigenesis, neuroblastoma, *MDM2*, *MYCN* amplification

Eur J Cancer, Vol. 31A, No. 4, pp. 520–523, 1995

INTRODUCTION

AMPLIFICATION OF cellular oncogenes is one of the major genetic alterations that appears to contribute to tumorigenesis [1, 2]. While most tumours show sporadic amplification of cellular oncogenes, human neuroblastoma tumours show frequent amplification of the *MYCN* gene, which correlates with aggressive tumour growth [3–7].

MYCN maps to chromosome 2 band p23-24 [8]. Amplified copies of *MYCN* localise to two types of chromosomal abnormalities, double minutes (DMs), which represent extrachromosomal elements [4] and homogeneously staining regions integrated within derivative chromosomes (HSRs) [8]. HSRs have never been observed at the normal position of *MYCN* at 2p23-24 [9], but the single copy *MYCN* is retained at its original site during amplification [10], suggesting transposition of *MYCN* during amplification.

While amplification of *MYCN* is well documented, amplification of other oncogenes in neuroblastoma cells has never been reported to date. We have recently observed that an HSR on chromosome 12 in neuroblastoma cell line LS contains amplified chromosome 12 DNA, in addition to amplified *MYCN*, suggesting that chromosome 12 DNA was co-amplified with *MYCN* [10]. In this study, we show by fluorescence *in situ* hybridisation (FISH) that the amplified chromosome 12 DNA is not the result of long-term *in vitro* cultivation of the cells, but was already present in early passages of the cell line and in the original tumour material. Moreover, we show that the amplified DNA encompasses the *MDM2* gene, which maps to 12q13-14. For a better understanding of the mechanism of amplification, we followed the cytogenetic evolution of the two amplified genes through the different passages of the cell line.

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MATERIALS AND METHODS

Cell lines and preparation of metaphases

Neuroblastoma line LS has been described elsewhere [11]. For cytogenetic analyses, the cells were arrested in metaphase with colcemid. Fixing of cells and Giemsa staining of chromosomes were performed according to routine procedures.

DNA extraction from paraffin-embedded tissue

For DNA extraction from formalin-fixed, paraffin-embedded tumour, the tissue was dewaxed by incubation in xylene (90, 15 and 15 min) and rehydrated in a descending ethanol series. After digestion with Proteinase-K (Boehringer Mannheim; starting concentration 200 μg per ml) for 5 days at 37°C (with daily addition of 100 μg), DNA was extracted with phenol, precipitated in 70% ethanol in the presence of 0.3 M sodium acetate and dissolved in distilled water.

DNA probes and labelling

Probe for *MDM2* (c14-2) was obtained from B. Vogelstein [12] and chromosome 12 library was a gift of Joe Gray (University of California, San Francisco). As the *MYCN* probe, we used cosmid pNb-101 [10]. For *in situ* hybridisation, the *MDM2* plasmid, total DNA from LS, and total chromosome 12 DNA were labelled with biotin-16-dUTP and the *MYCN* cosmid with digoxigenin-11-dUTP (Boehringer Mannheim) as previously described [13].

Fluorescence in situ *hybridisation (FISH)*

FISH was performed and evaluated as previously described [10]. Probe concentration was 6 ng per μl for *MYCN* and *MDM2* and 50 μg/μl for the chromosome 12 library. Repetitive sequences in pNb-101 or in the chromosome 12 library were suppressed with a 10-fold excess of *Cot-1* DNA (Bethesda Research Laboratories, Life Technologies). For reverse genomic hybridisation [14], 1 μg of biotin-labelled genomic DNA was cohybridised with 50 μg of *Cot-1* DNA in a final volume of 30 μl.

RESULTS

We recently identified an HSR on chromosome 12 in the neuroblastoma cell line LS that harbours both amplified *MYCN* and chromosome 12 DNA [10]. The line LS had been in culture for many years, but early passage cells (passage 4) and original tumour material, paraffin-fixed, were available. To test if chromosome 12 was amplified, DNA was extracted from both the early passage cells and the tumour sections, labelled and hybridised to normal lymphocyte chromosomes. In both cases two signals were seen, one on 2p23-24, which is the position of *MYCN*, and the other on 12q13-14 (Figure 1a, b),showing that the passage 4 cells and the initial tumour cells contain amplified DNA from both chromosome bands.

Chromosome band 12q13-14 is the position of the gene *MDM2*, which was originally discovered due to its amplification in a tumorigenic derivative of mouse BALB/c cells [15], and subsequently found amplified in human sarcomas [12]. To determine whether *MDM2* is contained in the chromosome 12 amplicons in early and late passages of the cell line, we used FISH analysis. *MDM2* clone c14-2 [12] was labelled with biotin, *MYCN* clone pNb-101 [10] was labelled with digoxigenin, and both were cohybridised to the metaphase chromosomes of LS. Fluorescence microscopy revealed amplification of both *MYCN* and *MDM2* (Figures 1c, d). In late passage cells, both *MYCN* and *MDM2* were amplified in the same chromosome 12 HSR (Figure 1c). In passage 4 cells, we observed two populations of cells: one with two HSRs, like the long term cell line, harbouring both amplified *MDM2* and *MYCN* (data not shown); the other with DMs carrying either *MDM2* or *MYCN* (Figure 1d).

For a better understanding of the amplification mechanism in cell line LS, a detailed cytogenetic analysis was carried out. A comparison between a normal G-banded chromosome 12, a derivative G-banded chromosome 12 and a derivative chromosome 12 that had been hybridised with a chromosome 12 total library and *MYCN* revealed that the HSR in LS is inserted at band 12q11, near the centromeric region (Figure 2). The different position of *MDM2* (12q13-14) indicates that the original gene is probably not encompassed by the HSR and that *in situ* amplification of the *MDM2* at its original position does not account for the molecular mechanism of *MYCN* and *MDM2* amplification in the LS HSR.

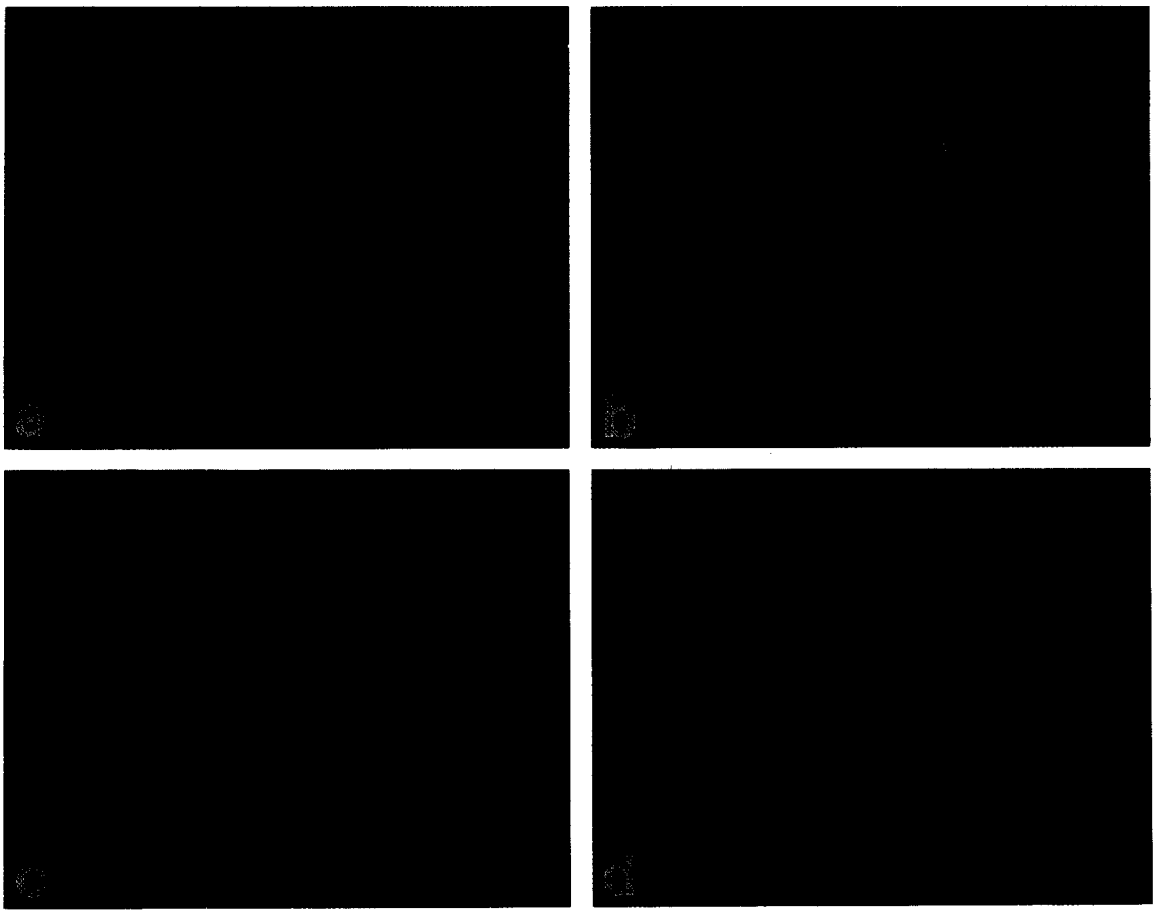

Figure 1. Results of fluorescence *in situ* hybridisation. DNA from passage 4 cells (a) and from original tumoral material (b) were labelled and hybridised to normal lymphocytes chromosomes. Signals were seen on the two copies of chromosome 2 at the position of *MYCN* and on both copies of chromosome 12 at q13-14 (indicated by arrows). Both *MYCN* (red) and *MDM2* (green) were amplified in the LS late passage cells (c). (d) LS passage 4 cells.

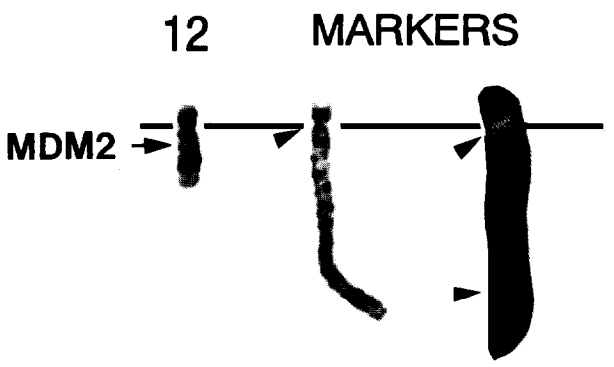

Figure 2. Comparison between a G-banded normal chromosome 12 and LS marker chromosomes with HSRs; the right marker chromosome was hybridised with chromosome 12 library (green) and *MYCN* (red). The HSR insertion position does not correspond to the *MDM2* position. The arrow indicates the *MDM2* gene position, and the arrowheads the two breakpoints in which the HSR is inserted.

DISCUSSION

Although amplification of *MYCN* is a common and well studied genetic alteration in neuroblastoma, little is known about amplification of other oncogenes. The presence of *MDM2* amplification in the early passage LS cells and probably in the original LS tumour, demonstrates that *MDM2* amplification had not developed during *in vitro* cell culture. This is the first report of *MDM2* amplification in a human neuroblastoma, both in a cell line and in the original tumour. Amplification of *MDM2* has been found in different types of sarcomas [12, 16–19] and gliomas [20], but in none of these has amplification of an additional non-syntenic gene been reported. Amplification of *MDM2* was accompanied by amplified *MYCN*. Amplification of two non-syntenic oncogenes has been previously reported occasionally for certain tumour cell lines, for instance *MYC* and RAS^K [21].

In LS, amplification of *MYCN* and *MDM2* seems to result from two independent molecular events. In later passages of LS, both genes are amplified in the same HSR on chromosome 12, although in passage 4 cells the amplified *MYCN* and *MDM2* were present on different DMs. In the original tumour, the two genes are probably amplified within DMs. In fact, the signal intensity in the reverse genomic hybridisation of tumour DNA was higher than that due to hybridisation of late passage cells indicating that the gene's copy number in the tumour cells was higher than in the cells with HSRs. It is possible that DMs integrated in chromosomes to form HSRs during the development of the cell line LS. This shift is in line with the fact that primary tumours usually carry DMs, while most *in vitro* cell lines have HSRs. One possibility leading to the amplification of *MYCN* and *MDM2* within the same HSR in later passage LS cells is that DNA of different DMs had recombined in the same cell to yield a larger structure containing amplified *MYCN* and *MDM2*, which then could have integrated into chromosome 12. The general possibility that DMs having more than a single copy of a particular oncogene can develop is also indicated by the large DMs in LS passage 4 cells which, judging by the fluorescence signal, carry more than one copy of *MYCN*. Further, recombination to yield DMs, each with two non-syntenic oncogenes, *MYC* (8q24) and *SAMK* (10q26), has been reported for the gastric cancer cell line SNU-16 [22]. Since we observed that the HSR in LS is not integrated at band 12q13-14, it is unlikely that the original *MDM2* gene had been amplified. We can, therefore, exclude the possibility that *MYCN* had been integrated in the vicinity of *MDM2* and that during further amplification *MDM2* had been co-amplified.

Malignant tumours characteristically arise from a multiplicity of events within the emerging cancer cell. Well characterised genetic alterations involved in neuroblastoma tumorigenesis are amplification of *MYCN* [3], deletion of the short arm of chromosome 1 [9, 23, 24] and rearrangements often involving chromosome 17 [25, 26]. In this study, we have detected one more genetic alteration: amplification of *MDM2* exclusively in combination with amplification of *MYCN*, as the result of independent molecular mechanisms.

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Acknowledgements—The technical assistance of B. Schneider and K. Reumann and the secretarial skills of I. Cederlund are greatly appreciated. This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Dr Mildred Scheel Stiftung, the Heidelberg-Mannheim Comprehensive Cancer Center, and the Israel-DKFZ Cooperation Program and is integrated into the frame of the European Communities Concerted Action "Molecular Cytogenetics of Solid Tumors" (BMHI-CT92-0156). We thank Bert Vogelstein for the *MDM2* probe. The advice of Dr Ivo Leuschner (University of Kiel) for extracting DNA from fixed tumour tissue has been of great help.

Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 523–526, 1995
Elsevier Science Ltd
Printed in Great Britain
0959–8049/95 $9.50+0.00

0959-8049(95)00012-7

Characterisation of the Chromosome Breakpoints in a Patient with a Constitutional Translocation t(1;17)(p36.31-p36.13;q11.2-q12) and Neuroblastoma

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Cytogenetic and molecular studies in neuroblastoma suggest the presence of a tumour suppressor gene at the distal chromosome band 1p36. Previously, we hypothesised that a constitutional translocation involving the region 1p36 [t(1;17)(p36;q12-q21)] in a patient with neuroblastoma predisposed him to tumour development. Here we report the molecular delineation of the translocation breakpoints. Somatic cell hybrids containing the derivative chromosomes were used to determine the position of chromosome 1p and 17q DNA probes respective to the breakpoints using fluorescence *in situ* hybridisation. The 1p breakpoint was localised between the PND and *D1S56* loci. The chromosome 17q breakpoint is flanked by *NF1* and *SCYA7*, as proximal and distal marker, respectively. We redefined the translocation as t(1;17)(p36.31-13;q11.2-q12). The identification of flanking markers of the breakpoints is a prerequisite for breakpoint cloning and identification of a putative neuroblastoma suppressor gene.

Key words: neuroblastoma, constitutional chromosome 1;17 translocation, suppressor gene
Eur J Cancer, Vol. 31A, No. 4, pp. 523–526, 1995

INTRODUCTION

CONSTITUTIONAL CHROMOSOME abnormalities in patients with a specific tumour were the first indications for the chromosomal location of tumour suppressor genes. The association of a constitutional deletion syndrome at the chromosomal band 13q14 and the retinoblastoma ultimately led to the cloning of the retinoblastoma gene *RB1* [1]. A constitutional deletion at chromosomal band 11p13 was found in the WAGR (Wilms' tumour, aniridia, genito-urinary malformations, retardation) syndrome and from this region the *WT1* gene was cloned [2]. Knudson suggested a similar two-hit mechanism as in retinoblastoma and Wilms' tumour for the origin of neuroblastoma [3], but a constitutional predisposing deletion syndrome was not described. Loss of heterozygosity studies in neuroblastoma tumours pointed to the chromosome 1p36 band as the candidate region for harbouring a neuroblastoma tumour suppressor gene [4, 5]. We have described a reciprocal constitutional chromosome translocation involving the same band 1p36 in a patient with neuroblastoma [6]. We hypothesised that this constitutional chromosome abnormality predisposed the patient to neuroblastoma development by disruption, dysregulation or deletion of one or more genes located at the translocation