Alterations of Microsatellites in Neurofibromas of von Recklinghausen’s Disease

Laura Ottini, Diana L. Esposito, Antonio Richetta, Marta Carlesimo, Raffaele Palmirotta, Maria Concetta Verli, Pasquale Battista, Luigi Frati, Felice Giacomo Caramia, Stefano Calvieri, Alessandro Cama, and Renato Mariani-Costantini

Department of Experimental Medicine [L. O., L. F., F. G. C.] and Institute of Dermatology [A. R., M. C., S. C.], University “La Sapienza,” 00161 Rome; Istituto Mediterraneo di Neuroscienze, 96077 Pozzilli, Isernia [L. F.]; and Institute of Human Pathology and Social Medicine, University “Gabriele D’Annunzio,” 66013 Chieti [D. E. L. R. F., P. M. V. P. B., A. C., R. M. C.]. Italy

ABSTRACT

von Recklinghausen’s disease, or type 1 neurofibromatosis, a common familial tumor syndrome, is characterized by the occurrence of multiple benign neoplasms of nerve sheath cells. The disease is caused by germ-line mutations of the NFl gene, which encodes a member of the GTPase-activating superfamily of Ras regulatory proteins. We analyzed 5 dinucleotide repeat loci in DNAs from neurofibromas and matched normal skin from 16 NFl patients. Eight cases (50%) manifested microsatellite alterations. Expansions or compressions of dinucleotide repeats were observed at one locus in four cases and at two loci in one case. Banding patterns compatible with the loss of a microsatellite allele were observed in four cases, including one that also presented microsatellite instability. The surprisingly high frequency of microsatellite alterations suggests that the NFl gene or another gene(s) contributing to the pathogenesis of neurofibromatosis might be directly or indirectly implicated in the control of genomic integrity.

INTRODUCTION

von Recklinghausen’s neurofibromatosis or NFl3 is one of the most common human genetic diseases, affecting approximately 1 in 3500 births (1). The disease is transmitted in a mendelian autosomal dominant fashion, and predisposes to a wide range of clinico-pathological manifestations, involving primarily tissues deriving from the neural crest. In adults, the most common clinical problems are related to the development of multiple cutaneous and more deeply placed neurofibromas. These benign, relatively circumscribed lesions, primarily composed of Schwann cells and fibroblasts, slowly but continuously increase in size and number and may eventually lead to severe local or systemic complications (1). NFl has been included among familial tumor syndromes because affected individuals are at an increased risk of developing a variety of neoplasms, particularly neurofibrosarcoma, glioma, pheochromocytoma, and myeloid leukemia (2).

NFl is caused by germ-line mutations that inactivate one allele of the tumor-suppressor NFl gene. This gene encodes neurofibromin, a member of the GTPase-activating protein superfamily of ras regulatory proteins (3).

Microsatellite instability, characterized by the presence of random shifts in the length of simple repeated sequences, or microsatellites, is a key feature of HNPCC and of a subset of apparently nonhereditary colorectal tumors (4—6). Microsatellite instability is known to occur in classic hereditary disorders associated with defects in DNA repair (28).

In this study, 5 microsatellite loci were analyzed in neurofibroma and normal skin DNAs from 16 NFl patients. Several microsatellite alterations, including mobility shifts, as detected by gain of novel microsatellite alleles missing in the patient’s constitutional DNA, and losses of constitutional microsatellite alleles, were observed in neurofibroma DNAs. Of the 16 individual cases studied, 8 manifested alterations at one or more microsatellite loci. Thus, our results indicate that microsatellite alterations occur at surprisingly high frequency in neurofibromas of NFl patients.

PATIENTS AND METHODS

Patients. Fourteen unrelated and two related NFl patients, diagnosed according to standard criteria (1), were analyzed. Four of the patients had a family history of disease, 12 were reportedly negative for family history and probably included de novo cases. All 16 patients presented with the typical clinical manifestations of NFl, including cafe-au-lait skin spots, axillary and groin freckling, and multiple cutaneous and s.c. neurofibromas (1).

DNA Analysis. Eight-μm sections of formalin-fixed, paraffin-embedded neurofibromas were collected on microscope slides and microdissected with sterile scalpels, referring to hematoxylin and eosin-stained sections to guide the separation of normal skin and of neurofibroma tissue into 1.5-ml polypropylene tubes, containing 1 ml xylene. No attempt was made to dissect out subsets of neurofibroma cells of a single type. The samples were incubated in xylene for 15 min and pelleted at full speed in a microfuge for 15 min. The xylene was then removed and the pellet was washed twice in absolute ethanol (1 ml). The samples were thoroughly dried under a sterile hood, and 100 μl of digestion buffer, containing 1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 0.02% Tween 20, and 100 μg/ml proteinase K were added to each tube. After an incubation of 3 h at 55°C, proteinase K was inactivated at 95°C for 10 min, and the samples were pelleted at full speed in a microfuge. The supernatant was stored at −20°C until use. One μl of a 1:10 dilution of each sample was used for each PCR reaction. DNA extractions and set-up of PCR reactions were performed in a laboratory distinct from that in which amplified DNAs were manipulated.

Two of the microsatellite markers analyzed in this study were those employed by Thibodeau et al. (5) to characterize genomic instability in colorectal cancer. These markers and their respective loci with chromosomal localizations were Mfd 41 (D17S250) (17p12—p11.1) and Mfd 27 (D5S107) (5q). The following microsatellite markers containing CA and GA repeats were also analyzed: Mfd 67 (D1S104) (1q21—q23; Weber et al., data obtained through GenBank); Mfd 39 (D8S87) (chromosome 8; Ref. 29); and D1S905 (chromosome 11; Ref. 30). All of these microsatellites are dinucleotide repeats. Primers and cycling conditions were as described in the relevant references (5, 29, 30). PCRs and electrophoretic separation and autoradiography were as described previously (31). By using the standard PCR protocol, the analysis of

Received 5/26/95; accepted 10/2/95.

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.

1 This study was supported by an Associazione Italiana per la Ricerca sul Cancro grant to R. M-C., by the Consiglio Nazionale delle Ricerche-Applicazioni Cliniche della Ricerca Oncologica Grant 94/01/16/1F39, and by Ministero dell’Università e Ricerca Scientifica e Tecnologica grants (40%) to P. B.

2 To whom requests for reprints should be addressed, at Istituto di Patologia Umana e Medicina Sociale, Università “Gabriele D’Annunzio,” Via dei Vestini 1, 66013 Chieti, Italy. Phone: +39 - (871) - 355-323 or 355-333; Fax: +39 - (871) - 355-322.

3 The abbreviations used are: NFl, type I neurofibromatosis; HNPCC, hereditary non-polyposis colorectal cancer; LOH, loss of heterozygosity.
RESULTS AND DISCUSSION

Neurofibromas from 16 NF1 patients were screened at 5 microsatellite loci (Table 1). Dinucleotide repeat markers were amplified by PCR, and polymorphic parental alleles were compared in paired typings of normal skin and neurofibroma DNAs, obtained from the same microdissected paraffin-embedded section. Several microsatellite alterations, including mobility shifts, as detected by microsatellite repeats missing in the patient's constitutional DNA, and losses of constitutional microsatellite repeats were observed in neurofibroma DNAs.

Eight of the sixteen individual cases studied manifested microsatellite alterations (Table 1). In particular, compressions or expansions of dinucleotide repeats were observed at one locus in four cases (cases 3, 4, 7 and 15) and at two loci in one case (case 6; Fig. 1). Only allele shifts >2 bp were detected in the six paired genotypings that resulted positive for microsatellite shifts. These alterations correspond to those typically associated with type I microsatellite instability (8).

Banding patterns suggesting loss of a microsatellite allele were obtained at one locus in four cases (cases 5, 6, 8, and 10), including one (case 6) that also presented size shifts at two other loci (Fig. 1). Without the analysis of close-flanking markers, it is not possible to determine whether these patterns correspond to extended chromosomal deletions or to limited losses involving a single microsatellite allele. In addition, because of the presence of genomic instability, the apparent loss of one microsatellite allele could have been due in some cases to comigration of the two alleles, following the size shift of one of them. However, it may be relevant that a recent study demonstrated somatic deletions, ranging from a single polymorphic locus to extensive regions of the NF1 gene, in benign neurofibromas of NF1 patients (32). This novel finding contrasts with the results of previous investigations of LOHs in neurofibromas, which were based on RFLP analysis (33–34).

The presence of widespread genomic instability in neurofibromas of NF1 patients raises several questions. Proteins directly or indirectly associated with DNA repair are involved in transcription, gene recombination, DNA replication, and cell cycle control (11–16). In hereditary nonpolyposis colorectal cancer, microsatellite instability has been detected in the majority of the cases (4) and has been firmly associated with mutations of genes that encode enzymes involved in mismatch repair (6, 14, 17–20). Microsatellite instability has also been demonstrated in variable proportions of several apparently nonhereditary types of solid tumor (8–10). Considering that multiple mechanisms appear to be implicated in the control of genomic integrity, it is conceivable that mutations in a variety of unrelated genes could be

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>ca/ga</th>
<th>ga/ca</th>
<th>ca</th>
<th>ga/ca</th>
<th>ca/ga</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>57</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>57</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>62</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>74</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>26</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11*</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>63</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>26</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14*</td>
<td>43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>44</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

a ND, not determined.
b a, daughter and father.
responsible for microsatellite alterations, particularly in extracolorectal tumors (11–16).

Indeed, genomic instability might lead to the accumulation of genetic lesions that favor tumor progression. It is intriguing that, although NFl is associated with inherited susceptibility to cancer, neurofibromas of NFl patients are benign tumors, which, in the vast majority of cases, do not evolve toward malignancy (1). In this respect, it should be noted that widespread mutations in normal tissues were demonstrated in a subset of HNPCC patients (35). Moreover, a surprisingly high frequency of LOH has been reported in atonic keratoses, which is usually a benign skin lesion (36). Finally, LOHs in the NFl region have been recently detected in benign neurofibromas of NFl patients (32). These observations suggest that a mutator phenotype or exposure to mutagenic agents per se may not be sufficient for carcinogenesis.

The monoclonal pattern of the microsatellite alterations is in apparent contrast with the presence of diverse cell types in neurofibromas and with the biochemical evidence for polyclonality (37). Indeed, the present study suggests that microsatellite alterations occurred in a progenitor cell ancestral to the majority of the cells present in the tumors. In this respect, it is interesting to note that another study reported evidence for a monoclonal origin of neurofibromas from female NFl patients by using an X-linked probe (34).

In addition to its functions as a negative regulator of Ras-mediated mitogenic signals and as downstream effector of Ras activity (3, 38), neurofibromin appears to have other functions, possibly linked to interactions with microtubules (3). To date, there is no evidence that relates the activities of neurofibromin to the efficiency of DNA repair. No evidence for excess of mutations at simple sequence repeats has been documented in blood samples from NFl patients relative to blood samples from controls (39). However, there is evidence that the Ras-dependent signal transduction pathway is involved in responses to DNA-damaging agents and could modulate genotoxic stress (11–12). Investigations of chromosomal aberrations and sensitivity to DNA damage in cells from NFl patients yielded conflicting results. Increased rates of spontaneous or X-ray-induced chromosomal aberrations and sister chromatid exchanges have been reported in neurofibroma-derived cells and in normal skin fibroblasts, melanocytes, and peripheral blood lymphocytes (22, 24, 27). However, other studies conducted on skin fibroblasts and on peripheral blood lymphocytes found no evidence for hypersensitivity to chromosomal aberrations, either spontaneous or induced by X-rays, UV light, or an alkylating agent (23, 25). The present study raises the possibility that either the NFl gene or another gene(s) plays a role in the pathogenesis of NFl-associated neurofibromas might be directly or indirectly implicated in pathways contributing to the control of genomic integrity.

ACKNOWLEDGMENTS

We express our gratitude to Giorgio Conconi (Institute of Dermatology, University “La Sapienza,” Rome, Italy); Dr. Angelo Del Nero (Dipartimento di Medicina Sperimentale, University “La Sapienza”); and our research students, Cristina Noviello, Gian Paolo Fava, and Mario Falchetti.

REFERENCES

Alterations of Microsatellites in Neurofibromas of von Recklinghausen's Disease

Laura Ottini, Diana L. Esposito, Antonio Richetta, et al.