Gain of 17q24–qter Detected by Comparative Genomic Hybridization in Malignant
Tumors from Patients with von Recklinghausen’s Neurofibromatosis

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ABSTRACT

The genetic changes leading to the development of malignant peripheral nerve sheath tumors (MPNSTs) are largely unknown. The few tumors that have been investigated cytogenetically had highly complex karyotypes and no consistent rearrangements, and the attempts to pinpoint consistent DNA-level changes have met with only limited success. We used comparative genomic hybridization to analyze seven MPNSTs and one dermatofibrosarcoma protubersans from eight patients with von Recklinghausen’s disease (neurofibromatosis type 1), as well as three sporadic MPNSTs.

Gains and losses of DNA sequences were found in all tumors, with an average of four losses (range, 0–14) and two gains (range, 0–5) per tumor. Two striking observations were made: (a) an increase in copy number of the distal part of the long arm of chromosome 17, with the smallest region of overlap 17q24–qter, was seen in five of seven MPNSTs and in the only dermatofibrosarcoma protubersans, all of which were from patients with neurofibromatosis, whereas none of the three sporadic MPNSTs had this alteration; and (b) loss of 13q, with the smallest region of overlap 13q14–q21, was found in 6 of 10 MPNSTs. The consistent involvement of these two chromosomal regions probably reflects two different pathogenetic mechanisms for MPNSTs.

INTRODUCTION

Individuals with von Recklinghausen’s NF,3 or NFl, vary considerably in the severity of their phenotypic features (1), including the tendency to develop malignant disease. Nevertheless, as many as half of all MPNSTs develop in NF patients.

The gene that in mutated form predisposes to NFl, NFl, maps to 17q11.2 and was cloned in 1990 by means of positional cloning (2—4). Deletions and point mutations within NFl have been reported both in solid tumors associated with NF and in other malignancies, such as colon cancer, malignant melanoma, and neuroblastoma (5–8), showing the suppressor nature of this gene as well as implying a more general role for it in carcinogenesis. Individuals with NF1 carry a germline mutation of NFl, and the deletion of 17q loci found in MPNSTs from such patients might reflect the complete inactivation of the gene. However, actual mutation of both alleles has been demonstrated in only one tumor (7).

Previous molecular and cytogenetic analyses have shown that many other genetic alterations are also likely to be important in the pathogenesis of MPNSTs. The MPNSTs characterized cytogenetically until now had complex karyotypes with no consistent aberration pattern (9, 10). Loss-of-function mutations of the TP53 gene have been suggested to contribute to MPNST development (11–13); however, very few tumors have been analyzed for such changes, and only a subgroup of them were shown to carry mutations within the examined domains (11–14).

CGH is a molecular cytogenetic technique that may be utilized to identify net gains and losses in the genome, thus establishing a “copy number karyotype” of each tumor (15). Although balanced or near-balanced chromosomal aberrations cannot be detected by this method, it provides a valuable tool to screen DNA from frozen or fixed tumor samples for genomic alterations.

MATERIALS AND METHODS

Patients and Tumors. Tissue specimens were taken from malignant tumors from eight patients with NFl (Table 1). Seven of the tumors were MPNSTs, and one was a DFSP. In addition, three MPNSTs from patients without evidence of NFl were also included. Additional information on the patients and tumors has been described elsewhere (9, 14). Cases 2, 3, 5, 6, and 8 in Ref. 9 correspond to samples 753, 2367, 32, 650, and 1347 in Table 1. In Ref. 14, patients 3, 5, 6, 7, and 8 correspond to samples of the same numbers in Table 1.

DNA Isolation. Normal DNA from all individuals was isolated from peripheral blood cells. Both normal and tumor DNA were extracted according to standard procedures using phenol and chloroform extraction followed by ethanol precipitation (this was either a manual procedure or automated by use of a DNA extractor from Applied Biosystems, Foster City, CA).

CGH. The original CGH method (15) has been modified, and the details of the procedure used in this study were described by Kalilioniemi et al. (16). Tumor DNA was labeled directly by nick translation with FITC-12-dUTP and normal DNA with Texas red-dUTP. The conditions of the nick translation reaction were modified to obtain by gel electrophoresis a smear of fragments between 500 and 2000 bp. The labeled DNA (400 ng tumor DNA and 400 ng normal DNA) and unlabeled Cot-1 DNA (10 μg) were mixed, ethanol precipitated, and dissolved in hybridization buffer (10 μl of 50% formamide, 10% dextran sulfate, and 2×SSC [1×SSC = 0.15 M NaCl and 0.015 M sodium citrate; pH 7]). After denaturation, the DNA was hybridized to normal denatured metaphase spreads and incubated for 1–2 days at 37°C. Finally, the slides were washed and mounted in an antifade-counterstain (DAPI) solution before microscopic examination.

Digital image analysis was used to identify chromosomal regions with abnormal fluorescence ratios (16, 17). Three single-color images, green (FITC) for tumor DNA, red (Texas red) for normal DNA, and blue (DAPI) for the DNA counterstain, were obtained from the selected metaphases using a fluorescence microscope (Olympus BX, Tokyo, Japan) and a cooled charge-coupled device camera (Xillix Technologies Corp., Vancouver, Canada) interfaced to a Sun workstation (Sun Microsystems Computer Corp., Mountain View, CA). The average number of metaphase cells analyzed per sample was six (range, 4–8). Chromosomes were identified based on their DAPI banding pattern. The relative green:red fluorescence ratio along the medial axis of each chromosome from the tip of the short arm to the tip of the long arm was calculated using software developed on the Scilimage analysis programming platform (TNO, Delft, The Netherlands). The fluorescence intensities were normalized so that the average green:red ratio for each metaphase was set to 1.0. The results from all analyzed metaphases (the total number of observations per chromosome) were combined, and profiles of the mean fluorescence ratio

Received 4/1/96; accepted 8/13/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: NF, neurofibromatosis; NFl, NFl type 1; MPNST, malignant peripheral nerve sheath tumor; CGH, comparative genomic hybridization; DFSP, dermatofibrosarcoma protubersans; DAPI, 4',6-diamidino-2-phenylindole; LOH, loss of heterozygosity.
and its SD were calculated and displayed. The set of chromosome profiles thus arrived at would be equivalent to the copy number karyotype of the tumor under investigation.

The CGH results were interpreted according to the guidelines described by Kallioniemi et al. (16). Normal FITC-labeled female DNA hybridized against normal Texas red-labeled male DNA was used as a negative control, whereas DNA from the MCF-7 breast cancer cell line hybridized with normal DNA was used as a positive control. Chromosomal regions showing a mean ratio and corresponding SDs less than 0.85 or greater than 1.15 were scored as lost or gained, respectively. Heterochromatin blocks (the entire Y chromosome, the whole arms of four different chromosomes) were excluded from the analysis.

**RESULTS**

**Overall Frequency of Genetic Changes Observed by CGH.** All analyzed tumors \((n = 11)\) showed one or more genetic aberrations, the average number of changes being six per tumor \((range, 1—17)\). Decreases in DNA sequence copy number were seen twice as often as gains \((Table 1)\). On average, four losses and two gains were observed per tumor \((range, 0—14 and 0—5, respectively)\). The copy number karyotype of case 753 is shown in Fig. 1.

**Chromosomes Exhibiting DNA Sequence Losses or Gains in MPNSTs.** The most frequent losses were from chromosome arms 13q \((6 of 10 tumors)\), 3q \((4/10), 9p \((4/10), 18p \((4/10), 10p \((3/10), and 11q \((3/10)\), with minimal common deleted regions in 13q14—q21, 3q11—q24, 9p, 18p, 10p, and 11q14—qter. Additional detected losses, involving altogether 22 different chromosome arms, were observed in one or two tumors each.

Chromosome arm 17q was the only consistently amplified region in several MPNSTs \((5 of 10)\), with 17q24—qter as the smallest region of overlap. All other chromosome arms showing an increase of DNA sequence copy number \((n = 11)\) did so in only one or two tumors.

**DISCUSSION**

The karyotypes of the 40 MPNSTs analyzed previously by chromosome banding have been highly complex, making complete identification of the aberrations impossible \((9, 10)\). Five of the tumors of the present series had previously been analyzed cytogenetically \((Ref. 9; see “Materials and Methods” for details)\), and all were found to have many seemingly unbalanced clonal aberrations, some of which could not be identified. This contrasts with the finding by CGH of only one, two, or three changes, respectively, in cases 650, 2367, and 32 \((Table 1)\). Presumably, the reason for the less-than-complete correspondence between the CGH and chromosome banding data should mainly be sought in the fact that the banding analysis left many aberrations unidentified. Another important factor could be that some.

**Table 1 Chromosomal copy number changes in 10 MPNSTs and 1 DFSP detected by CGH**

<table>
<thead>
<tr>
<th>Tumor sample</th>
<th>Diagnosis</th>
<th>Losses</th>
<th>Gains</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>NFI + MPNST</td>
<td>None</td>
<td>8p15, 8p12-q22, 17q22-qter</td>
</tr>
<tr>
<td>753</td>
<td>NFI + MPNST</td>
<td>1cen-p31, 3p21-qter, 6p21-qter, 8q23-qter, 10p, 13q, 18p, 20p, 22q</td>
<td></td>
</tr>
<tr>
<td>2367</td>
<td>NFI + MPNST</td>
<td>9p, 13q</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>NFI + MPNST</td>
<td>13q</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>NFI + MPNST</td>
<td>3, 9p, 11q14-qter, 14q31-qter, 18, 20p</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NFI + MPNST</td>
<td>3q, 5p14-qter, 10, 11q, 16q, 18p</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NFI + MPNST</td>
<td>1p35-pter, 1q32-qter, 3cen-q24, 4q21-qter, 5q14-q31, 7q31-qter, 9p, 10, 11, 13cen-q22, 14q24-q31, 16q, 18, Xq25-qter</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MPNST</td>
<td>1q32-qter, 2q35-qter, 7q31-qter, 9p, 13q14-qter</td>
<td></td>
</tr>
<tr>
<td>650</td>
<td>MPNST</td>
<td>8p</td>
<td></td>
</tr>
<tr>
<td>347</td>
<td>MPNST</td>
<td>2p16-pter, 8p22-pter, 12p, 13cen-q21</td>
<td></td>
</tr>
<tr>
<td>2362</td>
<td>NFI + DFSP</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

NFI = neurofibromatosis type 1; MPNST = malignant peripheral nerve sheath tumor; DFSP = dermatofibrosarcoma protuberans.

![Fig. 1](cancerres.aacrjournals.org)
of the cytogenetically aberrant clones may have been too small to make an impact in the CGH assay.

If the whole genome is multiplied, this will be normalized in the CGH analysis so that only deviations from the new norm will be registered as gains or losses. Karyotyping has shown that many MPNSTs are in the hypotriploid range, which may explain at least in part why losses are more frequent than gains as assessed by CGH. This fact and the fact that no high-level amplification was detected by CGH suggest that inactivation of suppressor genes may be more important than activation of proto-oncogenes in the development of MPNST. It should be noted that, although no apparent high-level amplifications (with green:red ratios exceeding 2) could be found, the exact degree of amplification cannot be measured by CGH. This is because the tumor:normal fluorescence ratio, obtained by measurements along the chromosomes, reflects both the level of amplification and the size of the amplified region.

The most frequently (6 of 10 tumors) lost region in the MPNSTs examined was 13q, with 13q14–21 as the minimal common deleted segment. Consistent genomic loss indicates that a pathogenetically important tumor suppressor gene is located in the lost segment. The delineation of the lost segment seems to rule out BRCA2 (in 13q12–13) as the target locus, but leaves RB1 (13q14) as a suppressor gene of possible pathogenetic importance. Loss of 13q is known to occur in several solid tumors, including musculoskeletal tumors, other than MPNSTs (18). In a subgroup of osteosarcomas, RB1 is homozygously inactivated (19). It should be noted that the loss of 13q was the only change or one of only two losses in tumors 32 and 2367, suggesting that this was an early event in tumorigenesis.

Three other regions, in chromosome arms 3q, 9p, and 18p, were lost in 4 of the 10 MPNSTs in our series. Although lack of an entire chromosome 3 is not uncommon in solid tumors, partial losses within the short arm, which contains several putative and known suppressor genes, are in many tumor types more frequent than losses restricted to 3q (20). Losses from chromosome arm 9p are frequent in many malignancies and often reflect inactivation of one or two cyclin-dependent kinase inhibitors (p16 and p15) important in the cell cycle regulation (21). The 9p loss is also a recurrent aberration in cytogenetic analysis of MPNST (9–10). Finally, there are no known candidate suppressor genes that may account for the observed deletions of 18p, a chromosome arm that is often lost during carcinogenesis because of loss of an entire copy of chromosome 18 (20).

The increase in copy number corresponding to the distal part of 17q is remarkable not only for its frequent occurrence in this series of MPNSTs, but also because this change only occurred in the familial cases, including the DFSP cases. The analyzed tumors were few, but one may nevertheless speculate that this particular aberration might be characteristic for the development of tumors in patients with NF1. Thus far, however, there is no obvious candidate target gene for the observed low-level amplification in this chromosome region. Molecular genetic analyses (Southern blot and/or fluorescence in situ hybridization) with probes to genes and loci along this 20-Mb region are required to assess the identity of the target gene(s) of amplification. Incidentally, roughly the same region of amplification was reported in breast carcinomas by CGH (22) as well as by chromosome microdissection (23). In ongoing fluorescence in situ hybridization studies, two independent regions of amplification have thus far been found in breast carcinoma cell lines.

Previous molecular genetic studies have shown LOH of 17q markers, including the NFI locus, in MPNSTs (7, 12, 14, 24, 25). This does not contradict our results, because the LOH studies have mainly used markers in the vicinity of the NFI locus, which is proximal to the region we observed as amplified. Indeed, retained heterozygosity has been seen at distal 17q loci, whereas other markers on chromosome 17 showed LOH (12, 14). Five of the tumors analyzed here (Table 1, tumors 8, 7, 6, 5, and 3) have previously been analyzed for LOH using chromosome 17 markers (14). Three of these tumors (7, 6, and 3) showed increased copy number at distal 17q in the CGH analysis, whereas the LOH findings were interpreted as retained heterozygosity, uninformative, and allelic gain, respectively, at distal 17q markers. Absence of one allele (LOH) at loci within the NFI gene was found in two of the five tumors (tumors 8 and 7), two were uninformative (tumors 6 and 3), and one (tumor 5) was unaltered. Thus, among the three tumors with distal 17q gain, by CGH analysis, one was informative (tumor 7) at the NFI locus by LOH studies, and exhibited loss of one parental allele.

DFSP is characterized karyotypically by supernumerary rings and marker chromosomes (18). Our findings are in full agreement with recent molecular cytogenetic data showing that sequences from chromosomes 17 and 22 are present in the rings and markers in these tumors (26, 27). It is tempting to speculate that the 22q amplification is of specific importance in DFSP tumorigenesis, because none of the MPNSTs exhibited amplification of this region. To our knowledge, none of the previously genetically analyzed DFSPs was obtained from patients with NF.

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