Human Neuroblastoma: Paradigm for a Tumor with Oncogene Amplification and Loss of a Putative Tumor Suppressor Gene

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Introduction

Human neuroblastoma cells often carry nonrandom chromosomal abnormalities signaling genetic alterations. Quite frequent are "double minutes" (DMs) and homogeneously staining regions (HSRs), both cytogenetic manifestations of amplified DNA, and chromosome 1p deletions indicating loss of genetic information. With the identification of amplified N-*myc* and the demonstration of a consensus deletion spanning the chromosome 1p36.1–2 region it now appears likely that both amplification of a cellular oncogene and loss of a tumor suppressor gene play important roles in neuroblastoma (Fig. 1).

N-*myc* Amplification

Biology of Amplified N-myc

N-*myc* was the first amplified oncogene that turned out to be of clinical significance due to its association with aggressively growing tumor phenotypes (for a review, see [1]). N-*myc* was originally identified when human neuroblastoma cells showing DMs or HSRs were analyzed with various oncogene probes [2, 3]. These surveys quickly established that, with few exceptions, cultured neuroblastoma cell lines carry the gene N-*myc* in an amplified form. At the same time neuroblastoma tumors were also found to carry amplified N-*myc* [3]. The initial surveys suggested that N-*myc* amplification was specific for neuroblastoma. It turned out later that N-*myc* amplification can be seen in small-cell lung cancer, retinoblastoma, and astrocytoma, although at much lower incidence. As a common feature, all these tumors have neural qualities. Until now N-*myc* has been the only gene, however, found amplified in neuroblastomas.

The oncogenic potential of enhanced expression of N-*myc* as the consequence of amplification has been addressed in various experimental systems. Enhanced expression, resulting from introduction of an N-*myc* expression vector, can assist mutationally activated H-*ras* in tumorigenic conversion of primary rat embryo cells [4], converts established cells of the rat [5] and of humans [6] to tumorigenicity, and rescues primary rat embryo cells from senescence [7]. Furthermore, N-*myc* has frequently been found activated by proviral insertion in murine leukemia virus (MuLV)-induced T cell lymphomas [8], and is involved in tumorigenesis in transgenic mice [9, 10]. These results clearly attest to the capacity of high N-*myc* expression to modulate the growth of cells, and it appears reasonable, therefore, to suggest that enhanced expression consequent on amplification contributes to tumorigenesis. The available evidence suggests that the nucleotide sequence of N-*myc* in neuroblastoma cells is unaltered compared to that of normal cells [11]. Consistent with this result, the biological activities of N-*myc* derived from normal or from neuroblastoma cells have not been found to differ [4, 7].

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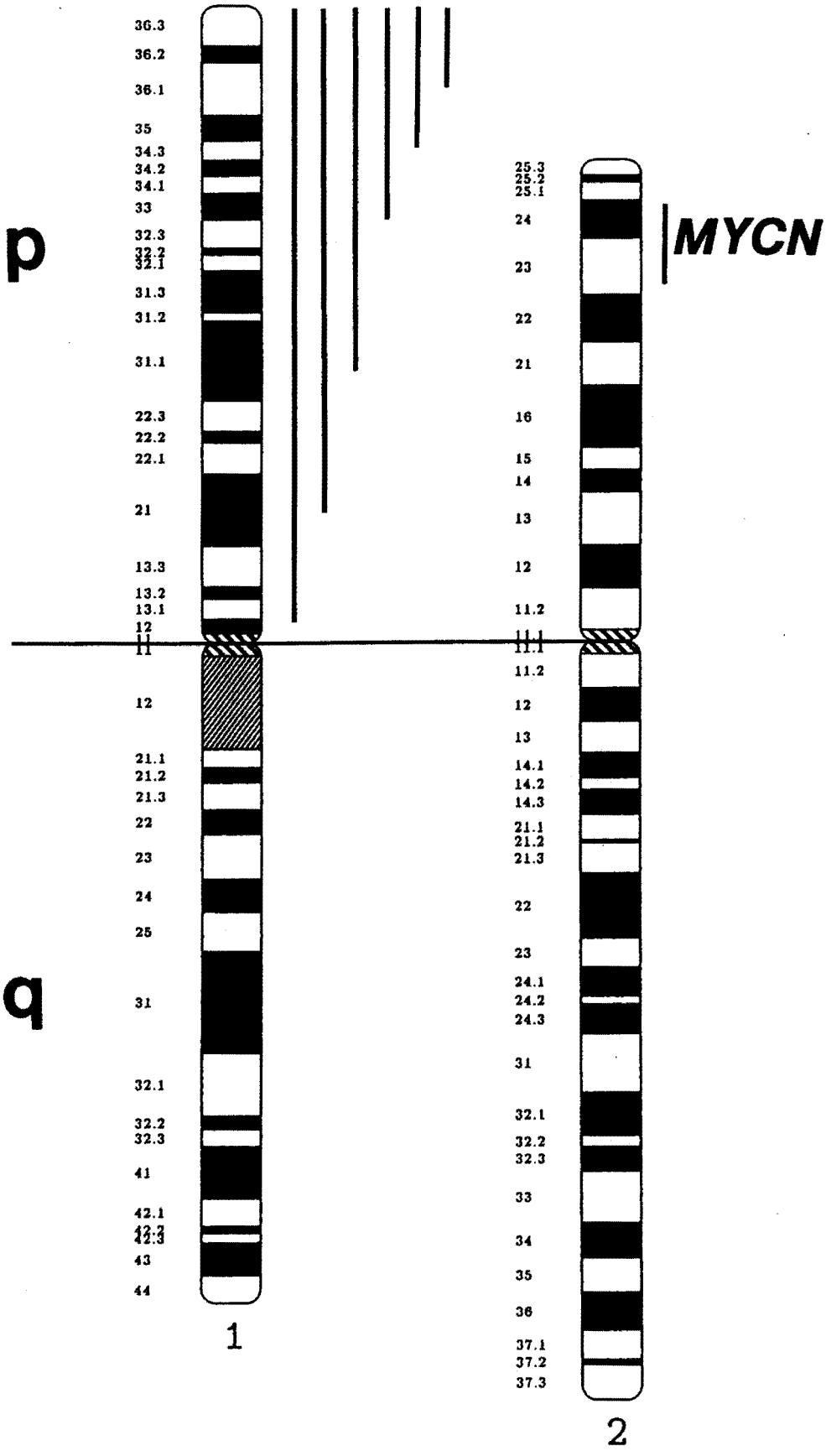

Fig. 1. Genes involved in human neuroblastoma. Loss of genetic material, possibly a tumor suppressor gene, from chromosome 1p36.1–2 and amplification of the oncogene *MYCN* from chromosome *2* seem to contribute in at least many cases to neuroblastoma. The *vertical lines* to the *right* of chromosome 1p indicate chromosomal deletions that vary in length in tumors of different patients. The region near the end of chromosome 1 has been found deleted in at least 90% of cases analyzed

Structural Arrangement of Amplified N-myc

There is little data available about the structure and the size of the amplified DNA encompassing cellular oncogenes, and what is known comes mostly from studies of N-*myc* amplification in neuroblastomas. By using random amplified probes isolated from flow-sorted chromosomes of the neuroblastoma line IMR-32 [12], it was observed that the amplified DNA encompassing N-*myc* differs when a series of neuroblastomas is analyzed [13, 14]. DNA amplified in IMR-32 was amplified to a lower degree or not at all in other neuroblastomas. The gene N-*myc* was amplified in all cases carrying amplified DNA, however. A similar observation was made by Zehnbauer et al. [15], who used a set of random probes from neuroblastoma line NGP. These authors also found that the amplification units in NGP cells and 12 different primary neuroblastoma tumors were similar over a contiguous region of at least 140 kb encompassing N-*myc*. In line with this, the gene encoding ornithine decarboxylase, which is linked to N-*myc*, has been found coamplified with N-*myc* in only one of six tumors [16].

The size of the amplified DNA containing N-*myc* and *myc* genes has been analyzed by employing denaturation and reassociation of DNA in agarose gels [17]. This approach involved cutting DNA with a restriction endonuclease, size fractionation through agarose gels, alkali denaturation, partial reassociation, and subsequent treatment with the single-strand-specific nuclease S1. Amplified sequences in tumor cells, due to their relative higher concentration, reassociate at a higher rate than their single-copy counterparts in normal cells and therefore become S1 nuclease-resistant when single-copy sequences are still sensitive. If the DNA is radioactively labeled, under suitable conditions the autoradiographs of the gels will reveal a banding pattern in cases of DNA amplification. This approach has indicated that the size of the amplified DNA encompassing N-*myc* ranges in different tumors from 290 to 430 kb, and that the DNA containing the *myc* units ranges in size from 90 to 300 kb [18].

A direct determination of the size and the structure of the amplified DNA in human neuroblastoma cells has been done by pulsed field gel electrophoresis, which is capable of fractionating DNA fragments in the size range from one hundred to several thousand kilobare pairs. The analysis was facilitated by the finding that the 5′-region of N-*myc* is in a CpG island and has recognition sequences for several rare cutting enzymes [19]. This situation made it possible to map the DNA encompassing N-*myc* over a distance of more than 1000 kb. By employing suitable N-*myc* probes derived from the 5′- and 3′-regions of recognition sites for rare cutting restriction endonucleases within N-*myc* the amplified DNA was found in many cases arranged in precise head-to-tail units. These units varied among different neuroblastomas and ranged from about 100 to 800 kb in size [19]. The precise and ordered head-to-tail arrangement is stable over long periods of time and does not change upon establishment of tumor cells into *in vitro* culture or during passages of tumor cells through athymic mice. There are principally two mechanisms that might be involved in initiation of amplification. Amplification may start with unscheduled replication of DNA encompassing N-*myc*, which maps to chromosome 2p23–24 [20] (Fig. 2A). Alternatively, amplification may start with loop formation and excision of the DNA (Fig. 2B). In either event, extrachromosomal DNA molecules that integrate into a distant chromosomal region and *in situ* amplification appear to result (Fig. 2) (for a detailed discussion see [1]). The amplified N-*myc* copies map in most instances to HSRs that are localized on different chromosomes in cells derived from different tumors. The direct repeat structure that in neuroblastomas is stable over many cellular divisions appears to be

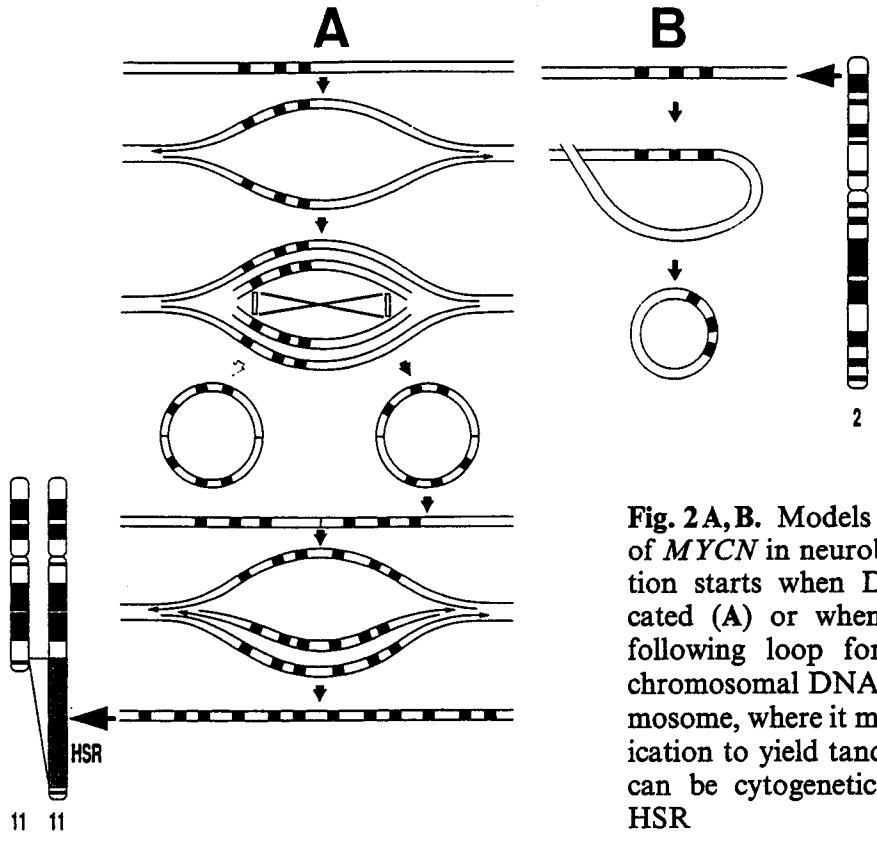

Fig. 2A,B. Models illustrating amplification of *MYCN* in neuroblastoma cells. Amplification starts when DNA becomes extrareplicated (**A**) or when DNA becomes excised following loop formation (**B**). The extrachromosomal DNA is integrated into a chromosome, where it may undergo in situ amplification to yield tandem DNA amplicons that can be cytogenetically demonstrated as an HSR

different from that of amplified DNA associated with drug resistance, where head-to-head arrangements with inverted repeats predominate and where high instability of the amplified DNA structure is observed [21]. It is not clear if these differences result from fundamental differences not clear if these differences result from fundamental differences of the mechanisms by which DNA in drug-resistant cells and in tumor cells become amplified, or if they are the consequence of exposure to cytotoxic drugs. It will be interesting to find out what the structure of the DNA encompassing other cellular oncogenes might be.

Clinical Significance of N-myc Amplification

An important prognostic variable for patients with neuroblastomas in the clinical stage. Patients with disease stages I and II have a good prognosis with 75%–90% 2-year disease-free survival, while patients with stages III and IV have a poor prognosis, with 10%–30% 2-year survival. Surveys of over 400 neuroblastomas revealed that a strong correlation exists between N-*myc* amplification and stage III and IV [22–25]. A number of patients with stage I or II have been identified carrying amplification. In all instances, these tumors, which on the basis of conventional diagnostic possibilities were of good prognosis, progressed later and turned out to be fatal. A peculiar stage IV-s characterized by frequent spontaneous regression rarely shows amplification (7%). Studies of three patients with N-*myc* amplification have been published [26–28]; all tumors progressed later. These observations clearly show that N-*myc* amplification is a reliable prognostic parameter for poor prognosis in patients with low stage of IV-s tumors.

The prognosis of patients over 1 year, mainly diagnosed with stage III or IV tumors, is particularly poor, and metastases occur predominantly in the bone, orbita, and distant lymph nodes. More than 50% of the patients over 1 year carried amplification of *myc*, while amplification was rarely seen in patients

below 1 year of age [24]. Altogether, N-*myc* amplification is associated with a higher malignant phenotype of neuroblastoma.

Current therapeutic strategies for treatment of neuroblastoma depend on the prognosis for survival which is evaluated in the basis of tumor stage, on the degree at which the tumor can be removed surgically and one the basis of genomic analyses of the tumor cells. The pilot study of the German Neuroblastoma Study Group advises treatment of patients according to protocols that are specific for each of four risk groups. Risk group A includes patients with a localized tumor that can be surgically removed to at least 90% (prognosis is 90%–100% for survival of patients). Risk group B includes patients with a localized tumor that extends beyond the area of the organ of origin and usually cannot be removed completely (prognosis 65%–80%). Risk group C includes patients that carry a metastatic tumor, or a localized tumor that cannot be removed after four cycles of chemotherapy (prognosis 20%–30%). Risk group D includes only patients with a IV-s tumor that frequently shows spontaneous regression (prognosis 75%–80%). Patients that on the basis of conventional parameters would be included in risk groups A and B are transferred to risk group C if there is N-*myc* amplification. Patients included in risk group C receive the most intensive therapeutic treatment. It remains to be seen if the same is advisable for patients of risk group D.

Chromosome 1p Deletion

Deletions of chromosome 1 were first described in 1975 [29]. Even though the breakpoints of the deletion were found to vary, the portion of the chromosome distal to band p32 seemed to be most consistently deleted. In all cases, the deletions appeared to involve only one chromosome. From cytogenetic analyses, the portion deleted appears to be lost from the genome, which means that the cells are monosomic for this genetic material [30].

At this point, the nature of the genetic material deleted and its significance to tumorigenesis can only be speculated upon. There is a good possibility that the deletion involves genetic information essential for normal differentiation of certain neutral cells. Loss could result in abnormal differentiation and could be a factor contributing to tumorigenesis. In a similar way, lack of genetic information identified by cytogenetic analysis in specific regions of other chromosomes seems to contribute to other types of tumors, in particular Wilm's tumor, retinoblastoma, lung cancer and certain forms of colon cancer (for a review see [31]). The general idea is that the presence of this genetic information supresses tumorigenesis and its lack allows tumors to develop. Genes behaving as suppressors of tumorigenesis have been termed "tumor suppressor genes," although their functions and the mechanisms by which they act are obscure as yet.

To define the role that loss of genetic information from 1p36 might have in neuroblastoma, we set out to determine a consensus deletion. Our approach depended on first generating a panel of DNA probes closely positioned to each other, and recently we reported on the generation of a microclone library specific for the very distal part of chromosome 1p [32]. The dense distribution of probes proved advantageous for the present study in different respects. Firstly, small deletions could be detected with a higher probability. Secondly, it provided the ability to detect allelic loss even in cases where adjacent loci were not informative due to homozygosity. Thirdly, it was necessary to define the borders of deleted regions within narrow limits and to determine a small region commonly deleted in different tumors.

Applying this strategy we were able to discover allelic deletions in a high proportion of tumors (90%) analyzed (Fig. 3). With respect to the cytogenetic data of

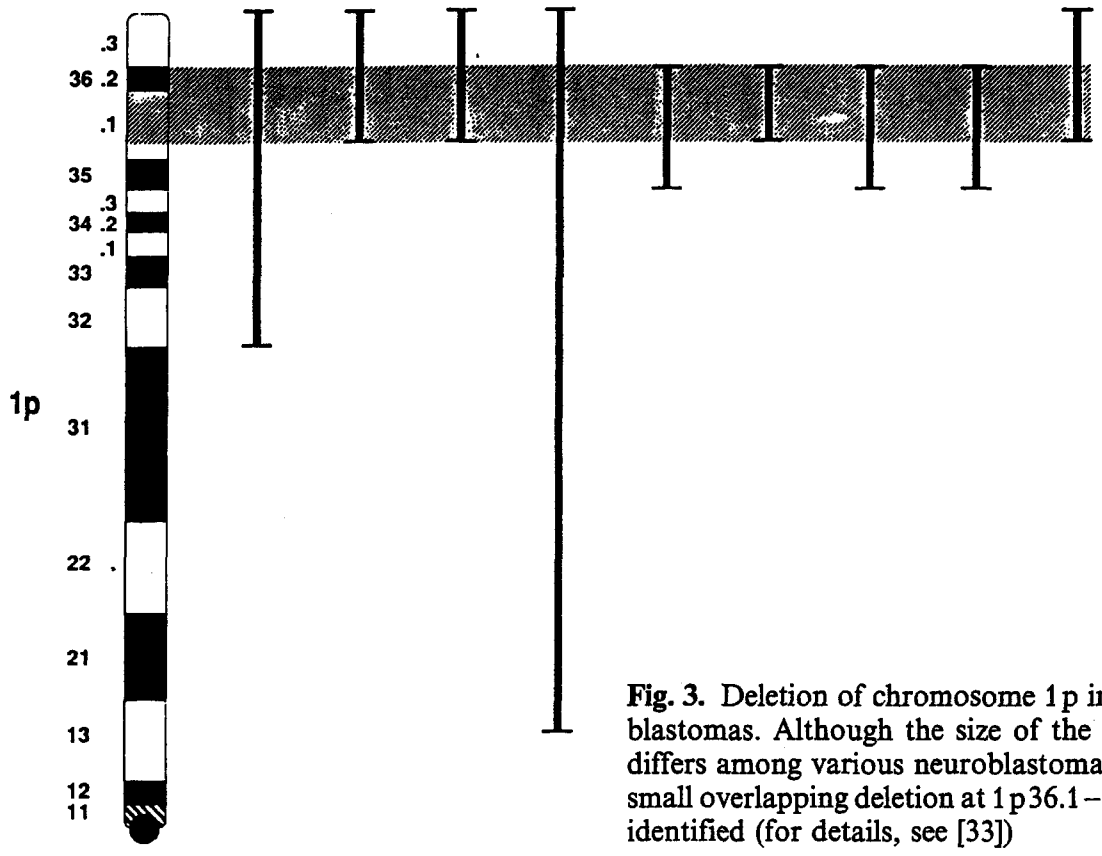

Fig. 3. Deletion of chromosome 1p in neuroblastomas. Although the size of the deletion differs among various neuroblastomas a very small overlapping deletion at 1p36.1–2 can be identified (for details, see [33])

neuroblastomas, our results revealed that distal 1p material was indeed lost from the genome of the tumor cells [33]. In another study of chromosomal deletions in neuroblastomas [34] loss of heterozygosity was also detected in 1p36, though the frequency described was lower (28%) than indicated by cytogenetic analyses. The higher overall frequency of loss of genetic material from the 1p region among stage III and IV tumors as compared to the frequency found by Fong et al. (28% vs. 90%) [34] is probably due to the fact that our microclone probes are from within the consensus deletion and are located closer to the genetic information related to neuroblastoma. The fraction of tumors displaying allelic loss with our probes exceeded that showing cytogenetically detectable rearrangements. This is not surprising, because the number of tumors displaying loss on the DNA level in the present study was bound to exceed that showing microscopically visible deletions. We suspect that small deletions are likely to remain undetectable upon microscopic investigation.

We found only one neuroblastoma that did not display any allelic loss. A deletion could probably not be detected in this case because two probes which resided in the area commonly lost in other tumors were not informative. Hence, it is still possible that in this particular case an as yet unidentified deletion is present. Alternatively, we must take into account that the tumor sample might have contained a high proportion of normal tissue, thus obscuring any loss of alleles. Yet another explanation may be that in this tumor the DNA in the consensus regions is altered by a subtle deletion or point mutation not detectable with our probes. Among the neuroblastomas analyzed we found evidence for interstitial deletions in four cases. Through these a consensus deletion could be defined which was commonly lost from the tumors. On this basis we have located a consensus deletion to the subbands 1p36.1–2. We are well aware of the limited resolution of *in situ* hybrid-

ization and of the uncertainty for an exact relative localization of DNA probes. We are in the process of defining the exact linkage relationship of these markers by long-range restriction mapping.

Even considering this uncertainty, a rough estimate can be made as to the size of the deleted DNA segment. The entire 1p36 band comprises about 0.7% of the total haploid chromosome length (for values of relative chromosome lengths, see [35]). Predicting an even distribution of the DNA along the chromosomes, 1p36 would contain about 20 Mbp (megabase pairs) of DNA (0.7% of the total haploid DNA content of 3×10^9). 1p36.1–2 represents roughly half of the 1p36 band. Hence the genomic region included in the consensus deletion would span about 10 Mbp of DNA. Since we can fall back on a large number of specific microcloned probes, we are in the process of establishing a long-range restriction map of this region with pulsed-field electrophoresis. This long-range map will provide information on the presence and location of CpG-rich islands, which often signify the 5′-regions of genes [36], and therefore will be a tool for the identification of coding DNA sequences in this region.

Recently, Suzuki et al. [37] reported on loss of heterozygosity in neuroblastomas using probes specific for various chromosomes. With a N-*myc*-specific probe they found only two out of 12 tumors to have lost alleles from 1p. This is in agreement with our data, assigning a putative consensus deletion more distal than the N-*myc* locus. In one tumor with an extended deletion we also found allelic loss involving the N-*myc*-region.

A significant correlation between the disease stage of neuroblastomas and N-*myc* amplification has been established in the past (for a review, see [38]). For the time being no data are available that elucidate the question of whether amplification of N-*myc* and loss of DNA sequences from chromosome 1 occur in association with each other or whether one of the mutational events precedes the other in neuroblastoma tumorigenesis. Our results do not support the idea of a correlation between these two events as has been proposed by Fong et al. [34].

At present, the molecular studies performed on human neuroblastoma indicate that the development of tumors may be a result of various genetic alterations involving amplification of the N-*myc* oncogene [38, 39], as well as loss of genetic information from 1p36 [33, 34] and 14q [37]. So far no evaluation of these mutational events can be made with respect to their function or to a hierarchy of events in the initiation and progression of human neuroblastoma.

Conclusions

Central issues in cancer research are (a) how genetic alterations contribute to tumorigenesis, (b) how specific genetic alterations can be turned into diagnostic tools to provide information on how to optimize existing therapeutic regimens, and (c) how to open up avenues for causal therapeutic strategies. During the past decade much has been learned about genetic alterations in tumor cells. The activation of the oncogenic potential of cellular genes can take different routes among which mutational alteration, translocation and amplification predominate (for a review, see [40]). In particular, amplification has found its way to practical clinical use due to its association with more aggressively growing types of human cancer. N-*myc* amplification in neuroblastoma has provided a paradigm for the prognostic significance of oncogene alteration, and at the same time has represented the clinical debut of oncogene research. The full significance of oncogene amplification as a predictor for poor prognosis became clear with the identification of amplified *erb*-B2 in aggressively growing breast cancers [41]. The state of the art is that amplified cellular oncogenes define cancer patients which have poor prognosis and may require specific therapeutic regimen.

Of great interest could be the identification of a gene that is commonly deleted in neuroblastomas from the chromosome 1p region. What will be the normal function of this putative tumor suppressor gene? And once the function is known, can this information be exploited to revert the malignant phenotype? The answer to these questions is not trivial, but the project is worth being pursued under this perspective.

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