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1p36: Every Subband a Suppressor?

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INTRODUCTION

MORE THAN 18 years ago, the first deletions of the p arm of chromosome 1 were detected in neuroblastomas [1]. Since then, cytogenetic analyses of many tumours and cell lines have revealed that these deletions can vary in size, some being very large, but all typically extending to the telomere. Loss of heterozygosity (LOH) studies later enabled the detection of smaller deletions, and defined the shortest region of overlap (SRO) to band 1p36 [2, 3]. It is, therefore, generally assumed that this region carries a neuroblastoma suppressor gene. However, recent analyses have challenged the simple concept of one single 1p36 neuroblastoma suppressor gene. Here, we review our recent data that suggest that 1p35-36 encodes four genes which may play a role in neuroblastoma tumorigenesis.

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1p DELETIONS DIFFER IN *MYCN* AMPLIFIED VERSUS SINGLE COPY CASES

LOH of the short arm of chromosome 1 is associated with amplification of the *MYCN* oncogene [2]. We found this association to be very strict, with analysis of 89 neuroblastomas showing that all 18 *MYCN* amplified cases had LOH of 1p [4, 5]. However, nearly 15% of the *MYCN* single copy neuroblastomas had also lost the distal 1p region. We now have convincing evidence that two different 1p suppressor genes are inactivated in *MYCN* amplified and *MYCN* single copy neuroblastomas.

The initial evidence supporting this hypothesis came from our studies on genomic imprinting in neuroblastoma. Genomic imprinting is the phenomenon whereby the two alleles of a gene are differentially expressed, depending on their parental origin. For instance, the *H19* gene on chromosome 11, that is implicated in tumour suppression in rhabdomyosarcoma [6], is expressed from the maternally inherited allele, but not from the paternal allele [7]. Preferential loss of one parental allele of a tumour suppressor region is considered an indication that the suppressor gene in question is imprinted. The allele that is preferentially lost is also the allele that would have been expressed, while the retained allele is the silent one.

We studied the parental origin of 1p36 deletions in neuroblas-

tomas. In an initial series, we found a strong preference for maternal loss, suggesting that the 1p36 suppressor is imprinted [4]. Concurrently, Cheng and associates reported a random parental origin of 1p deletions in neuroblastomas [8]. This series consisted of *MYCN* amplified neuroblastomas, while our series included *MYCN* amplified as well as single copy cases. We, therefore, analysed a much larger series, and discriminated between *MYCN* amplified and single copy neuroblastomas. In agreement with the results of Cheng's group, the 1p losses in the 18 *MYCN* amplified tumours were found to be of random parental origin. In contrast, in the *MYCN* single copy neuroblastomas, we found 16/17 deletions of 1p36 to be of maternal origin [5]. These results suggest that there is an imprinted suppressor lost in *MYCN* single copy cases and a non-imprinted suppressor that is lost in all *MYCN* amplified cases.

The concept of two different suppressors was further supported by the fine mapping of 1p deletions in cell lines and tumours [5, 9]. *MYCN* single copy cases have relatively small deletions, with an SRO in 1p36.2-3 (Figure 1). The smallest deletion was found in the cell line SK-N-AS, which has an interstitial deletion between the markers PND (1p36.23-31) and CEB15 (1p36.33) [9]. In contrast, *MYCN*-amplified tumours always had larger deletions, with an SRO between *D1S7* (1p35-36.1) and the telomere (Figure 1). *D1S7* was deleted in 17/18 neuroblastomas with *MYCN* amplification, and it was the most distal marker retained in the remaining *MYCN* amplified tumour. This suggests that a suppressor associated with *MYCN* amplification maps just distal of *D1S7*. However, as all deletions extend to the telomere, the formal SRO for a suppressor gene deleted in *MYCN*-amplified neuroblastomas is 1p35-pter.

A CONSTITUTIONAL BALANCED TRANSLOCATION MAPS TO 1P36.2

Balanced translocations are a classical tool for pinpointing disease genes. One neuroblastoma patient has been described with a constitutional balanced translocation in the 1p36 region [10]. The translocation was cytogenetically characterised as a t(1;17)(p36;q12-21). It is conceivable that the translocation inactivated one allele of a 1p36 suppressor gene, which predisposed the patient to the development of neuroblastoma. Somatic cell hybrids of the patient's fibroblasts and hamster A3 cells enabled the study of the translocation chromosomes [11]. We mapped the translocation breakpoint within a cluster of genes for small nuclear RNA-U1 and some tRNAs ([11, 12] and van der Drift *et al.*, Academic Medical Centre, Amsterdam). These RNAs are encoded by 15–30 gene copies each, which form an exceptional large cluster of 2–3 megabases in chromosomal band 1p36.2 [12]. The breakpoint is proximal to the marker PND, and, therefore, outside the SRO for the distal suppressor gene deleted in *MYCN* single copy neuroblastomas (see Figure 1). The translocation breakpoint could define the putative suppressor gene deleted in *MYCN*-amplified neuroblastomas, which we assume to map just distal to *D1S7* (see above). However, the cluster of *RNU*-1 genes maps about 28 cM distal to *D1S7* [12]. This large distance makes it unlikely that the balanced translocation has inactivated the suppressor associated with *MYCN* amplification. This suggests that band 1p36.2 could encode a third suppressor gene involved in neuroblastoma tumorigenesis.

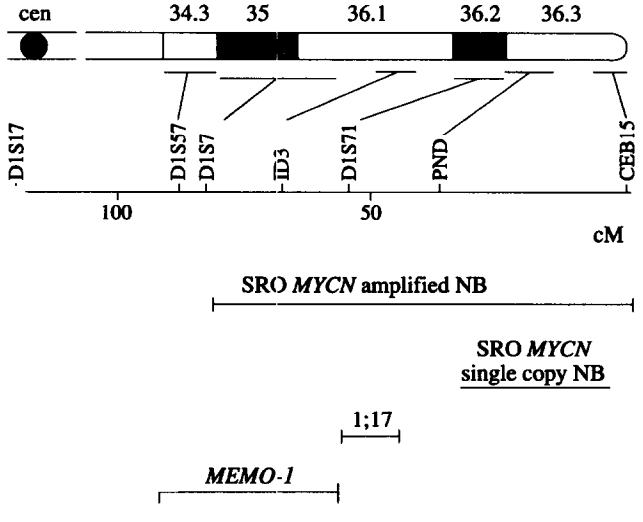

Figure 1. Map positions of the different loci on chromosomal bands 1p35-36 that play a role in neuroblastoma. The position of markers is given on a genetic map as well as on metaphase chromosomes as determined by FISH. NB, neuroblastoma; SRO, shortest region of overlap; *MEMO-1*, methylation modifier-1.

METHYLATION MODIFIER-1 (*MEMO-1*)

One of the characteristics of neuroblastomas is the low or absent expression of class I HLA antigens [13]. Class I HLA antigens are expressed on the surface of nearly all nucleated cells. They play an essential role in presentation of viral antigens and, probably, tumour-specific antigens to cytotoxic T cells. It has been shown that the expression of the rat homologue of class I HLA can be suppressed by *MYCN* [14]. However, transfection of *MYCN* to some human neuroblastoma cell lines does not reduce class I HLA expression levels [15, 16]. We therefore sought additional mechanisms that may regulate class I HLA expression in neuroblastomas. Tanaka and associates reported an unexpected correlation between DNA-methylation and the expression of the murine homologue of class I HLA: methylated genes were expressed, while hypomethylated class I genes were silent [17]. In an analysis of class I HLA methylation in a panel of human neuroblastoma cell lines, we found a highly variable methylation pattern. Many cell lines had hypomethylated class I HLA genes, while other cell lines showed hypermethylation of this region.

Genes that modify the methylation of specific target genes have not yet been isolated. However, one so-called "modifier of methylation" was genetically mapped to mouse chromosome 4, in a region that is syntenic to human chromosomal band 1p36 [18]. This modifier of methylation, *SSM*-1, regulates the methylation of a certain transgene construct. The map position of *SSM-1* prompted us to analyse a possible relationship between hypomethylation of class I HLA genes and deletions of 1p36 in neuroblastoma cell lines. This analysis showed that all cell lines with hypomethylation of class I HLA did indeed have 1p deletions. Cell lines without deletions or with very distal deletions have highly methylated class I HLA genes. This strongly suggests that a gene that modifies the methylation status of class I HLA genes maps in the region 1p35-36 (Cheng and associates, Academic Medical Centre, Amsterdam). We have called this locus *MEMO-1*, for methylation modifier-1.

A further analysis of the cell lines showed that the absence of class I HLA methylation is not strictly correlated with the presence of *MYCN* amplification (Cheng and associates, Academic Medical Centre, Amsterdam). This suggests that the *MEMO-1* gene is not identical to the 1p36 gene consistently deleted in *MYCN* amplified neuroblastomas.

So far, *MEMO-1* cannot be considered to be a tumour

suppressor gene. Although deletion of this gene causes hypomethylation and probably reduced expression of class I HLA, this phenotypic change is in itself not necessarily oncogenic. Deletion of the *MEMO-1* region could, in fact, have been driven by its localisation close to a true suppressor gene, for example, the gene associated with *MYCN* amplification. *MEMO-1* would, in that case, be a "bystander" gene that modifies some of the biological characteristics of neuroblastoma, but does not play a role in cellular transformation. Alternatively *MEMO-1* could have many other target genes besides class I HLA. Cellular proliferation and differentiation processes are associated with changes in the methylation level of many genes. In addition, genomic imprinting is mediated by differential methylation of the parental alleles of genes [19]. Relaxation of these patterns is thought to play a role in tumorigenesis [20]. As described above, the distal 1p36.3 suppressor gene in neuroblastomas is probably imprinted. Intriguingly, Cheng and associates reported preferential amplification of the paternally inherited copy of the *MYCN* oncogene, indicating that *MYCN* could also be imprinted [8]. This suggests that modification of the methylation of specific genes may play a role in neuroblast differentiation. It is, therefore, tempting to speculate that *MEMO-1* may be more than an innocent bystander.

EVEN MORE SUPPRESSOR LOCI?

Two earlier reports have suggested that 1p may harbour different neuroblastoma suppressor genes. Schleiermacher and colleagues [21] defined a region with interstitial deletions, proximal to the *LMYC* gene. This putative suppressor region (called NB-R2) is clearly proximal to the four regions reviewed here, as *LMYC* maps proximal to 1p35. Furthermore, Takeda and associates found that patients with very small distal 1p deletions had a better prognosis than patients with larger 1p deletions, which suggested the existence of two 1p suppressor genes [22]. These two types of deletions could reflect loss of a distal suppressor locus versus loss of a more proximally located suppressor gene, for example, the gene deleted in *MYCN*-amplified neuroblastomas. Remarkably, in a preliminary analysis of our series, we found that patients with LOH of 1p36, but no *MYCN* amplification also have a poor prognosis [23]. One characteristic of the series of Takeda and associates is that theirs included many patients detected by mass screening of infants. It is questionable as to whether such patients would ever develop clinically manifest neuroblastomas, implying that these tumours may be of a biologically distinct type.

Furthermore, several cloned 1p36 genes have been proposed as candidate neuroblastoma suppressor genes. They include [*ID-3* (formerly called *HEIR-1*) [24], a $P58^{cdc2L}$ gene [25] and *DAN* [26]. *ID-3* and $P58^{cdc2L}$ genes showed expression patterns in neuroblastoma cell lines that could suggest a role as suppressor genes, and $P58^{cdc2L}$ and *DAN* genes showed aberrant DNA bands in some neuroblastomas. The murine homologue of *DAN* can suppress tumorigenicity of *v-src* transformed fibroblasts [27]. However, assessment of a role for these genes in neuroblastoma tumorigenesis awaits further analysis.

IMPLICATIONS FOR NEUROBLASTOMA TUMORIGENESIS

Studies on familial retinoblastoma have shown that germ line mutations in the *RB* tumour suppressor gene are often mutations or microdeletions. Inactivation of the second, normal allele in retinoblast cells of these patients is often mediated by loss of a large chromosomal region encompassing the *RB* gene (LOH). This suggested that loss of such a large chromosomal region is just one means of inactivating a suppressor gene, and its effect is equivalent to a point mutation or microdeletion. The data described in this review suggest that the p arm of chromosome 1 probably harbours a series of suppressor genes that play a key role in neuroblast differentiation. Loss of the distal p arm of chromosome 1 would, in one step, render all genes hemizygous. This could immediately result in reduced expression levels or, in the case of imprinted genes, even in their complete silencing. It is possible that this change in expression already has some effect on the control over cellular proliferation. Perhaps of more importance is that, in the hemizygous state, these cells can easily accumulate mutations in the remaining alleles of suppressor genes. The cell hemizygous for 1p is like a time bomb, in which the intact alleles of the 1p suppressor loci can become mutated one after the other. Each single mutation results in complete loss of function of a given suppressor locus. In proliferating cells, the intact alleles of the remaining suppressor loci are at risk of subsequent inactivations. Various sequences and combinations of these inactivations could finally result in different types of neuroblastomas with subtle variations in clinical behaviour and outcome.

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Comparison of DNA Aneuploidy, Chromosome 1 Abnormalities, *MYCN* Amplification and CD44 Expression as Prognostic Factors in Neuroblastoma

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A comparison of the prognostic impact of five molecular variables in a large series was made, including tests of their nonrandom association and multivariate analysis. Molecular data were available for 377 patients and *MYCN* amplification, cytogenetic chromosome 1p deletion, loss of chromosome 1p heterozygosity, DNA ploidy and CD44 expression were investigated. Their interdependence and influence on event-free survival was tested uni- and multivariately using Pearson's χ^2-test, Kaplan–Meier estimates, log rank tests and the Cox's regression model. *MYCN* amplification was present in 18% (58/322) of cases and predicted poorer prognosis in localised ($P < 0.001$), metastatic ($P = 0.002$) and even 4S ($P = 0.040$) disease. CD44 expression was found in 86% (127/148) of cases, and was a marker for favourable outcome in patients with neuroblastoma stages 1–3 ($P = 0.003$) and 4 ($P = 0.017$). Chromosome 1p deletion was cytogenetically detected in 51% (28/55), and indicated reduced event-free survival in localised neuroblastoma ($P = 0.020$). DNA ploidy and loss of heterozygosity on chromosome 1p were of less prognostic value. Most factors of prognostic significance were associated with each other. By multivariate analysis, *MYCN* was selected as the only relevant factor. Risk estimation of high discriminating power is, therefore, possible for patients with localised and metastatic neuroblastoma using stage and *MYCN*.

Key words: *MYCN* amplification, chromosome 1, DNA ploidy, CD44, lactate dehydrogenase, prognostic factors, multivariate analysis
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INTRODUCTION

THE GOAL of investigations into neuroblastoma at a molecular level is to understand better the biological diversity of the disease. In the last 10 years, several factors have been described including deletions of the short arm of chromosome 1 (del 1p) [1–3], amplification of the oncogene *MYCN* [4–6], DNA ploidy [7–9], overexpression or absence of the *Ha-* and *NRAS* gene [10, 11], CD44 receptor [12, 13], multidrug resistance gene (*PGY*, previously MDR) [14, 15], the low affinity nerve growth factor receptor and *TRKA* gene [16, 17]. Since the presence